



Increased pro-inflammatory cytokine-secreting regulatory T cells are correlated with the plasticity of T helper cell differentiation and reflect disease status in asthma

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ARTICLE INFO

Keywords:

Asthma
Regulatory T cells
Th17
Th1

ABSTRACT

Background: Human regulatory T cells (Tregs) are a heterogeneous population which consists of three distinct subpopulations: CD25⁺CD45RA⁺ resting Treg (rTreg) cells; CD25^{hi}CD45RA⁺ activated Treg (aTreg) cells, which are both suppressive; and CD25⁺CD45RA⁺ cytokine-secreting T cells with pro-inflammatory capacity.

Objective: We investigated variation in peripheral Treg subpopulations of asthma and explored their potential roles in asthma inflammation.

Methods: Twenty-eight mild asthma patients, 26 moderate asthma patients, 18 severe asthma patients, and 36 healthy controls were recruited for a cross-sectional study. Phenotyping of peripheral CD4⁺ Tregs was performed based on flow cytometry results.

Results: The proportions of rTreg and aTreg cells among CD4⁺ T cells were higher in mild and moderate asthma patients than in healthy controls. All three groups of asthmatics had a higher proportion of pro-inflammatory Tregs than healthy controls, and these increased with asthma severity. The proportion of IL-17-producing Foxp3⁺ cells and IFN- γ -producing Foxp3⁺ cells strongly correlated with T helper 17 (Th17) cells ($r = 0.66$, $p < 0.001$) and Th1 cells ($r = 0.48$, $p < 0.001$). The pro-inflammatory Treg subpopulation was correlated with the severity of asthma and may be insensitive to corticosteroids.

Conclusions: Our data suggest that pro-inflammatory Treg subpopulations may be relevant to the plasticity of Th17 and Th1 differentiation and play an important role in the pathogenesis of asthma.

1. Introduction

Asthma is a complex and chronic disorder of conducting airways leading to variable airflow limitation, which is associated with airway hyper-responsiveness and ongoing active airway inflammation and tissue remodeling of the airway structure [1]. Airway inflammation, which is central to the pathophysiology of the disease, is related to activation of immune cells and the release of potent inflammatory mediators. Although classically thought to be a T helper 2 (Th2)-cell driven inflammatory disease, which is characterized by eosinophilic inflammation, Th2-cell associated cytokine production, and airway hyper-responsiveness (AHR), the immune response in asthma is

considered highly heterogeneous [2]. Other subsets, such as Th1, Th17, invariant natural killer T, and CD8⁺ T cells, likely contribute to corticosteroid sensitivity and the development and progression of the disease [3–5]. However, little is known about the regulatory mechanisms of inflammation that may affect the susceptibility to, severity of, or endotype of asthma.

There are numerous complex regulatory mechanisms for the maintenance of immune homeostasis, prevention of autoimmunity, and modulation of inflammation. Foremost amongst these are thymus-derived naturally occurring CD4⁺CD25⁺FOXP3⁺ regulatory T cells (nTreg), which are widely regarded as the primary forces in maintaining peripheral immune tolerance [6]. Studies show that FoxP3⁺

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natural Tregs are engaged in the prevention of immunopathological, auto-immune, and allergic diseases, and that their qualitative and/or quantitative defects can be a cause of these diseases [7,8].

FoxP3⁺Treg cells have long been considered an immunosuppressive population. However, human CD4⁺FoxP3⁺ T cells comprise three distinct subpopulations with precise phenotypes and fates [9]: CD25⁺⁺CD45RA⁺ FOXP3^{lo} resting Treg cells (rTreg cells), CD25⁺⁺⁺CD45RA⁺ (FOXP3^{hi}) activated Treg cells (aTreg cells), which represent different stages of Treg cell differentiation and are both suppressive *in vitro*, and CD25⁺⁺CD45RA⁺FOXP3^{lo} pro-inflammatory cytokine-secreting T cells, which lack suppressive activity and secrete IL17, IFN- γ , and IL-2. Since IL17 plays a key role in regulating neutrophilic and macrophage inflammation in the lung, which is insensitive to steroids [10], the cytokine-secreting Treg subpopulation should be considered when evaluating the immunologic mechanisms in asthma patients with different phenotypes. The delineation of Treg cell subpopulations would enable the assessment of the dynamics of Treg differentiation under distinct disease conditions.

Herein, we report that the pro-inflammatory subpopulation of peripheral Treg cells increases in asthma in correlation with Th17 and Th1 cell frequency and disease severity, suggesting that this subpopulation may be at least partially relevant to T helper cell differentiation and plays an important role in the pathogenesis of asthma. In this case, the pro-inflammatory cytokine-secreting Tregs appear to be insensitive to corticosteroids. A better understanding of this immunoregulatory process may facilitate the development of preventive or disease-modifying treatments for asthma.

