

CX3CR1 Contributes to Nasal Neutrophilia in Airways Allergy: Novel Role for IL-8 in Inducing CX3CR1 Expression by Neutrophils

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Abstract

Background: CX3CR1 is involved in promotion of inflammation by recruiting inflammatory cells to the inflamed tissue. However, no reports studied their expression by neutrophils infiltrating the nasal tissue in chronic rhinosinusitis (CRS) patients with or without associated airways allergy.

Objectives: Our objectives were to evaluate the expression of CX3CR1 by neutrophil infiltrating the inflamed nasal tissue in patients suffering from CRS, and to study the receptor gene induction in activated human neutrophils.

Methods: Immunohistochemistry were conducted to evaluate CX3CR1 expression by neutrophil cells infiltrating the middle turbinate from patients operated for endoscopic sinus surgery. The gene expression and the receptor surface expression in resting versus activated neutrophils by IL-8 were studied by Q-PCR and flow cytometry (FACS).

Results: It is shown that CX3CR1 was significantly expressed by nasal infiltrating leukocytes when compared to control group. This expression was higher in patients with CRS and airway allergy than those with CRS and no airway allergy. Neutrophils contributed largely to the sub-epithelial layer inflammatory cells expressing CX3CR1. Both the gene and the surface expression of CX3CR1 were significantly induced in activated neutrophils by IL-8.

Conclusion: CX3CR1 expression by neutrophils is expressed in CRS and the receptor's gene expression is induced in activated neutrophils by IL-8. These results further highlights and identifies an importance role for CX3CR1 in nasal inflammation.

Introduction

Neutrophils contribute to inflammatory cells influx in nasal cytology from allergic rhinitis (AR) patients and their increased blood ratio to lymphocytes seems to correlate to the disease severity [1,2]. We have reported recently a significant reduction in neutrophils influx in nasal secretions in early phase reaction to sodium cromoglycate treatment that correlated to the overall symptom score improvement in persistent AR [3]. All these evidences point to important role for neutrophils in the early phase reaction in the pathophysiology of AR. Nonetheless, few reports studied neutrophils infiltration of the nasal tissue in chronic inflammation of the nose. Recently, neutrophils were reported to be important local source of oncostatin M that disrupts the epithelial barrier of the airways highlighting their role in allergic asthma and chronic rhinosinusitis patients with nasal polyps [4]. This points to an important role for neutrophil recruitment to the chronically inflamed airways.

CX3CR1 is the receptor for its ligand CX3CL1 or fractalkine and also for eotaxin 3 [5,6] that is involved in adhesion and migration of leukocytes. CX3CR1 is expressed by lymphocytes, NK cells and monocytes. We have recently shown a novel role for CX3CR1 in recruiting NK cells to the epithelial layer of nasal mucosa in patients with chronic rhinosinusitis (CRS) and associated allergy [6]. However,

no reports exist to date regarding the role of CX3CR1 in neutrophils recruitment.

In the current study our objectives were 1) to evaluate the expression of CX3CR1 by neutrophil infiltrating the inflamed nasal tissue in patients suffering from CRS either with associated allergy, herein is referred to as allergic chronic rhinosinusitis (ACRS) or with no allergy referred to as non-allergic chronic rhinosinusitis (NACRS), and 2) to study the receptor gene induction in activated human neutrophils. To achieve our goals immunohistochemistry studies were conducted to evaluate CX3CR1 expression by inflammatory cells infiltrating the nasal tissue from patients operated for endoscopic sinus surgery. The gene expression and the receptor surface expression were also studied *in vitro* in normal resting peripheral neutrophils and activated neutrophils by IL-8 utilizing QPCR and flow cytometry. This study was approved by the Ethics Committee of the Liege University Hospital.

Material and Methods

Patients and volunteers

A total of 23 middle turbinate nasal biopsies from patients subjected to endoscopic sinus surgery for CRS; NACRS (n=11), ACRS without

asthma (n=6) and ACRS with asthma (n=6). As a control group, biopsies from the inferior turbinate of patients undergoing partial turbinectomy were obtained (n=5). Allergy was confirmed by positive prick skin tests and Radioallergosorbant test (RAST) to aeroallergens. All biopsies were subjected to immunohistochemistry.

As for the QPCR and FACS experiments healthy volunteers who do not suffer from upper airway inflammation participated by donating 60 ml of EDTA-anticoagulated peripheral venous blood. Neutrophils were isolated by dextran sedimentation and centrifugation in a Ficoll Hypaque gradient (Lymphoprep, Oslo, Norway). Erythrocytes were removed by hypotonic lysis. Cells were cultured in RPMI 1640 (Lifes Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Lifes Technologies).

Immunohistochemistry

Formalin-fixed paraffin embedded nasal specimens sections were deparaffinized in xylene, rehydrated in graded alcohols and washed in distilled water. To uncover antigenic sites, antigen retrieval was performed by heating the 4 µm-thick tissue sections in citrate buffer (pH6) for 11 min at 121°C (pressure cooking). The sections were incubated 1h with a rabbit polyclonal antibody directed against CX3CR1 (dilution 1/1000, Abcam ab8021, Cambridge, UK). Immunoperoxidase detection was performed using the Envision rabbit (Dako, Glostrup, Denmark). The number of neutrophils stained with the anti-CX3CR1 antibody was counted by an investigator who was blinded to the source of each sample, in 20 adjacent high power fields per sample (10 fields within the epithelium and 10 fields within the stroma).

