

Higher Frequencies of CD161⁺ Circulating T Lymphocytes in Allergic Rhinitis Patients Compared to Healthy Donors

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Key Words

CD161 • Th17 cells • CD3 cells • CD4 cells • CD8 cells • Allergic rhinitis

Abstract

Background: Th17 is a subset of T-helper lymphocytes that produce proinflammatory cytokines, mainly IL-17. Serum IL-17 is increased in allergic patients and relates to clinical severity. Recently, it has been reported that CD161 is a highly upregulated gene in Th17 clones and all IL-17-producing cells are contained in CD161⁺ T cells. This study aimed at comparing the frequency of peripheral CD161⁺ T cells in patients with allergic rhinitis (AR) and in healthy controls and at relating CD161 expression with symptom severity. **Methods:** Forty-four patients with AR and 29 healthy non-allergic subjects were evaluated. CD161 expression was evaluated on CD3⁺, CD4⁺ and CD8⁺ cells by double immunofluorescence staining and fluorescence activated cell sorter analysis. Symptom severity was assessed by the Visual Analogue Scale. **Results:** Allergic patients showed a significantly higher frequency of CD3⁺CD161⁺, CD4⁺CD161⁺ and CD8⁺CD161⁺ cells than healthy non-allergic subjects ($p < 0.0001$). Moreover, the expression of CD161 cells was significantly related to clinical severity. **Conclusions:** This study provides evidence that a higher frequency of CD161⁺ T cells is present in the peripheral blood of AR patients.

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Introduction

Allergic rhinitis (AR) is sustained by nasal mucosal inflammation dependent on a disorder of T-cell response. CD4⁺ T cells are divided into two main lineages: Th1 and Th2 cells [1]. Th1 cells typically produce interferon (IFN)- γ , whereas Th2 cells mainly produce interleukin (IL)-4, IL-5 and IL-13. Paradigmatically, allergy is characterized by a polarized Th2 response [1].

More recently, another T-lymphocyte subset, namely Th17 cells, has been discovered and may be involved in the pathogenesis of several immune-mediated disorders [2]. Th17 cells are characterized by the production of various cytokines, including IL-17, IL-6, tumor necrosis factor- α and IL-22 [2]. IL-17 is involved in tissue pathology in autoimmune disorders [2], and IL-17 modulation is involved in neutrophil and eosinophil infiltration in experimental murine asthma models [3, 4]. Furthermore, IL-17 induces recruitment and increased survival for airway macrophages [5]. These facts have initially suggested a regulatory role for IL-17 [6], even though the exact role of Th17 cells in nasal allergic inflammation is still debated. A first study showed that patients with viral rhinitis had elevated concentrations of IL-17 in nasal secretions, whereas allergic patients had normal levels [7]. However, it has been further evidenced that some patients, with AR due to birch allergy and evaluated out-

side the pollen season, may have detectable serum IL-17 levels which are related to some inflammatory parameters [8]. More recently, it was shown that serum IL-17 levels were significantly related to clinical symptoms, drug use and peripheral eosinophils in patients with pollen-induced AR evaluated during the pollen season [9]. Thus, serum IL-17 might be considered a biomarker of severe allergy in AR patients [8, 9]. In addition, two recent studies reported an association between serum IL-17 levels and symptom/medication score during sublingual immunotherapy, both in adults and children [10, 11]. Afterwards, the Th17 allergen-specific repertoire was investigated in both adults and children with AR during pollen exposure [12, 13]. Both studies reported that allergic patients showed higher frequencies of Th17 cells than normal controls after allergen-specific stimulus. Therefore, Th17 may play a pathogenic role also in allergy.

CD161 is the human homologue of the mouse NK1.1 which is expressed on almost all natural killer (NK) cells and also by a subset of T cells named 'NKT cells' [14]. Recently, it has been reported that CD161 was one of the most upregulated genes in human Th17 clones compared with Th1 or Th2 clones [15]. In addition, this study demonstrated that all IL-17-producing cells were included within the CD161⁺ fraction of adult circulating CD4⁺ T cells that were NKT-like cells [15]. Moreover, it was recently shown that all IL-17-producing cells were included within the CD161⁺ fraction of adult circulating lymphocytes and that CD161 expression is induced by RORc [16]. These authors concluded that CD161 may be a novel surface marker for human Th17 cells, and the exclusive origin of these cells is a CD161⁺CD4⁺ T-cell progenitor [15]. Then, the same research group provided another study of the relationship between Th17 and Th2 cells in asthmatic patients [17]. This study evidenced that: (1) a new T subset (Th17/Th2) able of producing both IL-4 and IL-17 can be obtained from the peripheral blood of healthy subjects; (2) Th17/Th2 cells are a small subset of circulating T CD4⁺ cells present in higher proportions in asthmatic patients; (3) Th17/Th2 cells originate from memory Th17 cells when exposed to IL-4, a typically allergic milieu, such as polarized Th2 [17]. Therefore, this study evidenced the plasticity of Th17 cells even toward the Th2 phenotype. In addition, it has been reported that the CD161 surface receptor is directly involved in transendothelial migration of CD4⁺CD161⁺ lymphocytes [18]. These findings would suggest that CD161 is a marker of the CD4⁺ lymphocyte subset which is ready to extravasate and tissue localize. Therefore, it is relevant to analyze

