

Isolation and phenotypic characterization of mucosal nasal lymphocytes by direct ex vivo analysis

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Abstract Cellular inflammation of the nasal mucosa demonstrates a local immune response which plays an important role in allergic rhinitis. The aim of the present study was to characterize nasal mucosal lymphocytes regarding their activation and differentiation state by direct ex vivo flowcytometric analysis. Lymphocytes from the inferior turbinates were isolated by a mechanical method of preparation and, for comparison, from peripheral blood by Ficoll gradient centrifugation. Patients suffering from rhinitis or difficulty in nasal breathing were divided into an allergic (pollen-allergy, $n = 13$) and non-allergic group ($n = 24$). Expression of different T- and B-cell markers was determined by flowcytometric analysis. CD4⁺ T-cells from the nasal mucosa exhibited a memory phenotype (CD45RO⁺, 97%), were highly activated (CD69⁺, 43–73%), and showed low expression of the cutaneous lymphocyte antigen (CLA⁺, 5%). Nasal CD20⁺ B-lymphocytes expressed significantly higher levels of mIgE and lower levels of CD23 and CD80 than peripheral B-cells. Subsets of CD80⁺ (4%) and CD86⁺ (6%) CD20⁺ B-lymphocytes were identified in the nasal mucosa. No significant differences between allergic and non-allergic individuals were determined. As expected, the data show profound phenotypical differences between circulating peripheral blood and

nasal mucosal lymphocytes. Activated memory lymphocytes are present in the nasal mucosa from allergic, but also non-allergic patients and may indicate to a significant role of a local inflammatory state without systemic criteria for allergy. In our study, we show that direct ex vivo isolation of lymphocytes is practicable method and offers a new technique to examine the local nasal allergic immune response using a multiparametric phenotypical analysis.

Keywords Allergic rhinitis · B cells · Costimulatory molecules · IgE · Lymphocytes

Introduction

Within the last years, the prevalence of allergic rhinitis is increasing globally, today affecting 25–35% of the worldwide population [1]. Allergic rhinitis is characterized by itching, sneezing, rhinorrhea and nasal obstruction. These symptoms result from cellular inflammation, as allergic rhinitis is understood as an immediate hypersensitivity reaction associated with an expansion of the Th2-type immune response and the production of allergen-specific immunoglobulin E (IgE) [2, 3].

In sensitized individuals, allergens cause mast cell degranulation and the release of mediators such as histamine and tryptase leading to vasodilation, plasma leakage and up-regulation of vascular cell adhesion molecule (VCAM) and E-selectin [2, 3]. Together with chemoattractants these events promote the late phase reaction which is characterized by the recruitment, activation and tissue infiltration of leukocyte populations, including eosinophils, basophils, neutrophils and CD4⁺ (Th2) lymphocytes [3, 4].

Previous studies analyzing the cellular infiltration and inflammation of the nasal mucosal tissue were performed

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by using nasal brushing [5], scraping [6], lavage techniques [7–9] or even biopsies [10–17]. These techniques have the disadvantage that epithelial or superficial mucosal cells can be investigated only whereas inflammatory cells from the submucosa are excluded. In the present study, a mechanical preparation method was used to isolate resident lymphocytes from the nasal mucosa to characterize them by subsequent flowcytometric analysis. This allowed an investigation of T- and B-lymphocytes resident in the site of the allergic reaction with regard to their state of activation and differentiation by direct ex vivo analysis.

