Bronchoepithelial Expression of CXCR1 and CXCR2 Does Not Facilitate Transepithelial Migration of Neutrophils

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Abstract
Background: Neutrophilic airway inflammation is one of the key features of chronic obstructive pulmonary disease (COPD). The chemokine receptors 1 (CXCR1) and 2 (CXCR2) are expressed in the bronchial mucosa during chronic inflammation and might be of importance for transepithelial migration of neutrophils. Objectives: This study addressed the role of bronchoepithelial CXCR1 and CXCR2 expression with respect to transepithelial migration of neutrophils. Methods: Primary bronchial epithelial cells (PBECs) derived from COPD patients and healthy controls as well as transiently CXCR1- and CXCR2-transfected Calu-6 cells were used for transepithelial migration assays of neutrophils under various conditions. Epithelial CXCR1 and CXCR2 expression was verified by means of flow cytometry. Results: Transepithelial migration of neutrophils was significantly increased following lipopolysaccharide pretreatment of epithelial cells. Transient transfection of CXCR1 and CXCR2 neither augmented the transepithelial migration of neutrophils, nor did the selective blockade of CXCR1 and CXCR2 have any significant effect on neutrophilic transepithelial migration. In addition, no differences were found in PBECs and neutrophils derived from healthy controls and COPD patients. Conclusions: The data of the present study do not support the hypothesis that bronchoepithelial expression of CXCR1 and/or CXCR2 facilitate transepithelial migration of neutrophils.

Introduction
The pathophysiology of chronic obstructive pulmonary disease (COPD) includes persistent inflammation of the airways and lung parenchyma, which is caused by an abnormal response of the lung to noxious gases and inhaled particles [1]. Typically, the cellular composition of the airway infiltrates consists of neutrophils, macrophages, and CD8+ T cells [1, 2]. There is convincing evidence that neutrophils are one of the primary effector cells in COPD [3–7]. The number of neutrophils is in-
increased in bronchoalveolar lavage fluid as well as in induced sputum samples and airways from COPD patients [6, 8, 9]. The amount of neutrophils correlates with disease severity and progression in these patients [3, 8]. Neutrophil recruitment to the airways is induced by chemotactic factors such as CXC ligand 8 (CXCL8) and leukotriene B4 (LTB4), and both mediators are often elevated in the airways of patients with COPD [10–13]. In addition, neutrophils were predominant in the airway lumen, but a lack of increase in these cells in the subepithelium can be noted in bronchial biopsies of COPD patients [14]. This observation may be due to accelerated transepithelial migration of neutrophils from the circulation into the airway lumen, although this has not been investigated in detail. It is well known that increased LTB4 and CXCL8 levels derive from alveolar macrophages and epithelial cells, respectively. This, however, should also result in increased levels of neutrophils in the subepithelium, which is not the case, and points towards potential local mechanisms that facilitate and regulate transepithelial migration of neutrophils to the site of inflammation.

CXCL8 and other neutrophil chemoattractants belong to the ELR-CXC chemokines, which are ligands of the CXC chemokine receptor 1 (CXCR1) or 2 (CXCR2). CXCR1 is the receptor for CXCL8 and CXCL6 (granulocyte chemoattractant protein-2), and CXCR2 binds these as well as all other ELR-CXC chemokines; CXCL8 binds with high affinity to both receptors, while the others are ligands of lower affinity [15–18]. Recent studies focusing on urinary tract infections demonstrated that transepithelial neutrophil migration is CXCR1 dependent in vitro and defective in CXCR1-knockout mice [19]. Along this line, it was hypothesized that CXCR1 and CXCR2 expression on bronchial epithelial cells is relevant for the interaction between neutrophils and the airway mucosal barrier. It was speculated that these receptors are of importance for neutrophil migration across airway epithelial layers, and, consequently, a potential future therapeutic target for the treatment of neutrophilic airway inflammation, e.g. in COPD patients.