2. Materials and methods

2.1. Subjects

The study included 36 healthy volunteers, 28 mild asthma patients, 26 moderate asthma patients, and 18 severe asthma patients. All patients met the criteria for the diagnosis of asthma as defined by current guidelines [11]. Of 54 patients with mild and moderate asthma, 26 reported using inhaled but not oral corticosteroids. Of 18 severe asthma patients, 9 reported using systemic corticosteroids and 1 reported using ICS (Table 1). Bronchodilator reversibility testing to salbutamol was performed at screening, and the asthma control questionnaire (ACQ) was administered. Exclusion criteria included respiratory tract infection, chronic sinusitis, acute asthma exacerbation, in-patient status, and current and ex-smokers. Patients with other active diseases such as COPD, tuberculosis, tumors, immune-modulatory treatment, pregnancy, and systemic infections were also excluded from this study. Healthy control subjects were excluded if they had a history of atopy, allergic rhinitis, or smoking. None of the asthmatics had experienced an exacerbation or respiratory infection for at least 4 weeks prior to the study. The recommendations for the classification of asthma severity of the GINA report [12] were used to classify asthma patients into three categories: mild, moderate, and severe. Written informed consent was obtained, and the local research ethics committee approved this study. Atopy status was judged by skin prick testing to at least one common inhalant allergen.

2.2. Cell collection

Peripheral blood samples were collected in EDTA-treated tubes from each subject and processed to measure peripheral blood mononuclear cells (PBMCs) for flow cytometry setup procedures. Blood samples were layered onto Ficoll-Paque Plus (Amersham Biosciences, Buckinghamshire, England), centrifuged (400 g for 20 min at 21 °C), and PBMCs were harvested. Cells were washed once in divalent cation-free Hanks balanced salt solution at 300 g for 5 min at 4 °C. PBMCs were resuspended and viable counts were obtained.

2.3. Flow cytometry

Freshly obtained human PBMCs were stained with anti-hCD4 (-FITC, SK3), anti-hCD25 (-PE-Cy5, BC69), anti-hCD45RA (-APC, OX33), anti-hHLA-DR (-PE, L243) or anti-hCD127 (-Alexa Fluor 700, A7R34). Cells for FoxP3 and CTLA-4 staining were prepared for intracellular flow cytometry with a “Fix and Perm” kit following the manufacturer's instructions. After permeabilization and washing, cells were labeled with FoxP3 (-PE, PCH101) and CTLA-4 (-APC, 14D3). Corresponding isotype controls were used as staining controls (BD Biosciences, San Jose, CA, USA and eBioscience, San Diego, CA, USA). Intracellular cytokine elaborations were determined in PBMCs and stimulated for 5 h with PMA (5 ng/ml) and calcium ionomycin (500 ng/ml) in the presence of GolgiStop. Cells were fixed, permeabilized, and stained with fluorochrome-conjugated mAbs against selected cytokines. Treg subpopulations were sorted by FACS Aria II (BD). All antibodies were purchased from BD or eBioscience.

Lymphocytes were gated using forward scatter and side scatter profiles while ensuring that first, second, and third fluorescence detector isotype control co-plots remained in the lower left quadrants. In our research, 300,000 lymphocytes were acquired for each sample to make sure that more than 100,000 CD4⁺ cells were gated on the FACS analysis. Data acquired by FCM Calibur (Becton Dickinson, San Jose, CA, USA) were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

2.4. Carboxyfluorescein succinimidyl ester (CFSE) staining and T-cell proliferation assay

CD4⁺CD25⁻ responder cells (5×10^4) from healthy controls were labeled with 1 μ M CFSE (Invitrogen, Carlsbad, CA, USA), then were cocultured with 5×10^4 Tregs or separate subsets sorted by the Aria sorter in anti-CD3-coated (5 μ g/ml OKT3 mAb; eBioscience) plates in the presence of soluble anti-CD28 (5 μ g/ml; eBioscience) for 72 h. CFSE-labeled cells were assessed by flow cytometry.

2.5. Cytokine measurements

The levels of transforming growth factor- β 1 (TGF- β 1), IFN- γ , interleukin (IL)-5, and IL-17A were measured in the serum supernatant using commercially available sandwich ELISA kits (R&D Systems Europe, Abingdon, UK) following the manufacturer's instructions.