Materials and methods

A total of 37 patients have been recruited from the Department of Otorhinolaryngology (Bundeswehrkrankenhaus, Berlin, Germany) and divided into an allergic (A; $n = 13$) and non-allergic (NA; $n = 24$) group. All patients suffered from difficulty in nasal breathing resulting from nasal obstruction due to anatomical abnormalities (e.g. septal deviation, post-traumatic) or hyperplasia of the turbinates due to seasonal or perennial rhinitis. All allergic subjects (A) suffered from seasonal rhinitis, responded positively to skin prick testing (ALK Scherax, Hamburg, Germany) and exhibited positive titers of specific IgE (Pharmacia, Freiburg) against grass pollen ($n = 11$) or birch pollen ($n = 2$). None of the non-allergic individuals (NA) with perennial symptoms had a positive skin prick test to house dust mite, alternaria, cat allergen and pollen or positive titers of specific IgE. None of the patients received antihistamines within the last 3 days or any immunosuppressive drugs (including topical or systemic steroids) 14 days ahead of surgery. Patients with a history of immunotherapy as well as atopic dermatitis were excluded. The study was performed outside pollen season. Written informed consent for participation in the study was obtained from each subject and the research protocol was approved by the local ethics committee.

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll gradient centrifugation as previously described [18]. Cells were washed twice with phosphate-buffered saline (PBS) before the staining for flowcytometric analysis.

The obtained inferior turbinates were kept in 0.9% saline under sterile conditions at 4°C from surgery until cell isolation and processed within 2 h after operation. After separation from the bone, the nasal mucosa was cut into 1–3 mm² sections and shredded in Medicons[®] with rotating knives (50 µm Syringe, DAKO, Hamburg, Germany) by the Medimachine (DAKO, Hamburg, Germany) under addition of medium (RPMI 1640 medium containing 10% fetal calf serum, 2% L-glutamine, 1% penicillin and 1% streptomycin).

The obtained cell suspension was filtrated using a 50 µm pore-size filter (DAKO, Hamburg, Germany) and finally washed with PBS. Erythrocytes were lysed with sodium chloride solution (0.2 and 1.6%).

Mucosal cells of allergic and non-allergic individuals were stained with monoclonal anti-CD3 and anti-CD20 antibodies (DAKO, Hamburg, Germany) as previously described [19]. For the prevention of unspecific binding, 1×10^6 PBMCs were preincubated with 100 µl staining buffer (2% bovine serum albumin, 0.02% sodium azide in PBS) supplemented with 3 µl beriglobin (Aventis-Behring, Marburg, Germany) for 10 min. For B-cell analysis 1×10^6 PBMCs were double stained with 2.5 µl FITC-labeled monoclonal anti-human IgE (DAKO, Hamburg, Germany) and 5 µl PerCP-labeled monoclonal anti-human CD20 (BD, Heidelberg, Germany); fourfold stained with 10 µl FITC-labeled monoclonal anti-human CD23 (Biosource, Solingen, Germany), 5 µl PE-labeled monoclonal anti-human CD80 (BD PharMingen, Heidelberg, Germany), 5 µl PerCP-labeled monoclonal anti-human CD20 (BD PharMingen, Heidelberg, Germany) and 5 µl APC-labeled monoclonal anti-human CD86 (BD PharMingen, Heidelberg, Germany). T-cell threefold staining was done with 10 µl FITC-labeled monoclonal anti-human CD45RO (Coulter Immunotech, Krefeld, Germany), 10 µl PE-labeled monoclonal anti-human CD45RA (Coulter Immunotech, Krefeld, Germany) and 10 µl PerCP-labeled monoclonal anti-human CD4 (BD, Heidelberg, Germany); threefold staining with 5 µl FITC-labeled monoclonal anti-human cutaneous lymphocyte antigen (CLA) (BD PharMingen, Heidelberg, Germany), 10 µl PerCP-labeled monoclonal anti-human CD4 (BD, Heidelberg, Germany) and 2.5 µl APC-labeled monoclonal anti-human CD69 (BD, Heidelberg, Germany). After incubation for 20 min cells were washed with PBS and fixed with 2% paraformaldehyde in PBS. Nasal cells were stained using similar conditions. Unstained cells served as controls. Phenotypic analyses were performed by four-color flowcytometry using a Becton Dickinson FACSCalibur flowcytometer and the Cell Quest software (BD). Following the identification of the lymphocyte population, T-cells were identified using the CD4+ staining (Fig. 1) and B-cells were identified using the CD20+ staining. The percentage of CD69+, CD45RA+, CD45RO+ and CLA+ T lymphocytes and CD23+, CD80+, CD86+ and IgE-bearing B cells was calculated based on the identified populations.

