

The presence of B7-H4+ macrophages and CD25+CD4+ and FOXP3+ regulatory T cells in the microenvironment of nasal polyps – a preliminary report

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Abstract: The nasal polyp (NP) seems to represent the end-stage of longstanding inflammation in patients with chronic rhinosinusitis. The aim of our study has been to evaluate the presence of two regulatory cell populations in the microenvironment of NP: CD4+CD25^{high} Foxp3⁺ (Treg) cells and B7-H4-expressing macrophages. Treg cells are actively able to inhibit T lymphocytes, while the population of B7-H4-expressing macrophages has recently been described as characterized by a regulatory function similar to that of Treg cells. For our study, we evaluated 14 NP tissue samples. The samples were divided into two main groups, eosinophilic (NP) and lymphocytic (NP), according to the predominant type of immune cell infiltration. The presence of Treg cells and B7-H4 positive macrophages in the samples was analyzed by FACS. Treg cells and B7-H4-expressing macrophages were identified in all the examined nasal polyps. The percentages of both Treg cells and of B7H4 positive cells found in the eosinophilic nasal polyps were higher than those found in the lymphocytic nasal polyps. Treg cells and B7H4+ macrophage subpopulations were present in the NP microenvironment and the alterations in their percentages were related to a distinct pattern of immune cell infiltration.

Key words: nasal polyps, nasal polyp microenvironment, Treg cells, B7-H4 expressing macrophages

Introduction

The nasal polyp seems to represent the end-stage of longstanding inflammation in patients with chronic rhinosinusitis [1]. The histomorphologic analysis of nasal polyps in patients suffering from chronic rhinosinusitis reveals frequent epithelial damage, thickened basement membrane, and mostly edematous to fibrotic stroma [2]. The accumulation of a variety of inflammatory cells (plasma cells, eosinophils, neutrophils, mast cells, macrophages, dendritic cells, monocytes, and lymphocytes and their subgroups) was also found in the nasal polyps [3,4]. Eosinophils made up the predominant cellular infiltration in about 80% of the nasal polyps occurring in members

of the Caucasian population. Additionally, increased numbers of eosinophils were observed in patients suffering from asthma and aspirin sensitivity, while neutrophils and lymphocytes were the two predominant types of cells infiltrating nasal polyps in patients with cystic fibrosis as well as the nasal mucosa of those suffering from chronic rhinosinusitis without nasal polyps [4].

Detailed histological examination enabled the selection of three different types of nasal polyps: those infiltrated predominantly by eosinophils with thickened basal membrane, those infiltrated by a mixed inflammatory cell pattern with a predominance of mononuclear cells without thickening of the basal membrane, and those infiltrated predominantly by neutrophils [5].

Sakaguchi and colleagues have identified a population of lymphocytes as CD4+ highly expressing CD25 (Treg) and able to prevent autoimmunity in a murine model; these are called T regulatory cells [6]. A tran-

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scription factor forkhead box P3 (Foxp3) has been determined to be more specific for the identification of Treg cells than other types of antigens [7,8,9]. The CD4+CD25^{high} Foxp3⁺ Treg cells are able to actively inhibit T lymphocytes, dendritic cells, natural killer cells (NK), and B cells in a cell to cell contact and dose-dependent manner [10]. Furthermore, Treg cells have been examined in patients with allergy and atopy. The cells were present in the majority of such patients and their regulatory functions were normal [11]. A break in Treg cell-mediated suppression has been observed at high doses of allergen concentration [12] or activation with superantigens [13]. The down-regulation of CD4+CD25+Foxp3+ T cells has been demonstrated in patients with nasal polyps when compared to healthy controls. Additionally, an elevation of the CD4+CD25+Foxp3+ T cells has been observed following steroid therapy [14].

