**Introduction**

Although considerable progress has been made in understanding the pathophysiology of acute lung injury (ALI) and despite all innovations in intensive care medicine, the mortality rate in ALI remains high [1]. Recruitment of neutrophils is a key event in the development of ALI [1, 2], leading to plasma leakage and deterioration of oxygenation. Classically, neutrophil tissue infiltration requires a sequential involvement of selectins, chemokines and cell adhesion molecules where tissue- and stimulus-specific recruitment patterns are just beginning to emerge [3]. In lipopolysaccharide (LPS)-induced ALI, the recruitment of neutrophils was found to depend on β₂-integrins [4] and P-selectin [5]. Although chemokines and their receptors have been shown to be important targets of anti-inflammatory strategies, only CXCR2 ligands such as IL8 in humans and KC or MIP2 in mice have been identified as important guiding cues of neutrophils during LPS-induced ALI [6]. Recent studies, however, point towards the importance of CC-chemokines and endogenous chemotactic ligands of formyl-peptide receptors in the recruitment of neutrophils during acute and chronic
inflammation [7, 8]. Thus, we here investigated the role of CCR2, CCR5, FPR1 and FPR2 in neutrophils lung tissue infiltration upon LPS inhalation.

Methods

Animals

Male wild-type (WT) C57Bl/6 J Ccr2−/− [9], Ccr5−/− [10], Fpr1−/− [11] or Fpr2−/− [12] mice, 8 weeks of age, were used for this study. C57Bl/6 J were treated with antagonists to CCR5 (maraviroc; Tocris Bioscience; 10 μg/g body weight, oral gavage, 30 min before or 30 min after LPS), FPR1 (cyclosporine H; Santa Cruz Biotechnology; 5 μg/g body weight, intraperitoneally, 30 min before or 30 min after LPS) or vehicle controls. All experiments were approved by the local ethical authorities.

LPS-Induced ALI

Aerosolized LPS from Salmonella enteritidis dissolved in 0.9% saline (500 μg/ml, 4 h) was utilized to induce neutrophil infiltration in the lung. Thirty minutes before euthanasia, 5 μl of anti-Ly6G and 100 μl of FITC-dextran (30 mg/ml, 70 kDa) were applied intravenously. The lungs were removed, minced, digested with liberase, and passed through a cell strainer (online suppl. fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000353229).

Flow Cytometry

Cell pellets were labeled with PerCP-Cy5.5 anti-mouse Ly-6G, PE anti-mouse CD115, APC-Cy7 anti-mouse CD45 and APC anti-Mouse F4/80. Neutrophils were identified by their typical appearance in the forward scatter-side scatter and as CD45+ CD115− and PerCP-Gr1+ cells (online suppl. fig. S2). Within the lung, FITC-Gr1 antibody was used to distinguish between interstitial neutrophils (CD45+, CD115−, PerCP-Gr1+, FITC-Gr1−) and intravascular neutrophils (CD45+, CD115−, PerCP-Gr1+, FITC-Gr1+).

Lung Permeability

FITC-Dextran (70 kDa; Sigma-Aldrich) was used to assess vascular leakage. One hundred microliters of FITC-dextran (30 mg/ml) were administered by tail vein injection 30 min prior to euthanasia and dye extravasation was used to assess change in vascular permeability. The fluorescence of the 100 μl bronchoalveolar lavage (BAL) supernatant (FluoBAL) and of 50 μl serum (FluoSerum) was measured and permeability volume was expressed in microliters (VPerm = (FluoBAL/100 μl)/(FluoSerum/50 μl) × BAL volume).

Histology

Paraffin-embedded lung sections were stained with Mayer’s hematoxylin and histologically examined. Scoring of histological sections was done in compliance with recommendations of the American Thoracic Society [13]. Criteria for scoring are detailed in online suppl. table S1.

Statistics

All data are expressed as mean ± SD. Statistical significance was tested using one-way analysis of variance with Dunnett’s post hoc test. p values <0.05 were considered statistically significant.

Results

CCR5 and FPR1 Orchestrate Neutrophil Recruitment in LPS-Induced Lung Inflammation

Recruitment of neutrophils, which is a key event in lung injury, is orchestrated by chemokines binding to G protein-coupled receptors during routine immune surveillance or inflammation. In order to investigate the role of different chemokine receptors in ALI, we exposed WT mice and mice lacking CCR2, CCR5, FPR1 or FPR2 to aerosolized LPS and monitored neutrophil recruitment by flow cytometry of digested lungs and BAL fluid. To discriminate between interstitial and intravascular neutrophils, an antibody to neutrophils was administered shortly before sacrifice, thus labeling adherent neutrophils. In this model neutrophil lung infiltration is a major contributor to subsequent lung damage [14]. Baseline counts of circulating white blood cells and platelets did not differ between the various strains (online suppl. table S2). LPS exposure increased the number of alveolar, interstitial and intravascular neutrophils in WT mice (fig. 1a–c). While neutrophil recruitment after LPS inhalation was not altered in Ccr2−/− mice, lung neutrophil infiltration in Ccr5−/− mice was significantly diminished in all three compartments (fig. 1a–c). Similarly, lack of FPR1 strongly reduced neutrophil accumulation in all lung compartments, while deletion of FPR2 was without effect (fig. 1a–c). With the importance of neutrophils in mediating lung damage we also investigated permeability changes and histological damages in these mouse strains. Changes in both parameters paralleled observations made for neutrophil recruitment, i.e. reduction of LPS-mediated permeability increases and structural damages in Ccr5−/− and Fpr1−/− mice, while no significant effects were observed in Ccr2−/− and Fpr2−/− mice (fig. 1d–f).

