

Abnormal Chemokine Receptor Profile on Circulating T Lymphocytes from Nonallergic Asthma Patients

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Key Words

Chemokine receptor · T lymphocyte · Nonallergic asthma

Abstract

Background: T lymphocytes are involved in the pathogenesis of nonallergic asthma. The objective of this study was to characterize the subset distribution and pattern of chemokine receptor expression in circulating T lymphocyte subsets from nonallergic asthma patients. **Methods:** Forty stable nonallergic asthma patients and 16 sex- and age-matched healthy donors were studied. Twelve patients did not receive inhaled steroids (untreated patients), 16 received 50–500 µg b.i.d. of inhaled fluticasone propionate (FP) (standard-dose patients), and 12 received over 500 µg b.i.d. of inhaled FP (high-dose patients) for at least 12 months prior to the beginning of this study and were clinically well controlled. Flow cytometry was performed using a panel of monoclonal antibodies (4 colors). **Results:** Nonallergic asthma patients treated with high doses of inhaled FP showed a significant reduction in the percentages of CD3+ T lymphocytes compared to healthy controls. Untreated patients

showed a significant increase in CCR6 expression in CD8+CD25+ and CD8+CD25+bright T cells compared to healthy controls. The results were similar for CXCR3 and CCR5 expression. In patients treated with standard doses of FP, CCR5 expression was significantly increased in CD3+ T lymphocytes relative to healthy controls. **Conclusions:** The different groups of clinically stable nonallergic asthmatic patients showed distinct patterns of alterations in subset distribution as well as CCR6, CXCR3, and CCR5 expression on circulating T lymphocytes.

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Introduction

The T lymphocyte compartment is characterized by the dynamic tissue distribution of its cells, which recirculate continuously in blood, body tissues, and secondary

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lymphoid organs. Antigen challenge in the tissues provokes localized preferential extravasation of memory/activated circulating T cells, which is largely driven by chemokine receptors (CKR) [1, 2]. The expression of CKR by different T lymphocyte subsets is heterogeneous and relies in part on their maturity and activation states [3]. Several CKR involved in the pathogenesis of T lymphocyte extravasation in the lung, such as CCR3, CCR4, and CCR8 (Th2-typical receptors), have been studied in asthmatic bronchial inflammation [4–6]. Nevertheless, the implication of CKR in nonallergic asthma remains to be established. We therefore studied the expression of Th1-typical CKR such as CCR2 [7], CCR5 [4], CCR6 [5, 8], and CXCR4 [4, 9], as well as CXCR3 (Th2- and Th1-typical CKR) [4, 10–13], in circulating T lymphocytes from nonallergic asthma patients.

Organ-specific or systemic immune system-mediated diseases are characterized by the involvement of circulating lymphocytes [14–16]. These findings would support the hypothesis of alterations in circulating T cells from nonallergic asthmatic patients. Asthmatic patients are heterogeneous not only in terms of their pathogenesis but also in terms of their clinical characteristics. The patient subset defined as well controlled is also heterogeneous and can be free of inhaled corticosteroid treatment or dependent on it [17]. The relevance of immune system alterations in patients with nonallergic asthma may be established by their presence in those with no clinical evidence of active bronchial inflammation. We studied in parallel a group of age- and sex-matched healthy individuals [13].

In the current study, we analyzed the distribution, activation stage, and pattern of CKR expression by circulating T lymphocytes and natural killer (NK) cells in a group of clinically stable, nonallergic asthma patients stratified according to their pharmacological treatment needs [18]. We excluded the potential immunoregulatory effects of allergic reactions and clinically active bronchial inflammation. In parallel, we studied a group of age- and sex-matched healthy individuals.

Materials and Methods

Study Design

A cross-sectional study was carried out in 40 well-controlled nonallergic asthma patients. The inclusion criterion was complete absence of exacerbation for at least the last 12 months [17]. The patients were classified into 3 groups according to the treatment prescribed by their doctors and received regularly for at least the 12 months prior to the study. Group 1 comprised 12 patients who

had not received fluticasone propionate (FP) (untreated patients), group 2 comprised 16 patients who had received doses of 50–500 µg b.i.d. of inhaled FP (standard-dose FP patients), and group 3, comprised 12 patients who had received over 500 µg b.i.d. of inhaled FP (1,500 µg/day) (high-dose FP patients) [18]. All patients included in this study were allowed to use rescue medication at the usual dose of one inhaled short-acting β_2 agonist (200 µg/6–12 h albuterol or 500 µg/6–12 h terbutaline) [17]. Patients were excluded if they had taken medications that might affect the immune system, such as salmeterol, formoterol, montelukast, omalizumab, oral steroids, or immunosuppressors, in the 3 months prior to the study.

All patients had positive results after exposure to a methacholine concentration which fulfilled a 20% FEV₁ decline (PC₂₀ <4 mg/ml), and all controls showed negative results (PC₂₀ >16 mg/ml). Sixteen unrelated, nonsmoker, sex- and age-matched, healthy controls were also studied (table 1). Neither the patients nor the controls had any concomitant allergic, autoimmune, infectious, or psychiatric disease, a previous tumor history, immunodeficiency disease, or renal, heart, or liver failure or received any treatment other than that described for asthma. Patients with any other lung or upper-airway disease, those with an FEV₁ ≤80% and/or an FVC ≤80%, and those with upper-airway disease (including nonallergic rhinitis, nasal polyps, and the acetylsalicylic acid triad) were excluded. Pregnant women were also excluded. Patients and healthy controls had normal levels of total serum immunoglobulin E (IgE) and skin prick tests were negative against a subset of foods, and indoor and outdoor common aeroallergens. Peripheral blood (PB) samples were obtained between 4 and 5 h after the last dose of inhaled FP. All subjects gave written informed consent for inclusion into this study.