the pathophysiological role of CD161⁺ cells in allergic disorders.

The aims of this study were: (1) to investigate the frequency of CD161⁺ cells in patients with AR, (2) to compare these findings with normal controls, and (3) to relate CD161 expression to clinical severity.

Methods

The study included 44 patients (14 males and 30 females, mean age 35.9 years) with AR, and 29 healthy non-allergic subjects (10 males and 19 females, mean age 32.3 years).

The patients were included in the study on the basis of a clinical history of AR, positive skin prick test (table 1) and the presence of nasal symptoms according to validated criteria [19].

All subjects were healthy, except for AR, without any active disorder, including rhinosinuitis, and asthma diagnosis was excluded in all patients.

Skin prick test and blood sampling were performed in all patients. The skin prick test was performed according to the guidelines of the European Academy of Allergy and Clinical Immunology [19] and using a common panel as previously reported [8, 9]. The study was approved by the local ethics committee and performed with written informed consent. The Visual Analogue Scale (VAS) was used to quantify the subjective feeling of nasal symptoms, including itching, sneezing, rhinorrhea and obstruction, as previously reported [21]. It ranges from 0 (no symptoms) to 10 (very troublesome symptoms). Patients were asked to position a cross on a line corresponding to their own perception of nasal symptom severity.

Peripheral blood mononuclear cells (PBMC) were obtained from Ficoll-Hypaque gradient of heparinized venous blood, as described [18]. PBMC were stained with anti-CD161 monoclonal antibody (mAb; 191b8, IgG2a) and anti-CD3 (UCHT-1, IgG1; Serotec, UK) or anti-CD4 (Leu3a, IgG1; Becton Dickinson, Palo Alto, Calif., USA) or anti-CD8 (leu2a, IgG1; Becton Dickinson), incubated for 30 min at 4°C and then washed and incubated with anti-isotype specific goat anti-mouse (GAM) antiserum conjugated with phycoerythrin (PE) (Invitrogen, Milan, Italy; for staining anti-CD161 mAb) or with allophycocyanin (APC) (Invitrogen; for anti-CD3 or anti-CD4 or anti-CD8 mAb). After 30 min at 4°C, samples were washed and resuspended in RPMI-1640 medium and run on a CyAN ADP (Beckman-Coulter) cytofluorimeter equipped with an argon laser (488 nm) and an He-Ne laser exciting PE or APC at 488 or 647 nm, respectively. As negative controls, cells were stained with an unrelated mAb (Becton Dickinson) matched for the isotype followed by PE or APC-conjugated specific anti-isotype GAM. At least 20,000 events for each sample were analyzed. Data are expressed as percentage of positive cells or as log far red (APC) or red (PE) fluorescence intensity versus number of cells, as described [18].

Statistical analysis was performed using the statistical software package MedCalc 9 (Frank Schoonjans, Belgium). Descriptive statistics were performed first, and quantitative parameters were reported as the means with interquartile ranges. Comparison of the serotonin levels in the two groups of patients was performed by the non-parametric Wilcoxon test. Correlation be-

Table 1. Patients with respective sensitizing allergens

Patient No.	Sensitizing allergens
1	birch
2	mites
3	mites
4	birch, parietaria, mites
5	parietaria
6	birch, parietaria
7	birch
8	birch, parietaria
9	birch, cypress, olive, parietaria
10	grasses, parietaria
11	birch, parietaria, mites
12	cypress
13	birch, grasses, parietaria, mites
14	birch
15	birch
16	birch, parietaria
17	parietaria
18	parietaria
19	parietaria
20	mites
21	birch
22	birch
23	mites
24	grasses, parietaria
25	parietaria
26	mites
27	grasses, mites
28	grasses, olive
29	parietaria, cat
30	mites
31	parietaria, mites
32	birch, grasses
33	birch, olive
34	parietaria, mites
35	grasses, parietaria
36	birch, parietaria, mites
37	birch, parietaria
38	grasses, parietaria
39	birch
40	mites
41	birch, mites
42	parietaria
43	birch, parietaria
44	grasses, olive, mites

tween quantitative variables was evaluated by means of the Spearman correlation coefficient. For the purpose of this analysis, correlation coefficients ≥ 0.8 were considered as very strong, from 0.6 to 0.79 as strong, from 0.4 to 0.59 as moderate, from 0.2 to 0.39 as weak, and <0.2 as very weak [22].