For the detection of vital cell amount, PI staining (100 µl, 1:1,000) was performed. Additionally, mucosal material was fixed with liquid nitrogen and cut into 5-µm slices. Staining was performed using ChemMate Detection Kit (DakoCytomation GmbH, Hamburg, Germany) following incubation with CD4 (1:3,000) and CD20 (1:300) antibody (Dako, Hamburg, Germany) and routine staining with Papanicolaous 1:4 (MERCK, Darmstadt, Germany).

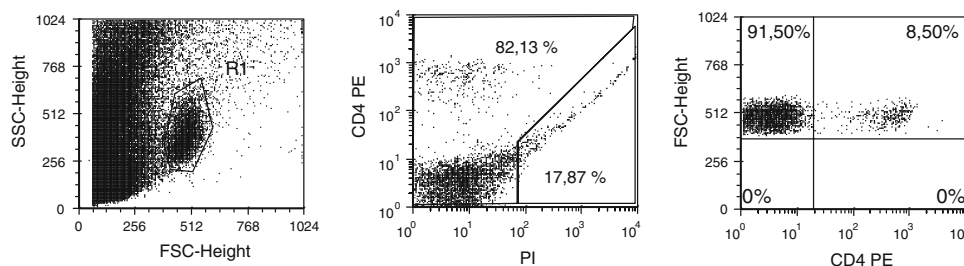


Fig. 1 Identification of vital nasal T helper lymphocytes by flowcytometric analysis. The *dot plots* show a representative example of the nasal mucosal lymphocytes from an inferior turbinate of a grass pollen

Statistics

The Wilcoxon test was used for non-parametric paired comparisons, and the Mann–Whitney *U* test for unpaired comparisons between groups.

Results

Cell survival after preparation and detection of lymphocytes within the nasal mucosa

Staining for vital cells using PI revealed about 80% vital cells within the lymphocytic population, less than 20% were dead cells due to preparation (see Fig. 1).

Preceding immunohistochemistry of the turbinates showed that the localization of CD4+ T-lymphocytes at the epithelial barrier and close to the nasal glands, mainly (see Fig. 2a). By contrast, CD20+ B lymphocytes were distributed diffusely in the submucosa without a clear association to a defined anatomical structure (see Fig. 2b).

In general, the flow cytometric analysis of two turbinates from one individual revealed approximately 10,000 CD4+ T- and 7,000 CD20+ B-lymphocytes. The number of

allergic individual following flowcytometric analysis with PI and CD4, displaying 82% of vital cells within the lymphocyte population

isolated cells coming from nasal turbinates of allergic individuals was about 20% higher than from non-allergic individuals.

Phenotype of nasal and peripheral T lymphocytes

CD69, a membrane molecule being highly expressed on T lymphocytes after activation [20] showed a low expression on PBMCs from allergic and non-allergic individuals (median: A 1.5%, NA 1.9%) whereas CD4+ T lymphocytes from the nasal mucosa were CD69-activated to a great extent ($P < 0.05$; Fig. 3). Although not reaching statistical significance due to a wide range of activation within both groups, expression of CD69 on T cells from the nasal mucosa was higher in allergic (median: A 73.4%, NA 43.4%).

