Title:
A novel cooperation between CX3CL1 and CCL26 inducing NK cells chemotaxis via CX3CR1: a possible mechanism for NK cell infiltration of the allergic nasal tissue

Running head: NK recruitment via CX3CR1

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Abstract

Background: Recent data indicated that Natural killer (NK) cells and chemokines could play a pivotal role in nasal inflammation. CX3CR1, the only receptor for fractalkine/CX3CL1, is abundantly expressed by NK cells, and was recently shown to be also a receptor for eotaxin-3/CCL26. However, no reports explored the NK cells-CX3CL1-CCL26 axis via CX3CR1 in allergy.

Objective: Our goals were first to determine specifically NK cells recruitment pattern in nasal tissue of allergic chronic rhinosinusitis (ACRS) and non-allergic chronic rhinosinusitis (NACRS) patients in comparison with healthy controls, and secondly to investigate the function of CX3CR1 in NK cell migration.

Methods Immunohistochemistry, microchemotaxis chambers, flow cytometry and confocal microscopy were used in this study.

Results: Herein, we showed that NK cell infiltrated the epithelial layers of nasal tissue only in ACRS patients and not in NACRS patients or controls. NK cells were also more numerous in the stroma of the nasal tissue from ACRS patients compared to NACRS patients or controls. This migration could be mediated by both CXCL1 and CCL26, since these two chemokines induced NK cell migration. Moreover, both molecules stimulated also cytoskeleton changes and F-actin reorganization in NK cells. Chemotaxis and cytoskeleton changes were sensitive to genistein, a tyrosine kinase inhibitor. By flow cytometry, we demonstrated that single antigen nasal provocation challenge increased the expression of CX3CR1 on NK cells in allergic rhinitis (AR) patients. The function of this receptor was associated by a significant augmentation of NK cells chemotaxis against the optimal doses of CX3CL1 and CCL26.

Conclusion: Our results point out a novel role for CX3CR1 in NK cell migration that may contribute to the NK cells trafficking to the allergic upper airway. This could be mediated largely by CX3CL1 and CCL26 stimulation of tyrosine kinase pathway. If so, then a potent CX3CR1 antagonist would be a suitable therapeutic modality in allergic rhinitis and allergic chronic rhinosinusitis.

Key Words: NK cell, CX3CR1, CX3CL1, CCL26, Chemotaxis, Allergic rhinitis.

Introduction

Chemokines that attract inflammatory cells play a critical role in promoting nasal inflammation and the development of nasal polyps. CX3CL1 that belongs to CX3C chemokine family is expressed as a membrane bound form and under suitable conditions CX3CL1 is cleaved to its soluble form that has been reported to be higher in the plasma of patients with AR. The specific receptor for CX3CL1 is the CX3CR1 that is expressed on monocytes, NK cells, T cells and mast cells, mediating adhesion and migration of these leukocytes [1-3]. Moreover, segmental allergen challenge up-regulates the function of CX3CR1 in peripheral blood CD4 T cells [4]. CX3CL1 protein detection in nasal polyps was reported by Danielsen et al [5].

CC-chemokines are another group of chemokines also implicated in allergic inflammation. CCL26 or eotaxin-3 stimulates CCR3 receptors together with eotaxin 1 and 2. Eotaxin genes are located in the region of chromosome 17q that is linked to atopy and total IgE levels [6, 7]. Further, the role of CCL26 in AR and asthma is well reported [8-11]. However, recently it has been shown that among eotaxin family only CCL26 has the ability to stimulate CX3CR1 receptor and attract not only eosinophils but also NK cells [12]. Nonetheless, the function of CX3CR1 in recruiting NK cells to the allergic nose via CCL26 is not yet demonstrated.

Classically NK cells are defined as CD3- CD16+ CD56+ lymphocytes subpopulation, but recently NKp46 has been described as a specific marker for the detection of both human and mouse NK cells [13]. These cells are mainly found in the peripheral blood, but they are also present in tissues, for example in the lung [14]. NK cells play a pivotal role in immune response against viruses and tumours by killing infected or transformed cells [15, 16]. However, NK cells can also interact with various components of the immune system and secrete cytokines and thus regulate or exacerbate the immune responses [17, 18]. Some investigators demonstrated the ability of NK cells to differentiate in the presence of IL-4 into a cell subset, secreting distinct cytokines patterns similar to T_{H2} profile such as IL-5 and IL-13 [19, 20]. Other studies demonstrated the existence of type 2 cytokine-secreting NK cells in AR and showed increased number and enhanced cytotoxicity of NK cells [21]. Moreover, we recently reported a novel role for NK cells in the pathophysiology of AR by recruiting eosinophils

through its IL-8 secretion. This activity is modulated by IL-15 and vitamin D_3 [22]. Despite of these evidences indicating a role for NK cells in inflammation, little is known about NK cells migration in AR and its associated condition, the ACRS.

Accordingly, our goal was to investigate NK recruitment in ACRS and in NACRS. To determine the mechanism of this migration, the expression of CX3CR1 by NK cells after nasal allergen challenge and the NK chemotaxis induced by CX3CL1 and CCL26 were studied.

Materials and Methods

Patients and control subjects

A total of 58 subjects participated in the current study. Out of those, 22 were subjected to endoscopic sinus surgery and their nasal biopsies were obtained by trimming of the middle turbinate as a part of the surgery. As control group, biopsies from the inferior turbinate of patients undergoing partial turbinectomy were obtained (n=5). All biopsies were subjected to immunohistochemistry. The 22 patients with chronic rhinosinusitis (CRS) with or without nasal polyps were divided into 2 groups: group 1 is formed by11 patients with non allergic CRS (NACRS, patients who had no history of allergy and was proven negative to prick skin tests and allergosorbant test (RAST)) while group 2 is formed by 11 patients with allergic CRS (ACRS, patients with long history of poorly controlled AR with positive skin tests and RAST to aeroallergens and in whom nasal tissue swelling resulted into obstruction of the ostiomeatal complex and the development of CRS). The latter was further subdivided into ACRS without asthma (n=6) and ACRS with asthma (n=5). The other 31 subjects were 27 allergic (AR) patients who were recruited from the rhinology outpatient clinics (patients who are allergic to aeroallergens

and who had neither associated sinusitis nor nasal polyps) and 4 healthy volunteers who served as control group. These patients contributed to the nasal allergen challenge tests and chemotaxis assays. This study was approved by the Ethics Committee of the Liege University Hospital.