Assessment of Atopy

For each subject, skin prick tests were performed and total serum IgE and specific serum IgE against a panel of common foods and common indoor and outdoor aeroallergens were examined to exclude atopy. We used a subset of 15 allergens (ALK-Abelló, Madrid, Spain) and all were measured by ImmunoCAP® (Phadia, Uppsala, Sweden) following the manufacturer's instructions. Patients and healthy controls had normal levels of total serum IgE, and skin prick tests and specific IgE were negative (table 1).

Blood Samples

Fresh blood samples were collected from the antecubital vein into 10-ml heparinized tubes (Becton-Dickinson Vacutainer System; Meylan, Cedex, France). Blood was diluted to one third with 0.9% saline solution (Fresenius Kabi, Barcelona, Spain). PB mononuclear cells (PBMC) were recovered via Ficoll-Hypaque density gradient centrifugation (Lymphoprep™; Axis-Shield, Oslo, Norway) using the Boyüm method [19]. PBMC were resuspended in RPMI 1640 medium (BioWhittaker Products, Verviers, Belgium) and added to 10% heat-activated fetal calf serum, 25 mM Hepes, and a 1% antibiotic mix (penicillin-streptomycin) (both from BioWhittaker Products) [14].

Blood Lymphocyte Count Calculation

Blood lymphocyte counts of T lymphocyte subsets were calculated according to standard flow cytometry criteria for lymphocyte subset identification and lymphocyte counts obtained in conventional hemograms as previously described [20]. We calculated the

Table 1. Clinical status data of clinically stable nonallergic asthma patients and data of healthy controls

	Untreated ^a (n = 12)	Standard-dose FP ^b (n = 16)	High-dose FP ^c (n = 12)	Healthy controls (n = 16)
Mean age ± SD, years	48.4±9.8	47.8±10.0	54.4±11.2	54.9±14.5
Male/female ratio	7/5	7/9	6/6	9/7
FVC, % predicted	94	91	83	98
FEV ₁ , % predicted	92	88	81	95
Methacholine concentration that fulfilled a 20% FEV ₁ decline (PC ₂₀)	<4 mg/ml	<4 mg/ml	<4 mg/ml	>16 mg/ml
SPT	negative	negative	negative	negative
Mean total IgE ± SD, kU/l	75.0±5.8	58.3±2.0	72.7±3.5	49.9±4.5
Specific IgE, kU _A /l	<0.35	<0.35	<0.35	<0.35
Family history of atopy	negative	negative	negative	negative
Mean age of onset of asthma ± SD, years	27.2±4.2	23.4±3.7	20.51±2.4	–
Mean duration of asthma ± SD, years	20.1±1.5	25.5±3.2	30.7±5.8	–
Response to typical aggravants of asthma (viral infections, exercise, extreme cold weather, aspirin, and strong chemical odors)	broncho constriction (FEV ₁ <80% predicted)	broncho constriction (FEV ₁ <80% predicted)	broncho constriction (FEV ₁ <80% predicted)	
Other medications received prior to blood collection	none	none	none	

^a Patients who received no FP. ^b Patients treated with standard FP doses (100–1,000 mg/day) who showed mild or moderate clinical severity. ^c Patients with a severe clinical condition who received high-dose FP treatment (1,500 mg/day).

percentage of CD3-expressing cells in the total lymphocyte gate defined by forward and side scatter in PBMC. The blood lymphocyte count of circulatory T lymphocytes was calculated as the percentage of CD3+ cells in PB lymphocytes multiplied by the total number of lymphocytes per microliter measured by a Coulter counter. After simultaneous staining of PBMC with anti-CD3, anti-CD4, and anti-CD8 antibodies, we calculated the absolute number of CD4+ and CD8+ T lymphocytes by multiplying the total number of T lymphocytes previously calculated by the percentage of cells positive for each of the two antigens in CD3+ T cells. Finally, we calculated the absolute number of CD3+CD4+ and CD3+CD8+ T cell subsets defined by the expression of CD45 isoforms CD45RA+CD45RO–, CD45RO+CD45RA–, and CD45RA+CD45RO+ and the expression of CD28+, CD25+, and HLA-DR antigens. For these calculations, we multiplied the percentage of each subset in the parent CD3+CD4+ or CD3+CD8+ populations by the absolute count of CD3+CD4+ and CD3+CD8+ T cells, respectively. In addition, we analyzed cell surface CCR2, CCR5, CXCR4, CCR6, and CXCR3 CKR for these T cell populations and subpopulations. All lymphocyte counts are expressed as cells per microliter.