Based on the expression of the distinct CD45 isoforms, naive CD45RA+ subsets of CD4+ T lymphocytes were

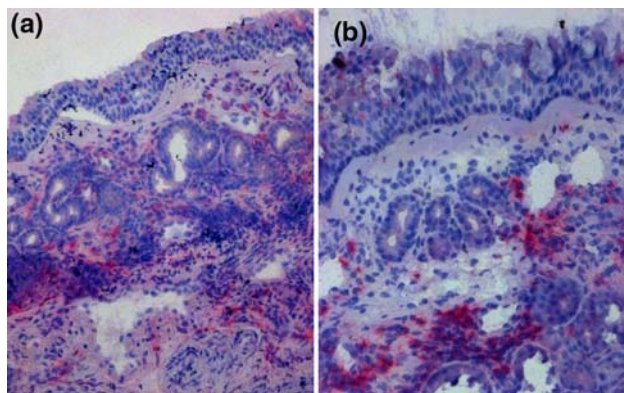


Fig. 2 Identification of CD4 (a) and CD20 (b) positive lymphocyte subsets using immunohistochemical staining. The examples represent the distribution of lymphocytes within the tissue setting of a grass pollen allergic individual

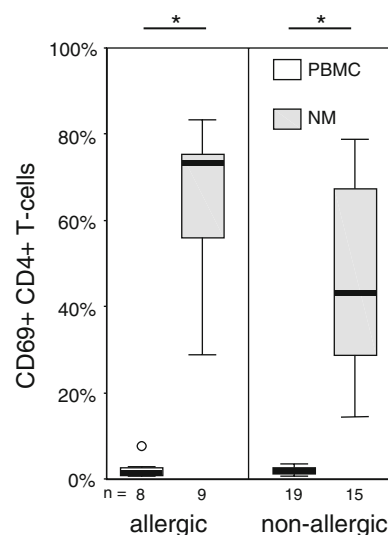


Fig. 3 Significant up-regulation of CD69 on CD4+ T cells of the nasal mucosa (NM). CD69+ T-cell determination using flowcytometric analysis (percentage of CD4+) after mechanical preparation of nasal lymphocytes (NM) and Ficoll gradient centrifugation of peripheral blood mononuclear cells (PBMC). Both done in allergic or non-allergic rhinitis patients. Mean values are represented by *horizontal bars*. Asterisks $P < 0.05$

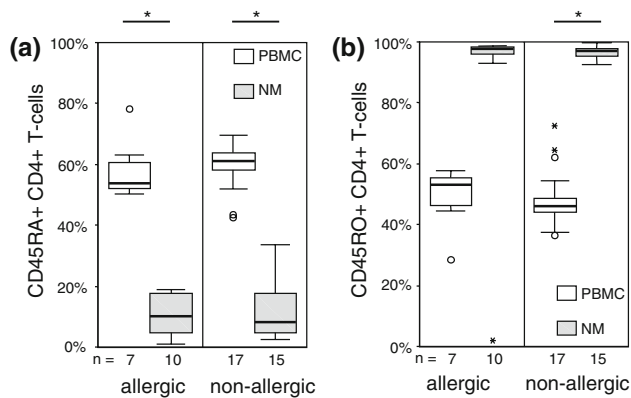


Fig. 4 Memory CD4+ T cells predominate in the nasal mucosa (a) compared to a balanced proportion of CD45RO+ and CD45RA+ T-lymphocytes in the peripheral blood (b). CD45RO and CD45RA positive T-cell determination using flowcytometric analysis (percentage of CD4+) after mechanical preparation of nasal lymphocytes (NM) and Ficoll gradient centrifugation of peripheral blood mononuclear cells (PBMC). Both done in allergic or non-allergic rhinitis patients. Mean values are represented by horizontal bars. Asterisks $P < 0.05$

distinguished from CD45RO+ memory T cells [21]. In both groups the majority of nasal mucosal CD4+ T cells exhibited the memory phenotype (median: A 97.7%, NA 97.2%; Fig. 4b). By contrast, about one half each of the peripheral blood CD4+ T-cells belonged to the naive subset (median: A 53.8%, NA 61.2%; Fig. 4a) and the memory subset (median: A 53.2%, NA 46.3%; Fig. 4b). No significant differences were determined between the allergic and non-allergic group.

