

ORIGINAL ARTICLE

Basic Mechanisms in Allergic Disease

Impaired immunoregulatory network of the CD4 T lymphocytes in refractory asthma

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Summary

Background: The immunopathogenesis of severe asthma has been associated with an inefficient regulatory response. There are a few studies about the CD4 T cells profile among individuals with severe asthma refractory to treatment.

Objective: To evaluate the CD4 T lymphocyte profile from individuals with severe asthma according to their response to treatment, relating to their atopy status and age of asthma onset.

Methods: We evaluated nineteen individuals with severe asthma refractory to treatment (SAR), 21 with well-controlled or partly controlled severe asthma (CSA) and 23 with mild-to-moderate asthma (MMA). Lymphocytes were obtained from PBMC, and the frequency of expression of different molecules in this population was assessed using the flow cytometry.

Results: We observed the frequency of CD4⁺IFN- γ ⁺T cells was higher in atopic individuals with SAR than with CSA. In addition, among the atopic and early-onset asthma (EOA), the frequency of CD4⁺CTLA-4⁺T cells was lower in the SAR group than the CSA group. In relation to non-atopic and late-onset asthma (LOA) phenotypes, we noted the frequency of CD4⁺FoxP3⁺T cells was lower in individuals with SAR than with CSA. We also observed among the LOA patients, the frequency of CD4⁺TGF- β ⁺ T cells was decreased in SAR group than the in CSA group.

Conclusion and Clinical Relevance: Our data suggest that refractoriness to treatment in asthma is associated with a lower expression of distinct regulatory molecules by CD4 T cells between those who are atopic and have EOA and those who are non-atopic and have LOA. Thus, these results may contribute to the identification of new regulatory strategies to treat asthma according to their phenotypes.

KEYWORDS

asthma, CD4 T lymphocyte, refractoriness, regulatory molecules

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1 | INTRODUCTION

Asthma affects more than 358 million people world-wide.¹ The disease is usually characterized by chronic inflammation of the lower airways and recurrent episodes of wheezing, dyspnoea, chest tightness and cough, which vary over time, together with variable expiratory airflow limitation.²

Asthma is a heterogeneous disease characterized by a variety of phenotypes related to age of onset, triggering factors, comorbidities, response to treatment, as well as the pattern of inflammatory cells involved in the pathophysiology of the disease. This heterogeneity makes it difficult to choose a treatment tailored to the patients' endophenotype, unless one searches for biomarkers. In most patients, the disease can be controlled by the combined use of inhaled corticosteroids (ICS), short- and/or long-acting β_2 adrenergic agonist bronchodilators. However, it is estimated that 5%-10% of asthmatic individuals have severe asthma,^{3,4} which requires treatment with high-dose ICS plus a second controller (and/or systemic corticosteroids) to prevent or which remains "uncontrolled" despite this therapy.⁵

In addition of the atopic (AT) and non-atopic (NA) phenotypes that are well known and defined, the age of asthma onset is another phenotype that has been widely used to characterize asthma. Usually, early-onset asthma (EOA) refers to asthma that begins before adolescence and persists in adulthood, besides being characterized by having allergic symptoms, atopy and consequently high levels of IgE.⁶ Late-onset asthma (LOA), in turn, is defined as asthma that begins in adolescence⁷ or in adulthood and seems to be less associated with the atopic profile.⁶

The immunopathogenesis of asthma has been linked to activation of a Type 2 response with production mainly of IL-4, IL-5 and IL-13 by Th2 cells⁸ and innate immune cells (ILCs).⁴ These cytokines are responsible for recruiting and activating cells such as mast cells^{9,10} and eosinophils¹¹ and stimulating the production of IgE by B lymphocytes,¹² contributing to the inflammatory process observed in the disease. Furthermore, Th1- and Th17-type responses have been associated with asthma severity. Studies have shown that the Th1 response, mainly through the production of IFN- γ , can cause smooth muscle contraction and apoptosis of bronchial epithelial cells,^{13,14} while the Th17 response, with IL-17A production, contributes to bronchial remodelling¹⁵ and to the resistance to corticosteroid treatment.¹⁶ The regulatory T cells, which are well known by modulating the immune response, are in lower numbers in blood and sputum of patients with severe asthma refractory to treatment and present impaired suppressive activities.^{17,18}

The aim of this study was to evaluate the CD4 T lymphocyte phenotypic profile in patients with severe asthma according to atopy status and age of asthma onset. Investigating the immunological

markers in different phenotypes in the clinical context will help understanding the mechanism involved in the pathogenesis of the disease and clarify essential questions that may improve the treatment of asthma patients.

2 | METHODS

2.1 | Characteristics of the studied subjects

This study evaluated 73 individuals: 19 subjects with severe asthma refractory to treatment (SAR), 21 with well-controlled/partially controlled severe asthma (CSA), 23 with mild-to-moderate asthma (MMA) and 10 individuals without asthma (WA). Severe asthma (SA) patients should have been followed up in the Program for the Control of Asthma of Bahia—ProAR, for at least six months. They were classified as CSA by the Global Initiative for Asthma (GINA, 2006) criteria¹⁹ and as SAR by American Thoracic Society (ATS)³ major and minor criteria. At the time of blood harvest, all subjects with SA were using medium or high dose of ICS (800-1600mcg budesonide or equivalent) combined to long-acting β_2 agonist (12-24 mcg of formoterol or 100 mcg of salmeterol). The subjects were not included if they had ≥ 1 exacerbation episode in the previous month.

The control groups (MMA and WA) were recruited consecutively from the same communities where patients with SA live. They were invited to volunteer by public advertisement in health facilities and public transportation. Individuals with MMA were identified based on their history of asthma. Their diagnosis was validated by an expert on asthma, and they were not included if they were using ICS.

All individuals were older than 18 years, and we did not include smokers, pregnant women and those with serum positive for Chagas disease, HIV, HTLV-1, hepatitis B and C virus, diabetes and autoimmune diseases, or any other conditions that might interfere with the immune response.

The study was approved by Ethics Committee of Maternity Clímerio of Oliveira, Federal University of Bahia (License Number: CONEP n°15782, CEP 095/2009 and 095/2012). All individuals who were recruited and agreed to participate in the study signed an informed consent form.

2.2 | Atopy status and age of asthma onset

The atopy status was defined by a positive skin prick test (SPT) and/or presence of specific IgE to at least one aeroallergen in serum. The response to *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Alternaria alternata*, *Cladosporium herbarum*, *Dermatophagoides farinae*,

Dermatophagoides pteronyssinus, *Blomia tropicalis*, cat epithelium, dog epithelium, *Blattella germanica*, *Periplaneta americana*, *Paspalum notatum* and *Cynodon dactylon* was assessed by SPT, and specific IgE to *Dermatophagoides pteronyssinus* and *Blomia tropicalis* was quantified by solid-phase fluorescence immunoassay (ImmunoCAP™) (Thermo Fisher Scientific, Uppsala, Sweden) considering the cut-off of 0.70 KU/L.

Regarding the age of asthma onset, we choose the age limit of 12 years to define the two phenotypes: EOA (<12 years) and LOA (≥ 12 years).^{7,20}

2.3 | Obtaining peripheral blood mononuclear cells (PBMC) and flow cytometry assay

The PBMC were obtained through separation by Ficoll-Hypaque gradient and adjusted to a concentration of 3×10^5 cells/mL in RPMI 1640 medium containing 10% normal human AB serum, 100 U/mL of penicillin, 100 mg/mL of streptomycin, 2 mmol/L of L-glutamine and 30 mmol/L of HEPES (Life technologies GIBCO BRL, Gaithersburg, MD).