**Materials and Methods**

**Flow Cytometry**

CXCR1 and CXCR2 receptor proteins were detected on the cell surface of primary cells and cell lines using specific antibodies (anti-CXCR1 R&D Systems, MAB330, and anti-CXCR2 R&D Systems, MAB331). Primary cells or transfected cell lines were harvested and washed once and resuspended in PBS. Specific primary antibodies were added (1:50) and the suspension was incubated for 30 min on ice. After additional washing with PBS, labeled secondary antibodies were added to the suspension (1:50). The suspension was incubated for 30 min at 4°C in the dark. After incubation, cells were washed in PBS and resuspended in PBS prior to analysis. Fluorescence was measured with a BD FACS-Calibur flow cytometer (BD, Franklin Lakes, N.J., USA) using a 488-nm excitation laser. The number of cells stained was calculated using CellQuest software (BD).

**Cloning of CXCR1 and CXCR2 and Transfection of Calu-6 Cells**

For transient and stable transfection, the coding sequences of CXCR1 and CXCR2 were cloned in the pDSred-N1 backbone (Clontech, Mountain View, Calif., USA) or pcGFP-N1 (Clontech). The coding sequences from CXCR1 (NM_000634.2) and CXCR2 (NM_001557.3) were amplified using CXCR1_fw 5’-GCTAGCCGTGAAACTGAAAGAGCAGATG-3’ and CXCR1_rev 5’-CTCGAGAAAGAGACATTGACA-3’ with attached Nhel and Xhol restriction sites. A specific CXCR2 sequence was amplified using CXCR2_fw 5’-CTCGAGAAGGTATGGAGAATGTCGCC-3’ and CXCR2_rev 5’-CTCGAGAAGGTATGGAGAATGTCGCC-3’ with attached Nhel and Xhol restriction sites. cDNA from neutrophilic granulocytes served as template DNA. Amplicons were subcloned into the pcCR2.1 backbone using the TOPO-Cloning kit (Invitrogen, Darmstadt, Germany). After digestion with Nhel and Xhol, the fragments were cloned into the expression vectors. The vectors are named CXCR1-pDSred-N1 and CXCR2-pcGFP-N1. The accuracy of inserts was checked by sequencing.

For transient overexpression of CXCR1 and CXCR2, Calu-6 cells were transfected with the CXCR1-pDSred-N1 and CXCR2-pcGFP-N1. After reaching 80% confluency, cells were transfected with 4 µg plasmid DNA in a cell culture flask. DNA was mixed in a 1:2 ratio with FuGENE6 transfection reagent (Roche, Mannheim, Germany). After 30 min of incubation in serum-free media, the mix was added to the cells. After 24 h, cells were checked for CXCR1 and CXCR2 expression using flow cytometry. Positive cells were used for transmigration assays.

**Granulocyte Isolation**

Informed consent was obtained from all patients and healthy volunteers according to the procedures approved by the local ethics committee. Neutrophilic granulocytes were isolated from healthy volunteers and COPD patients; 20 ml of whole blood was transferred to a dextran gradient [18 ml Deltalex 60 (DeltaSelect) plus 2 ml citrate dextrose solution; Sigma, Taufenkirchen, Germany]. Erythrocytes were removed by centrifugation. The supernatant containing lymphocytes and sera was transferred to 20 ml Ficoll (Ficoll-Paque Plus). After 20 min of centrifugation at 600 g (no break), the neutrophilic granulocytes were at the bottom of the gradient. The supernatant containing the plasma and lymphocytes was removed. Remaining erythrocytes were removed from the pellet with erythrocyte lysis buffer (Buffer EL; Qiagen, Hilden, Germany) plus a 5-min incubation at 4°C and centrifugation. The neutrophilic granulocytes were resuspended in normal growth media. Cell numbers were determined using trypan blue exclusion (Sigma) and a Neubauer chamber.
Transmigration Assay