The cutaneous lymphocyte-associated antigen (CLA) is a unique skin-homing receptor expressed by memory T cells infiltrating the skin [22], e.g. in atopic patients [23]. CLA expression was similar in nasal mucosal T cells from allergic and non-allergic individuals. A higher expression of CLA was detected in peripheral blood (median: A 14%, NA 12.8%) than in nasal mucosa (median: A, NA 5%; data not shown).

Phenotype of nasal and peripheral B lymphocytes

CD23, the low affinity receptor for IgE (FcεRII), plays an important role in the regulation of IgE production and thus in the development and maintenance of allergic inflammation [24]. The up-regulation of CD23 has been described previously to be increased on B-lymphocytes and monocytes from atopic individuals and is modulated by cytokines such as interleukin 4 (IL-4) and interferon γ (IFN- γ) [24–26]. Our data show that CD23 expression on peripheral B-lymphocytes was higher in the allergic group (median: A 55%, NA 25.5%; Table 1); although this was not significant. Nasal B-lymphocytes expressed significantly lower levels of CD23 in allergic and non-allergic individuals (median: A 13.2%, NA 21.7%) with a slightly higher expression in B cells from the control group.

IgE binds to the membrane of allergen-specific B lymphocytes via CD23 (FcεRII) [27]. In peripheral blood we identified only small subsets of IgE binding B-lymphocytes (median: A 4.6%, NA 3.5%; Table 1). In sensitized and non-sensitized individuals the expression of membrane immunoglobulin E (mIgE) was significantly higher in the nasal mucosa with a slight increase in the control group (median: A 10.5%, NA 19%).

Up-regulation of CD80 and CD86 on B cells indicates activation in the context of immune responses, as these costimulatory molecules and their counter-receptors CD28 and CD152 play an important role in T cell-mediated immune responses [10, 28]. In the present study, we identified subsets of CD80 (median: A, NA 4%; Fig. 5a) as well as CD86 (median: A 6.4%, NA 5%; Fig. 5b) positive B-cells in the nasal mucosa. CD80 expression was significantly higher in the peripheral blood of allergic (median: A 20.7%) and non-allergic (median: NA 16.6%) individuals than in the nasal mucosa. By contrast, peripheral CD86 expression was about only 1.1% (median A, NA) in both groups.

Table 1 Expression of CD23 and membrane immunoglobulin E (mIgE) on peripheral blood and nasal mucosal CD20+ B cells in allergic and non-allergic rhinitis

	Nasal mucosa		Peripheral blood	
	A	NA	A	NA
CD23+ CD20+ B lymphocytes	13.2 ± 5.7	21.7 ± 4.1	55.0 ± 8.0	25.5 ± 5.7
mIgE+ CD20+ B lymphocytes	10.5 ± 7.0	19.0 ± 4.9	4.6 ± 0.5	3.5 ± 0.4

CD20+ B-cells from the nasal mucosa express significantly ($P < 0.05$) lower levels of CD23 and higher levels of mIgE than peripheral B-cells without differences between the allergic (A) and non-allergic (NA) group

CD23+ and mIgE + B-cell determination using flowcytometric analysis (percentage of CD20+) after mechanical preparation of nasal lymphocytes and Ficoll gradient centrifugation of peripheral blood mononuclear cells. Results are expressed as mean ± SEM