After separation, the cells were stained with fluorochrome-conjugated antibody and acquired by flow cytometry. Briefly, in

suspensions of 3×10^5 cells/mL were added brefeldin A (10 μ g/mL; Sigma, St. Louis, MO), in 96-well plates for 4 hours at 37°C, 5% CO₂. Next, we performed the staining of surface markers with monoclonal antibodies specific for CD4 T lymphocytes: CD3 FITC (clone OKT3), CD4 PerCPCy5.5 (clone OKT4), CD28 PECy7 (clone CD28.2) and CTLA-4 PE (clone 14D3). After that, the plates were incubated with 150 μ L/well of permeabilization buffer for 10 minutes at room temperature. The intracellular staining was performed using monoclonal antibodies such as IL-5, IFN- γ and IL-10 PE (clones TRFK5, 45.B3 and JES3-9D7, respectively), IL-17A PECy7 (clone eBio64-DEC17), FoxP3 APC (clone 236A/E7), IL-13 FITC (clone PVM13-1) and TGF- β APC (clone 9016, R&D System), except TGF- β , all other antibodies are from eBioscience. The acquisition was performed using FACSCanto (Becton Dickinson) in a total of 100,000 events. The isotype controls were used to define background staining.

The lymphocytes were analysed according to the frequency of the cell surface markers using the FlowJo™ software (Tree Star, Ashland, OR). Lymphocyte region was defined by non-specific fluorescence from the forward scatter (FSC) and side scatter (SSC) (Figure 1A). The CD4 T lymphocytes were selected through CD3 marker

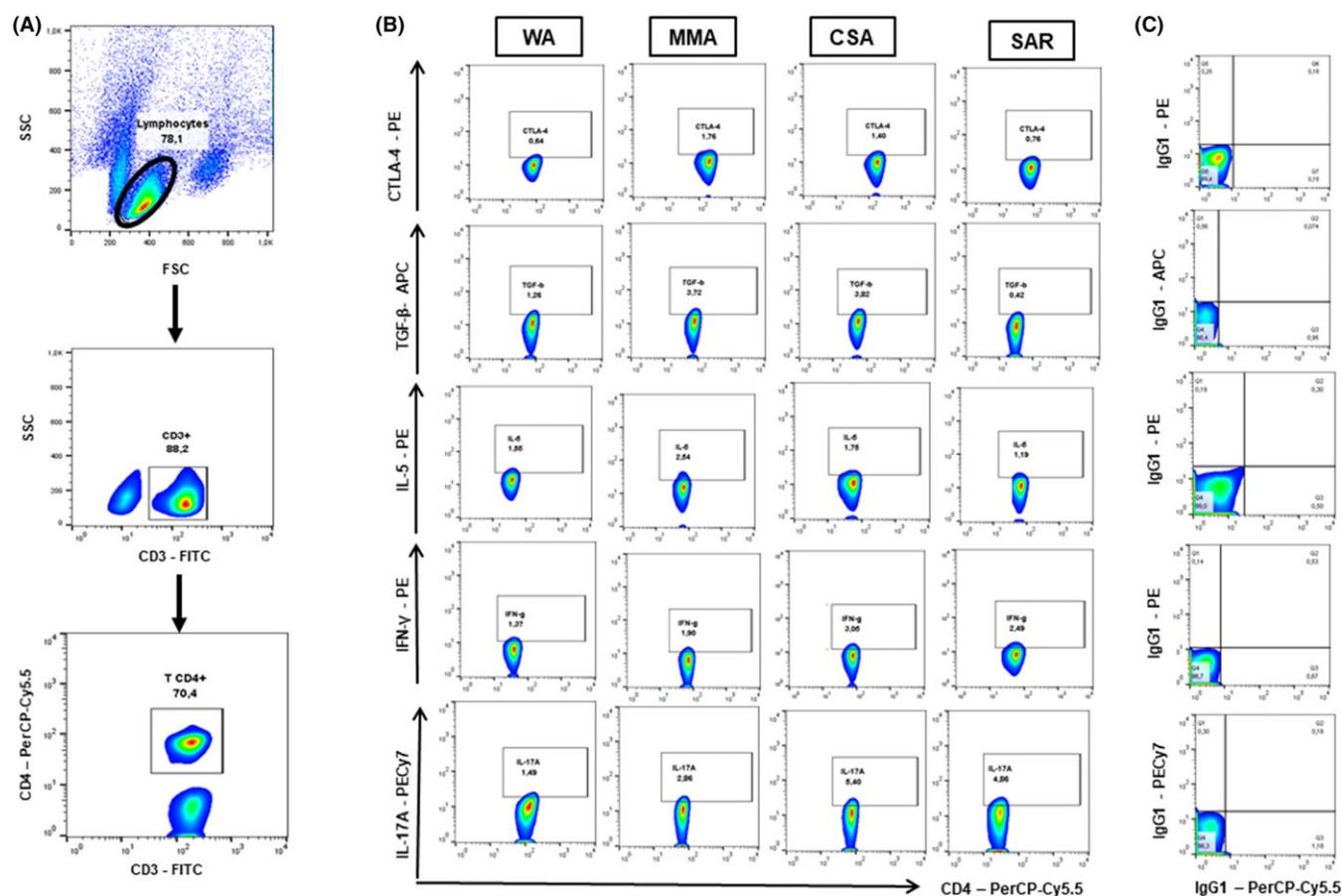


FIGURE 1 Densitometric image of non-specific fluorescence from the forward scatter (FSC) and side scatter (SSC), identifying the lymphocyte population and then selection of T lymphocyte population with CD3 marker and the subpopulation CD4⁺ gated within the T lymphocytes (A). Representative plots of CD4 T cells expressing CTLA-4, TGF- β , IL-5, IFN- γ and IL-17A from a patient of each group (B) and their corresponding isotype controls (C). Representative graphs of one experiment. Legends: WA (without asthma), MMA (mild-to-moderate asthma), CSA (well-controlled/partially controlled severe asthma), SAR (severe asthma refractory to treatment)

and then the CD4⁺ marker (Figure 1A). After this selection, we evaluated the expression of surface markers and intracellular cytokines.

2.4 | Vitamin D analysis

Vitamin D was evaluated in serum by the chemiluminescence method (Architect i1000, ABBOTT, Illinois, USA).

2.5 | Statistical analysis

Most of the statistical analysis and graphical representation were performed using the GraphPad PRISM 5.0 software (La Jolla, CA), and linear regression analysis was performed using the SPSS Statistics Software (version 17.0, SPSS Inc, Chicago, IL). Comparisons of continuous variables between 2 or more groups were performed using nonparametric methods, such as Mann-Whitney test and Kruskal-Wallis test, respectively. For categorical data and correlation analysis were used the chi-squared test and Spearman correlation, respectively. The results are expressed in median (first and third quartiles). All statistical tests were two-tailed, and the value $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Characteristics of study population

Table 1 shows the characteristics of the population. The mean age of the SAR group was higher than the MMA group ($P < 0.05$). We

also observed a significant difference in relation to the frequency of female sex. The pre- and post-bronchodilator FEV₁ percentage was lower in the SAR and CSA groups compared to the WA group, and it was also decreased in the SAR group in relation to the MMA group. The BMI was higher in patients with SAR compared to patients with CSA, MMA and WA, and in the CSA group in relation to WA group. We did not observe differences among groups related to the positivity of SPT and/or specific IgE, age of asthma onset, total IgE levels, eosinophil and neutrophil count in peripheral blood nor serum vitamin D.

Evaluating phenotypes of asthma according to atopy status and age of asthma onset (Figure 2), we observed that in the MMA group, 14 subjects were AT (eight EOA and six LOA) and nine were NA (six EOA and three LOA); in the CSA group, 14 were AT (11 EOA and three LOA) and seven were NA (three EOA and four LOA); and in the group with SAR, 13 were AT (eight EOA and five LOA) and six were NA (two EOA and four LOA).