Macrophages and dendritic cells have also been identified in nasal polyps [15,16]. The accumulation of mannose-receptor positive macrophages reported in nasal polyps in cell aggregates suggests that they play a key role in the pathogen-macrophage interaction within nasal polyps [15]. The number of macrophages has been noted to be significantly higher in eosinophilic than in lymphocytic nasal polyps [5]. The disturbance of the immune cell activity regulation that has been observed in nasal polyps is also typical of the tumor microenvironment. Reportedly, tumor-associated macrophages play an important role in immune suppression. Certainly, it has been demonstrated that tumor-associated macrophages are able to promote tumor growth and metastasis acting on tumor cells [17]. B7-H4, as expressed by certain macrophages, consists of co-stimulatory molecules, members of the membrane protein B7 family that is responsible for the negative regulation of T-cell-mediated immune response [18,19,20]. Kryczek and coworkers have recently demonstrated the presence of a population of B7-H4 positive macrophages in cases of human ovarian carcinoma. These cells are characterized by a suppressive function in a manner similar to CD4+ Treg cells. Furthermore, these two populations of suppressive cells interact with each other--CD4+ Treg cells stimulate APC (antigen presenting cell) B7-H4 expression and enable APC suppressive activity through B7-H4 induction. It has also been shown that tumor environmental IL-6 and IL-10 stimulate monocyte/ macrophage B7-H4 expression. Treg cells can trigger APC IL-10 production which in turn stimulates B7-H4 expression and renders APCs suppressive through B7-H4 [21,22]. Recently, we have identified the presence of B7-H4-expressing macrophages in decidua during pregnancy when the mechanisms regulating the local immune tolerance develop [23,24].

As macrophages probably participate in immune tolerance in nasal polyps [5], it is likely that there is a relationship between the development and persistence of nasal polyps and the emergence of the local suppressive environment found in these polyps.

To reiterate, the aim of our present study has been to evaluate the presence of two regulatory subpopulations of cells, namely Treg cells and B7-H4-positive macrophages, in the microenvironment of nasal polyps.

Methods

Clinical material. All the tissue samples were derived from the Otolaryngology Department of the Jagiellonian University during routine endonasal sinus surgery. Thirty patients were recruited for the study from those who had undergone functional endoscopic sinus surgery between January 2005 and November 2008; from this group patients who had undergone steroid treatment (inhaled and/ or oral steroids) during the 3 months prior to the surgery were automatically excluded from the study. Only those patients who had not been treated with steroids of any kind were considered since according to the literature, steroids can influence the number of Treg cells in nasal polyps. All tissue samples were histopathologically verified. Pathological analysis using the classical hematoxylin and eosin staining techniques after fixation in a formalin of the surgically removed material was performed in the Pathology Department of the Jagiellonian University by an experienced pathologist. The predominant immune cell infiltration in the nasal polyps was then determined by histopathological examination. Based on this examination, two distinct types of polyps were selected---eosinophilic and lymphocytic. The criteria for the selection of the different types of nasal polyps were as follows:

- Lymphocytic nasal polyps have a predominant infiltration of mononuclear cells; the percentage of eosinophils in these polyps should not exceed 10%, and the basal membrane should not be thickened. Eosinophilic nasal polyps, on the other hand, have an inflammatory infiltrate composed mainly of eosinophils (more than 50% of the total number of inflammatory cells), and the basal membrane is thickened.
- The tissue samples were thus divided into two main groups according to the predominant type of immune cell infiltration: eosinophilic nasal polyps (ENP) predominantly infiltrated by eosinophils (8 cases), and lymphocytic nasal polyps (LNP) predominantly infiltrated by lymphocytes (6 cases).

The clinical characteristics of the subjects are presented in Table 1.

Isolation of mononuclear cells from nasal polyps. The nasal polyps were cut into small fragments and disintegrated by smashing these through a 40 µm cell-strainer. The analyzed cells were studied after freezing -following process. Briefly, the tissue was dissociated and the cell pellet was treated with 1X ammonium chloride (8.99 g NH₄Cl, 1.0 g KHCO₃, 37 mg tetrasodium EDTA dissolved in 11 H₂O, pH 7.3) in order to get rid of any erythrocytes. The cells were then frozen in a freezing medium (10% DMSO, 20 % FBS, 70% DMEM). Prior to staining the cells were fow, spun down, washed in cold 1×DPBS, and counted. 1×10⁶ cells were used for staining.