Nasal allergen provocation

AR patients who presented to our rhinology clinics with clinical symptoms of AR and were allergic to aeroallergens (house dust mites, grass pollens, fungal spores or animal danders) participated in the allergen nasal challenge. The allergen was chosen according to the results of skin prick tests and RAST. None of them were taking antihistaminic or nasal/systemic cortisone therapy. After obtaining their consent, 10 µl of the diluent were introduced into the nose to the anterior part of the inferior turbinate unilaterally on impregnated discs. After 15 min and after excluding the nonspecific hyperresponsiveness to diluent, nasal lavage with saline was done and then the purified and standardized allergen dilution (stallorgenes 100 IR/ml) was introduced into the nose in a similar fashion. After 1-2 min the patients were asked to blow their nose and saline nasal irrigation was done. Patients' vital signs were monitored for at least 30 min after challenge. The control groups consisted of AR patients who were challenged with the diluent solution only.

Cell preparation

Eosinophils and NK cells were isolated by Percoll (66%) solution separation. Eosinophils and NK cells were further purified by negative immunomagnetic cell separation (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). For eosinophil purification, anti-CD16 magnetic beads were used for the depletion of neutrophils and NK cells. For NK cells, a cocktail of biotin-conjugated antibodies against non NK cells lineage followed by a cocktail of magnetic microbeads conjugated to antibodies against biotin were used to obtain a population with more than 95% of CD56+ve CD3-ve NK cells (NK Cell Isolation Kit-human, Miltenyi Biotec). Both kits were used according to the

manufacturer's instructions. Cell viability was tested by Trypan blue and was always >98%.

Chemotaxis assay

Chemotaxis assays were performed in triplicate in a 48-well microchemotaxis Boyden chamber incubated in 5% CO_2 at 37°C for 90 min. Aliquots of 29 µl of the recombinant human chemotactic agent CX3CL1 or CCL26 (R&D systems) were placed in the lower wells and 50 µl of NK cells suspension (10^6 cells/ml) were placed in the upper wells. The two chambers were separated by a 5.0 µm pore polycarbonate membrane (Nuclepore, Whatman, Middlesex, UK). Migrated cells adherent to the lower surface were counted in 5-selected high power fields/well under a light microscope (5 hpf; X400). As for blocking experiments the NK cells were pre-treated with 1µM genistein (Sigma), for 60 min at 37^0C. Cells were then washed twice, their viability were rechecked by Trypan blue staining and was always>98%. Cells were re-suspended in buffer medium and their chemotaxis was checked as stated above.

Confocal microscope images

Thirty µl of cell suspensions at a concentration of 10^6 cells/ml were placed in 1 μ-Slide coated (collagen IV) cell microscopy chamber (Ibidi Integrated BioDiagnostics, Munich-Germany) and left to adhere for 1h. After two washes, stimulation with buffer, CX3CL1 or CCL26 was then performed for 5 minutes. Cells were then permeabilized by 0.1% saponin buffer (Sigma Aldrich) for 15 min, washed twice and stained with Alexa fluor 488® – Phalloidin diluted 40X (Invitrogen Molecular probes®) for 1h in the dark, on ice. Cells were then washed twice with PBS and analysed by confocal microscopy (Leica TCS SP2). As for blocking experiments adhered cells were incubated for 1h with 1 µM genistein before being stimulated with CX3CL1 and CCL26.

Flow cytometry

Staining of PBMC using the following antibodies: CD3-perCP, CD56-PE, CD16-Horizon V450, isotype control (BD Biosciences, Erembodegem, Belgium), anti-NKp46-APC

(Miltenyi Biotec.) and anti-CX3CR1 FITC (MBL, Woburn, MA USA) were analysed with FACS Canto II and Diva software (BD Biosciences).

Immunohistochemistry

The density of NK cells was assessed by immunohistochemistry in formalin-fixed paraffin-embedded nasal tissue biopsies. In order to uncover antigenic sites, antigen retrieval was performed by heating 6 min the 4 µm-thick tissue sections in citrate buffer (pH 6) at 121°C (pressure cooking). Then the sections were incubated overnight with a mouse mAb directed against NKp46/NCR1 (dilution 1/100, clone195314, R&D Systems, Oxon, UK). Immunoperoxidase detection was performed using the LSAB2 kit (Dako, Glostrup, Denmark). The number of cells stained with the anti-NKp46 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 within the subepithelial stroma).

Statistical analysis

Statistical significance was performed by paired t-test and ANOVA. Values of *P*<0.05 were considered significant.

Results

NK cells recruitment to nasal tissue in CRS

Surgical nasal biopsies of the middle turbinate of patients undergoing surgery for ACRS or NACRS with or without nasal polyps were investigated for the infiltrating numbers of NK cells, by immunohistochemistry with antibody against NKp46 (n=22). The results were compared to control samples obtained from patients undergoing surgery for reduction of inferior turbinates (n=5), who were negative for skin and RAST tests. Interestingly, as shown in Fig. 1, patients with ACRS without asthma had strikingly more

NKp46+ NK cells infiltrating the nasal tissues than both the NACRS and the control groups (Fig. 1C, F-G). An increased number of NK cells in the epithelium were only observed in ACRS patients without asthma (Fig. 1F). This infiltration was not observed in ACRS patients with asthma (Fig 1E-G). These results indicate a specific recruitment pattern of NKp46+ cells to the nasal epithelial layer of patients with ACRS, and that this histological feature is lost in ACRS with asthma.