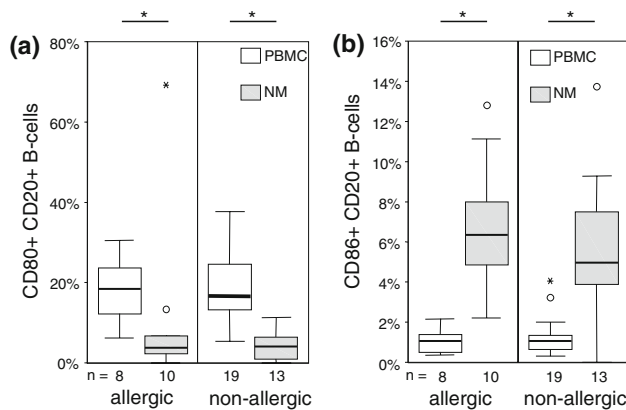


Fig. 5 Predominance of CD80+ CD20+ B-cells in the peripheral blood (a) versus higher expression of CD86+ on nasal CD20+ B-lymphocytes (b). CD80 and CD86 positive B-cell determination using flowcytometric analysis (percentage of CD20+) after mechanical preparation of nasal lymphocytes (NM) and Ficoll gradient centrifugation of peripheral blood mononuclear cells (PBMC). Both done in allergic or non-allergic rhinitis patients. Mean values are represented by horizontal bars. Asterisks $P < 0.05$

Discussion

In the present study, we examined the phenotype of nasal mucosal lymphocytes by direct ex vivo flowcytometric analysis. By using a cell isolation method which is mainly based on mechanical preparation, supplementary techniques, e.g. enzymatic isolation techniques, which may have an impact on the lymphocytic phenotype, have been avoided. Flowcytometric analysis was performed on the cell suspension, which was originally obtained by mechanical tissue disaggregation to reduce cells loss due to density centrifugation technique. As a sufficient direct ex vivo flowcytometric analysis requires a maximum count of solitary cells which are altered minimally, our method is exclusive compared with lymphocyte isolation techniques described recently [29].

In consistence with previous studies we show a high tissue infiltration of memory CD45RO+ and activated CD4+ T-lymphocytes in allergic rhinitis [13, 14, 17, 30, 31]. These findings imply that resident memory helper T-cells are involved in allergic inflammation of the nose.

Furthermore, several adhesion molecules, e.g. E-selectin, are up-regulated in the nasal mucosa in allergic rhinitis [3, 4]. With regard to this, we investigated CLA, the unique skin-homing receptor and the major ligand for E-selectin, which plays an important role in IgE-mediated atopic dermatitis [23, 32]. In our study, we aimed at determining whether CLA might also function as a homing receptor for mucosal T-cell migration in allergic rhinitis, an IgE-mediated atopic disease of the mucosa. As previously described [33, 34], no significant differences in CLA expression on

peripheral blood T-lymphocytes were found between allergic and non-allergic individuals. In contrast to atopic dermatitis in which CLA expression is about 85% on inflammatory skin lymphocytes [33], we did not find an enrichment of CLA+ T-cells in the nasal mucosa from allergic rhinitis patients. This indicates that CLA does not function as a homing receptor for nasal memory T-cells in the context of the IgE-mediated mucosal inflammation.

The finding of IgE+ B-cells in nasal mucosa is consistent with previous studies which demonstrated the enrichment of IgE+ B-cells and IgE+ plasma cells in the nasal mucosa from symptomatic allergic rhinitis patients [16, 27, 35] and suggests a local allergen-specific IgE response [11, 16]. Due to the low count of plasma cells in the obtained mucosal samples, most IgE+ B-cells might relate to IgE bound to membrane CD23. However, Cameron et al. demonstrated a local induction of IgE switching in B-cells after ex vivo allergen exposure [36, 37]. This is promoted by IL-4 and IL-13 de novo production in loco [37] with T-cells but also mast cells being the most potential source of IL-4 and IL-13 production [36]. Recently, Takhar et al. demonstrated that local B-cell class switch recombination to IgE is also stimulated in allergic rhinitis after in vivo allergen exposure [38].