Immunostaining and Flow Cytometric Data Acquisition

Fresh purified PBMC were counted by light microscopy using trypan blue to assess cell viability. In each well, 10⁵ cells were pelleted in a 96-well V-bottom plate and one anti-human CKR monoclonal antibody was added (biotinylated for CCR2, CCR5, and CXCR4; unlabeled for CCR6 and CXCR3). Cells were incubated

(20 min) and washed with phosphate-buffered saline before the addition of fluorescein isothiocyanate (FITC)-avidin to detect biotinylated antibodies or FITC-goat-anti-mouse (Becton-Dickinson Biosciences, San Jose, Calif., USA) to detect unlabeled antibodies. Cells were then incubated and washed once with phosphate-buffered saline before the addition of combinations of fluorochrome-conjugated anti-human monoclonal antibodies: phycoerythrin (PE)-antiCD28, allophycocyanin (APC)-antiCD45RO, peridinin chlorophyll protein (PerCP)-antiCD3, PerCP-antiCD4, PerCP-antiCD8, PerCP-antiHLA-DR, and PE-antiCD56 (Becton-Dickinson), and PE-antiCD45RA, APC-antiCD4, APC-antiCD8, APC-antiCD19, and PE-antiCD25 (Caltag Laboratories, Burlingame, Calif., USA). Mouse monoclonal antibodies against human CCR2, CCR5, CXCR4, CCR6, and CXCR3 were generously provided by the National Center for Biotechnology (Madrid, Spain) [21, 22]. The cells were again incubated and then washed once with phosphate-buffered saline. Flow cytometry was performed using a FACSCalibur apparatus (Becton-Dickinson) with CellQuest software for data acquisition and analysis.

Statistical Analysis

All analyses were performed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, Ill., USA). Since the data distribution was adjusted to a normal distribution after a Kolmogorov-Smirnov test, differences between groups were compared using the Student t test. Moreover, an ANOVA test followed by a post hoc analysis was used for multigroup comparisons. *p* < 0.05 was considered statistically significant.

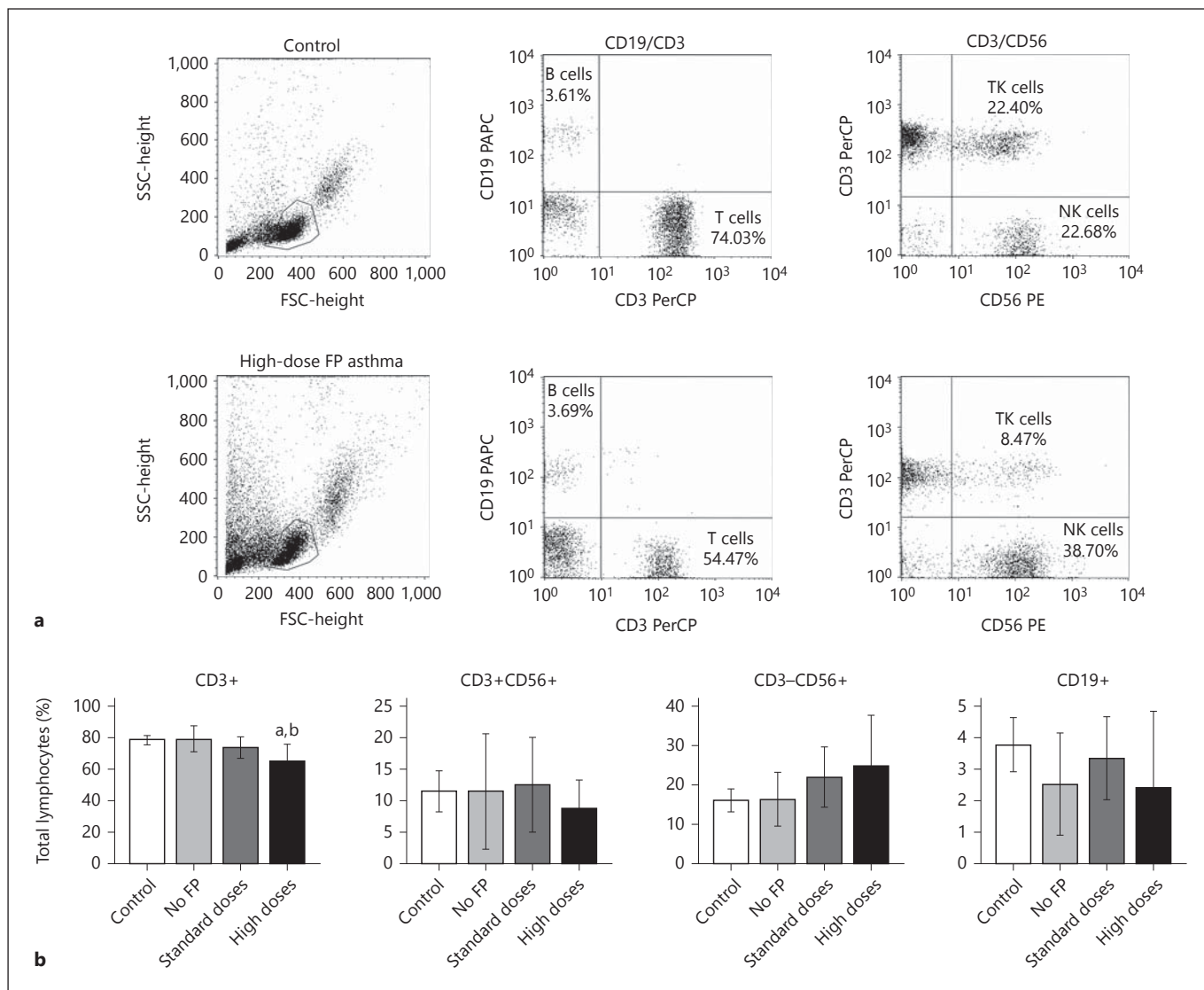


Fig. 1. Distribution of T (CD3+), NKT (CD3+CD56+), NK (CD3-CD56+), and B (CD19+) lymphocytes in PB from clinically stable nonallergic asthma patients and healthy donors. **a** Dot plots of circulating CD3+, CD19+, CD3+CD56+, and CD3-CD56+ cells from a representative asthma patient dependent on inhaled high-dose FP treatment and a healthy control. **b** Percentages of cells that express CD3, CD56, and CD19 antigens on PBMC. Bars indicate