3.2 | The refractoriness in individuals with early-onset asthma and atopic is associated with a lower frequency of CD4 T lymphocytes expressing CTLA-4

The frequency of CD4 T lymphocytes expressing their costimulatory molecules, CD28 and CTLA-4, are shown in Table 2 and Figure 3. Regarding the activation molecule CD28, we observed lower numbers of CD4⁺CD28⁺ T lymphocyte in CSA group when compared to WA group (Table 2). We did not observe difference in the

TABLE 1 Characteristics of the study population

	WA (n = 10)	MMA (n = 23)	CSA (n = 21)	SAR (n = 19)	P
Age (years) ^{e,g}	46 (37.5-56.2)	36 (28-50)	46 (30.5-51)	53 (36-59)	<0.05 ^a
Female sex n (%) ^f	10 (100)	18 (78.3)	11 (52.3)	16 (84.2)	<0.05
Positive SPT n (%) ^f	3 (30)	12 (52.2)	13 (61.9)	12 (63.1)	0.46
Positive specific IgE n (%) ^f	2 (20)	14 (60.8)	12 (57.1)	10 (52.6)	0.17
Age of asthma onset (y) ^{e,g}	NA	7 (1-25)	6 (1.5-16)	6 (2-27)	0.81
IgE total (UI/mL) ^{e,g}	192 (31-832)	163 (44.3-675.6)	293.4 (73.3-847.3)	261.7 (164.7-480)	0.42
Pre-BD FEV ₁ (%) ^{e,g}	89.8 (86.9-110.7)	85.1 (79.1-90.9)	70.7 (63.2-83.6)	64 (55.3-74.73)	<0.05 ^{a,b,c}
Post-BD FEV ₁ (%) ^{e,g}	99 (91.6-109.3)	88.5 (81.2-92.9)	79.3 (68.2-91.9)	72.5 (63.7-79.3)	<0.05 ^{a,b,c}
BMI ^{e,g}	25 (24.3-29.3)	27.8 (25-29.3)	27.2 (24-29.4)	30.2 (29.3-34.8)	<0.05 ^{a,b,d}
Eosinophils/mm ³ ^{e,g}	148 (102.3-313)	271 (119-433)	272 (162.5-368)	185 (96-256)	0.32
Neutrophils/mm ³ ^{e,g}	4093 (3424-4317)	3730 (2883-5345)	3018 (2374-3756)	3581 (2500-4676)	0.27
Vitamin D (ng/mL)	32.5 (22.1-39.9)	29.1 (26.7-36.4)	29.5 (26.8-35.2)	29.6 (19.6-35.6)	0.84

BMI, body mass index; CSA, well-controlled/partially controlled severe asthma; MMA, mild-to-moderate asthma; NA, not applicable; Pre- and Post-BD FEV₁, pre- and post-bronchodilator forced expiratory volume in one-second; SAR, severe asthma refractory to treatment; SPT, skin prick test; WA, without asthma.

^aSAR vs MMA.

^bSAR vs WA.

^cCSA vs WA.

^dSAR vs CSA.

^eKruskal-Wallis, Dunn's post-test.

^fChi-square.

^gMedian (1st quartile-3rd quartile).

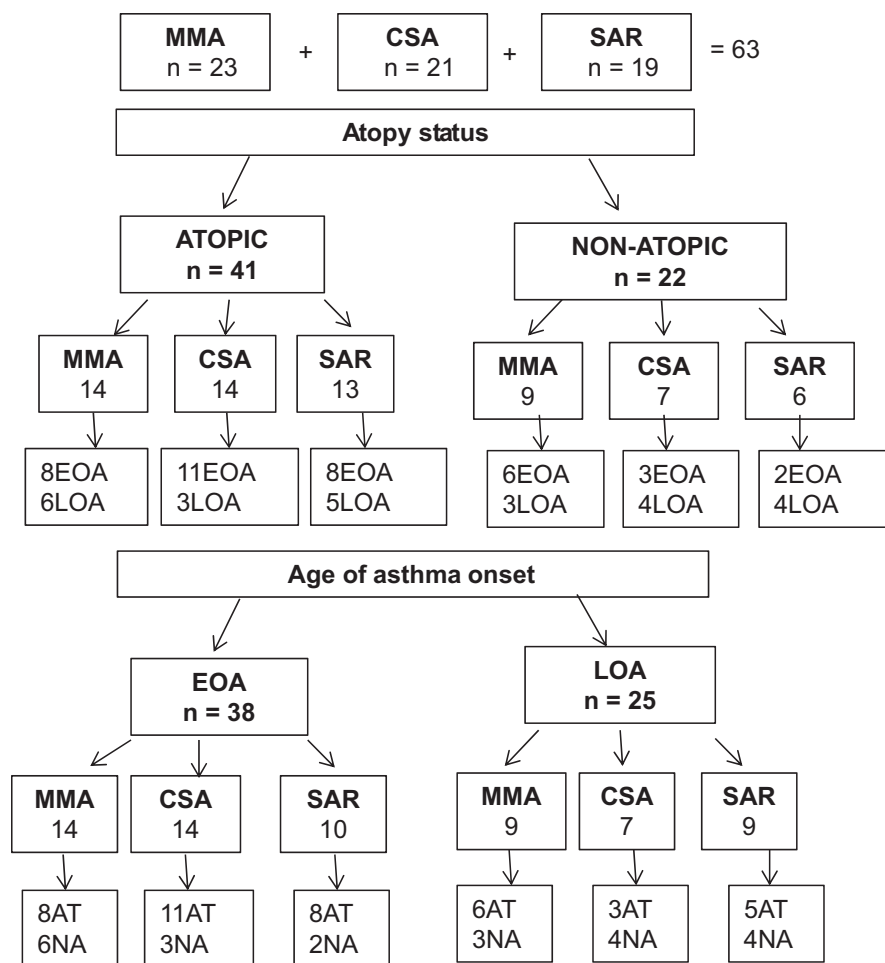


FIGURE 2 Subgroups of the study population regarding the atopy status and age of asthma onset. Legends: MMA (mild-to-moderate asthma), CSA (well-controlled/partially controlled severe asthma), SAR (severe asthma refractory to treatment), AT (atopic), NA (non-atopic), EOA (early-onset asthma), LOA (late-onset asthma)

expression of this molecule among the groups according to atopy status and age of asthma onset (Figure 3A,D).

Concerning the CTLA-4, we found a higher expression of this regulatory molecule by CD4⁺ T lymphocytes from patients with CSA and MMA when compared to cells from subjects with SAR and WA (Table 2 and Figure 1B). Furthermore, we also noted that in the atopic group, the frequency of CD4⁺CTLA-4⁺ T cells was lower in the SAR patients [0.7% (0.3-1.1)] than in CSA patients [2.03% (0.6-2.4)], $P < 0.05$, and in non-atopic asthmatics, the frequency of this marker was lower in subjects with SAR [0.7% (0.5-0.7)] when compared to the MMA subjects [1.8% (0.9-2.8)], $P < 0.01$ (Figure 3B). Similarly, we also observed a lower frequency of CD4⁺CTLA-4⁺ T cells in subjects with EOA [0.7% (0.3-0.8)] in the SAR group when compared with CSA [1.6% (0.7-2.4)] and MMA subjects [1.0% (0.6-1.8)] (Figure 3E). No significant difference was found in CD4⁺CTLA-4⁺ T cells among the individuals with LOA. Moreover, the linear regression analysis showed that the decrease in frequency of CD4⁺CTLA-4⁺ T cells appears to be related to high dose of ICS (Table S1).

To better understand the behaviour of these molecules, we made a ratio of the frequency of CD4⁺CD28⁺ T cells and CD4⁺CTLA-4⁺ T cells. We noticed a higher ratio in the SAR group compared to the CSA and MMA groups (Table 2). Regarding the atopy status, we observed a higher ratio in individuals with SAR, in both atopic [76.3

(45.1-134.7)] and non-atopic individuals [112.7 (92.6-145.4)] when compared to subjects with CSA [AT 38.7 (27.6-90.6) and NA 58.7 (42.2-106.5)]. We also observed that among non-atopic asthmatics, this ratio was higher in the SAR group than in the MMA group [35.4 (19.0-89.9)] (Figure 3C). In relation to the age of asthma onset, we found that among the individuals with EOA, this ratio was higher in SAR group [99.7 (40.4-144.7)] than in the CSA group [41.2 (28.4-100.4)] (Figure 3F). No significant difference was observed among the LOA individuals.