To test granulocyte migration, transmigration assays were performed. Tissue culture (TC) inserts with 3.0-μm pore size (Greiner Bio-One, Frickenhausen, Germany) were coated with rat tail collagen I (Sigma-Aldrich). CXCR1- and CXCR2-positive Calu-6 cells were seeded on the outer side of the TC insert membrane, which represents the apical side in the migration assays. The TC inserts were placed into 24-well plates filled with normal growth media (Calu-6 medium). A volt ohmmeter (World Precision Instruments, Berlin, Germany) was used to verify the tightness of the cell layer; 12 h before the assay, half of the TC inserts were pretreated from the apical side with 5 μg/ml lipopolysaccharide (LPS; source: Escherichia coli 055:B5; Sigma-Aldrich). LPS was removed completely before the migration assay was performed. Isolated granulocytes (250,000) were added to the basal side of each TC insert membrane. After 3 h of incubation at 37°C, the TC inserts were removed from the wells. The remaining growth medium was transferred to fresh cups. The number of migrated cells was assessed using the CyQuant Cell Proliferation Kit (Invitrogen, Darmstadt, Germany) according to the manufacturer’s protocol. Specific antibodies (anti-CXCR1 R&D Systems, MAB350, and anti-CXCR2 R&D Systems, MAB331) were used to block the receptors. Antibodies were applied 12 h before the assay was performed to the apical side of the TC inserts. The assay antibodies had been completely removed in advance. Untransfected Calu-6 cells or naive primary bronchial epithelial cells (PBECs) served as controls. Experiments were performed twice for each condition and granulocyte isolation.

Subjects

Patients who were referred to our clinic for flexible bronchoscopy for various reasons were screened to be included in the present study. Selection of COPD patients was based on the definition and classification given by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) [1]. In brief, the following inclusion criteria had to be met: stable airflow limitation, FEV₁ <70% of predicted, reversibility <10% of predicted FEV₁ after 200 μg inhaled salbutamol and a smoking history of >10 pack years. None of the patients with COPD had a history of atopy or evidence of atopy on skin prick testing for common aeroallergens. Control subjects were nonsmokers with normal lung function and no history of airway disease. Exclusion criteria were a history of respiratory tract infection or exacerbation of airway disease within the previous 8 weeks, and systemic or topical corticosteroid treatment during the previous 12 weeks. All patients gave written informed consent, and the study was approved by the local ethics committee (approval No. 03/180).

Patients underwent fiberoptic bronchoscopy under light sedation. Bronchial epithelium was obtained by gentle brushing of segmental and subsegmental bronchi under direct visual guidance by means of a protected brush. Each patient had 20–30 brushes of the bronchial epithelium. Brushing was accomplished by a very light gliding along the surfaces of the airways. Brushes were immediately placed in ice-cold bronchial epithelial growth medium and transported directly to the laboratory for further processing.

Culture of PBECs

Brushes were vortexed vigorously, and the cell suspension harvested was filtered through a 200-μm filter (Millipore; Billerica, Mass., USA) to remove mucus and cellular debris, and then treated with 4.8 U/ml Dispase II (Roche, Mannheim, Germany) to eliminate cell clumping. The cells were then centrifuged at 150 g for 10 min, and the pellet was resuspended in cell culture medium. Cells were counted using a hemocytometer. Cell viability was determined by trypan blue exclusion. Cells were resuspended in serum-free bronchial epithelial cell growth medium (Promocell, Freiburg, Germany) supplemented with a variety of growth factors, including bovine pituitary extract (0.052 mg/ml), recombinant human epidermal growth factor (0.5 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), epinephrine (0.5 μg/ml), triiodothyronine (6.5 ng/ml), transferrin (0.01 mg/ml) and retinoic acid (0.1 μg/ml). In addition, the medium contained penicillin G (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml; Invitrogen, Darmstadt, Germany). Cultures were maintained in a humidified atmosphere at 37°C in air/carbon dioxide (95/5% vol/vol). The identity of the epithelial cells was confirmed in all cultures by light microscopy and in randomly selected cultures by immunocytochemical staining for cytokeratin expression using a pancytokeratin antibody (clone KLI) directed against cytokeratin types 1, 2, 5–8, 11, 14, 16, and 18 (Immunotec, Marl, Germany). The monoclonal antifibroblast antibody FibAS02 (Dianova, Hamburg, Germany) was used to exclude contamination by fibroblasts [20]. The staining was performed according to Kunz-Schughart et al. [21], as described in detail previously. In general, cell culture experiments were performed after the first passage and no higher passage cell cultures were used.

Calu-6 Cell Culture

Calu-6 cells (human lung fibroblasts) were maintained in MEM with EBSS and l-glutamine (PAN Biotech) supplemented with sodium pyruvate, non-essential amino acids (1 mM; Biochrome, Berlin, Germany), penicillin (50 U/ml), streptomycin (50 μg/ml) and 10% FCS (PAA Laboratories, Pasching, Austria). Cells were grown at 37°C in humidified air with 5% CO₂.