Flow cytometry. The cell phenotype was analyzed with the panel of mAb – CD4 FITC, CD25 APC, and FOXP3 PE (Pharmingen). Briefly stated 1×10⁶ cells were suspended in 60 µl of 1×DPBS. 20 µl of CD4FITC and CD25 APC was added to the tube and it was incubated in the dark for 30 min at 4°C. After incubation the cells were washed twice in cold 1×DPBS. After a second wash the cell pellet was resuspended in 1× buffer A and incubated at room temperature for 10 min. The cells were then spun down and the liquid

Table 1. The clinical characteristics of the subjects

Groups	Eosinophilic nasal polyps (8)	Lymphocytic nasal polyps (6)
Age average (years)	48 (26-67)	24 (7-60)
Sex female: male	6/2	3/3
Asthma	4/4	1/5
Aspirin intolerance	2/6	0/6
CT score (Lund-Mackay)	31 (14-44)	16,5 (13-19)

decanted. Next, the cells were washed twice in PERM WASH buffer. For permeabilization they were suspended in 0.5 ml of buffer C and incubated for 30 min. Following permeabilization the cells were washed twice again in PERM WASH. In order to perform the staining the cells were suspended in 80 µl of PERM WASH buffer and 20 µl of PoxP3 PE antibody was added and the staining was carried on for 30 min at room temperature. Following staining, the cells were washed twice again in PERM WASH buffer. After incubation the cells were yet again washed twice in PBS and were permeabilized with FoxP3 permeabilization buffer (Becton Dickinson; USA) for 10 min at room temperature in the dark. Following this, they were stained with anti-FOXP3 antibodies for 30 min at 4°C in the dark. The stained cells were then washed and collected using the FACSCanto flow cytometer (Becton Dickinson; USA). Finally, they were analyzed with FACS Diva software (Becton Dickinson; USA). Each time 3×10^4 events from the lymph gate were saved for analysis. Logical gates were then used to analyze particular populations of cells. At first, from the population of lymphocytes, the subpopulation of double CD25CD4 positive cells was gated and the number of Foxp3 positive lymphocytes among this population of cells (Gate P2) was estimated.

The CD14/B7H4 cells were stained using monoclonal antibodies--CD14 FITC and B7H4 PE (E-Bioscience)--in the same way as described for the Treg cells above, only the permeabilization step was omitted. The analysis was also performed in the same way: first the population of monocytes was determined (Gate 5), 5×10^4 events were collected from the gate set on CD14 positive cells (Gate P8), and then the percentage of CD14/B7H4 positive cells in the population of CD14⁺ cells was estimated.

Ethical issues. The patient's consent was obtained in each case. Additionally, approval for the research program was granted from the Ethical Committee of the Jagiellonian University in Krakow: KBET/90/B/2005.

Statistical analysis. The distribution of variables in the study groups of patients checked with the use of the Shapiro-Wilk test showed that each of the patients was in fact different from normal. The statistical significance between the groups was determined by the Kruskal-Wallis test, one-way analysis of variance by ranks. The Mann-Whitney U test was then used as applicable. All statistical analyses were carried out with the Statistica 8.0 software program. A p value <0.05 was considered indicative of statistical significance.

Results

The two groups of nasal polyps were compared with respect to the percentages of both FOXP3⁺ cells in the subpopulations of CD25⁺CD4⁺ T regulatory cells and B7H4⁺ cells in the larger subpopulation of macrophages.