3.3 | The refractoriness in individuals with late-onset asthma and non-atopic is associated with a lower frequency of CD4⁺ T lymphocytes expressing FoxP3

Table 2 and Figure 4 show the frequency of CD4⁺ T cells expressing regulatory molecules such as IL-10, FoxP3 and TGF- β among asthmatic groups. We did not observe significant difference in the frequency of CD4⁺IL-10⁺ T cells among the groups (Table 2) nor even when we considered the atopy status (Figure 4A). However, among LOA patients, we observed a lower frequency of CD4⁺IL-10⁺ T cells in the SAR group [1.4% (0.7-1.6)] than in the MMA group [2.9% (1.4-4.2)] (Figure 4D).

TABLE 2 Frequency of CD4 T cells expressing costimulatory, regulatory, Type 2 and Th1 molecules and ratio of the costimulatory molecules in individuals with asthma

	WA (n = 10)	MMA (n = 23)	CSA (n = 21)	SAR (n = 19)	P
T CD4 ⁺ (%) ^{f,g}	70.4 (65.2-74.4)	66.7 (56.8-73.4)	73.1 (55.1-77.9)	69.1 (62.5-75.5)	0.53
T CD4 ⁺ CD28 ⁺ (%) ^{f,g}	80.6 (59.9-90.5)	68.9 (46.6-80.8)	62.3 (50.3-76.9)	66.1 (33.4-76.8)	<0.05 ^a
T CD4 ⁺ CTLA-4 ⁺ (%) ^{f,g}	0.6 (0.4-0.7)	1.2 (0.9-2.1)	1.3 (0.7-2.2)	0.7 (0.4-0.9)	<0.01 ^{a,b,c,d}
CD28/CTLA-4 ^{f,g}	111.8 (77-161.5)	50.3 (31.5-77.5)	43.6 (29.7-93.7)	86.9 (48.5-118.1)	<0.05 ^{c,d}
T CD4 ⁺ IL-10 ⁺ (%) ^{f,g}	1.1 (0.7-1.9)	1.7 (1.0-3.6)	1.4 (0.9-1.9)	1.3 (0.7-1.7)	0.06
T CD4 ⁺ FoxP3 ⁺ (%) ^{f,g}	3.4 (2.8-5.7)	3.7 (2.2-6.7)	4.9 (2.9-7.0)	3.5 (2.2-5.5)	0.49
T CD4 ⁺ TGF-β ⁺ (%) ^{f,g}	1.1 (0.6-1.2)	2.3 (1.4-5.1)	5.1 (1.7-6.6)	1.3 (0.6-2.2)	<0.05 ^{a,b,c,d}
T CD4 ⁺ IL-5 ⁺ (%) ^{f,g}	1.1 (0.7-1.6)	2.1 (1.4-4.2)	1.6 (1.0-2.1)	1.2 (1.0-2.5)	<0.05 ^{b,d}
T CD4 ⁺ IL-13 ⁺ (%) ^{f,g}	1.3 (0.5-1.7)	1.3 (0.8-1.8)	1.4 (0.7-2.4)	0.9 (0.5-1.7)	0.70
T CD4 ⁺ IFN-γ ⁺ (%) ^{f,g}	1.2 (0.5-1.7)	1.6 (1.1-2.7)	1.9 (0.9-3.3)	2.3 (1.6-2.6)	<0.05 ^{a,e}
T CD4 ⁺ IL-17A ⁺ (%) ^{f,g}	1.3 (0.9-2.8)	2.6 (1.3-5.3)	4.5 (1.9-6.5)	2.6 (1.9-6.3)	<0.05 ^{a,e}

CSA, well-controlled/partially controlled severe asthma; MMA, mild-to-moderate asthma; SAR, severe asthma refractory to treatment; WA, without asthma.

^aCSA vs WA.

^bMMA vs WA.

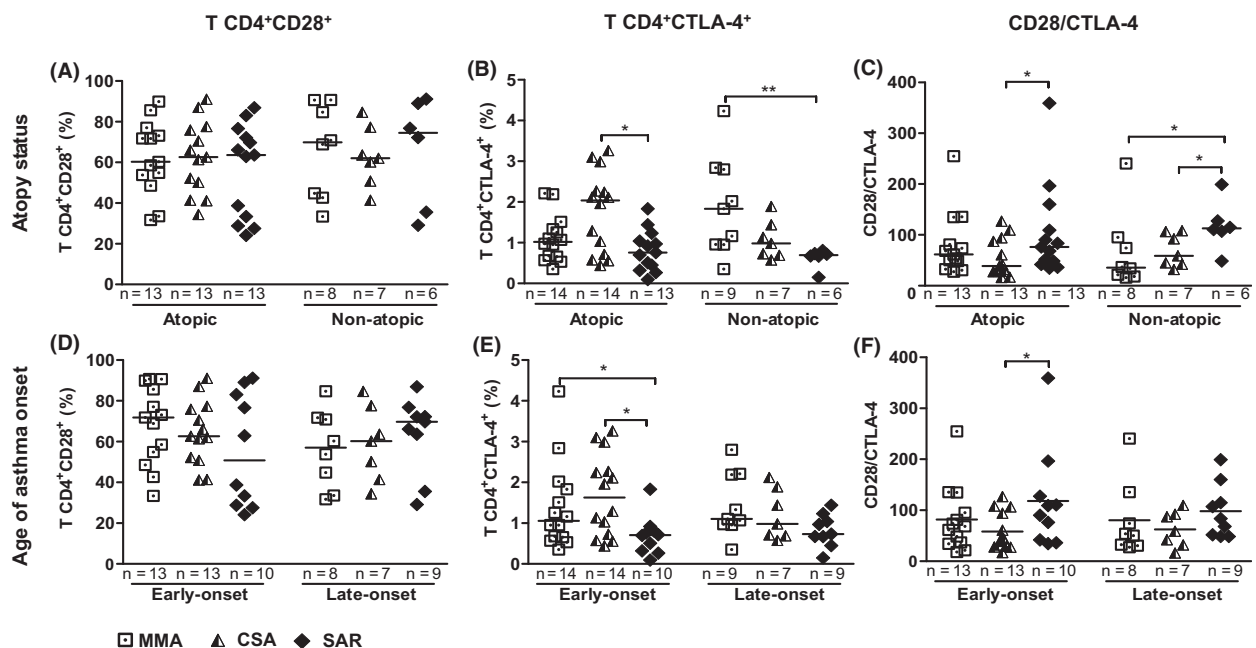
^cSAR vs CSA.

^dSAR vs MMA.

^eSAR vs WA.

^fKruskal-Wallis, Dunn's post-test.

^gMedian (1st quartile-3rd quartile).

**FIGURE 3** Frequency of CD4 T lymphocytes expressing costimulatory molecules, CD28 and CTLA-4, and the ratio of these molecules, CD28/CTLA-4, regarding their atopy status (A-C) and age of asthma onset (D-F). Legends: MMA (mild-to-moderate asthma), CSA (well-controlled/partially controlled severe asthma), SAR (severe asthma refractory). The bars represent the median values of each data set.