Inhibition of CCR5 and FPR1 Prevents Lung Neutrophil Recruitment

Based on the results obtain from various gene-targeted strains, we further aimed at investigating the effect of antagonists to CCR5 and FPR1 on neutrophil lung tissue infiltration upon LPS inhalation. To test the importance of these receptors, mice were treated with maraviroc, a specific CCR5 antagonist, or cyclosporine H, an antagonist to FPR1. Pretreatment of mice before challenge with LPS abolished neutrophil adhesion and interstitial tissue infiltration (online suppl. fig. S3A–C). Interestingly, similar results were obtained when mice received the antagonists after LPS inhalation (fig. 2a–c), supporting the therapeutic relevance of these approaches.
CCR5 and FPR1 Mediate Lung Neutrophil Recruitment

**Fig. 1.** Neutrophil recruitment in response to LPS is reduced in Ccr5<sup>−/−</sup> and Fpr1<sup>−/−</sup> mice. Mice were challenged with LPS by inhalation and sacrificed 4 h later. The quantifications of alveolar (a), interstitial (b) and intravascular (c) neutrophils within the lungs of WT, Ccr2<sup>−/−</sup>, Ccr5<sup>−/−</sup>, Fpr1<sup>−/−</sup> and Fpr2<sup>−/−</sup> mice are displayed. *n* = 8–10 for each bar. 

Microvascular permeability was assessed by measurement of FITC-dextran clearance. Structural analyses of histological lung sections were made based on HE staining (e, f). Scale bar = 100 μm. Statistical significance was tested using one-way analysis of variance with Dunnett’s post hoc test. Asterisk indicates significant difference compared with LPS-treated WT mice.
CCR5 and FPR1 Neutralization Attenuates Endotoxin-Induced Lung Injury

Lung injury is characterized by an increased permeability of the alveolar-capillary barrier, resulting in lung edema with protein-rich fluid. Permeability was quantified by measurement of BAL protein concentration and the clearance of fluorescent dextran. Both parameters were found to be increased upon LPS treatment indicative of elevated plasma leakage (fig. 2d, e). Treatment of mice with antagonists to CCR5 or FPR1 either before or after...
LPS exposure largely inhibited lung edema formation (fig. 2d, e; online suppl. fig. S3D, E).

Histological analyses of lungs following LPS exposure revealed alveolar septal thickening, accumulation of inflammatory cells in the interstitium and alveoli, and influx of protein-rich fluid into the alveolar space as compared to control mice. Antagonists to CCR5 and FPR1 administered before or after LPS inhalation abrogated histological changes indicating protective effects in ALI (fig. 2f, g; online suppl. fig. S3F).

Discussion

In the study reported here, we identify the importance of CCR5 and FPR1 in neutrophil recruitment to inflamed lungs. CCR5, a receptor known for its importance in the recruitment of monocytes [15, 16], was recently also appreciated for its importance in the recruitment of neutrophils to atherosclerotic lesions or sites of ischemia [8, 17]. In these settings as well as in models of ALI, CCL5, a major CCR5 ligand, was found to be platelet derived [8, 14]. Indeed, neutralization of CCL5 prevents LPS and acid-induced ALI, whereas overexpression of CCL5 in murine lungs increases neutrophil accumulation altogether, supporting an important role for CCL5 in lung neutrophil recruitment [14, 18]. While CCR2 has been reported to be important in the recruitment of neutrophils to atherosclerotic lesions and in ischemia [8, 17], our study, as well as previous work [19], exclude a major role for CCR2 in lung neutrophil recruitment upon LPS stimulation. In fact, the impact of CCR2 on neutrophil recruitment at later time points following LPS inhalation appear indirect, as CCR2 primarily impairs the accumulation of monocytes and macrophages which create a milieu that favors the recruitment of neutrophils [19]. In addition to the importance of CCR5, we identify a crucial role for FPR1 but not FPR2 in neutrophil lung infiltration following LPS inhalation. The high affinity receptor FPR1 recognizes formylated peptides released from bacteria or from the mitochondria of necrotic cells. Recent studies propose a hierarchical gradient of guidance cues where chemokines bring the neutrophils to the vicinity of the site of inflammation and formylated peptides from either origin direct the neutrophils the final few hundred microns into the injury [20, 21]. In contrast to FPR1, FPR2 responds to both proinflammatory and proresolving ligands [22, 23], which may at least in part explain the different outcome of FPR2 deletion observed here. Specifically, the lipid metabolites lipoxin A4 and resolvin D1, as well as the peptide annexin A1 were shown to interact with FPR2, resulting in attenuated leukocyte recruitment [24, 25], and may hence neutralize the chemotactic activity exerted by ligands such as formylated peptides or cathelicidin. Taken together, our data provide novel insights into the chemokine-driven neutrophil recruitment in ALI and may allow for the design of specific neutralizing strategies.

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Disclosure Statement

None.

References