Expression pattern of CX3CR1 by NK cells in AR

To determine which mechanism could be responsible of this recruitment, we analysed the expression of chemokine receptor CX3CR1 on peripheral blood lymphocytes from AR patients by flow cytometry. As shown in Fig. 2, CX3CR1 was mostly detected on cells expressing NK cell markers such as NKp46 (Fig. 2A), CD56 (Fig. 2B) or CD16 (Fig. 2C) and not on T cells (CD3+, Fig. 2D). Moreover, we observed an up-regulation of CX3CR1 on NK cells after nasal challenge with aeroallergens (Fig. 2E and Table 1), but not in AR control group who were challenged with the diluents only (Fig. 2F and Table1). The expression of CX3CR1 was not modulated on NK cells from healthy donor challenged with histamine (Fig. 2G). Other markers of NK cells such as 2B4 did not show change in its expression after nasal challenge (data not shown). Although the number of NK cells expressing CX3CR1 increased after nasal allergen challenge in AR patients, the percentage of NK cells among the lymphocyte population was not modified by the nasal challenge (Table 2). This CX3CR1 up-regulation after aeroallergen challenge suggests a modification of NK migration in the pathophysiology of AR. To test this hypothesis, we performed chemotaxis experiments.

CX3CL1 and CCL26 induced-NK cells chemotaxis is tyrosine kinase dependent and is increased after nasal allergen challenge

Peripheral blood NK cells from 12 AR patients were tested for their chemotaxis response against CX3CL1 and CCL26. As seen in figure 3A and B, both chemokines demonstrated a classical bell shape chemotactic dose response, with the optimal activity being at 50 ng/ml for CX3CL1 ($p< 0.05$, Fig 3A) and 1µg /ml for CCL26 ($p<0.05$, Fig 3B). The chemotactic activity of the optimal doses of CX3CL1 and CCL26 against NK cells, were

blocked when NK cells were pre-treated with a tyrosine kinase inhibitor (genistein, 1µM, p <0.0001, Fig. 3C and D). Confocal microscopy studies confirmed that both chemokines induced cytoskeletal changes with F-actin reorganization that was sensitive to 1µM genistein treatment (Fig. 4).

To test the impact of CX3CR1 up-regulation after aeroallergens challenge (Fig. 2 E), we challenged 6 AR patients sensitive to house dust mites (HDM) with HDM allergen. NK cell, chemotactic activity against the optimal doses of CX3CL1 and CCL26 were measured before and 6 hours post challenge. As shown in Fig. 5, 6 hours after challenge, both chemokines increased the migration activity of NK cells significantly (p< 0.0001).

Discussion

NK cells recently gained a lot of attention as helper cells in promoting allergic inflammation of the airways. The ability of these cells to secrete eosinophilotactic mediators provided further insights in the possible cross talk between NK cells and eosinophils in promoting allergic inflammation. On other hand, NK could contribute to allergen-specific immune suppression (for review [23]). For example, the fact that NK1 cells inhibit IgE production by B cells [24] and the negative correlation between the percentage of NK cells and total IgE levels found in serum of severe atopic dermatitis patients [25] suggested a role of NK cells in IgE regulation in these patients. Moreover, a

partial impairment of the NK cell capability of promoting and maintaining appropriate TH1 responses has been reported in the blood of respiratory allergic patients [26].

To our knowledge, this is the first report that investigating NK cells in nasal mucosa of allergic patients. Previously, NK cells have been studied only in the blood or skin of allergic patients [21, 26, 27] and in mouse models of asthma (lung and lymph nodes) [28]. With a specific marker for NK cells, NKp46 [13], we showed a recruitment of NK cells in nasal biopsies of AR patients (Fig. 1). Nothing was reported about the mechanisms that recruit NK cells to the allergic nose. Herein, we demonstrate a novel functional up-regulation of CX3CR1 on NK cells in AR patients. This results in increased migratory response to the competitive agonistic activity of CX3CL1 and CCL26. The chemotactic index (CI) of CX3CL1 was almost comparable to CCL26, but the amount of CCL26 needed to induce a migration was higher. This is consistent with a recent report by Nakayama et al [12] showing that CCL26 is 10 fold less potent than CX3CL1. Since these two mediators are well known to be up-regulated in AR, our results provide a possible mechanism of NK cell recruitment to the allergic nose, and strongly indicate a novel bi-agonistic effect for CCL26 in recruiting both eosinophils and NK cells through CCR3 and CX3CR1 receptors, respectively to the allergic inflamed nose.

Investigation of nasal biopsies from patients undergoing surgery for CRS demonstrated a specific recruitment of NK cells in the epithelial layers of the allergic nose but not in other non-allergic inflammatory pathologies. An accumulation of NK cells was also reported in the superficial dermis and in the epidermis of allergic contact dermatitis skin [27]. Interestingly, this histological feature was lost in patients having upper airway allergy worsened by asthma. This may be linked to the fact that, in a mouse model of self-limited allergic airway inflammation, NK cells were recently implicated in the resolution of inflammation [29]. Moreover, attenuation airway hyperresponsiveness induced in mice by IL-2 and IL-18 treatment required NK cells [30]. In advanced inflammation cascade observed in asthmatic patients NK cells would be less able to migrate to the nose, perhaps because they express lower levels of adhesion molecules. This is in agreement with the decreased expression of CD54 and CD62L adhesion molecules observed on blood NK cells from children with acute exacerbation of asthma compared to children with stable asthma after prednisolone therapy [31]. NK cell

trafficking in inflamed tissues in mouse model was dependent of CD62L expression [29]. Another possible mechanism is the inhibition of NK cells by activin-A [32]. Activin-A seems to be a critical regulator of allergic asthma [33] and higher level of activin-A is observed in the serum and in bronchial biopsies of asthmatic patients compared to controls [33, 34]. Infiltration of NKT cells, another innate cell population, was also different in the nasal mucosa of asthmatic and non-asthmatic patients. NKT cells express NK markers and an invariant T cell receptor and can also play a role in the pathogenesis of asthma (for review [35]). These cells were only detected in the sinus mucosa of asthmatic patients and not in non-asthmatic patients [36]. In a mouse model, NKT cells played a role in eosinophilic inflammation in acute asthma phase [37].