*P < 0.05, **P < 0.01. Kruskal-Wallis, Dunn's post-test

In relation to CD4⁺FoxP3⁺ T cells, we did not observe significant differences among groups either (Table 2). However, when we analysed according to the atopy status and age of asthma onset, we

noticed that among NA and LOA individuals, the frequency of these cells was lower in SAR group [NA 3.1% (2.1-4.5) and LOA 3.5% (2.1-4.0)] than in CSA group [NA 6.1% (4.9-8.1) and LOA 5.6% (4.6-8.0)]

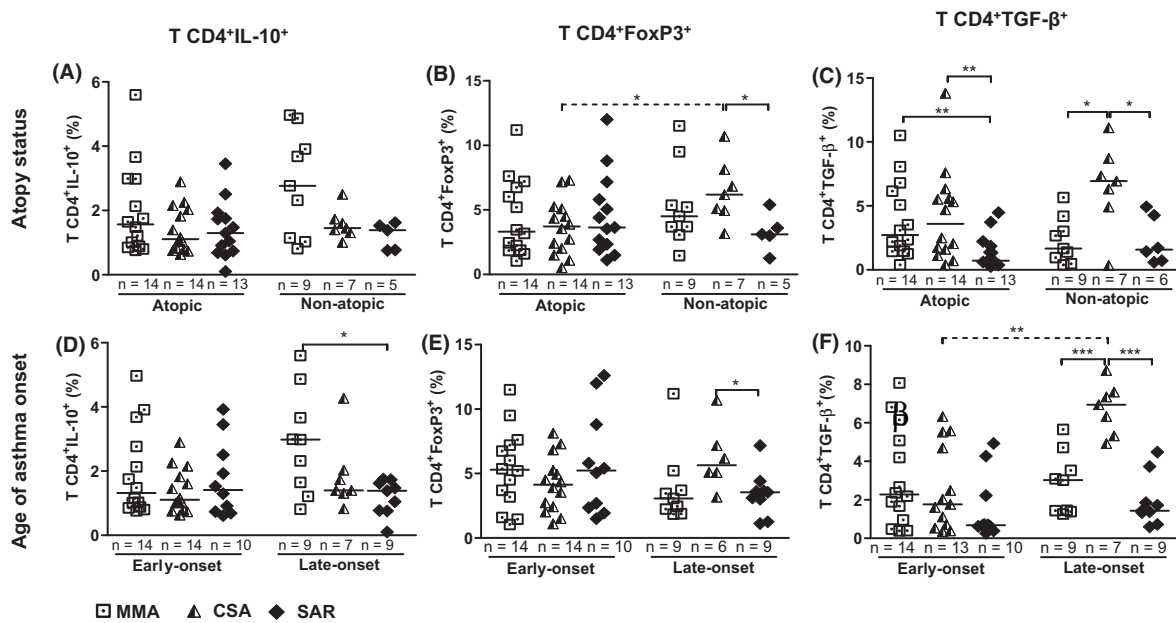


FIGURE 4 Frequency of CD4 T lymphocytes expressing IL-10, FoxP3 and TGF-β among asthmatic groups (A-C), regarding their atopy status (A-C) and age of asthma onset (D-F). Legends: MMA (mild-to-moderate asthma), CSA (well-controlled/partially controlled severe asthma), SAR (severe asthma refractory). The bars represent the median values of each data set. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Filled line: Kruskal-Wallis, Dunn's post-test and dotted line: Mann-Whitney Test

(Figure 4B,E). In addition, we found that in the non-atopic CSA subjects, the frequency of CD4⁺FoxP3⁺ T cells was higher than in atopic CSA [3.7% (1.9-5.1)]. No significant difference was found in this population among atopic and EOA individuals.

We also observed that the frequency of CD4⁺TGF-β⁺ T lymphocytes in the group of individuals with CSA and MMA was higher than in individuals with SAR and WA (Table 2 and Figure 1B). Moreover, the frequency of CD4⁺TGF-β⁺ T cells was similar among AT and NA asthmatics, and lower in individuals with SAR [AT 0.7% (0.6-2.0) and NA 1.5% (0.7-4.4%)] than in CSA subjects [AT 3.5% (1.4-5.7) and NA 6.9% (4.9-8.7)] (Figure 4C). Among AT asthmatics, the frequency of these cells was also lower in the SAR group when compared to the MMA group [2.7% (1.4-6.3)], and in NA asthmatics, the frequency of CD4⁺TGF-β⁺ T cells was increased in subjects with CSA compared to individuals with MMA [1.6% (0.7-3.6)]. Regarding LOA subjects, the frequency of CD4⁺TGF-β⁺ T cells was higher in CSA group [6.9% (5.3-7.6)] when compared to SAR and MMA groups [1.4% (1.0-2.8) and 3.0% (1.4-4.1), respectively; $P < 0.001$]. These cells were also elevated in CSA individuals with LOA when compared to EOA [1.7% (0.6-5.1), $P < 0.01$] (Figure 4F).

After the linear regression analysis, we observed that only the decrease in frequency of CD4⁺TGF-β⁺ T cells appears to be influenced by high doses of ICS (Table S1). Regarding the vitamin D, we did not observe a significant correlation of serum vitamin D levels with the frequency of CD4 T lymphocytes expressing TGF-β, IL-10 or FoxP3 (Figure S1A-C).

3.4 | The refractoriness in atopic individuals is associated with a higher frequency of CD4 T lymphocytes expressing IFN-γ

The frequencies of CD4⁺ T cells expressing Th2 (IL-5 and IL-13), Th1 (IFN-γ) and Th17 (IL-17A) cytokines are in Table 2 and Figure 5.

In patients with MMA, the frequency of CD4⁺IL-5⁺ T cells was increased when compared to individuals with SAR and WA (Table 2 and Figure 1B). We did not observe a significant difference in these cells among asthmatics considering their atopy status and age of asthma onset (Figure 5A,E). Furthermore, we found no significant differences in the frequency of CD4⁺IL-13⁺ T cells among asthmatics groups neither (Table 2), not even when considered their atopy status (Figure 5B) and age of asthma onset (Figure 5F).

On the other hand, we observed a higher frequency of CD4⁺ T lymphocytes expressing IFN-γ or IL-17A in individuals with SAR and CSA when compared to WA individuals (Table 2 and Figure 1B). Although we did not observe a significant difference in correlation between the frequency of CD4⁺IL-17A⁺ T and CD4⁺IFN-γ⁺ T cells in each group (Figure S2A-D), we observed a moderately positive correlation when both severe groups (CSA and SAR) were evaluated together (Figure S2E). We observed a similar correlation between the frequency of CD4⁺IL-17A⁺ T and CD4⁺IL-5⁺ T cells (Figure S2F-J). Concerning the atopy status, we observed that among the atopic patients, individuals with SAR showed a higher frequency of CD4⁺IFN-γ⁺ T cells [8.1% (2.6-10.5)] than those with CSA [4.3% (2.9-5.9)] (Figure 5C). We did not observe difference in frequency of