2.6. Data analysis

Group data were expressed as the mean and SEM or as the median and interquartile range where appropriate. For data not distributed normally, comparisons between three groups were made using one-way Kruskal-Wallis tests, and a p-value of < 0.05 was considered significant. If this test indicated significance, the Mann-Whitney test was used for post-hoc analysis for comparisons between two groups, with corrections of p-values according to Bonferroni (a p-value < 0.017 was considered significant). Correlation was assessed by calculating Spearman's rank correlation coefficient. Here, a p-value of < 0.05 was considered significant. Statistical analyses were performed using SPSS for GraphPad Prism 5.0 software.

3. Results

3.1. Subject characteristics

The demographics, lung function, and ACQ scores of the study groups are summarized in Table 1. The patients with moderate to severe asthma had lower FEV₁ and higher ACQ scores than the other groups, indicating a higher severity of disease than that of patients with mild asthma. One-half of the moderate to severe group was using long-

Table 1
Characteristics of subjects.

Characteristic	Healthy (n = 36)	Asthmatics		
		Mild asthma (n = 28)	Moderate asthma (n = 26)	Severe asthma (n = 18)
Age (year)	30.6 (20–43)	34.5 (21–55)	36.2 (19–57)	38.8 (23–58)
Sex (M/F)	20/16	12/16	15/11	11/7
BMI (kg/m ²)	23.7 (21.0–25.1)	24.0 (22.4–25.9)	25.2 (22.7–29.4)	25.7 (23.1–28.8)*
Onset of asthma (childhood/adulthood), n	0/0	18/10	11/15	8/10
Positive family history of asthma*	0	16	13	11
Atopic status, Subjects with > one positive SPT	0	24	25	12
Blood eosinophils, × 10 ⁹ /L	0.11 (0.04–0.19)	0.27 (0.06–0.52)	0.33 (0.12–0.62)#	0.20 (0.05–0.48) *†
Blood neutrophil, × 10 ⁹ /L	3.2 (1.8–5.7)	2.9 (1.4–5.0)	3.9 (1.6–6.3)#	4.8 (2.9–8.9) *†
IgE, IU/ml	28.1 (5.2–79.8)	178.0 (48.0–398.0)§	195.0 (57.8–426.0)#	165.0 (30.4–326.0) *†
FEV ₁ , L	3.8 (2.6–4.6)	3.1 (2.5–3.8)	3.0 (2.4–4.0)	2.4 (1.3–4.1) *†
ΔFEV ₁ with albuterol (% of baseline)	2.6 ± 3.4%	12.3 ± 8.5%§	10.8 ± 6.9%#	8.6 ± 6.5% *†
FEV ₁ /FVC %	77.3 (71.8–84.2)	73.3 (68.2–79.4)	58.4 (49.1–64.8)#	38.57 (30.2–49.3) *†
FEV ₁ % predicted	97.2 (88.0–112)	90.4 (84.4–98.2)§	76.2 (69.1–84.3)#	62.5 (52.4–80.2) *†
ACQ	N/T	0.61 (0.2–1.5)	0.58 (0–1.9)	1.8 (0.6–3.8) †
On ICS/OCS	0/0	12/0	14/0	1/9
ICS (μg budesonide equivalent/day)	0	320 ± 107	639 ± 188	N/A
OCS (mg prednisone equivalent/day)	0	0	0	33.6 ± 14.2
LABA use	0	6	14	9
LTRA use	0	13	16	13
SABA use	0	10	14	13
On theophylline	0	12	17	14

Data are expressed as mean ± SD for ΔFEV₁ and ICS dose, median (interquartile range) for others.

Definition of abbreviation: SPT: skin prick test; ICS: inhaled corticosteroids; OCS: oral corticosteroid; LTRA: leukotriene receptor antagonist; LABA: long-acting beta₂-agonists. *Family history defined as at > 1 first-degree relative.

Comparisons between asthmatic groups and the control group: *P < 0.05.

Comparison with moderate asthma: † P < 0.05.

Comparisons with mild asthma: #P < 0.05.

Comparisons with healthy: §P < 0.05.

acting β agonists in addition to corticosteroids. The majority of asthmatics were atopic. All three groups of asthmatics had a higher blood eosinophilia and Serum IgE levels than healthy control.