means $\pm$ SD for data obtained from healthy controls ($\square$) and untreated ($\blacksquare$), standard-dose FP-treated ($\blacksquare$), and high-dose FP-treated ($\blacksquare$) nonallergic asthma patients and represent significant differences between the patient subset and healthy controls. We only found statistically significant differences between high-dose FP patients with respect to healthy controls (a) and standard-dose FP patients (b) in the CD3+ population using Student's t test.

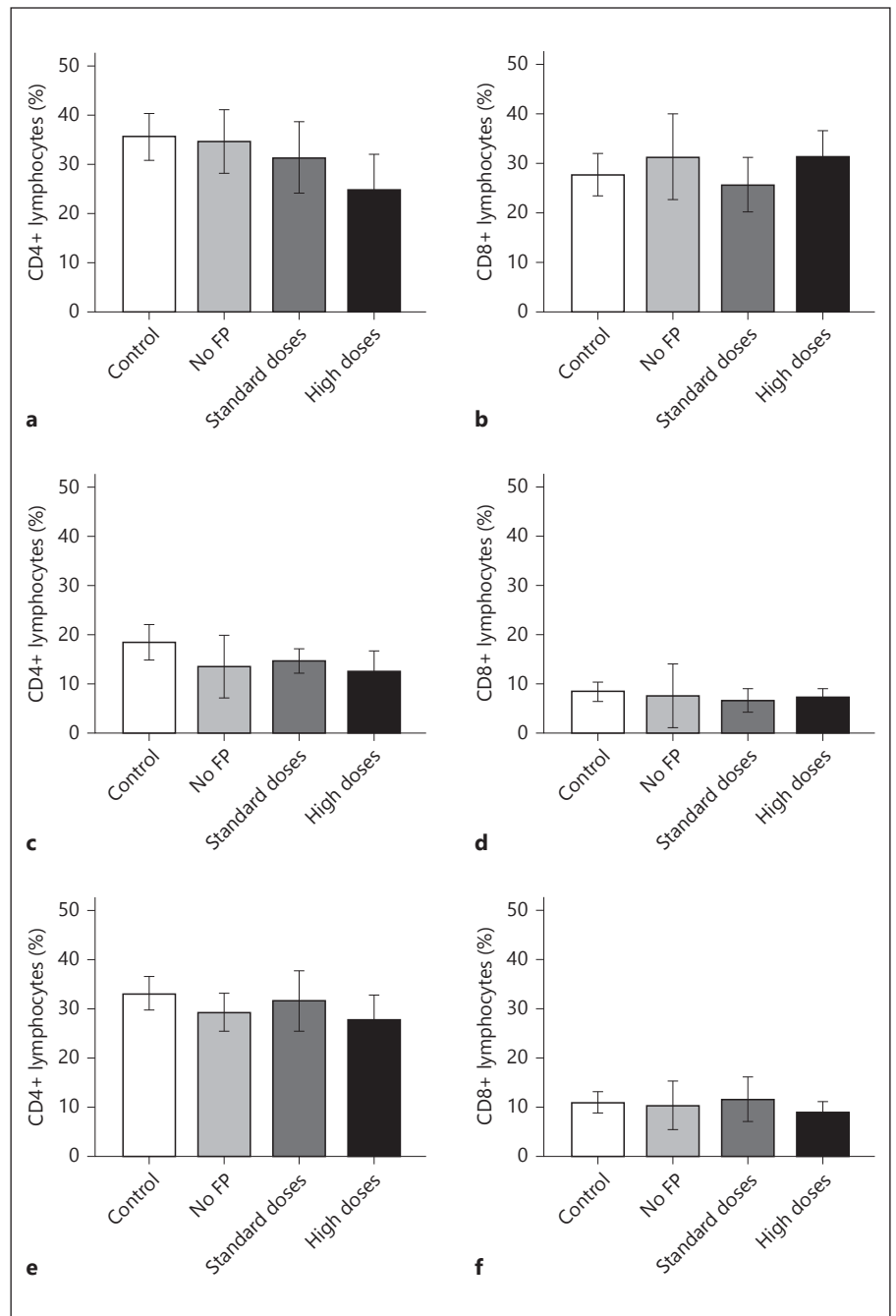
Results

Nonallergic Asthma Patients Treated with High Doses of Inhaled FP Showed Marked Changes in Circulating T Lymphocyte and NK Cell Subpopulations

Compared to the healthy controls, untreated and standard-dose FP-treated stable nonallergic asthma patients showed no significant alterations in the percentages or ab-

solute numbers of CD3+ T lymphocytes, CD3+CD56+ killer T (NKT) cells, CD3-CD56+ NK cells, or CD19+ B lymphocytes in PB (fig. 1). A significant reduction was observed in the percentages of CD3+ T lymphocytes in high-dose FP-treated stable nonallergic asthma patients compared to healthy controls (fig. 1). We also found a significant reduction in the percentages and absolute numbers of CD3+ T lymphocytes in high-dose FP patients relative to