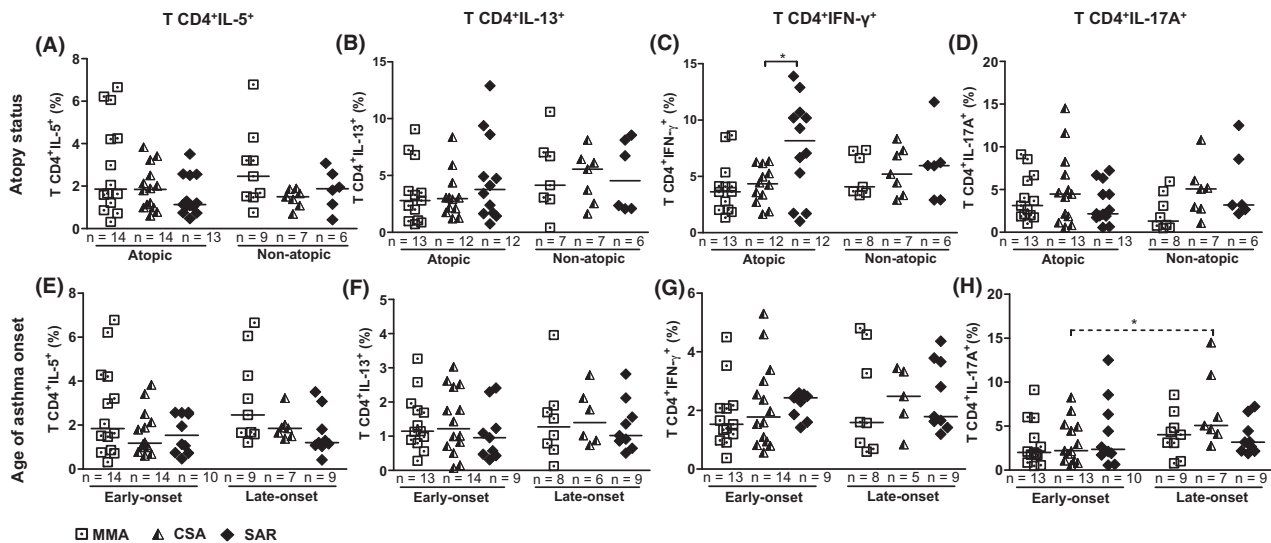


FIGURE 5 Frequency of CD4 T lymphocytes expressing Type 2 (IL-5 and IL-13), Th1 (IFN-γ) and Th17 (IL-17A) cytokines regarding their atopy status (A-D) and age of asthma onset (E-H). Legends: MMA (mild-to-moderate asthma), CSA (well-controlled/partially controlled severe asthma), SAR (severe asthma refractory). The bars represent the median values of each data set. * $P < 0.05$, ** $P < 0.01$. Filled line: Kruskal-Wallis, Dunn's post-test and dotted line: Mann-Whitney Test

CD4⁺IFN-γ⁺ T cells among the NA asthmatics. We also did not find difference in the frequency of CD4⁺IL-17A⁺ T cells regarding the atopy status (Figure 5D). In relation to the age of asthma onset, we did not find any significant difference in the frequency of CD4⁺ T cells expressing IFN-γ or IL-17A among groups (Figure 5G,H) either. However, we observed that subjects with CSA with LOA phenotype showed a higher frequency of CD4⁺IL-17A⁺ T cells [5.0% (4.1-10.8)] than EOA phenotype subjects [2.2% (1.1-5.0)] (Figure 5H).

4 | DISCUSSION

In this study, we evaluated the phenotypic profile of CD4 T lymphocytes of individuals with severe asthma according to their response to treatment, atopy status and age of asthma onset. Despite the well-known involvement of eosinophils and/or neutrophils in asthma, we found no significant difference between the groups. What could explain our results is that a greater recruitment of these cells locally in the lower airways may be occurring, whereas one cannot see differences in the blood. The sputum may be the ideal sample for observing these phenomena. Another possible explanation is the small sample size. One can notice that the interval estimates of eosinophils and neutrophils are overlapping in the groups. Regarding the expression of costimulatory molecules by T lymphocytes, we evaluated CD28, a positive regulator of T cell activation, and CTLA-4, an inhibitory signalling molecule that regulates the immune response and maintains the peripheral tolerance.²¹ In our study, we observed that individuals with SAR had a lower frequency of CD4⁺CTLA-4⁺ T lymphocytes than MMA and CSA groups, mainly in those atopic and with EOA, and that this decrease seems to be influenced by the high dose of ICS. Study of Pietruczuk et al²² observed that expression of

CTLA-4 in regulatory T cells was decreased in subjects with SA compared to subjects with MMA. Another study showed that CTLA-4 polymorphisms may play an important role in atopy in asthmatic subjects.²³ Our study, in turn, shows that the lower frequency of CD4⁺CTLA-4⁺ T cells in individuals with SAR may be associated with a reduced ability to regulate the immune response, suggesting a role of this molecule in refractoriness to treatment of asthma. The ratio of these costimulatory molecules also reinforces this possibility.

Other molecules associated with the regulatory response, such as IL-10, FoxP3 and TGF-β, were also evaluated. Studies have shown that both inhaled and systemic glucocorticoid treatment, in moderate and severe asthmatics, increased mRNA expression for Foxp3 and IL-10 in CD4⁺ T cells from the peripheral blood,²⁴ although TGFβ expression remained unchanged.^{25,26}

We did not observe significant differences in IL-10 expression by CD4⁺ T lymphocytes among groups with different phenotypes associated with asthma severity. One possible explanation is that there are other cellular sources, in addition to lymphocytes, involved in the expression of this cytokine. A study evaluating the expression and production of IL-10 by mRNA and ELISA showed that monocytes but not T or B lymphocytes would be involved in the production of IL-10 after stimulation with dexamethasone.²⁷

When evaluating the phenotype subgroups, we found that individuals with late-onset SAR had less CD4⁺IL-10⁺ T cells when compared to individuals with MMA. There are no studies addressing the frequency of expression of IL-10 in these cells considering the age of asthma onset phenotype in patients with refractory asthma.

There was also no difference in the expression of FoxP3 by CD4⁺ T lymphocytes in groups with different phenotypes associated with asthma severity. When we evaluated the expression of FoxP3 in the subgroups of phenotypes, we observed in NA and LOA groups

a lower frequency of FoxP3 expression in SAR patients. There is a previous work studying the FoxP3 mRNA expression showing an increase in the expression of this molecule in asthmatics patients with severe disease treated with glucocorticoids.²⁴ We described that in SAR patients, there are less FoxP3⁺ CD4⁺ cells compared with CSA group, and we concluded that FoxP3 would be a predictor of treatment refractoriness.

The role of TGF- β remains controversial in asthma, since this cytokine plays a dual role in the disease, both regulating the immune response and remodelling of the airways.^{28,29} In the present study, we observed that the frequency of CD4⁺TGF- β ⁺ T lymphocytes was decreased in subjects with SAR when compared to individuals with CSA and MMA, and this low frequency also appears to be associated with the high dose of ICS used by these individuals. Moreover, we also observed a lower frequency of CD4⁺TGF- β ⁺ T cells in atopic, non-atopic and LOA individuals in the SAR group than in the CSA group, thus indicating that a lower expression of TGF- β by CD4 T cells may also contribute to poor response to treatment in severe asthma.

It is known that vitamin D induces immunomodulatory effects.^{29,30} Although several studies have shown a relationship of vitamin D with frequencies of IL-10⁺ Treg,³¹ TGF- β ⁺ Treg³² and FoxP3⁺ Treg cells,³³ we did not observe a significant correlation of serum vitamin D levels with frequency of CD4 T lymphocytes expressing these markers. Therefore, this nutrient does not appear to be significantly influencing the regulatory cells. Reid et al³⁴ also observed that in vivo treatment of vitamin D-insufficient asthmatic patients with vitamin D had no detectable effect on the expression of regulatory cytokines, as IL-10, as well as expression of IL-4, IFN- γ and IL-17A by CD4⁺ T cells.

Regarding the Th2 cytokines, we found a higher frequency of CD4⁺IL-5⁺ T lymphocytes in the MMA group than in the SAR group, similarly reported by Smith et al³⁵ but no significant difference was noted regarding the expression of IL-13. Perhaps in individuals with MMA, the Th2 response may be more evident, at the same time, that it appears to be modulated easily by regulatory molecules that are upregulated in that group. Furthermore, no difference was observed in the expression of IL-5 and IL-13 by CD4⁺ T cells according to atopy status and age of asthma onset. Smith et al³⁵ found that ILCs2 of sputum samples from patients with SA produced more Th2 cytokines, such as IL-5 and IL-13, than the CD4 T cells. Another study showed a higher frequency of IL-13⁺ILCs2 cells in individuals with uncontrolled and partially controlled asthma when compared to the individuals with controlled asthma and healthy controls, and that these cells were more resistant to the action of glucocorticoids than Th2 cells in vitro.³⁶ Although we have not observed increased expression of Th2 cytokines by CD4 T lymphocytes in individuals with SA, we cannot be confident there is no Th2 response in the disease, since most patients with severe asthma are atopic according to the positivity of SPT and/or specific IgE. Therefore, innate cells may be a major driver of Th2 response, playing a more important role in the severity of asthma than CD4 T cells.