3.2. Separation of CD4⁺FoxP3⁺ T cells into three subpopulations according to the expression of FoxP3, CD25, and CD45RA

Human CD4⁺FoxP3⁺ T cells can be divided into three functional subsets based on the expression of FoxP3 and CD45RA, namely, rTreg (FoxP3^{lo}CD45RA⁺, Fr. I), aTreg (FoxP3^{hi}CD45RA⁺, Fr. II) and pro-inflammatory cytokine-secreting subsets (FoxP3^{lo}CD45RA⁺ cells, Fr. III) as described previously [9]. These subsets can be prepared as live cells by cell sorting as CD25⁺⁺CD45RA⁺, CD25⁺⁺⁺CD45RA⁺, and CD25⁺⁺CD45RA⁺ cells, respectively (Fig. 1A). As shown in Fig. 1B, aTreg cells expressed Foxp3 and intracellular CTLA-4 to the highest degree, whereas Fr. III expressed those proteins to the lowest degree. Similarly, the highest level of HLA-DR was observed in the aTreg subpopulation. Analysis of cytokine production by each fraction showed that aTreg and rTreg cells produced low levels of IL-17 and IFN-γ, whereas Fr. III produced high amounts of these cytokines (Fig. 1C). The extent of CFSE dilution of labeled naive CD4⁺CD25⁺ T cells (hereafter called responder cells) cocultured with an equal number of each fraction was measured to assess the *in vitro* suppressive potency of each fraction. As shown in Fig. 1D, rTreg and aTreg cells suppressed the proliferation of responder cells, whereas Fr. III had no suppressive effect.

3.3. Variations in three subpopulations under normal and asthma conditions

We next applied the new definition of FoxP3⁺ T-cell subsets to the analysis of asthma states that reportedly show different patterns of Treg cell involvement [13–17]. The proportions of rTreg and aTreg cells

among CD4⁺ T cells were higher in mild and moderate asthma patients than in healthy controls (median, rTreg: 2.19%, 3.42%, and 3.41%, p < 0.0001; aTreg: 1.21%, 1.52%, and 1.85, p < 0.0001; for healthy, mild, and moderate asthma, respectively). Severe asthma patients showed significantly lower proportions of rTreg cells than the other three groups (median, 1.42%, p < 0.0001) and lower proportions of aTreg cells (median, 1.46%, p < 0.0001) than moderate asthma patients (median, 1.46% vs. 1.85%, p < 0.05), but these proportions were still significantly higher than those in the healthy group (median, 1.46% vs. 1.21%, p < 0.05). All three groups of asthmatics had a higher proportion of FrIII than healthy control subjects, with a progressive increase associated with asthma severity (Fig. 2A).

3.4. Tregs from patients with severe asthma exhibit weakened suppressive activity on effector T cells

To investigate whether Tregs from patients with asthma exhibited altered immunosuppressive capacity due to disturbed suppressive and pro-inflammatory subsets, the protein levels of Foxp3 and CTLA-4 in CD4⁺CD25⁺CD127⁺ T cells as determined by median fluorescence intensity (MFI), were examined because the transcription factor, Foxp3, is essential for Treg cell development and function [18], and CTLA-4 is expressed in highly immunosuppressive Tregs [19–21]. As shown in Fig. 2B, all three groups of asthmatics had higher Foxp3 and CTLA-4 MFI values than healthy controls (median, Foxp3: 2590, 3580, 3825, and 3054, p < 0.0001; CTLA-4: 28.3, 81.3, 89.1, and 46.9, p < 0.0001; for healthy, mild, moderate, and severe asthma, respectively). However, severe asthma patients showed significantly lower Foxp3 and CTLA-4 MFI values than mild and moderate asthma patients (p < 0.01).

Disturbed homeostasis in immune suppressive subsets and the pro-inflammatory subset and altered functional protein levels of Treg cells such as CTLA-4 in asthma patients prompted us to perform *in vitro*