All tests were two sided and a p value <0.05 was considered statistically significant.

Table 2. Cell frequencies according to positivity for CD3, CD4 or CD8 and CD161 antigens in controls and patients with AR

Variable	Control (n = 29)	AR (n = 44)	p value
CD3 ⁺			
CD161 ⁺	8.04 (5.72–13.24)	20.66 (15.86–24.02)	<0.0001
CD161 [−]	91.96 (86.77–94.28)	79.35 (75.98–84.14)	<0.0001
CD3 [−]			
CD161 ⁺	13.64 (4.44–20.73)	29.6 (16.76–42.53)	0.0001
CD161 [−]	85.49 (79.17–95.56)	70.41 (57.48–83.24)	0.0001
CD4 ⁺			
CD161 ⁺	8.23 (3.78–12.01)	20.06 (14.24–24.35)	<0.0001
CD161 [−]	91.77 (87.99–96.22)	79.94 (75.66–86.22)	<0.0001
CD8 ⁺			
CD161 ⁺	7.8 (5.33–11.88)	16.91 (10.47–25.26)	<0.0001
CD161 [−]	92.2 (88.13–94.68)	83.7 (73.76–89.54)	<0.0001

Data are medians given as percentages, with interquartile ranges in parentheses.

Results

CD161 Expression in AR Patients and Healthy Subjects

As reported in table 2, patients suffering from AR had higher frequencies in PBMC of CD3⁺CD161⁺ cells than healthy subjects ($p < 0.0001$). Frequency of CD4⁺CD161⁺ cells was higher in allergic patients than in healthy controls ($p < 0.0001$). Finally, CD8⁺CD161⁺ cells were more elevated in allergic patients than in healthy controls ($p < 0.0001$). A representative example of fluorescence activated cell sorter plots of expression of CD161 antigen on CD3⁺, CD4⁺ or CD8⁺ cells in a patient with AR or a healthy donor is shown in figure 1. It is evident that the fraction of CD3⁺ cells expressing the CD161 antigen is strongly increased in PBMC of allergic patients. This was confirmed for CD4⁺ cells as well. A negligible fraction of cells (range 0.5–5% of CD4⁺ cells) expressing CD4 at a low level (CD4^{dull}) were CD161. These cells were monocytes as they expressed CD14 antigen (data not shown). The expression of CD161 was markedly increased on peripheral lymphocytes expressing high levels of CD8 (fig. 1) as well. Lymphocytes with CD8^{high} were CD3⁺ ($>99\%$ in all donors analyzed; data not shown). It should be noted that the expression of CD161 antigen was increased also in CD8^{dull} cells. These cells did not express CD3 (data not shown) but were CD16⁺CD56⁺, conceivably representing the NK cell fraction of PBMC.