Migration of NK cells involved chemokine receptor activation [38] and classically chemokine signal transduction pathway is mediated by Ca^{2+} mobilization, protein kinase C and heterotrimeric GTP-binding proteins [39], but as alternative pathway kinases and phosphatases, adaptor proteins, and a small GTP-binding proteins can be also implicated chemokine signalling [37, 39-41]. In this study, genistein, a tyrosine kinase inhibitor, abolished cytoskeleton changes and chemotaxis of NK cells suggesting a tyrosine kinase signal transduction pathway involved in CX3CR1 stimulation by both CX3CL1 and CCL26. This is supported by the fact that NK cell trafficking has been shown to depend largely on tyrosine kinase-linked receptors [42].

In conclusion, our results highlight the role for NK cells recruitment in the pathophysiology of AR and point out CX3CR1 as a potential therapeutic target in allergic inflammation of the airway.

Figure legends:

Figure 1: NKp46+ cell infiltration in nasal biopsies. NKp46+ cells (stained in brown) in biopsies from (A) control, (B) NACRS and (C) ACRS patients without asthma, (D) isotype control on biopsy from NACRS patient. NKp46+ cell counting, (E) in nasal biopsies (means ± SEM), (F) in nasal epithelium and (G) in subepithelial stroma (bars represent the mean values). Original magnification: A-D, X200. Control subjects (n=5), NACRS subjects (n=11), CRS subjects without asthma (n=6), ACRS subjects with asthma (n=5). NS= not significant. Asterisk indicates $p<0.0001$ and $p=0.0236$ by paired *t*-test, for bars 1&3 and bars 3&4, respectively.

Figure 2: CX3CR1 expression on NK cells. CX3CR1 FACS staining versus NKp46 (A), CD56 (B), CD16 (C) and CD3 (D) staining. CX3CR1 expression on NK cells after nasal challenge (E-H) from AR patients challenged with aeroallergen (E), challenged with aeroallergen diluent (F) and healthy control challenged with histamine (G).

Figure 3: Chemotactic activity of CX3CL1 and eotaxin-3 on human NK cells, dose-response experiments. (A) CX3CL1 and (B) CCL26 in inducing NK cell chemotaxis. (C&D) Blockade effect of 1µg/ml genistein in NK cell chemotaxis against the optimal doses of CX3CL1 and CCL26. Asterisk indicates $P<0.05$ (A&B) and $p<0.0001$ (C&D), by paired t-test & ANOVA. Results are means ± SEM of 12 independent experiments performed in triplicate.

Figure 4: Confocal images of NK cell showing shape changes and F-actin reorganisation (in green). Results are from one experiment representative of 3, (A) stimulation with buffer, (B) stimulation with CX3CL1 and CCL26. Genistein treatment alone had no effect on NK cell shape or F-actin (C), but blocked the F-actin reorganisation and shape changes induced by CX3CL1 and CCL26 (D).

Figure 5: Effect of nasal allergen challenge with HDM on (A) CX3CL1- and (B) CCL26-induced NK cells chemotaxis. Results are means ± SEM of 6 independent experiments performed in triplicate. Asterisk indicates p<0.0001 by paired t-test & ANOVA.

References

1. Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, Greaves DR, Zlotnik A, Schall TJ, A new class of membrane-bound chemokine with a CX3C motif. Nature 1997;385: 640-4.
2. El-Shazly A, Berger P, Girodet P-O, Ousova O, Fayon M, Vernejoux J-M, Marthan R, Tunon-de-Lara JM, Fraktalkine produced by airway smooth muscle cells contributes to mast cell recruitment in asthma. J Immunol 2006;176: 1860-8.
3. Imai T, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M, Kakizaki M, Takagi S, Nomiyama H, Schall TJ, Yoshie O, Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. Cell 1997;91: 521-30.
4. Rimaniol A-C, Till SJ, Garcia G, Capel F, Godot V, Balabanian K, Durand-Gasselin I, Varga EM, Simonneau G, Emilie D, Durham SR, Humbert M, The CX3C chemokine fractalkine in allergic asthma and rhinitis. J Allergy Clin Immunol 2003;112: 1139-46.
5. Danielsen A, Tynning T, Brokstad KA, Olofsson J, Davidsson A, Interleukin 5, IL6, IL12, IFN-gamma, RANTES and Fractalkine in human nasal polyps, turbinate mucosa and serum. Eur Arch Otorhinolaryngol 2006;263: 282-9.
6. Koppelman GH, Stine OC, Xu J, Howard TD, Zheng SL, Kauffman HF, Bleecker ER, Meyers DA, Postma DS, Genome-wide search for atopy susceptibility genes in Dutch families with asthma. The Journal of allergy and clinical immunology 2002;109: 498-506.
7. Barnes KC, Mathias RA, Nickel R, Freidhoff LR, Stockton ML, Xue X, Naidu RP, Levett PN, Casolaro V, Beaty TH, Testing for gene-gene interaction controlling total IgE in families from Barbados: evidence of sensitivity regarding linkage heterogeneity among families. Genomics 2001;71: 246-51.
8. Berkman N, Ohnona S, Chung FK, Breuer R, Eotaxin-3 but not eotaxin gene expression is upregulated in asthmatics 24 hours after allergen challenge. American journal of respiratory cell and molecular biology 2001;24: 682-7.
9. Gu Z, Jin M, Cao Z, Role of eotaxin-3 in chronic rhinosinusitis with nasal polyps. Otolaryngol Head Neck Surg 2011;145: 324-6.
10. Ravensberg AJ, Ricciardolo FL, van Schadewijk A, Rabe KF, Sterk PJ, Hiemstra PS, Mauad T, Eotaxin-2 and eotaxin-3 expression is associated with persistent

eosinophilic bronchial inflammation in patients with asthma after allergen challenge. The Journal of allergy and clinical immunology 2005;115: 779-85.

11. Olze H, Forster U, Zuberbier T, Morawietz L, Luger EO, Eosinophilic nasal polyps are a rich source of eotaxin, eotaxin-2 and eotaxin-3. Rhinology 2006;44: 145-50.

12. Nakayama T, Watanabe Y, Oiso N, Higuchi T, Shigeta A, Mizuguchi N, Katou F, Hashimoto K, Kawada A, Yoshie O, Eotaxin-3/CC chemokine ligand 26 is a functional ligand for CX3CR1. Journal of immunology 2010;185: 6472-9.