Statistical Analysis

For statistical analyses, the Kruskal-Wallis rank sum test was used to compare data between groups, followed by the Mann-Whitney rank sum test using SigmaStat for Windows V2.03. Differences were considered to be statistically different if p < 0.05. x-fold migration was calculated for each value compared to the mean of the control group.

Results

Subjects

Nine COPD and 21 control subjects without airflow limitation were included in the study. All patients were referred for bronchoscopy for clinical reasons. All patients underwent preoperative diagnostic procedures for suspected peripheral or central carcinoma of the bronchus. In addition, based on the patients’ history and files, no other conditions known or suggested to be associated with altered immune responses were detected. In particular, no signs of diabetes, rheumatoid arthritis, in-
flammmatory bowel diseases, or myeloproliferative syndromes were found. The COPD patients were classified as stage I (n = 3), stage II (n = 2), stage III (n = 1) and stage IV (n = 3) based on the GOLD classification [1]. All were exsmokers and aged between 45 and 70 years. COPD patients were clinically stable for at least 8 weeks prior to bronchoscopy without any changes in medication. Control subjects, aged between 42 and 68 years, had no evidence of chronic disease and were free of medication.

CXCR1 and CXCR2 and Bronchial Epithelial Cells

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First we confirmed the expression of CXCR1 and CXCR2 on the surface of PBECs using flow cytometry. PBECs from COPD patients and controls (non-COPD patients) were stained for CXCR1 and CXCR2 using specific antibodies. In Figure 1a, c, representative FACS analyses are shown. On PBECs from COPD patients, expression of CXCR1 was increased compared to CXCR2 expression on PBECs: 60% of PBECs analyzed were positive for CXCR1 whereas only 3% of the cells were CXCR2 positive. In PBECs from control patients, 37% of the cells expressed CXCR1, whereas CXCR2 expression amounted to 3%. All of the PBECs showed similar mean fluorescence intensity ratios; the calculated ratio for CXCR1 on PBECs was 22.92 (control 1) and 25.53 (control 2) compared to 26.1 on PBECs from a COPD patient. For CXCR2, the respective ratios were 4.26 (control 1) and 4.53 (control 2) compared to 4.82 (COPD patient).

**Surface Expression of CXCR1 and CXCR2**

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**Surface Expression of CXCR1 and CXCR2 in Calu-6 Cells following Transient Transfection**

Calu-6 cells were transiently transfected with CXCR1 or CXCR2 expression vectors. Before using these cells in the migration assay, surface expression of CXCR1 and CXCR2 was confirmed by means of FACS analysis. The analysis was performed three times with cells from independent transfections with similar results. In Figure 2, representative FACS analyses of the expression of both receptors on naive Calu-6 and following transient transfection are shown. Naive Calu-6 cells show neither...
CXCR1 nor CXCR2 expression (fig. 2a, c). CXCR1 expression was higher on the Calu-6 cells compared to CXCR2. After transient transfection, 40% of the Calu-6 cells showed CXCR1 expression, with 18% of the cells showing CXCR2 expression. This expression pattern is comparable with the expression on the surface of naive PBECs. Subsequently, the transiently transfected Calu-6 cells were used in transmigration assays in combination with neutrophilic granulocytes from either healthy controls or COPD patients.

Transmigration Assays of Neutrophilic Granulocytes in Combination with Transiently CXCR1- and CXCR2-Transfected Calu-6 Cells

The resistance of the transiently transfected Calu-6 cell layer was measured using a voltmeter to confirm the tightness of the cell layer (data not shown). Neutrophilic granulocytes from COPD patients or healthy volunteers were added to the basal side of the transfected cells and cocultured for 3 h. In figure 3, the x-fold migration of neutrophils from control patients is shown. LPS stimulation significantly increased the transmigration of neutrophils resulting in a 1.4-fold (range 1.05–1.87) increase. Similar findings were seen for CXCR1 overexpression following LPS stimulation (1.3-fold; range 1.08–2.11) and unstimulated conditions. For CXCR2, transmigration of neutrophils was significantly lower following LPS treatment (median = 0.98; range 0.89–1.48) compared to control cells and unstimulated conditions. When neutrophils from COPD patients were used instead, no significant increase in the transmigration of neutrophils was observed with respect to CXCR1 and CXCR2 overexpression under either condition (fig. 4). In summary, as shown in figures 3 and 4, there is no augmentation in the migration of neutrophilic granulocytes from either healthy donors or COPD patients following CXCR1 and CXCR2 overexpression.