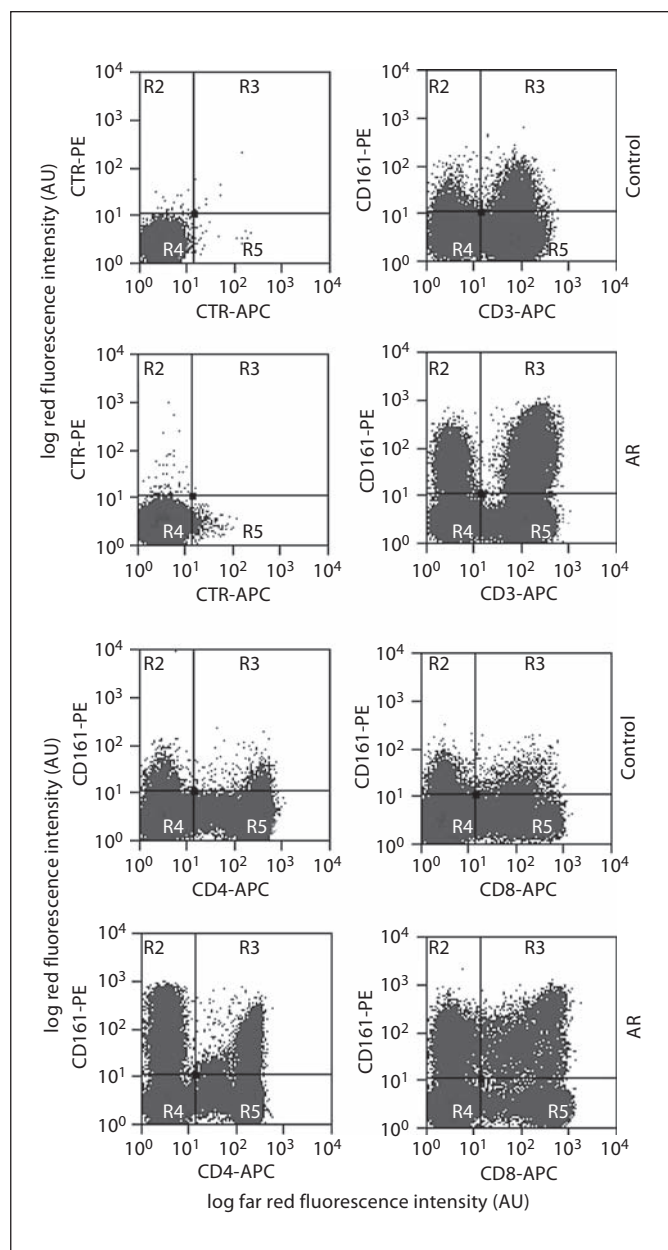


Fig. 1. Fluorescence activated cell sorter plots for the expression of CD161 on CD3⁺, CD4⁺ or CD8⁺ PBMC. PBMC isolated from patients suffering from AR or healthy donors as controls were stained with the indicated mAbs followed by anti-isotype specific GAM conjugated with PE (for CD161) or with APC (for CD3, CD4 or CD8). CTR-PE and CTR-APC: PBMC stained with an unrelated mAb (Becton Dickinson) matched for the isotype. Each plot is subdivided into four quadrants. In the upper right quadrant, cells are shown simultaneously expressing the indicated surface markers. AU = Arbitrary units.

Relationship between CD161 Expression and Clinical Severity

The clinical severity assessed by VAS was significantly related to CD3⁺CD161⁺ cell frequency, and the relationship was strong ($r = 0.851$), as reported in figure 2a. The expression level of CD4⁺CD161⁺ cells (calculated bona fide within the CD4^{high} T-cell fraction) was significantly related to clinical severity, and the relationship was of moderate grade ($r = 0.580$; fig. 2b). On the other hand, the correlation between CD8⁺CD161⁺ cells (calculated bona fide within the CD8^{high} T cells) and VAS was the lowest detected among CD3⁺ and CD4⁺ lymphocyte subsets ($r = 0.530$; fig. 2c).

Discussion

Recently, it has been reported that CD161 is a marker for IL-17-producing cells, mainly the Th17 subset. In fact, over the past 20 years, the dichotomic functional Th1-Th2 paradigm has dominated the attention on CD4⁺ T cells [23]. Afterwards, Th1 and Th2 have had to make way for a third effector lineage, Th17, the discovery of which was sustained by the identification of new cytokines: IL-17 and IL-22 [24]. With the characterization of the Th17 lineage, a more unified model of CD4 orchestration of monocytic (Th1), mast cell/basophilic/eosinophilic (Th2) and neutrophilic (Th17) patterns of inflammation by distinct CD4⁺ T-cell subpopulations is now apparent. This unified understanding of the inductive-effector cytokines specific for each effector subset has been evidenced. Indeed, IFN- γ is a characteristic Th1-dependent cytokine, whereas IL-4, IL-5 and IL-13 are Th2 cytokines. The Th17-associated cytokines may be subdivided as inductive (IL-6, IL-1 β , transforming growth factor- β and IL-23) and effector (IL-17A, IL-17F and IL-22) cytokines.

Recently, a growing list of chronic inflammatory disorders has been linked to Th17. In this regard, the recent progresses in characterizing the proinflammatory IL-17 cytokine family have added an additional layer of complexity to the regulation of allergic inflammation [25]. Further, IL-17A may play roles in the development of various allergic diseases that have classically been considered to be Th2-mediated disorders [26]. Moreover, IL-17A may be considered a new biomarker of disease progression and allergy severity as recently evidenced in AR [8, 9]. Ex vivo analysis of peripheral CD3⁺CD4⁺ T lymphocytes suggested that allergic patients (both adults and children) may have more elevated percentages of both CD4⁺IL-17⁺ and CD4⁺IFN- γ ⁺IL-17⁺ cells than normal