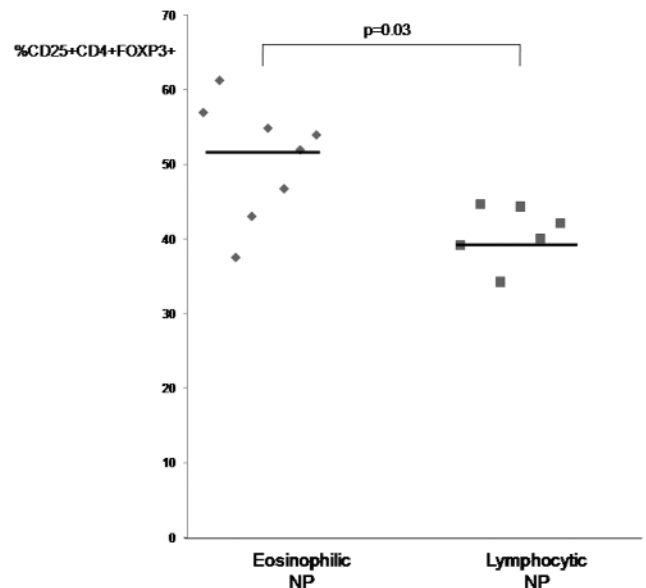


Fig. 1. The percentages of FOXP3⁺ cells in the subpopulation of CD25⁺CD4⁺ lymphocytes in lymphocytic and eosinophilic nasal polyps (NP). The cells isolated from both the lymphocytic NP (n=6) and eosinophilic NP (n=8) were stained and analyzed for the presence of regulatory T cells. The percentage of CD4⁺ CD25⁺ FOXP3⁺ cells was significantly higher in the eosinophilic NP than the lymphocytic NP (p=0.03).

The analysis of the CD25⁺CD4⁺FOXP3⁺ T regulatory cells and suppressive B7H4⁺ macrophages in nasal polyps

We found statistically significant differences in the percentages of FOXP3⁺ cells in the subpopulation of CD25⁺CD4⁺ lymphocytes when the lymphocytic and eosinophilic nasal polyps were compared.

We have noted a comparable percentage of CD4⁺ lymphocyte T in both groups of patients (respectively as follows: Group ENP – 76.9% (±20.8); Group LNP 68.5% (±39.3). Additionally, we have also found a statistically significantly higher percentage of CD4⁺CD25⁺ cells within the CD4⁺ T lymphocyte subpopulation in ENP groups than in LNP groups of patients (respectively Group ENP – 9.9% (±4.3); Group LNP – 4.4% (±3.13); p=0.03). We also observed a statistically significantly higher percentage of Foxp3 positive cells within CD4⁺ lymphocytes in eosinophilic nasal polyps than in lymphocytic nasal polyps (respectively Group ENP – 2.3% (±2.00); Group LNP – 1.2% (±0.36)).

Likewise, we have found differences in the percentages of B7-H4 positive cells within the subpopulation of CD14 positive macrophages in the different types of nasal polyps.

No differences in the percentage of the CD14 positive macrophages were observed in the examined nasal polyps (respectively Group ENP – 50.09% (±26.9); Group LNP – 42.7% (±28.09).

Discussion

In our present study, we found that the percentages of CD4+CD25+Foxp3+ Treg cells and of the B7-H4-expressing macrophages were significantly higher in the eosinophilic nasal polyps than in the lymphocytic nasal polyps.

To our knowledge this is the first investigation to focus on the alterations in both Treg cells and B7-H4 macrophages in the stroma of nasal polyps with respect to the predominant type of immune cell.

Both Th1- and Th2-type cytokines have been found to be up-regulated in nasal polyps, regardless of the atopic status of the patient [25,26]. CD4+CD25+ Treg cells have been identified as a subpopulation of T cells capable of down-regulating antigen-specific T-cell responses [27]. It has recently been discovered that the forkhead transcription factor Foxp3 is a more specific marker for Tregs [8]. Moreover, Foxp3 may be naturally induced in human T lymphocytes following their activation, and this induction is related to the acquisition of the Treg phenotype in these cells [28]. The down-regulation of Foxp3 has been observed in patients suffering from nasal polyps, and an increase in the number of these cells has been found following steroid therapy for nasal polyps [14]. It has been proposed that low levels of Foxp3 expression in nasal polyps may be related to the persistence of the inflammation [14,29]. Moreover, it has been demonstrated that allergic rhinitis is related to a lower number of Foxp3 positive cells, which could in turn be associated with the impaired regulatory activity of CD4+CD25+ T cells [30]. In fact, the effectiveness of grass-pollen immunotherapy has been found to be related to an increase in the number of CD25+, Foxp3+, Foxp3+CD25+, and Foxp3+CD4+ cells in the nasal mucosa of such patients in comparison with the nasal mucosa of untreated patients [31]. Finally, it has also been shown that the Treg cell CD4+CD25+Foxp3 has an intact regulatory function in atopic and allergic patients [11]. As the percentage of Treg CD4+CD25+Foxp3 cells was shown to be decreased in nasal polyps, we were interested in any measurable differences in the percentages of these cells with respect to the different nasal polyp types. In the present study it appeared that the percentage of the Treg CD4+CD25+Foxp3 cells was significantly higher in eosinophilic nasal polyps than in lymphocytic polyps. The number of mononuclear cells, however, remained at a comparable level in both groups of nasal polyps. It seems that an aggressive inflammation in eosinophilic nasal polyps elicits a higher infiltration of Treg cells which, even though they are supposed to control and restrict inflammation, seem to become ineffective. As long as inflammation develops, an immunosuppressive microenvironment is being created, and this will most likely result in an ineffective immune response and continuing inflammation. The actual etiology of nasal