In relation to the cytokines of the Th1 and Th17 profile, we found a higher frequency of CD4 T lymphocytes expressing IFN- γ or IL-17A in both the severe asthma groups compared to individuals WA, suggesting that both cytokines are involved in the immunopathogenesis of severe asthma, regardless the treatment response pattern. Our correlation findings on the frequency of CD4⁺IFN- γ ⁺ T and CD4⁺IL-17A⁺ T cells reinforce this possibility, as well as our correlation of the frequencies of CD4⁺IL-17A⁺ T cells with CD4⁺IL-5⁺ T cells. However, Hasegawa et al³⁷ observed that high serum levels of IL-17A in combination with upregulated Th2, IL-10 and IFN- γ cytokines may indicate the refractory endotype of asthma. Beside this, Chambers et al³⁸ have also shown high levels of both cytokines IFN- γ and IL-17A in PBMC of patients with corticosteroid-resistant severe asthma compared to patients who are sensitive to treatment. Considering the atopy status, we observed in our study a higher frequency of CD4⁺IFN- γ ⁺ T cells in atopic SAR individuals, confirming that refractoriness to treatment in atopic patients is associated with increased Th1 response. Some authors have also observed an elevated frequency of IFN- γ ⁺ T cells in atopic asthmatic children,³⁹ while others did not observe difference between atopic asthmatic children and healthy controls.⁴⁰

Moreover, though we observed a higher frequency of CD4⁺IL-17A⁺ T cells in the CSA group with LOA than in those with EOA, we also found a higher frequency of CD4⁺TGF- β ⁺ T cells in this same group, suggesting an attempt to balance the immune response in these individuals.

Thus, our findings suggest that refractoriness is probably associated with impaired regulatory functions of CD4 T cells. In atopic and EOA asthmatics patients, refractoriness seems to be associated with lower expression of the regulatory molecule CTLA-4 and higher expression of IFN- γ , the latter being only observed among atopic individuals. In non-atopic and LOA individuals, refractoriness is associated with lower expression of FoxP3, whereas lower TGF- β was only observed in the LOA phenotype. These data suggest refractoriness to treatment is associated with reduced distinct regulatory molecules by CD4 T cells in those who are atopic and EOA and those who are non-atopic and LOA.

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CONFLICT OF INTEREST

All authors declare that there are no conflicts of interests to report in relation to this manuscript.

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REFERENCES

- GBD Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet*. 2016;388:1545-1602.
- GINA Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention, 2017.
- ATS Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. *Am J Respir Crit Care Med*. 2000;162: 2341-2351.
- Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol*. 2015;16:45-56.
- Chung KF, Wenzel SE, Brozek JL, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J*. 2014;43:343-373.
- Wenzel SE. Asthma: defining of the persistent adult phenotypes. *Lancet*. 2006;368:804-813.
- Holguin F, Bleecker ER, Busse WW, et al. Obesity and asthma: an association modified by age of asthma onset. *J Allergy Clin Immunol*. 2011;127:1486-1493. e1482.
- Robinson DS, Hamid Q, Ying S, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med*. 1992;326:298-304.
- Wenzel SE, Schwartz LB, Langmack EL, et al. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am J Respir Crit Care Med*. 1999;160:1001-1008.
- Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med*. 2002;346:1699-1705.
- Bousquet J, Chanez P, Lacoste JY, et al. Eosinophilic inflammation in asthma. *N Engl J Med*. 1990;323:1033-1039.
- Gascan H, Gauchat JF, Roncarolo MG, Yssel H, Spits H, de Vries JE. Human B cell clones can be induced to proliferate and to switch to IgE and IgG4 synthesis by interleukin 4 and a signal provided by activated CD4+ T cell clones. *J Exp Med*. 1991;173:747-750.
- Klunker S, Trautmann A, Akdis M, et al. A second step of chemotaxis after transendothelial migration: keratinocytes undergoing apoptosis release IFN-gamma-inducible protein 10, monokine induced by IFN-gamma, and IFN-gamma-inducible alpha-chemoattractant for T cell chemotaxis toward epidermis in atopic dermatitis. *J Immunol*. 2003;171:1078-1084.
- Holgate ST, Polosa R. Treatment strategies for allergy and asthma. *Nat Rev Immunol*. 2008;8:218-230.
- Zhao J, Lloyd CM, Noble A. Th17 responses in chronic allergic airway inflammation abrogate regulatory T-cell-mediated tolerance and contribute to airway remodeling. *Mucosal Immunol*. 2012;6:335-346.
- McKinley L, Alcorn JF, Peterson A, et al. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J Immunol*. 2008;181:4089-4097.
- Macessier E, Nieves A, Lorec AM, et al. T-cell activation during exacerbations: a longitudinal study in refractory asthma. *Allergy*. 2008;63:1202-1210.
- Chambers ES, Nanzer AM, Richards DF, et al. Serum 25-dihydroxyvitamin D levels correlate with CD4(+)Foxp3(+) T-cell numbers in moderate/severe asthma. *J Allergy Clin Immunol*. 2012;130:542-544.
- GINA Global Strategy for Asthma Management and Prevention. Global Initiative for Asthma (GINA), 2012.
- Tan DJ, Walters EH, Perret JL, et al. Age-of-asthma onset as a determinant of different asthma phenotypes in adults: a systematic review and meta-analysis of the literature. *Expert Rev Respir Med*. 2015;9:109-123.
- Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol*. 2005;23:515-548.
- Pietruczuk M, Eusebio M, Kraszula L, Kupczyk M, Kuna P. Phenotypic characterization of ex vivo CD4+CD25highCD127low immune regulatory T cells in allergic asthma: pathogenesis relevance of their FoxP3, GITR, CTLA-4 and FAS expressions. *J Biol Regul Homeost Agents*. 2012;26:627-639.
- Munthe-Kaas MC, Carlsen KH, Helms PJ, et al. CTLA-4 polymorphisms in allergy and asthma and the TH1/TH2 paradigm. *J Allergy Clin Immunol*. 2004;114:280-287.
- Karagiannis C, Akdis M, Holopainen P, et al. Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. *J Allergy Clin Immunol*. 2004;114:1425-1433.
- Hartl D, Koller B, Mehlhorn AT, et al. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *J Allergy Clin Immunol*. 2007;119:1258-1266.
- Stelmach I, Jerzynska J, Kuna P. A randomized, double-blind trial of the effect of glucocorticoid, antileukotriene and beta-agonist treatment on IL-10 serum levels in children with asthma. *Clin Exp Allergy*. 2002;32:264-269.
- Mozo L, Suarez A, Gutierrez C. Glucocorticoids up-regulate constitutive interleukin-10 production by human monocytes. *Clin Exp Allergy*. 2004;34:406-412.
- Schmidt-Weber CB, Blaser K. The role of TGF-beta in allergic inflammation. *Immunol Allergy Clin North Am*. 2006;26:233-244. vi-vii.
- Makinde T, Murphy RF, Agrawal DK. The regulatory role of TGF-beta in airway remodeling in asthma. *Immunol Cell Biol*. 2007;85:348-356.
- Ginde AA, Sutherland ER. Vitamin D in asthma: panacea or true promise? *J Allergy Clin Immunol*. 2010;126:59-60.
- Urry Z, Xystrakis E, Richards DF, et al. Ligation of TLR9 induced on human IL-10-secreting Tregs by 1alpha,25-dihydroxyvitamin D3 abrogates regulatory function. *J Clin Invest*. 2009;119:387-398.
- Kreindler JL, Steele C, Nguyen N, et al. Vitamin D3 attenuates Th2 responses to *Aspergillus fumigatus* mounted by CD4+ T cells from cystic fibrosis patients with allergic bronchopulmonary aspergillosis. *J Clin Invest*. 2010;120:3242-3254.
- Chauhan A, Singh M, Agarwal A, Sachdeva N, Attri SV. Interplay of vitamin D with T regulatory cells (FOXP3+Treg) and thymic stromal lymphopoietin (TSLP) in children with atopic diseases. *MOJ Immunology*. 2018;6:95-98.
- Reid B, Girodet PO, Boomer JS, et al. Vitamin D3 treatment of vitamin D-insufficient asthmatic patients does not alter immune cell function. *J Allergy Clin Immunol*. 2016;138:286-289. e289.
- Smith SG, Chen R, Kjarsgaard M, et al. Increased numbers of activated group 2 innate lymphoid cells in the airways of patients with severe asthma and persistent airway eosinophilia. *J Allergy Clin Immunol*. 2016;137:75-86. e78.
- Jia Y, Fang X, Zhu X, et al. IL-13(+) Type 2 Innate Lymphoid Cells Correlate with Asthma Control Status and Treatment Response. *Am J Respir Cell Mol Biol*. 2016;55:675-683.
- Hasegawa T, Uga H, Mori A, Kurata H. Increased serum IL-17A and Th2 cytokine levels in patients with severe uncontrolled asthma. *Eur Cytokine Netw*. 2017;28:8-18.
- Chambers ES, Nanzer AM, Pfeffer PE, et al. Distinct endotypes of steroid-resistant asthma characterized by IL-17A(high) and IFN-gamma(high) immunophenotypes: Potential benefits of calcitriol. *J Allergy Clin Immunol*. 2015;136:628-637. e624.
- Brown V, Warke TJ, Shields MD, Ennis M. T cell cytokine profiles in childhood asthma. *Thorax*. 2003;58:311-316.