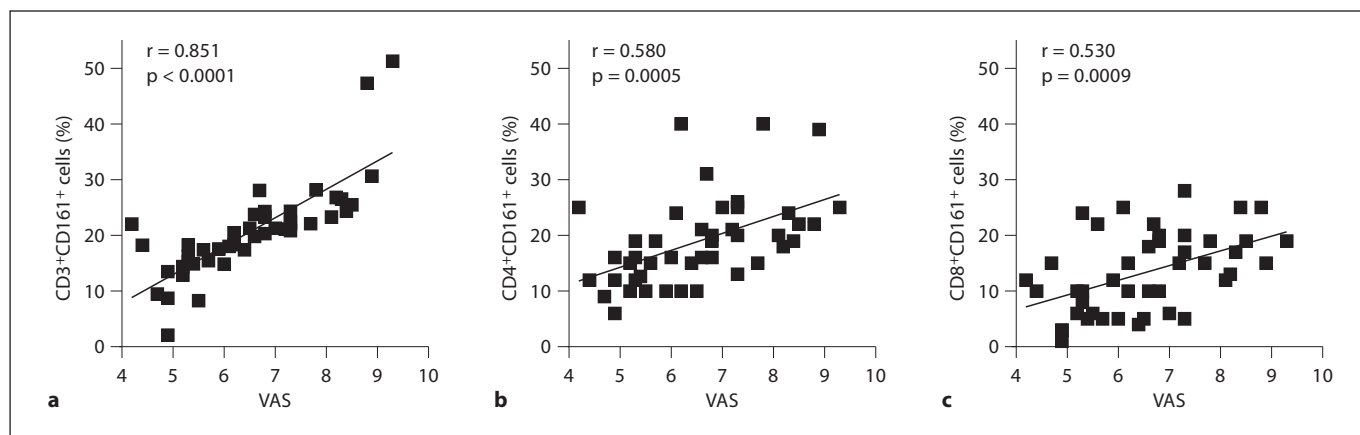


Fig. 2. Relationship between symptom severity assessed by VAS and frequency of CD161⁺ cells in patients with AR on CD3⁺ cells (a), CD4^{high+} cells (b) or CD8^{high+} cells (c).

controls [12, 13]. This underlines the possible role of Th17 in allergic inflammation.

Recently, there has been evidence that asthmatic patients had a higher frequency of CD161⁺ cells compared to normal subjects [17]. Therefore, this study was designed to preliminarily confirm these findings in an AR model.

Firstly, this study showed that patients with AR had a higher frequency of CD3⁺CD161⁺, CD4⁺CD161⁺ and CD8⁺CD161⁺ cells compared to healthy subjects.

Secondly, the expression of CD161⁺ cells was significantly related to symptom severity, mainly concerning CD3⁺ cells.

These findings might suggest that also in AR, CD161⁺ cells may play a pathogenic role, as there is a significant relationship with clinical severity.

CD161 is a surface receptor involved in transendothelial migration of CD4⁺ T cells and T lymphocytes bearing $\gamma\delta$ T-cell receptor [18, 27, 28]. $\gamma\delta$ T cells are thought to play a role in mucosal immunity, response to fungi and intracellular pathogens [29, 30]. Although not shown, we could not detect a statistically significant increment of CD3⁺ $\gamma\delta$ T cells expressing the CD161 receptor in patients suffering from AR. We have previously reported that in multiple sclerosis during the 'pousse' of this disease, a selective increase in CD3⁺CD161⁺ $\gamma\delta$ T cells can be detected [27, 28]. This would indicate that CD161 is a receptor expressed on different T-cell subsets which play a pathogenic role in a given disease. On this basis, the observed increment of CD161⁺CD3⁺ cells in AR would indicate that these cells are devoted to extravasate and localize into inflamed tissues to exert their functional ac-

tivities. It is evident from our results that the expression of CD161 appeared to be upregulated on CD8^{dull} cells which are conceivably NK cells. It remains to be determined whether these cells play a role in the outcome of AR or whether they are associated with an increase in peripheral blood content of the CD161-inducing cytokine IL-12, as reported in the literature [27].

Of course, this study has some limitations: (1) the number of patients is quite restricted; (2) cytofluorometric analyses have been performed on total CD4⁺ and CD8⁺ PBMC subsets without actually gating on CD3⁺CD4⁺ and CD3⁺CD8⁺ memory or naïve subsets; (3) it was conducted only when patients were symptomatic; (4) there are no data on allergic inflammation, and (5) there are no functional data. Further studies should be performed to respond to these unanswered questions.

However, this study provides evidence that the increase in the percentages of CD161⁺ peripheral T cells correlates well with AR severity, and thus, may be involved in the clinical outcome of this disease.

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