CX3CR1 gene and surface expression by Q-PCR and FACS

Peripheral blood isolated neutrophils were used immediately or were incubated 1 hour in the presence of 10ng of recombinant human

IL-8 (CXCL8 (Peprotech, Rocky Hill, NJ, USA). Total cell RNA was isolated from 10⁶ neutrophils or lymphocytes using a NucleoSpin RNA kit (Macherey-Nagel, Duren, Germany). cDNA of the samples was prepared from the same starting amount of RNA using a SuperScript First-strand Synthesis System (Lifes Technologies). The relative gene expression of *CX3CR1* (forward primer: CCC TGA ATC AGT GAC AGA AAA CT; reverse primer: ACG GAG TAG AAT ATG GAC AGG AA) was measured using SYBR Green master mix (Roche, Mannheim, Germany) in a ABI 7900HT Sequence Detection System (Applied Biosystem). A house keeping gene, GAPDH (forward primer: ACC AGG TGG TCT CCT CTG AC, reverse primer: TGC TGT AGC CAA ATT CGT TG) was used as reference gene to normalized all values. As for FACS experiments, the following antibodies were used: CD16-APC (Miltenyi Biotec, Auburn, USA), isotype control ((RatIgG2b K) BD Biosciences (Franklin Lakes, NJ, USA)) and anti-CX3CR1-FITC (BioLegend, San Diego, USA) were used to stain neutrophils. Analysis was performed with FACS Canto II and Diva software (BD Biosciences).

Results and Discussion

Figure one shows the expression pattern of CX3CR1 by inflammatory cells infiltrating the nasal tissue. As seen in Figure 1a, nasal biopsies from CRS patients demonstrated significant recruitment by paired t-test of CX3CR1 positive cells when compared to control group. There was no difference in the expression between the ACRS and the NACRS group. However as seen in Figure 1b, dividing the ACRS group into two subgroups; ACRS with AR only and ACRS with AR and concurrent asthma, we were able to demonstrate that the maximum expression was seen in ACRS and asthma group. Moreover, in the latter group, CX3CR1 positive cells were higher in the epithelial layer than the sub-epithelial layer (Figure 1c). This is in consistence with our earlier report that showed significant infiltration of the epithelial layer by NK cells expressing CX3CR1 in ACRS [5].

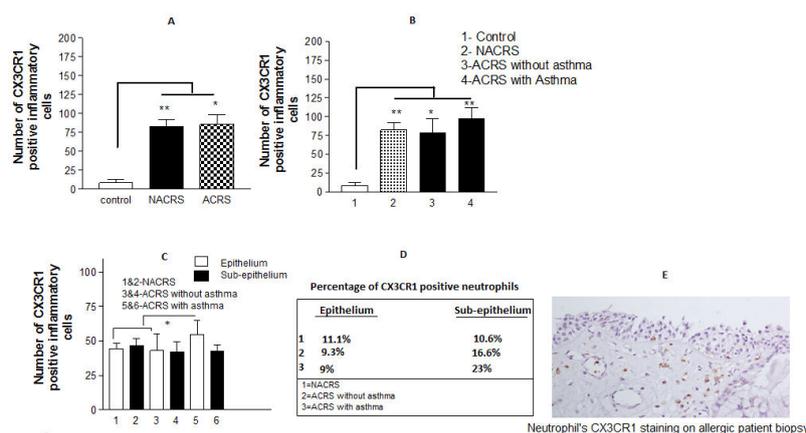


Figure 1: Data represent a total of 23 middle turbinate nasal biopsies from patients subjected to endoscopic sinus surgery for CRS; NACRS (n=11), ACRS without asthma (n=6) and ACRS with asthma (n=6); As a control group, biopsies from the inferior turbinate of patients undergoing partial turbinectomy were obtained (n=5); The number of neutrophils stained with the anti-CX3CR1 antibody was counted in 20 adjacent high power fields per sample (10 fields within the epithelium and 10 fields within the stroma).

To evaluate whether neutrophils contributed to CX3CR1 positive infiltrating inflammatory cells, the number of neutrophils stained with the anti-CX3CR1 antibody was counted by an investigator who was blinded to the source of each sample, in 20 adjacent high power fields per sample (10 fields within the epithelium and 10 fields within the stroma). The percentage of positive CX3CR1 neutrophils were comparable in the epithelial layer between all groups being 11.1%, 9.3% and 9% in NACRS, ACRS without asthma and ACRS with asthma, respectively (Figure 1d). However, there was much higher percentage of CX3CR1 positive neutrophils in the sub-epithelial layer

in the ACRS group, being 10.6%, 16.6% and 23% in NACRS, ACRS without asthma and ACRS with asthma, respectively (Figure 1d).