Fig. 2. Distribution of activation stage CD4 and CD8 T lymphocyte subsets in nonallergic asthmatic patients and healthy donors. The percentages of CD4+ (**a, c, e**) and CD8+ (**b, d, f**) T lymphocytes that express different combinations of the CD45 antigen are shown. **a, b** Naïve T cells (CD45RA+CD45RO–). **c, d** Recently activated T cells (CD45RA+CD45RO+). **e, f** Memory T cells (CD45RA–CD45RO+). Bars indicate means $\pm$ SE for data obtained from healthy controls ($\square$) and non-inhaled FP-treated ($\blacksquare$), standard-dose inhaled FP-treated ($\blacksquare$), and highest-dose inhaled FP-treated ($\blacksquare$) nonallergic asthmatic patients. The percentages are relative to the subset of reference CD4 or CD8 T cells.



standard-dose FP patients. There were no significant alterations in the percentages of CD3+CD4+ or CD3+CD8+ T cell subsets in any patient group (data not shown).

To characterize the CD3+CD4+ and CD3+CD8+ T cell subset distribution according to previous antigenic stimulation, we studied the percentages of naïve (CD45RA+CD45RO–), recently activated (CD45RA+CD45RO+), and memory (CD45RA–CD45RO+) T cells.

Untreated patients showed significantly higher percentages and absolute numbers of naïve CD3+CD4+ T cells compared to those treated with standard or high doses of FP and compared to healthy controls (fig. 2). The absolute numbers of CD45RA+CD45RO+ and CD45RA–CD45RO+ subsets in CD4+ T lymphocyte populations were significantly decreased in high-dose FP patients with respect to healthy controls.

Table 2. CCR6 and CXCR3 expression in circulating T lymphocytes of clinically stable nonallergic asthma patients

	Untreated (n = 12)	Standard- dose FP (n = 16)	High- dose FP (n = 12)	Healthy controls (n = 16)	ANOVA test (significance level)	Post hoc ^c (95% CI)
CCR6						
CD4+	29.0±2.0	28.4±4.7	26.6±4.4	26.1±2.8	0.075	
CD4+CD45RA+CD45RO+	58.4±3.1	42.8±6.8	44.7±6.8	39.3±4.5	0.028	
CD8+	12.7±2.0	8.5±1.4	18.5±4.4	15.8±2.9	0.272	
CD8+CD45RA+CD45RO+	28.8±3.1	9.7±2.9	21.4±4.3	15.3±2.9	0.296	
CD8+CD25+	63.1±7.8 ^{a, b}	20.1±4.3	32.1±4.5	31.6±4.1	0.002	0.006 (–55.3 to –7.6) ^a 0.003 (10.2 to 64.3) ^b
CD8+CD25+bright	69.5±6.0 ^{a, b}	18.1±4.1	28.1±3.7	33.1±5.2	0.000	0.001 (–64.5 to –13.2) ^a 0.001 (17.9 to 75.9) ^b
CXCR3						
CD4+	7.1±1.5	7.1±2.1	8.8±2.7	3.5±0.7	0.164	
CD4+CD28+	8.4±3.8	4.9±0.5	13.1±7.2	3.1±0.9	0.067	
CD8+	14.6±3.6	11.3±2.1	16.4±4.6	13.4±2.1	0.250	
CD8+CD25+	48.9±10.1 ^{a, b}	19.7±3.7	19.2±4.5	17.9±4.5	0.003	0.004 (–60.8 to –9.2) ^a 0.033 (1.9 to 60.3) ^b
CD8+CD25+bright	53.0±1.3 ^{a, b}	17.1±0.6	17.8±0.5	20.6±1.1	0.034	0.064 (–66.4 to 1.4) ^a 0.057 (–0.8 to 75.9) ^b

Data are represented as percentages with respect to total lymphocytes (means ± SD). The post hoc analysis in the CXCR3+CD8+CD25+bright subpopulation nearly reached statistical significance. Numbers represent cell percentages expressing CCR6 or CXCR3 chemokine receptors.

^a Statistical significance between standard-dose FP-treated patients and healthy controls.

^b Statistical significance between untreated patients and standard-dose FP-treated patients.

^c Sheffe.

Increased CCR6, CXCR3, and CCR5 Expression in Several Circulating T Cell Subpopulations from Nonallergic Asthma Patients

We analyzed CCR2, CCR5, CCR6, CXCR3, and CXCR4 expression by circulating T lymphocytes in the different groups of nonallergic asthma patients and in healthy controls. Untreated patients showed a significant increase in CCR6 expression in CD8+CD25+ and CD8+CD25+bright T cells compared to healthy controls (table 2). These untreated patients also showed a significantly increased CCR6 expression in CD8+CD25+ and CD8+CD25+bright T cells compared to those treated with standard FP doses. The results were similar for CXCR3 expression. We found no significant differences in CCR2, CCR5, and CXCR4 expression in the lymphocyte subsets analyzed for these patients and healthy controls (not shown).

In patients treated with standard FP doses, CCR5 expression was significantly increased in CD3+ T lymphocytes, as well as in the CD4+, CD4+CD45RA–CD45RO+, CD4+CD28+, and CD8+CD28+ T lymphocyte subsets,

relative to healthy controls (table 3). These standard-dose FP-treated patients also showed a significant increase in CCR5 expression in CD8+CD25+ and CD8+CD25+bright T lymphocytes compared to healthy controls (table 3). There was also a statistically significant difference in CD3+ T lymphocytes between standard-dose FP- and high-dose FP-treated patients, as well as in CD8+CD25+ T lymphocytes in untreated patients compared to healthy controls (table 3). We found no significant differences between these patients and healthy controls in terms of CCR2 and CXCR4 expression in the lymphocyte subsets analyzed (data not shown).