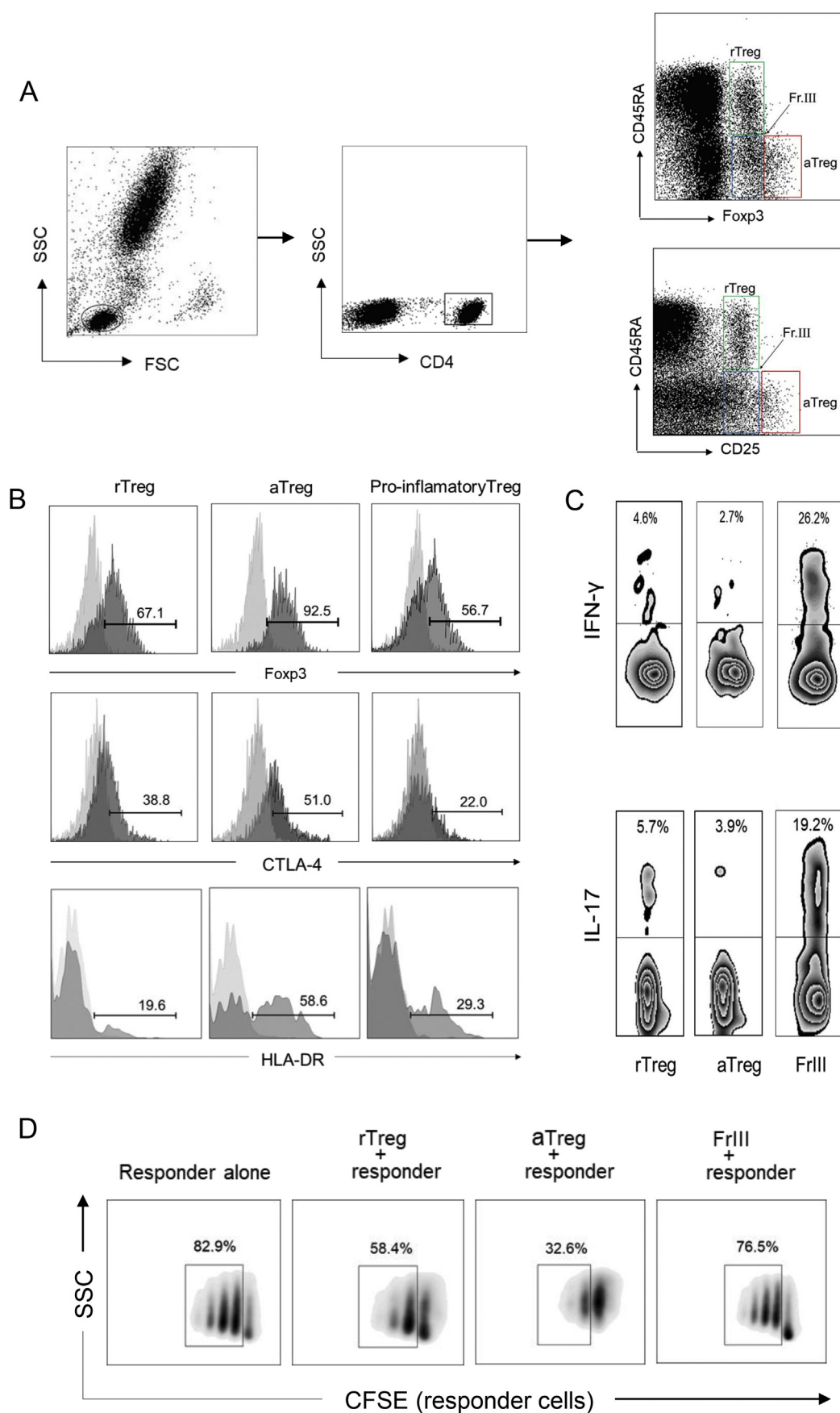
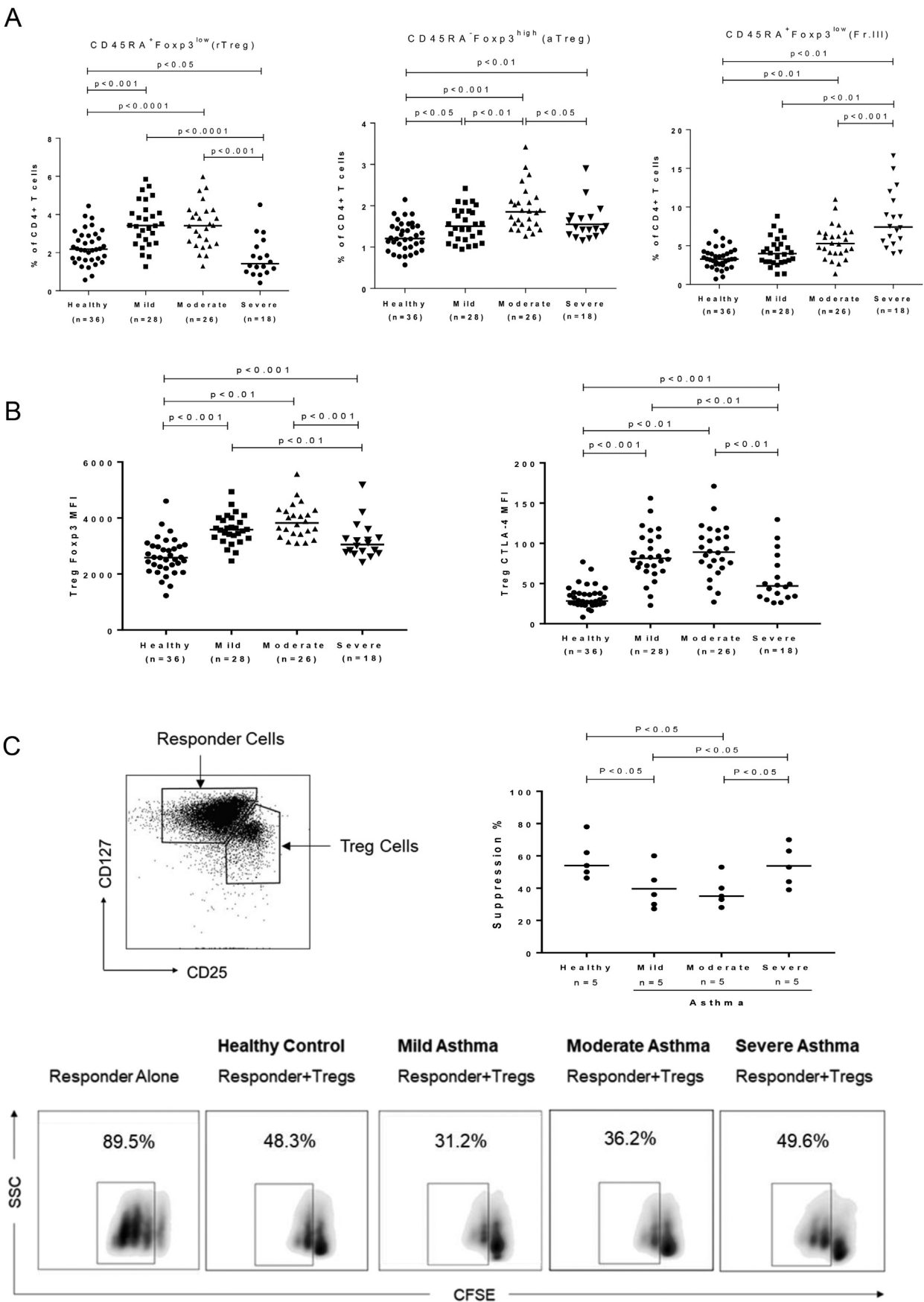