polyps, however, still remains unclear. Chronic rhinosinusitis with nasal polyps is associated with a number of diseases, including Samter's triad (aspirin sensitivity, asthma, and nasal polyps), cystic fibrosis, and asthma. The prevalence of chronic rhinosinusitis in the general population is 4% [4]. Asthma has been reported in patients with nasal polyps more frequently than in controls (6%); 26% of patients with nasal polyps also have asthma. Additionally, nasal polyps have been observed in 36-96% of patients with aspirin sensitivity [4]. In our present study, 35.7% of the patients had asthma; from this group of patients 40% represented aspirin sensitive individuals. We observed a statistically significantly higher percentage of CD4+CD25+Foxp3+ Treg cells in the nasal polyps derived from patients with bronchial asthma (NPA) in comparison to the percentage found in nasal polyps obtained from patients who did not suffer from bronchial asthma (NPWA). No such difference, however, was observed in the percentages of B7-H4-expressing macrophages (the respective percentages of B7-H4 positive macrophages within CD14 positive cells were as follows: Group NPA- 31% (± 20.99), Group NPWA 24.3% (± 16.9), $p=0.6$; respectively percentage of CD4+CD25+Foxp3+ Treg cells within CD4+ lymphocytes Group NPA -54% (± 7.78), Group NPWA -39.7% (± 5.06), $p=0.01$). Moreover, in our present study, patients suffering from eosinophilic nasal polyps also had bronchial asthma and aspirin sensitivity more frequently than patients with lymphocytic nasal polyps (Table 1). Moreover, the stage of the disease, as determined by the CT score (Lund-Mackay), was more advanced in patients with eosinophilic nasal polyps than in patients with lymphocytic nasal polyps (Table 1). Higher Treg and B7H4 positive cell percentages in eosinophilic nasal polyps were therefore related to a more clinically severe course of the disease. The suppressive microenvironment in tumors is created not only by the regulatory activity of Treg CD4+CD25+Foxp3+ cells, but also by macrophages [32]. With regard to nasal polyps, suppressive macrophages expressing RCAS1 have been identified only in eosinophilic nasal polyps [5]. RCAS1 is responsible for the regulation of the immune cell activity that inhibits the growth of receptor expressing cells (T and B lymphocytes and NK cells) both *in vitro* and *in vivo* and is also responsible for inducing apoptosis [33]. In lymphocytic nasal polyps the local immunosuppressive microenvironment may not yet have been fully developed due to the duration of the inflammation or because a different mechanism of the disease was involved. In cases of autoimmune liver diseases it has been demonstrated that the number of RCAS1-expressing macrophages increased as the inflammation and the severity of the disease progressed (measured here by the AlAt, Aspat levels) [34].

In our present study we identified a new subpopulation of regulatory macrophages expressing the B7-