The increased CD23 expression on peripheral B-lymphocytes in atopic patients [25] is consistent with our findings and is a result of an unbalanced IL-4/IFN- γ ratio and higher serum IgE levels compared to the non-atopic group. The identification of a CD23+ subset in nasal B-cells suggests the presence of activated B-lymphocytes which may act as regulators in IgE synthesis and play a role in local IgE-dependent antigen presentation [24, 27]. HLA-DR expression on B-cells stabilizes T–B-cell interactions, whereas CD23 enhances IgE-dependent T-cell activation and stimulates the immune responses to allergens [27, 39]. Switching of B-cells to IgE in the Th2 microenvironment of mucosal tissues may also amplify the preexisting IgE response.

Interestingly, in our study the expression of CD23 was significantly lower on nasal than peripheral B-cells which seems to be contradictory assuming a local IgE synthesis in the nasal mucosa as well as an up-regulation of CD23 in the presence of IgE and IL-4. One explanation might be that all patients were recruited outside pollen season and without any clinical symptoms. Thus, activated CD23+ B-cells would occur in nasal mucosa after allergen challenge either via migration from the peripheral blood or due to activation of B-cells resident in nasal mucosa tissue, only. Up-regulation of CD23 would thereby contribute to the local IgE-dependent CD23-mediated allergic immune response. Recently, Horiguchi et al. found increased nasal mucosal CD23+ B-cells from symptomatic perennial allergic rhinitis patients—assuming a permanent allergen exposure—compared with non-allergic rhinitis patients [29].

Another explanation for the lower CD23 expression on mucosal B-cells might be that allergens promote nasal B-cells to loose CD23 and differentiate into another phenotype, eventually plasma cells which may represent a local source for IgE synthesis. This assumption is supported by an immunohistochemical study in which tissue sections from patients with allergic rhinitis showed a decrease of nasal CD23+ B-cells after allergen provocation in vivo [12].

Concerning the costimulatory molecules CD80 and CD86 Hattori et al. found that CD86 expression occurs in the majority of nasal B-cells from perennial allergic rhinitis patients with severe symptoms and increases after allergen challenge in vivo. In our study, no differences in CD86 as well as CD80 expression on nasal B-lymphocytes were observed comparing seasonal allergic and non-allergic individuals. This might be due to the lack of allergen exposure since our study was performed outside pollen season. After allergen provocation, Hattori et al. also observed up-regulation for CD80 and the counter-receptors CD152 suggesting a local amplification of allergen-specific immune responses via these costimulatory molecules [10].

In our study, we found remarkable differences between circulating peripheral blood lymphocytes and lymphocytes resident in nasal mucosa. However, no significant differences between the allergic and non-allergic group were determined which may be due to the lack of major differences between the groups. Thus, our controls may form a heterogeneous group consisting of individuals suffering from difficulties in nasal breathing due to anatomical abnormalities or trauma as well as perennial or seasonal rhinitis without positive skin prick testing and without elevated levels of total or specific IgE. Furthermore, some non-allergic individuals had no significant infiltrates and less resident cells for sufficient cell isolation and FACS-analysis, therefore individuals with nasal mucosa tissue containing large amounts of lymphocytes were included into our control group. Our findings of highly activated memory T cells, activated CD23+ and IgE+ B-cells in allergic and non-allergic individuals support previous studies suggesting that “idiopathic” rhinitis represents a “localized” form of allergic rhinitis in the absence of systemic atopic markers [40, 41]. In future, nasal provocation testing may be an additional method to differentiate such patients who may suffer from a “local” allergic response, only.

Taken together, the data shows phenotypical differences between circulating peripheral blood lymphocytes and lymphocytes localized in the nasal mucosa. Activated memory T-lymphocytes and IgE+ B-cells are present in the nasal mucosa from allergic, but also non-allergic patients and may contribute to the local inflammatory state. In our study, we introduced direct ex vivo flowcytometric analysis of lymphocytes isolated by a mechanical method of prepara-

tion for the first time and offer a new tool to examine the local nasal allergic immune response.

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