Fig. 1. Delineation of $CD4^+CD25^+$ T cells into three subsets according to cell surface markers and phenotypic characterization of regulatory T-cell subsets. (A) Gating strategy and representative dot plots of three subsets of $CD4^+$ Tregs defined by expression of CD45RA and Foxp3 (right upper panel) or CD45RA and CD25 (right lower panel). (B) Each subset expressed different levels of Foxp3, cytotoxic T-lymphocyte antigen 4 (CTLA-4), and HLA-DR. Protein expression is represented by light grey lines and isotype by solid grey profiles. (C) Flow cytometry of the production of interleukin 17 (IL-17) and interferon γ (IFN- γ) by three subsets after stimulation with phorbol-12-myristate-13-acetate (PMA) and ionomycin for 5 h. Percentages of cytokine-secreting cells are shown. Data are representative of five independent experiments. (D) CFSE dilution by 10^4 labeled $CD4^+CD25^-$ responder T cells assessed after 3 days of TCR-stimulated coculture with each subset at a 1:1 ratio. Cell number and percentage of dividing cells per well are indicated. Data are representative of six separate experiments. All the cells in this section are derived from healthy controls.



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Fig. 2. Variations in subpopulations of Treg cells lead to altered functional protein expression and immunosuppressive capacity. (A) Frequency of each subpopulation in healthy controls and asthma patients of different severities. (B) Foxp3 and CTLA-4 expression levels measured by mean fluorescence intensity (MFI) on Tregs defined as $CD4^+CD25^+CD127^-$ from healthy subjects and patients with asthma. (C) Suppression of T-cell proliferation by whole Treg cells was assessed by carboxyfluorescein succinimidyl ester (CFSE) staining. CFSE-labeled $CD4^+CD25^-$ T cells with anti-CD3/CD28 stimulation were cultured alone or with Treg cells at a ratio of 1:1. Percentages of proliferating cells from four groups were compared. Data are representative of five independent experiments.