Transmigration Assays of Neutrophilic Granulocytes Using PBECs

PBECs derived from control subjects and COPD patients were used in combination with neutrophils from healthy controls and COPD patients to perform transmigration assays in both the absence and presence of spe-
specific CXCR1 and CXCR2 antibodies. The antibodies were applied to PBECs 12 h prior to incubation with neutrophilic granulocytes.

As shown in figure 5a, LPS stimulation of PBECs derived from non-COPD patients resulted in a 1.9-fold (median = 1.89, range 0.24–4.70) increase in transepithelial migration of neutrophils. In the presence of anti-CXCR1 and anti-CXCR2 antibodies, the increase in LPS-stimulated transepithelial migration of neutrophils derived from control subjects was 1.5-fold (range 0.62–3.26; non-significant compared to LPS stimulation). In the presence of anti-CXCR1 or anti-CXCR2, the increase was 1.9-fold (range 0.88–3.42) and 1.08-fold (range 1.0–1.14), respectively (data not shown).

Similar results (fig. 5b) were obtained when PBECs derived from COPD patients were used. LPS stimulation resulted in a 1.8-fold (range 0.88–4.62) increase in the transmigration of neutrophils derived from COPD patients (p < 0.05 vs. unstimulated control). Blockade of both receptors revealed a 2.1-fold increase in the transepithelial migration of neutrophils (range 1.47–3.13; non-significant compared to LPS stimulation), whereas a 1.4-fold (range 1.22–1.96) increase for CXCR1 and a 2.1-fold (range 1.47–3.13) increase were observed when only CXCR1 or CXCR2 were blocked (data not shown).

Figure 5 summarizes the results demonstrating that the CXCR1 and/or CXCR2 blockade had no significant effect on transepithelial migration of neutrophilic granulocytes under either condition.

Discussion

In the present report, the potentially CXCR1- and CXCR2-dependent transepithelial migration of neutrophils was investigated by using different cell culture models as well as airway epithelial cell lines and PBECs derived from healthy nonsmokers and COPD patients. Recent studies suggest an accelerated transepithelial migration of neutrophils from the circulation to the airway lumen, which could, at least in part, explain why airway inflammation dominated by neutrophils is often found in COPD patients.

Significant CXCR1 and CXCR2 expression has already been demonstrated in bronchial epithelial cells [22–24]. Qiu et al. [23] showed upregulation of epithelial CXCR2 mRNA expression in COPD patients with severe exacerbation. However, in the same paper, neither in stable COPD patients nor in patients with severe COPD exacerbation CXCR1 expression was detected. In a more recent paper, a robust bronchoepithelial expression of both receptors was seen in COPD patients across all stages of severity [22]. This is in line with the results of the present study demonstrating cell surface expression of CXCR1 and CXCR2 in PBECs from healthy donors and COPD patients, respectively. Therefore, it is evident that the bronchial mucosa expresses both chemokine receptors, which may be involved in the transepithelial migration of neutrophils.

Godaly et al. [19] were able to demonstrate that the expression of functional IL-8 receptors is crucial for neutrophils to cross the epithelial barrier. This was shown in IL-8 receptor-knockout mice with urinary tract infection as well as in human uroepithelial cell lines. In addition, it was speculated that IL-8 dimer formation, which is required for IL-8 receptor activation, forms a bridge between the neutrophils and the epithelial cells, thereby enabling transepithelial cell migration. This hypothesis was supported by a recently published paper using molecular dynamic simulations, thereby showing that electrostatic interactions are favorable for CXCL8 homodimer formation, which could have substantial consequences biologically [25].