In patients treated with high doses of FP, we observed a significant reduction in CCR2 expression in circulating CD3+ T cells (33.85 ± 6.13%) and CD3–CD56+ NK cells (35.27 ± 5.49%) compared to healthy controls (52.09 ± 4.79 and 56.11 ± 6.05%, respectively). There were no significant differences in CXCR4 expression in the lymphocyte subsets analyzed between these patients and healthy controls (data not shown).

Table 3. CCR5 expression in circulating T lymphocytes from clinically stable nonallergic asthma patients

	Untreated (n = 12)	Standard- dose FP (n = 16)	High- dose FP (n = 12)	Healthy controls (n = 16)	ANOVA test (significance level)	Post hoc ^d (95% CI)
CD3+	70.5±5.0	81.5±2.8 ^{a, b}	59.7±7.8	62.5±4.3	0.003	0.012 (–46.1 to –4.4) ^a 0.044 (0.6 to 58.7) ^b
CD4+	66.5±5.9	73.2±3.9 ^a	56.8±7.6	51.7±6.1	0.016	0.036 (1.1 to 47.2) ^a
CD4+CD45RA–CD45RO+	65.7±6.1	78.9±2.9 ^a	57.6±7.9	51.1±6.4	0.007	0.014 (–53.3 to –4.6) ^a
CD4+CD28+	69.1±7.1	78.7±4.1 ^a	57.0±8.3	51.1±4.2	0.002	0.009 (–53.9 to –6.1) ^a
CD8+	76.5±4.0	82.1±2.9	64.5±7.0	64.7±6.9	0.038	
CD8+CD28+	75.9±5.5	84.1±3.2 ^{a, b}	64.2±8.5	64.1±4.3	0.002	0.007 (–56.3 to –7.2) ^a
CD8+CD25+	73.9±7.7 ^c	82.8±2.0 ^a	60.6±8.6	49.4±5.7	0.004	0.017 (–57.2 to –4.2) ^a 0.047 (–64.1 to –0.3) ^c
CD8+CD25+bright	72.5±8.4	86.4±3.2 ^a	69.4±6.0	53.8±6.7	0.007	0.012 (–61.3 to –5.7) ^a

Data are presented as percentages with respect to total lymphocytes (means ± SD). Numbers indicate cell percentages that express the CCR5 chemokine receptor.

^a Statistical significance between standard-dose FP-treated patients and healthy controls.

^b Statistical significance between standard- and high-dose FP-treated patients.

^c Statistical significance between untreated patients and healthy controls.

^d Sheffe.

Discussion

In this study, we demonstrated that clinically stable nonallergic asthma patients show marked alterations in subset distribution and CKR expression in circulating T lymphocytes. The pattern of circulating T cell involvement differs in untreated patients and in those who require chronic FP treatment for disease control.

Although T lymphocytes have a central role in the pathogenesis of allergic asthma [1], the knowledge on the T lymphocyte compartment in nonallergic asthma patients is limited. The aim of this study was to characterize subset distribution and CKR expression by circulating T lymphocytes of nonallergic asthma patients. We studied a population of nonallergic asthma patients with good clinical control of the disease [17], an inclusion criterion to avoid potential T lymphocyte abnormalities secondary to the characteristic bronchial inflammation of active disease. The population of clinically well-controlled nonallergic asthma patients was stratified according to the patients' established long-term requirement for FP treatment [18]. The results showed a significant decrease in the CD3+ population in FP-treated patients with respect to healthy controls. This decrease was more marked in high-dose FP patients. These findings might be explained by several mechanisms. Naïve T cells are 100-fold more sensitive to corticosteroids than are memory T cells [23]; it is thus possible that the reduction in naïve CD4+ T lymphocytes in

FP-treated patients with respect to untreated patients was due to the FP treatment. Moreover, a dose-dependent effect was observed. There were no significant differences in the percentage of naïve CD4+ T cells in patients treated with standard or high FP doses. The reduction in naïve CD4+ T cells might also be related to the activation stage secondary to a subclinical inflammatory stage of the bronchial tree. Alteration of the circulating T lymphocyte compartment in clinically well-controlled nonasthmatic patients is further supported by the reduction in memory CD4+ T cells in patients treated with high FP doses.

CKR expression by T lymphocytes is critical for regulation of their migration to secondary immunological organs and tissues in inflammatory diseases [3]. We focused on analysis of CCR2, CCR4, CCR5, CXCR3, and CXCR4 expression by circulating T lymphocytes from clinically well-controlled nonallergic asthmatic patients. Untreated nonallergic asthmatics showed CCR6 overexpression in CD8+CD25+ and CD8+CD25+bright T cells. CCR6 has been implicated in the regulation of lymphocyte migration into lung tissue in patients with asthma [5, 24], and untreated patients also showed CXCR3 overexpression driven by the CD8+CD25+ subset. These modifications in the CKR expression pattern by circulating T cells are not explained by a generalized nonspecific abnormality. Indeed, CCR2 and CXCR4 expression in T cells from untreated patients remained normal. The increase in CCR5 expression observed in circulating activated T cells from