Taken collectively, it could be deduced from the immunohistochemistry analysis, those patients who have combined upper and lower airways allergy and CRS express the highest percentage of CX3CR1 positive inflammatory cells infiltration to the inflamed nasal tissue. There is also interesting preference for neutrophils to infiltrate the sub-epithelial layer.

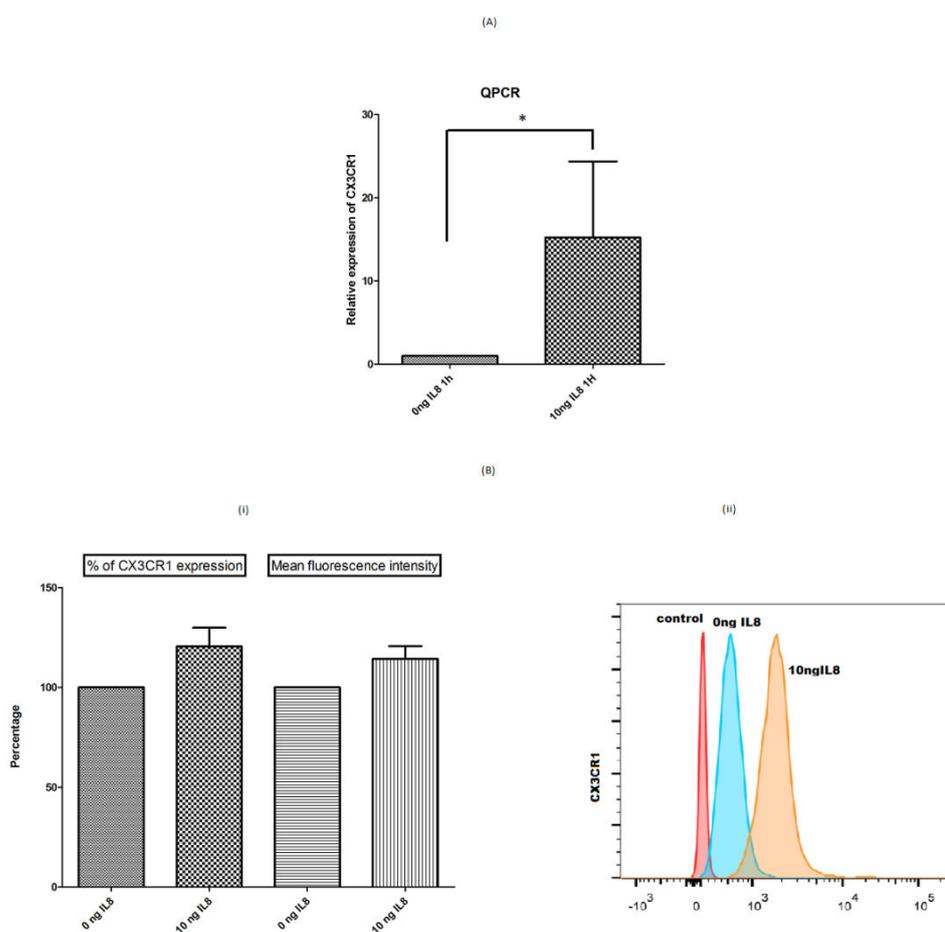


Figure 2: (a) QPCR of *CX3CR1* gene expression in absence and presence of 10 ng/ml IL-8 (N=8); (b) Percentage and mean fluorescence of neutrophils expressing *CX3CR1* in absence and presence of 10 ng/ml IL-8 (N=7); (c) Histogram of one experiment representative of 7 experiments.

Current opinion indicates that after allergen exposure, cytokine inducing neutrophil recruitment such as IL-8, play a pivotal role in airways inflammation especially the severe neutrophil phenotype of asthma [7-10]. Therefore, we next investigated the *in vitro* effect of IL-8 on the gene expression pattern of CX3CR1 by neutrophils. As seen in Figure 2a, treatment of neutrophils by 10 ng/ml IL-8 for 1 h largely induced the gene expression of *CX3CR1*. This was functional as treated neutrophils demonstrated increase in the number and intensity of surface CX3CR1 expression (Figures 2b (i) and 2b (ii)). This may indicate a novel role for IL-8 as an important inflammatory mediator in inducing neutrophil recruitment to the sub-epithelial layer of the allergic inflamed nose *via* CX3CR1 up-regulation.

In conclusion, we show the first evidence of the expression of CX3CR1 by neutrophils infiltrating allergic inflamed nasal tissue, and the induction of the receptor by IL-8. These results highlight a novel role for CX3CR1-neutrophil axis in ACRS and open channels for pharmacological therapy targeting neutrophils trafficking in chronic inflammatory diseases of the airways. Further investigations are needed to elaborate on the signal transduction mechanisms involved in *CX3CR1* gene regulation in activated human neutrophils and their implications in the allergic inflammatory cascade reaction.

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Authors Contribution

Lejeune T and Roncontrati P: Contributed to data collection.

Lefebvre P: Acquisition of data, Fund raising.

Delvenne P: Acquisition of data, Critical review of manuscript.

El-Shazly AE: Designed the study and wrote the manuscript, acquisition of data, fund raising and Critical review of the work.

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