40. Machura E, Mazur B, Rusek-Zychma M, Barc-Czarnecka M. Cytokine production by peripheral blood CD4+ and CD8+ T cells in atopic childhood asthma. *Clin Dev Immunol*. 2010;2010:606139.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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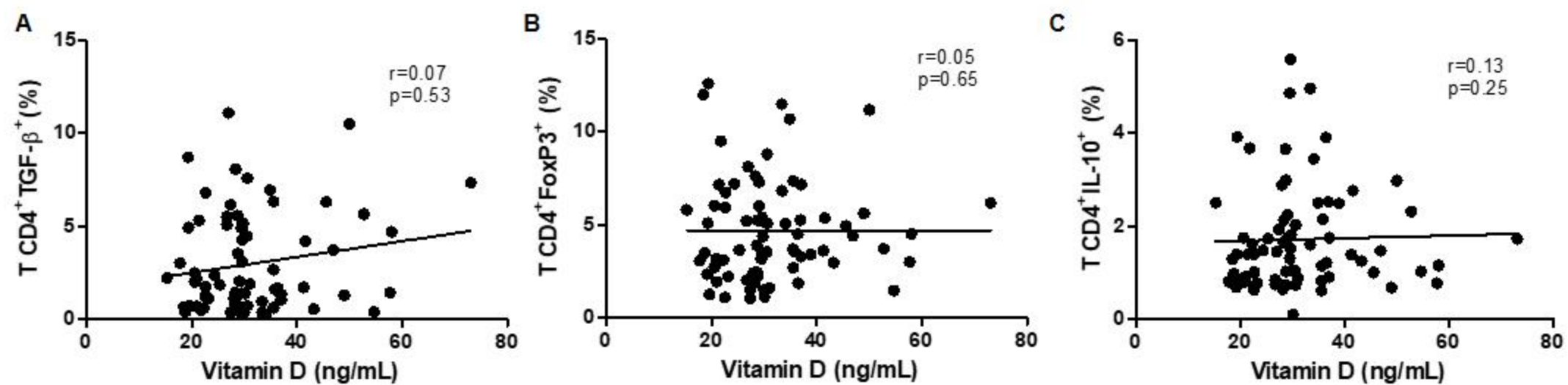


Fig S1: Correlation of serum vitamin D levels with frequency of CD4 T cell expressing the regulation markers TGF- β (A), FoxP3 (B) and IL-10 (C).

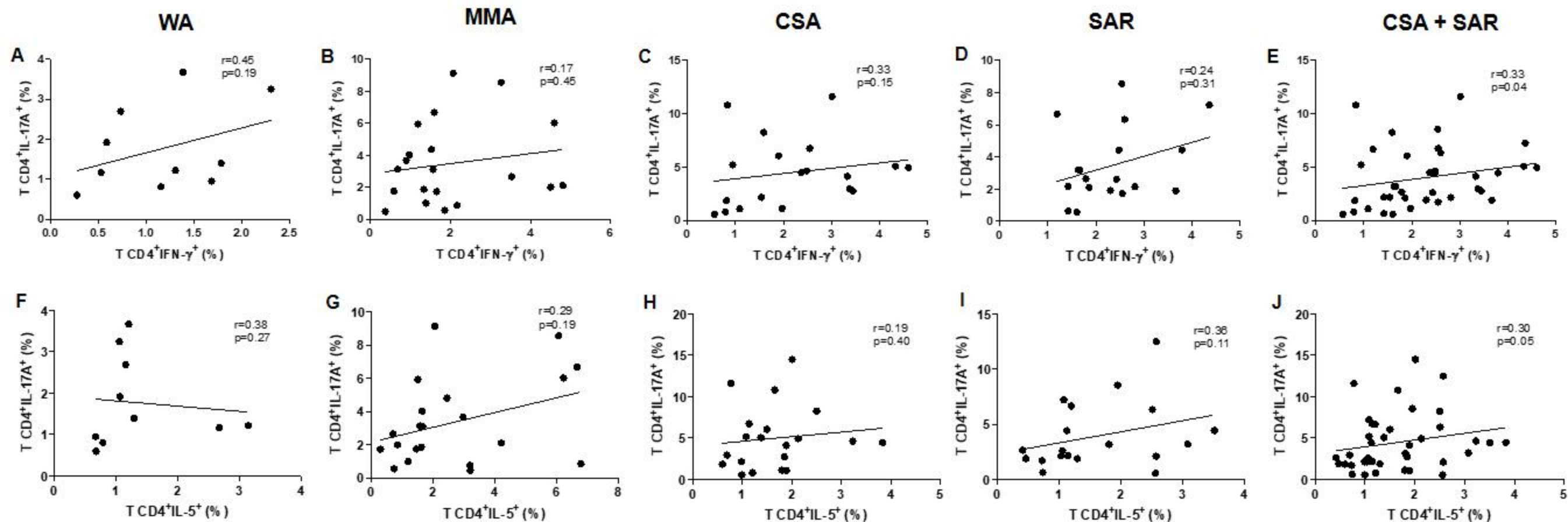


Fig S2: Correlation of the frequency of CD4⁺IL-17A⁺ T cell with the frequency of CD4⁺IFN- γ ⁺ T cell and CD4⁺IL-5⁺ T cell in individuals WA (A and F), MMA (B and G), CSA (C and H), SAR (D and I) and CSA + SAR (E and J). Legends: WA (without asthma), MMA (mild to moderate asthma), CSA (well-controlled/partially controlled severe asthma), SAR (severe asthma refractory to treatment).

Table S1. Linear regression: Influence of ICS dose on Treg cell populations in subjects with severe asthma (CSA - Well-controlled/partially controlled severe asthma and SAR - Severe asthma refractory to treatment).

T reg cells	β	CI	<i>P</i> value
T CD4 ⁺ CTLA-4 ⁺	-0,460	1.453 – 2.719	0,003
T CD4 ⁺ TGF- β ⁺	-0,350	3.343 – 7.917	0,027
T CD4 ⁺ FoxP3 ⁺	-0,20	2.440 – 7.458	0,90
T CD4 ⁺ IL-10 ⁺	0,10'	0.572 – 1.957	0,52

CI: Confidence interval