patients treated with standard doses of FP supports this selective lymphocyte extravasation. Normalization of CCR6 and CXCR3 expression by T cells was observed in both groups of FP-treated patients. CXCR3 has been described as a stable marker of activated memory T cells in allergic asthmatics [4], and CXCR3 has also been implicated in allergic airway disease in murine models [10–12]. Our untreated patients showed CXCR3 overexpression driven by the CD8+CD25+ T cell subset. We observed decreases in the percentage of CXCR3 and CCR6 on peripheral CD8+CD25+ and CD8+CD25+bright T cells in FP-treated compared to untreated patients. These modifications in CKR expression by circulating T cells might be explained by a lack of control by inhaled corticosteroids in severely affected patients, rather than by a generalized nonspecific abnormality. The CCR2 and CXCR4 expression in patient T cells remained normal. A decrease in CXCR3 expression on CD4+ and CD8+ T cells has been reported after 20 mg/day prednisolone therapy for 2 weeks [25] and after FP therapy [26]. These different CKR expression patterns in circulating T lymphocytes in well-controlled asthmatic patients would indicate the heterogeneity of immune system abnormalities shown by nonallergic asthma patients.

This pattern of abnormalities in the circulating T cell compartment in asthma patients varies according to the stage of disease severity [27]. In severe persistent asthma, there is a predominantly Th1 cytokine pattern, with greatly expanded memory T cells (CD8+CD45RO+) [27]. CD8+ T cells have also been implicated in allergic pulmonary inflammation [12]; in contrast, we found involvement of CD4+ T cell subpopulations but not of CD8+ T cells. CD4+CD45RA+ T cells are 100-fold more sensitive to corticoids than are CD4+CD45RO+ T cells [23], which might explain the decrease in the percentage and absolute numbers of the CD4+CD45RA+CD45RO– subpopulation in our high-dose FP patients compared to the healthy controls. A percentage reduction in circulating T cells was also observed among the group of patients receiving standard and high FP doses.

Systemic steroid administration is followed by a reduction in circulating CD4+ T cells lymphocytes, while CD8+ T lymphocyte levels remain unaltered [28], which coincides with the findings in our patients. This study provides evidence of reduced percentages of CD4+ T cell subpopulations in nonallergic asthmatics treated with high doses of inhaled FP. The observed reduction in recently activated T cells (CD45RA+CD45RO+) within the CD3+CD4+ T cell subsets in nonallergic asthma patients, which was statistically significant in the group treated

with high FP doses, might be linked to the preferential extravasation of activated T cells in nonallergic asthma patients and/or inhaled corticosteroid treatment effects. Both mechanisms might be also involved in the CR expression pattern observed in circulating T cells. The CD8+CD25+ and CD8+CD25+bright T cell subsets were involved in 3 of the CKR studied, i.e. CCR6, CXCR3, and CCR5. Alterations in the circulating lymphocyte subset distribution could be related to preferential extravasation of certain subsets which would then be depleted in PB, with the degree of depletion proportional to the intensity of extravasation to inflamed end organs, including the lungs [3]. Selective extravasation of cells that express a specific CR can cause their depletion in PB [2].

In summary, the disturbance observed in T cell compartment and Th1-typical CKR in nonallergic asthma patients indicates the importance of the immune system in the pathogenesis of this disease. Because our findings are derived from a cross-sectional analysis, they are insufficient to link these T cell behavior abnormalities to the pathogenesis of nonallergic asthma. Further prospective analyses are needed to validate the underlying mechanisms of this complex disease, particularly for their potential application as future therapeutic targets.

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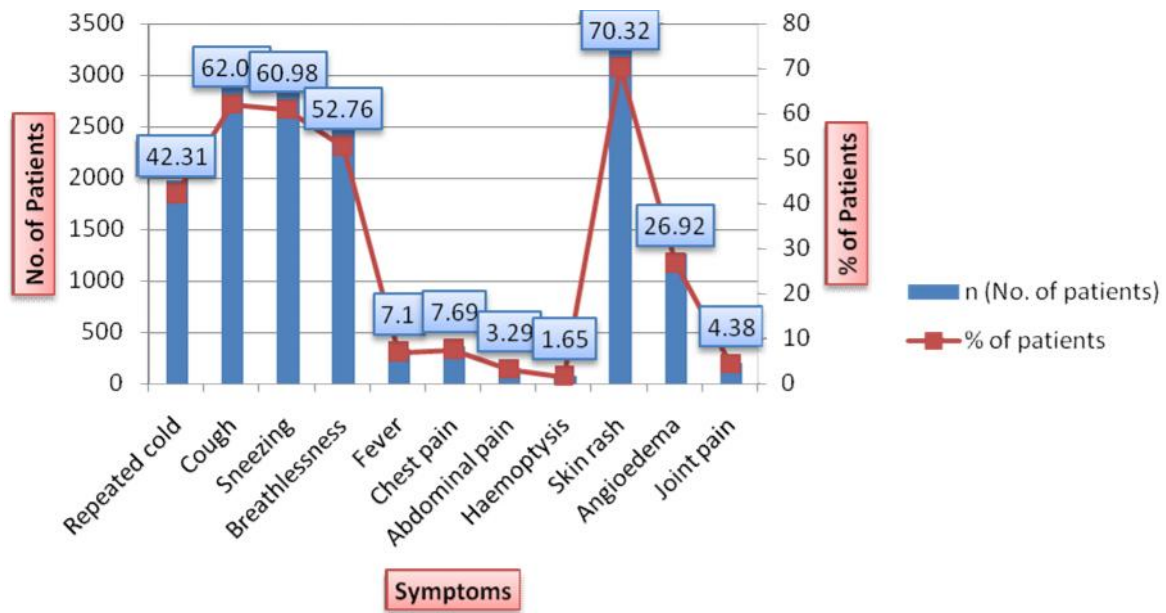


Fig S1. Number and percentage of patients having symptoms due to food allergy.