However, the results of the present study do not reinforce this view with respect to the transepithelial migra-
tion of neutrophils across the bronchoepithelial barrier. Different experimental approaches were realized to substantiate the hypothesis. Transient CXCR1 and CXCR2 transfections of Calu-6 cells, neither of which express CXCR1 or CXCR2 in the control situation, were performed and used for transmigration experiments. In addition, PBECs from healthy nonsmokers and COPD patients, with confirmed IL-8 receptor expression, as well as neutrophils from both cohorts were differentially applied for migration assays. Anti-CXCR1 and anti-CXCR2 control experiments confirmed the negative results found. Therefore, several lines of evidence were generated demonstrating that the migration of neutrophils through airway epithelial cell layers is not controlled and/or facilitated by CXCR1 and CXCR2 expression.

Based on the results presented here and available data in the literature, the effects of CXCR1 and CXCR2 on respiratory epithelial cells remain obscure. According to the evidence presented for IL-8 receptor-expressing intestinal epithelial cells, one might speculate that CXCR1 and/or CXCR2 play an important role in the maintenance of the mucosal epithelial surface barrier, thus affecting the infiltration of inflammatory cells into the host that may maintain airway inflammation. This was at least demonstrated in an in vitro migration model using intestinal Caco-2 and HT-29 cells [26]. Whether the same holds true for respiratory epithelial cells requires further investigation and was beyond the scope of the present study.

Neutrophil recruitment during COPD exacerbations appears to be partially mediated by the two important neutrophil chemoattractants CXCL5 (ENA-78) and CXCL8 (IL-8). Upregulation of their receptors, CXCR1 and CXCR2, has been observed in bronchial biopsy specimens in severe COPD exacerbations [23], which is in line with our results. In addition, CXCR1 and CXCR2 are expressed on a broad range of leukocytes, most notably neutrophilic granulocytes. The physiological role of these receptors is mediating neutrophil recruitment, which has been described in humans as well as in primates, rodents and rabbits. Recent data provide evidence that the expression of CXCR1 and CXCR2 in neutrophils is maintained in COPD patients when compared to healthy controls or healthy smokers, indicating that COPD per se does not alter the expression of chemoattractant receptors [27].

Importantly, treatment with a CXCR2 antagonist (SCH527123) blocked pulmonary neutrophilia associated with the repeat bronchoscopy procedure [28], suggesting that CXCR2 inhibition is important for the inflammatory events occurring in the distal lungs of human COPD patients. Similar results were found in ozone-induced neutrophil recruitment to the lungs in healthy humans [29]. Thus, there is overwhelming evidence that IL-8 receptor expression on neutrophils is important for the development of the neutrophilic lung inflammation present in COPD. The aforementioned studies in animals and humans have clearly demonstrated that CXCR1/CXCR2 antagonism inhibits pulmonary and airway neutrophilia [28, 29]. Since the present study shows that the blockade of bronchoepithelial CXCR1/CXCR2 expression has no impact on transepithelial neutrophil migration, the important site of CXCR1/CXCR2 blockade has to be the neutrophil itself. This hypothesis is further supported by the observation that the CXCR2 antagonist SCH527123 reversibly decreases peripheral neutrophil counts in humans. This implies that the CXCR2 antagonism inhibits neutrophil egress from the bone marrow and migration from peripheral blood to tissue [29]. Neutrophils are generated in large numbers in the bone marrow; mature neutrophils circulate in the bloodstream for several hours and are recruited to tissues and sites of infection via a multistep process [30, 31]. There is convincing evidence that the mechanisms of neutrophil trafficking are complex and probably disease and stimulus specific in connection with a certain degree of redundancy in the mechanisms involved [30]. To further address this issue in the future, it would be important to use disease-specific models in conjunction with disease- and phenotype-specific neutrophils.

There are some limitations of the present study. Firstly, the data presented are from in vitro cell culture experiments, which do not necessarily reflect the complex in vivo situation seen in COPD patients. Secondly, CXCR1 and CXCR2 expression of neutrophils was not assessed on a regular basis before each experiment. Although unlikely, this could also have influenced our results.

In summary, the present study generated substantial evidence indicating that airway epithelial expression of both IL-8 receptors CXCR1 and CXCR2 does not facilitate transepithelial migration of neutrophilic granulocytes. This, however, does not contradict the importance of CXCR1 and CXCR2 in neutrophilic lung inflammation when expressed on polymorphonuclear granulocytes.

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References