13. Walzer T, Blery M, Chaix J, Fuseri N, Chasson L, Robbins SH, Jaeger S, Andre P, Gauthier L, Daniel L, Chemin K, Morel Y, Dalod M, Imbert J, Pierres M, Moretta A, Romagne F, Vivier E, Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. Proc Natl Acad Sci U S A 2007;104: 3384-9.

14. Culley FJ, Natural killer cells in infection and inflammation of the lung. Immunology 2009;128: 151-63.

15. Langers I, Renoux VM, Thiry M, Delvenne P, Jacobs N, Natural killer cells: role in local tumor growth and metastasis. Biologics 2012;6: 73-82.

16. Colonna M, Jonjic S, Watzl C, Natural killer cells: fighting viruses and much more. Nat Immunol 2011;12: 107-10.

17. Hanna J, Mandelboim O, When killers become helpers. Trends Immunol 2007;28: 201-6.

18. Reschner A, Hubert P, Delvenne P, Boniver J, Jacobs N, Innate lymphocyte and dendritic cell cross-talk: a key factor in the regulation of the immune response. Clinical and experimental immunology 2008;152: 219-26.

19. Peritt D, Robertson S, Gri G, Showe L, Aste-Amezaga M, Trinchieri G, Differentiation of human NK cells into NK1 and NK2 subsets. Journal of immunology 1998;161: 5821-4.

20. Deniz G, Akdis M, Aktas E, Blaser K, Akdis CA, Human NK1 and NK2 subsets determined by purification of IFN-gamma-secreting and IFN-gamma-nonsecreting NK cells. European journal of immunology 2002;32: 879-84.

21. Mesdaghi M, Vodjgani M, Salehi E, Hadjati J, Sarrafnejad A, Bidad K, Berjisian F, Natural killer cells in allergic rhinitis patients and nonatopic controls. Int Arch Allergy Immunol 2010;153: 234-8.

22. El-Shazly AE, Lefebvre PP, Modulation of NK cell autocrine-induced eosinophil chemotaxis by interleukin-15 and vitamin D(3): a possible NK-eosinophil crosstalk via IL-8 in the pathophysiology of allergic rhinitis. Mediators Inflamm 2011;2011: 373589.

23. Deniz G, Akdis M, NK cell subsets and their role in allergy. Expert Opin Biol Ther 2011;11: 833-41.

24. Aktas E, Akdis M, Bilgic S, Disch R, Falk CS, Blaser K, Akdis C, Deniz G, Different natural killer (NK) receptor expression and immunoglobulin E (IgE) regulation by NK1 and NK2 cells. Clinical and experimental immunology 2005;140: 301-9.

25. Wehrmann W, Reinhold U, Kukel S, Franke N, Uerlich M, Kreysel HW, Selective alterations in natural killer cell subsets in patients with atopic dermatitis. Int Arch Allergy Appl Immunol 1990;92: 318-22.

26. Scordamaglia F, Balsamo M, Scordamaglia A, Moretta A, Mingari MC, Canonica GW, Moretta L, Vitale M, Perturbations of natural killer cell regulatory functions in respiratory allergic diseases. The Journal of allergy and clinical immunology 2008;121: 479-85.

27. Carbone T, Nasorri F, Pennino D, Donnarumma M, Garcovich S, Eyerich K, Bergamo F, Cavani A, CD56 highCD16 - NK cell involvement in cutaneous lichen planus. Eur J Dermatol 2010;20: 724-30.

28. Ishikawa H, Sasaki H, Fukui T, Fujita K, Kutsukake E, Matsumoto T, Mice with asthma are more resistant to influenza virus infection and NK cells activated by the induction of asthma have potentially protective effects. J Clin Immunol 2012;32: 256-67.

29. Haworth O, Cernadas M, Levy BD, NK cells are effectors for resolvin E1 in the timely resolution of allergic airway inflammation. Journal of immunology 2011;186: 6129-35.

30. Matsubara S, Takeda K, Kodama T, Joetham A, Miyahara N, Koya T, Swasey CH, Okamoto M, Dakhama A, Gelfand EW, IL-2 and IL-18 attenuation of airway hyperresponsiveness requires STAT4, IFN-gamma, and natural killer cells. Am J Respir Cell Mol Biol 2007;36: 324-32.

31. Lin S-J, Chang L-Y, Yan D-C, Huang Y-J, Lin T-J, Lin T-Y, Decreased intercellular adhesion molecule-1 (CD54) and L-selectin (CD62L) expression on peripheral blood natural killer cells in asthmatic children with acute exacerbation. Allergy 2003;58: 67-71.

32. Robson NC, Wei H, McAlpine T, Kirkpatrick N, Cebon J, Maraskovsky E, Activin-A attenuates several human natural killer cell functions. Blood 2009;113: 3218-25.

33. Kariyawasam HH, Semitekolou M, Robinson DS, Xanthou G, Activin-A: a novel critical regulator of allergic asthma. Clin Exp Allergy 2011;41: 1505-14.

34. Semitekolou M, Alissafi T, Aggelakopoulou M, Kourepini E, Kariyawasam HH, Kay AB, Robinson DS, Lloyd CM, Panoutsakopoulou V, Xanthou G, Activin-A induces regulatory T cells that suppress T helper cell immune responses and protect from allergic airway disease. The Journal of experimental medicine 2009;206: 1769-85.

35. Umetsu DT, Dekruyff RH, Natural killer T cells are important in the pathogenesis of asthma: the many pathways to asthma. The Journal of allergy and clinical immunology 2010;125: 975-9.

36. Yamamoto H, Okamoto Y, Horiguchi S, Kunii N, Yonekura S, Nakayama T, Detection of natural killer T cells in the sinus mucosa from asthmatics with chronic sinusitis. Allergy 2007;62: 1451-5.

37. Koh YI, Shim JU, Lee JH, Chung IJ, Min JJ, Rhee JH, Lee HC, Chung DH, Wi JO, Natural killer T cells are dispensable in the development of allergen-induced airway hyperresponsiveness, inflammation and remodelling in a mouse model of chronic asthma. Clinical and experimental immunology 2010;161: 159-70.

38. Walzer T, Vivier E, G-protein-coupled receptors in control of natural killer cell migration. Trends in immunology 2011;32: 486-92.