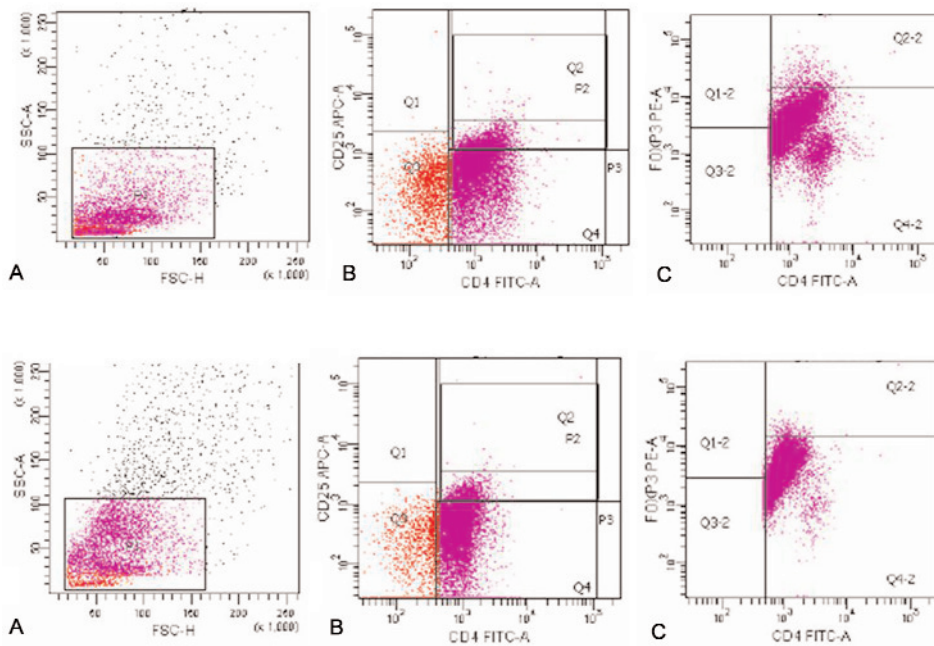


Fig. 2. Treg CD4+CD25+Foxp3+ cells in eosinophilic nasal polyps. The logical gating strategy for the estimation of CD4+ CD25+ FOXP3+ Tregs in eosinophilic NP: A. gating on the population of small lymphocytes (Gate P1); B. gating on the population of double positive CD4+CD25+ cells (Gate P2); C. population of FoXP3 positive cells within CD4+CD25+ cells from Gate P2.

Fig. 3. Treg CD4+CD25+Foxp3+ cells in lymphocytic nasal polyps. The logical gating strategy for the estimation of CD4+ CD25+ FOXP3+ Tregs in lymphocytic NP: A. gating on the population of small lymphocytes (Gate P1); B. gating on the population of double positive CD4+CD25+ cells (Gate P2); C. population of FoXP3 positive cells within CD4+CD25+ cells from Gate P2.

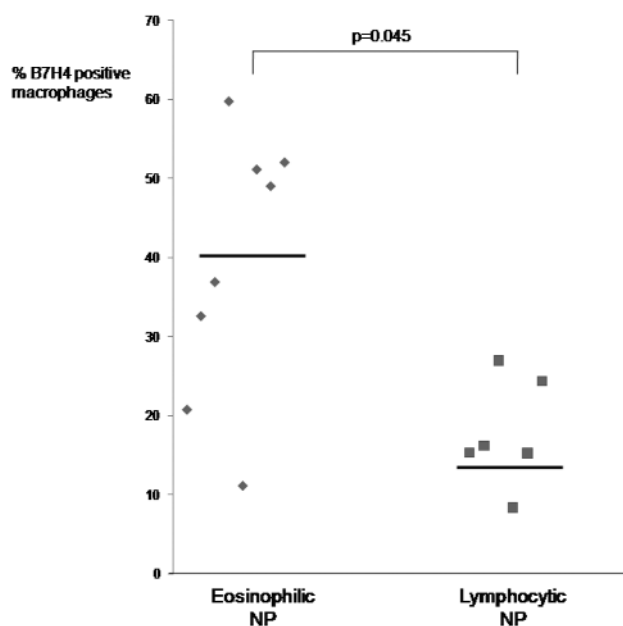


Fig. 4. The percentages of B7-H4-positive cells within the subpopulation of CD14 positive macrophages in the lymphocytic and eosinophilic nasal polyps. The cells isolated from the lymphocytic NP (n=6) and eosinophilic NP (n=8) were stained and analyzed for the presence of B7-H4 macrophages. The percentage of CD14+ B7-H4+ cells was significantly higher in the eosinophilic NP than the lymphocytic NP ($p=0.045$).