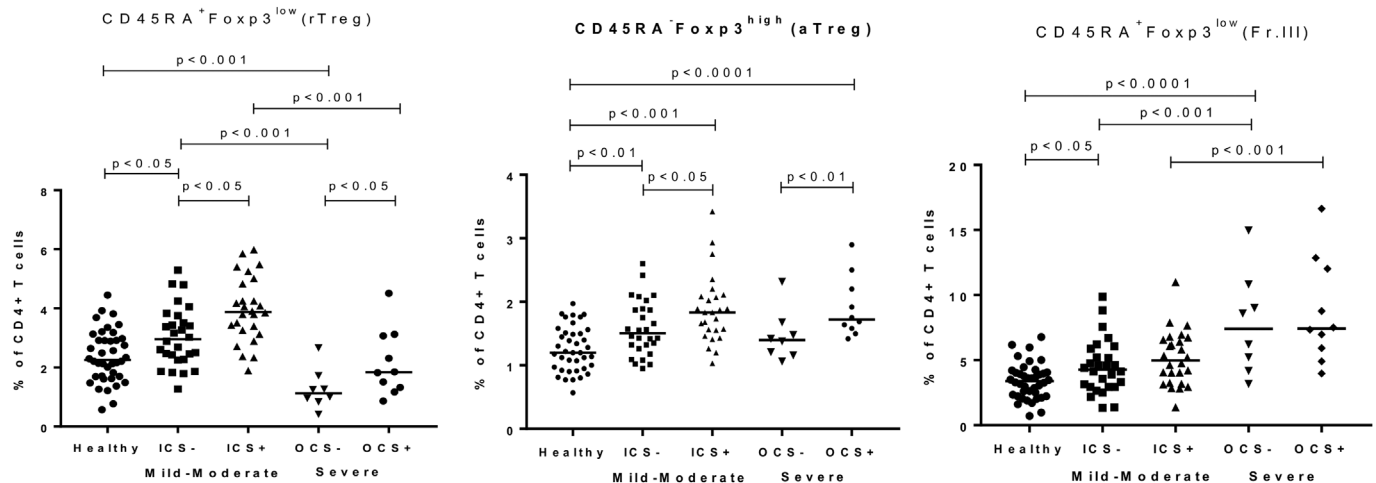


Fig. 3. Proportions of Treg subpopulations in healthy controls, non-corticosteroid treated, and corticosteroid treated asthma patients: (A) resting Treg cells ($CD45RA^+FOXP3^{low}$); (B) activated Treg cells ($CD45RA^-FOXP3^{high}$); and (C) non-suppressive $FOXP3^+$ T cells ($CD45RA^+FOXP3^{low}$).

experiments to test the immune suppressive function of Tregs in asthma patients. As illustrated in Fig. 2C, patients with severe asthma showed a lower suppressive capacity than mild and moderate asthma patients.

3.5. Corticosteroids promote the differentiation of rTreg and aTreg but not the pro-inflammatory cytokine producing Treg cells in asthma patients

Corticosteroids can promote regulatory T cells by a Foxp3-dependent mechanism [22,23]. Therefore, we sought to determine whether corticosteroid treatments can affect the differentiation of the three subsets of Treg cells in asthma patients. Patients with mild and moderate asthma were treated with inhaled but not systemic steroids. A significant increase in rTreg and aTreg cells was observed in steroid-treated patients with asthma compared with those in untreated patients regardless of disease severity ($p < 0.05$) (Fig. 3). The proportion of Fr III did not differ significantly between steroid-treated patients and untreated patients, suggesting that the pro-inflammatory subset of Tregs is not sensitive to corticosteroids.

3.6. Pro-inflammatory cytokine producing $FOXP3^+$ regulatory T cells increase in asthma patients

To confirm that FrIII cells in asthma patients are functional pro-inflammatory cytokine-producing T cells, we stimulated $CD4^+$ T cells with PMA/ionomycin. Consistent with the phenotyping results, compared with healthy controls and patients with mild asthma, the numbers of $CD4^+FOXP3^+IL-17^+$ cells (median, 0.11%, 0.12%, 0.17%, and 0.26% for healthy, mild, moderate, and severe asthma, respectively, $p < 0.001$) and $CD4^+FOXP3^+IFN-\gamma^+$ cells (median, 0.38%, 0.55%, 0.75%, and 1.02% for healthy, mild, moderate, and severe asthma, respectively, $p < 0.001$) were dramatically increased higher in moderate and severe asthma patients (Fig. 4A–D). The number of IL-17-producing $FOXP3^+$ cells and IFN- γ -producing $FOXP3^+$ cells strongly correlated with T helper 17 (Th17) cells ($CD4^+FOXP3^-IL17^+$) and Th1 cells ($CD4^+FOXP3^-IFN-\gamma^+$), respectively ($r = 0.66$, $p < 0.001$; $r = 0.48$, $p < 0.001$; Fig. 4E and F). The proportion of $CD4^+FOXP3^-IL17^+$ and $CD4^+FOXP3^-IFN-\gamma^+$ cells showed the same trend as the proportion of IL-17-producing $FOXP3^+$ cells and IFN- γ -