Table 1. Age group-wise distribution of patients showing sensitivity to different food allergens

Allergen	Below 15, n = 611 (M:F = 298:313)				15–40, n = 2,265 (M:F = 1,067:1,198)				Above 40, n = 1,804 (M:F = 1,178:626)			
	M	F	Total	%	M	F	Total	%	M	F	Total	%
<i>Fruit</i>												
Banana	156	78	234	38.3	391	408	799	35.3	260	223	483	26.8
Papaya	18	27	45	7.4	90	82	172	7.6	66	47	113	6.3
Cucumber	25	18	43	7.03	65	74	139	6.1	43	39	82	4.5
Pumpkin	9	21	30	4.9	62	103	165	7.3	66	51	117	6.5
Mango	16	11	27	4.4	70	76	146	6.4	35	36	71	3.9
Guava	11	13	24	3.9	56	73	129	5.7	35	17	52	2.9
Grapes	14	3	17	2.8	23	21	44	1.9	18	9	27	1.5
Citrus fruits other than orange	5	4	9	1.5	33	35	68	3	13	11	24	1.3
Orange	4	5	9	1.5	23	30	53	2.3	16	15	31	1.71
Groundnut	5	3	8	1.3	34	18	52	2.3	18	5	23	1.3
Coconut dry	5	2	7	1.14	20	21	41	1.8	9	12	21	1.16
Chikoo	3	3	6	0.98	34	30	64	2.8	18	12	30	1.7
Apple	2	2	4	0.65	30	21	51	2.3	17	10	27	1.5
<i>Pulses</i>												
Lentil (<i>Lens culinaris</i>)	46	27	73	11.9	127	150	277	12.23	74	60	134	7.4
Soybean (<i>Glycin max</i>)	38	23	61	9.98	98	88	186	8.2	70	41	111	6.1
Chick pea (<i>Cicer arietinum</i>)	22	25	47	7.7	99	84	183	8.07	71	29	100	5.5
Moong bean(<i>Vigna radiata</i>)	13	8	21	3.4	56	71	127	5.6	52	35	87	4.8
Black lentil (<i>Vigna mungo</i>)	10	10	20	3.3	57	63	120	5.3	44	24	68	3.8
Pigeon pea (<i>Cajanus cajan</i>)	3	1	4	0.65	9	29	38	1.7	14	10	24	1.3
Moth bean (<i>Vigna aconitifolia</i>)	1	0	1	0.16	20	24	44	1.9	18	15	33	1.8
<i>Vegetables</i>												
Brinjal	87	71	158	25.9	347	397	744	32.8	248	227	475	26.3
Lady's fingure	65	55	120	19.6	273	316	589	26	194	153	347	19.2
				4								
Tomato	57	51	108	17.7	230	263	493	21.8	172	142	314	17.4
Cauliflower	27	22	49	8.01	135	142	277	12.2	94	69	163	9.03
Cabbage	16	19	35	5.7	86	69	155	6.8	70	42	112	6.2
Spinach	16	17	33	5.4	79	111	190	8.4	64	29	93	5.1
Bitter gourd	13	13	26	4.2	57	68	125	5.5	43	43	86	4.8
Mustard leaves	12	8	20	3.2	62	46	108	4.8	36	21	57	3.2
Beans	10	8	18	2.9	51	56	107	4.7	40	22	62	3.4
Potato	10	7	17	2.8	33	32	65	2.9	25	17	42	2.3
Radish	8	2	10	1.64	36	34	70	3.09	26	15	41	2.3
Onion	7	2	9	1.5	21	25	46	2.03	14	18	32	1.7
Pointed gourd	3	5	8	1.3	18	28	46	2.03	20	12	32	1.7
Basella leaves	3	4	7	1.15	33	31	64	2.8	26	20	46	2.5
Garlic	3	2	5	0.82	29	24	53	2.3	18	9	27	1.5
Mushroom	2	3	5	0.82	25	21	46	2.03	13	15	28	1.5
Ginger	2	2	4	0.65	44	45	89	3.9	20	9	29	1.6
<i>Cereals</i>												
Wheat	70	63	133	21.8	170	306	476	21.01	281	129	410	22.7
Rice	9	4	13	2.1	33	23	56	2.5	31	21	52	2.9
<i>Animal Protein</i>												
Egg	120	83	203	33.2	263	298	561	24.8	186	135	321	17.8
Milk	62	56	118	19.3	166	190	356	15.7	107	86	193	10.7
Chicken	26	13	39	6.4	66	87	153	6.8	64	33	97	5.4
Prawn	8	13	21	3.4	46	57	103	4.5	25	28	53	2.9
Raohu fish(<i>Labeo rohita</i>)	9	7	16	2.6	31	43	74	3.3	31	26	57	3.1

Hilsa fish(<i>Tenua</i> <i>losa ilisha</i>)	4	6	10	1.6	31	28	59	2.6	18	17	35	1.9
Mutton	5	5	10	1.6	23	17	40	1.8	25	15	40	2.2
Beef	2	2	4	0.65	40	39	79	3.5	23	11	34	1.9