H4 molecule that interact with Treg cells and create the suppressive microenvironment found in nasal polyps. Treg cells induce the secretion of high levels of IL-10 by APCs; IL-10 in turn stimulates the expression of APC B7-H4 on APCs and renders APCs immunosup-

pressive through B7-H4 expression [22]. B7-H4-expressing macrophages have also been found in patients with ovarian cancer. These cells were able to inhibit TAA (tumor associated antigen)-specific T-cell proliferation, cytokine production, and cytotoxicity *in vitro*. *In vivo*, B7-H4-expressing macrophages both inhibited TAA-specific immunity and promoted tumor growth [21,22]. Since the population of Treg cells is limited and the mechanism of suppression is carried out by a Treg to T cell contact, this may not provide sufficient suppression *in vivo*. It has therefore been postulated that Tregs are able to inhibit the function of APCs, and the induction of B7-H4 macrophages may be an important mechanism of this Treg-mediated regulation [22]. While Treg cells would seem to be the first line of tolerance at the inflammation site, the range of tolerance is actually determined by the Treg cell number (in dose-dependent manner) [21,22]. Beyer and coworkers have suggested that Treg cells are able to carry out their regulatory function in a cell-contact and dose-dependent manner [10]. According to other reports [21,22] B7-H4 macrophages may constitute the second line of tolerance when Treg suppression is inefficient or exhausted as they can continue the suppressive mode and recruit Treg cells. In a similar way, the nasal polyp microenvironment may control the growth and persistence of nasal polyps through the development of local immune tolerance by recruiting cells with regulatory activity, such as Treg cells and B7-H4 macrophages.

Immune suppression seems to be a complex phenomenon involving Treg cells and B7-H4-expressing macrophages. In conclusion, the alterations in both the Treg cell and suppressive B7H4+ macrophage subpop-

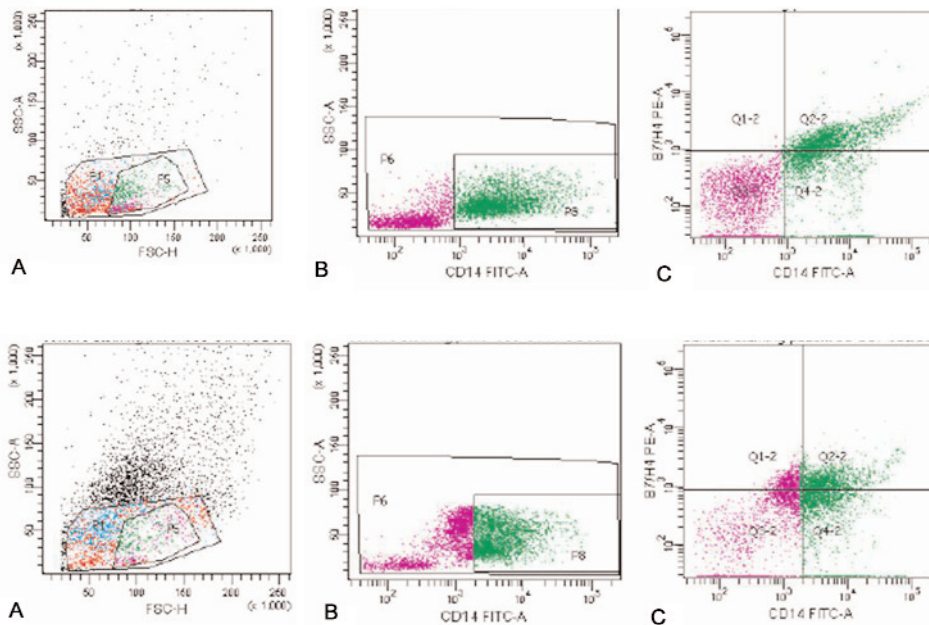


Fig. 5. B7-H4-positive macrophages in eosinophilic nasal polyps. The logical gating strategy for the estimation of CD14⁺ B7-H4⁺ macrophages in eosinophilic NP: A. gating on the population of monocytes (Gate P5); B. gating on the population of strongly positive CD14⁺ cells (Gate P8); C. population of B7-H4 positive cells within CD14⁺ cells from Gate P8.

Fig. 6. B7-H4-positive macrophages in lymphocytic nasal polyps. The logical gating strategy for the estimation of CD14⁺ B7-H4⁺ macrophages in lymphocytic NP: A. gating on the population of monocytes (Gate P5); B. gating on the population of strongly positive CD14⁺ cells (Gate P8); C. population of B7-H4 positive cells within CD14⁺ cells from Gate P8.

ulations in the mucosa of nasal polyps may be related to the restriction of an activation of an excessively stimulated immune response.

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