39. Snyderman R, Pike MC, Chemoattractant receptors on phagocytic cells. Annu Rev Immunol 1984;2: 257-81.

40.   Bacon KB, Szabo MC, Yssel H, Bolen JB, Schall TJ, RANTES induces tyrosine kinase activity of stably complexed p125FAK and ZAP-70 in human T cells. The Journal of experimental medicine 1996;184: 873-82.

41.   Knall C, Worthen GS, Johnson GL, Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. Proc Natl Acad Sci U S A 1997;94: 3052-7.

42.   Saudemont A, Garcon F, Yadi H, Roche-Molina M, Kim N, Segonds-Pichon A, Martin-Fontecha A, Okkenhaug K, Colucci F, p110gamma and p110delta isoforms of phosphoinositide 3-kinase differentially regulate natural killer cell migration in health and disease. Proc Natl Acad Sci U S A 2009;106: 5795-800.
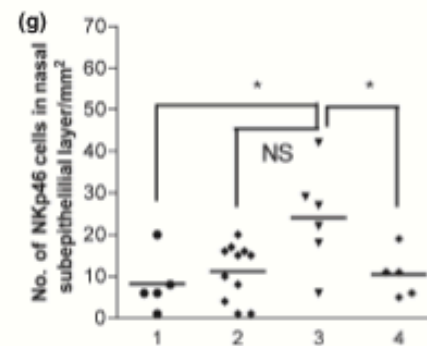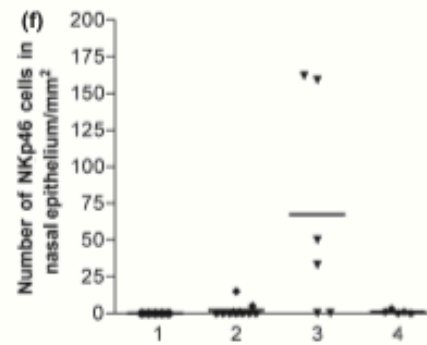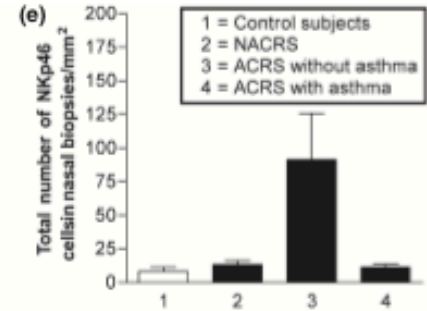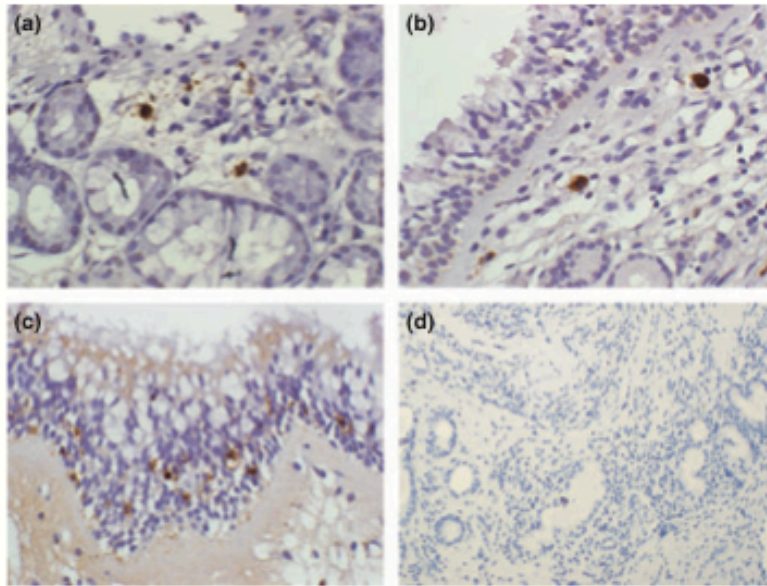
Fig. 1. Natural killer (NK)p46+ cell infiltration in nasal biopsies. NKp46+ cells (stained in brown) in biopsies from (a) control, (b) non-allergic chronic rhinosinusitis (NACRS) and (c) ACRS patients without asthma, (d) isotype control on biopsy from NACRS patient. NKp46+ cell counting, (e) in nasal biopsies (means ± SEM), (f) in nasal epithelium and (g) in subepithelial stroma (bars represent the mean values). Original magnification: a–c, X400; d, X100. Control subjects ($n = 5$), NACRS subjects ($n = 11$), CRS subjects without asthma ($n = 6$), ACRS subjects with asthma ($n = 5$). NS = not significant. Asterisk indicates $P < 0.0001$ and $P = 0.0236$ by paired t-test, for bars 1 & 3 and bars 3 & 4 respectively.
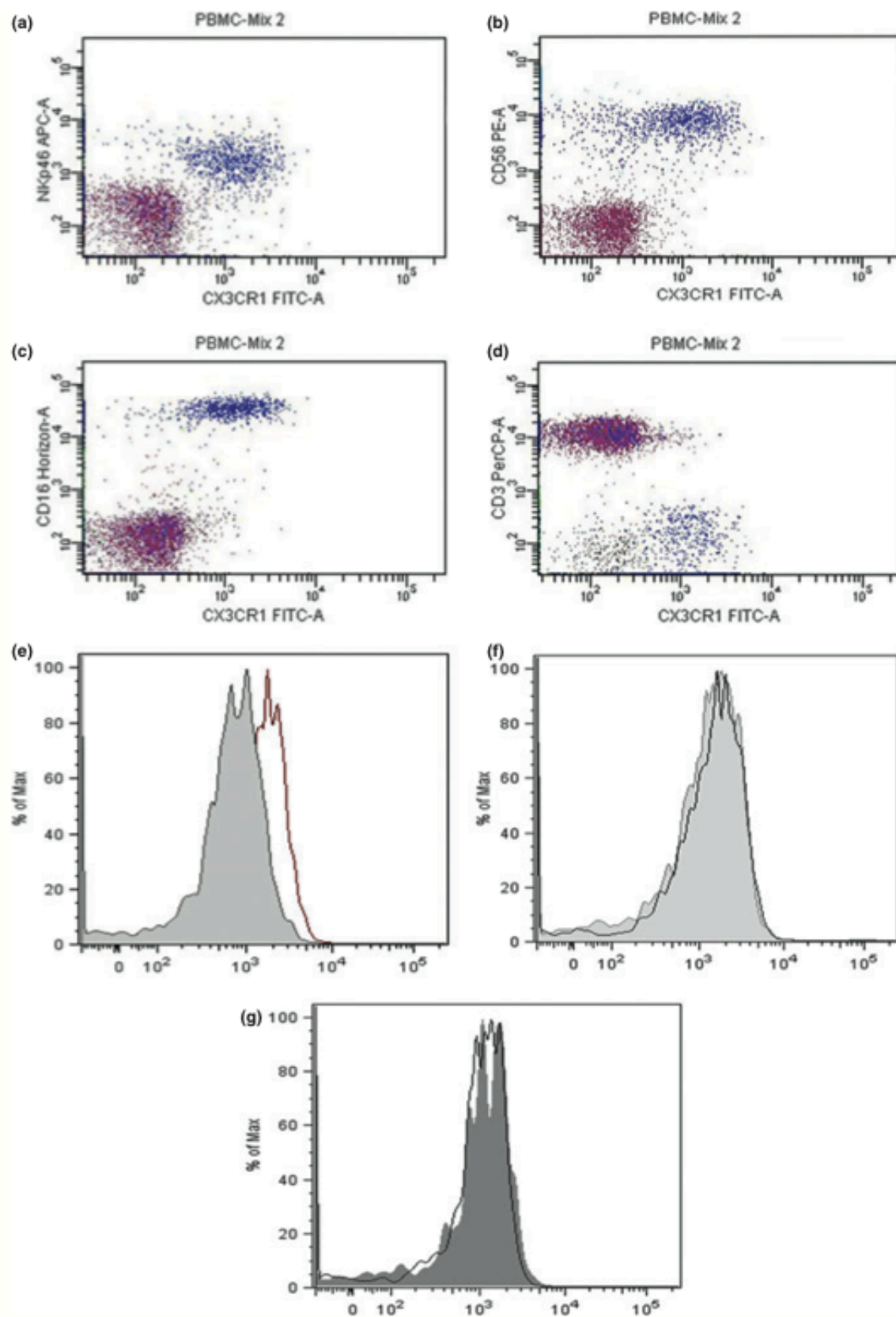
Fig. 2. CX3CR1 expression on natural killer (NK) cells. CX3CR1 FACS staining vs. NKp46 (a), CD56 (b), CD16 (c) and CD3 (d) staining. CX3CR1 expression on NK cells after nasal challenge (e–g) from AR patients challenged with aeroallergen (e), challenged with aeroallergen diluent (f) and healthy control challenged with histamine (g).
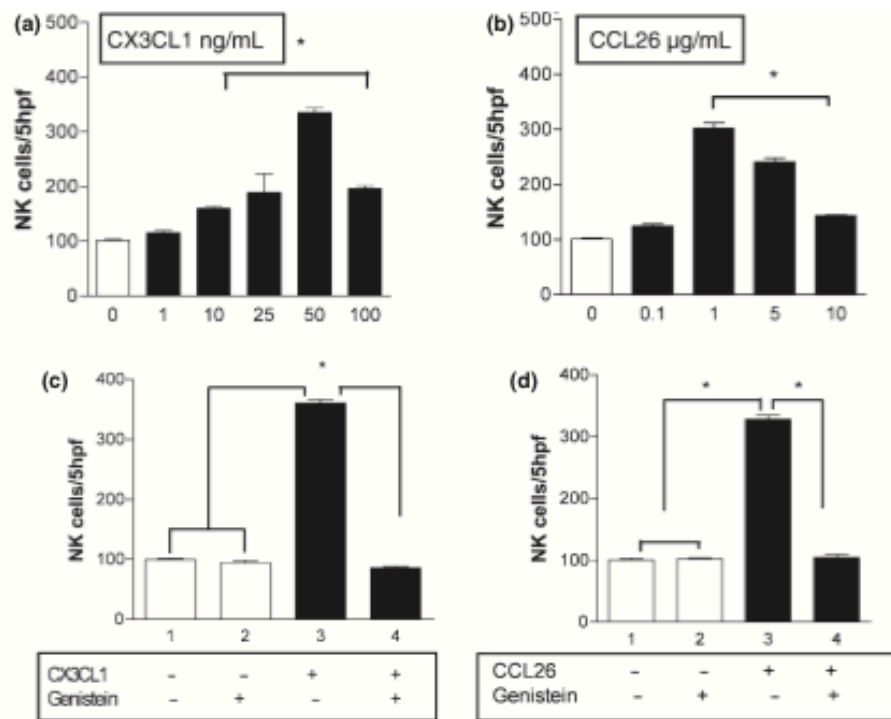
Fig. 3. Chemotactic activity of CX3CL1 and eotaxin-3 on human natural killer (NK) cells, dose-response experiments. (a) CX3CL1 and (b) CCL26 in inducing NK cell chemotaxis. (c and d) Blockade effect of 1 μg/mL genistein in NK cell chemotaxis against the optimal doses of CX3CL1 and CCL26. Asterisk indicates $P < 0.05$ (a and b) and $P < 0.0001$ (c and d), by paired t-test & ANOVA. Results are means ± SEM of 12 independent experiments performed in triplicate.

(a)

Buffer

Buffer

(b)

CX3CL1 50 ng/mL

CCL26 0.1 µg/mL

(c)

Genistein 1 µM

Genistein 1 µM

(d)

CX3CL1 50 ng/mL
+
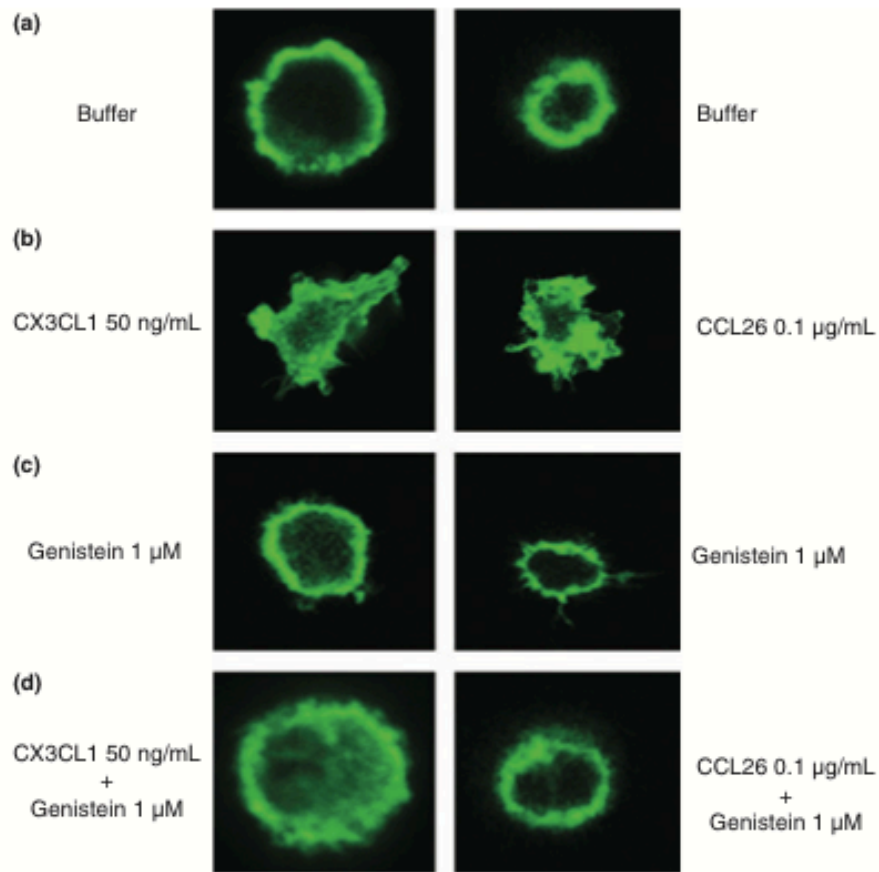Genistein 1 µM

CCL26 0.1 µg/mL
+
Genistein 1 µM

Fig. 4. Confocal images of natural killer (NK) cell showing shape changes and F-actin reorganisation (in green). Results are from one experiment representative of 3, (a) stimulation with buffer, (b) stimulation with CX3CL1 and CCL26. Genistein treatment alone had no effect on NK cell shape or F-actin (c), but blocked the F-actin reorganisation and shape changes induced by CX3CL1 and CCL26 (d).
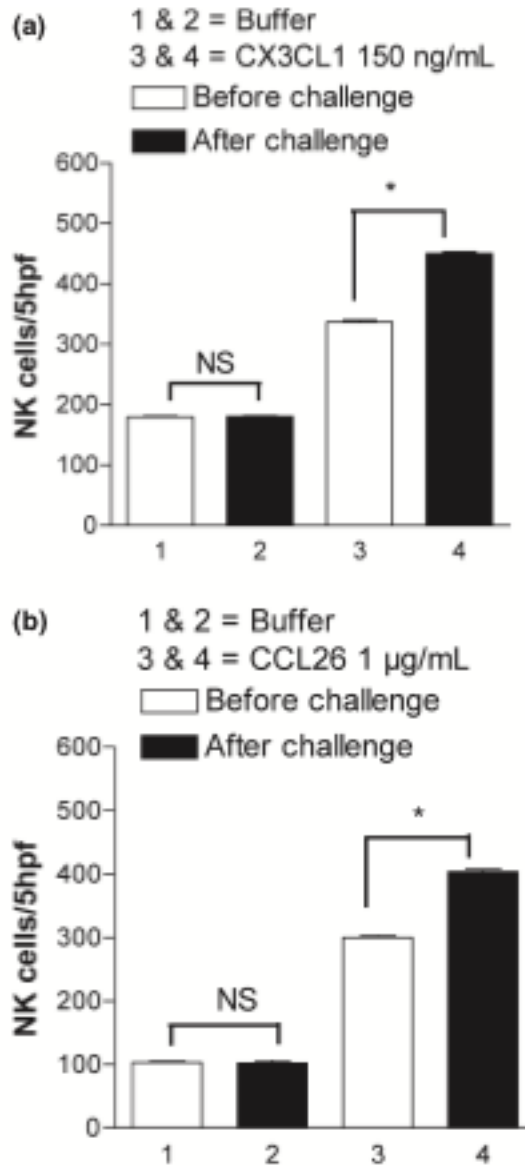
Fig. 5. Effect of nasal allergen challenge with HDM on (a) CX3CL1- and (b) CCL26-induced natural killer (NK) cells chemotaxis. Results are means ± SEM of six independent experiments performed in triplicate. Asterisk indicates $P < 0.0001$ by paired t-test & ANOVA.

Table 1. Mean fluorescence intensity (MFI) of CX3CR1

Patients	% of MFI ratio of NK-CX3CR1+		MFI of NK-CX3CR1+	
	Before challenge	After challenge	Before challenge	After challenge
AR patients	100	114.2 ± 9.1	1434.3 ± 63.1	1632.2 ± 63.1
AR control	100	96.6 ± 8.3	1819 ± 221.5	1776.5 ± 364.5

Table 2. Dynamics of natural killer (NK) cells before and after allergen nasal challenge in AR patients

Patients	% of NK CX3CR1+/total NK population		% of total NK cells/total lymphocytes	
	Before challenge	After challenge	Before challenge	After challenge
AR patients	55 ± 7.3	64.7 ± 8.8	18.2 ± 3.9	15.2 ± 1.9
AR control	69.2 ± 3.4	70 ± 6.3	21.2 ± 3.7	17.6 ± 2.4

% of sub-populations of NK cells before and after challenge

	CD56 bright		CD56 dim	
	Before challenge	After challenge	Before challenge	After challenge
AR patients	5.3 ± 2.6	6.9 ± 7	93.7 ± 4.6	92.4 ± 6.5
AR control	8.2 ± 2.4	4.8 ± 1.3	91.9 ± 2.3	95.2 ± 1.4