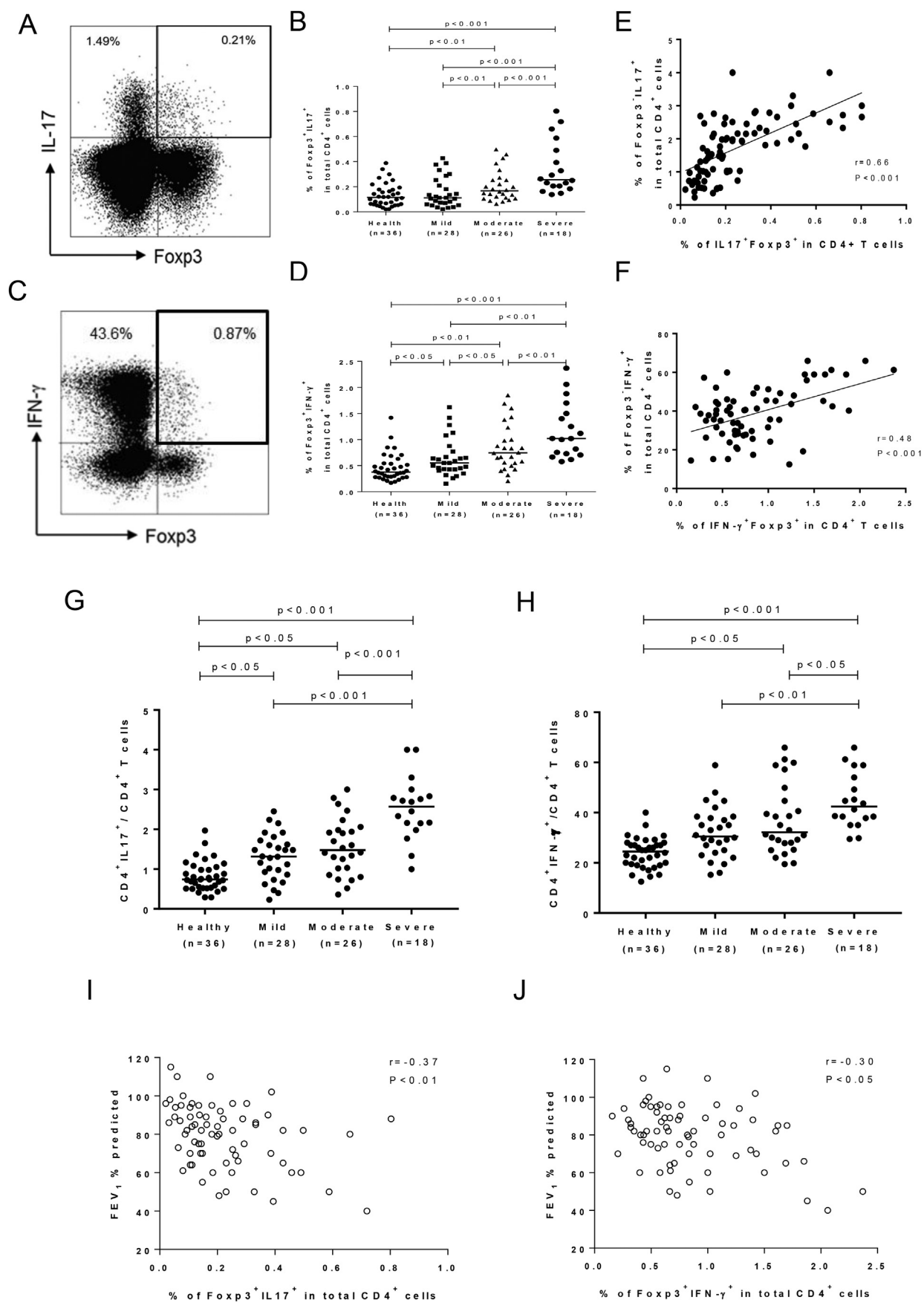
producing $FOXP3^+$ cells in the different groups. All three groups of asthmatics had a higher proportion of $CD4^+FOXP3^-IL17^+$ (Th17; Fig. 4G) and $CD4^+FOXP3^-IFN-\gamma^+$ cells (Th1; Fig. 4H) than healthy control subjects, with a progressive increase associated with asthma severity (median, Th17: 0.63%, 1.48%, 1.34%, and 2.33%, $p < 0.0001$; Th1: 25.2%, 35.4%, 33.5%, and 42.6%, $p < 0.0001$; for healthy, mild, moderate, and severe asthma, respectively). The increased frequencies of the $CD4^+FOXP3^+IL-17^+$ and $CD4^+FOXP3^+IFN-\gamma^+$ populations were inversely associated with lung function (FEV_1 predicted) (Fig. 4I and J), suggesting that the pro-inflammatory subpopulation of Treg cells was correlated with the severity of asthma.

3.7. Dysregulated cytokine profile in patients with asthma

The increased pro-inflammatory potential of Tregs, Th1, and Th17 cells in patients with asthma was also reflected in the significantly increased levels of IFN- γ and IL-17, which are Th1- and Th17-associated cytokines, respectively. All asthmatic patients showed increased levels of IL-17 and IFN- γ , with a progressive increase related to disease severity ($p < 0.001$ and $p < 0.0001$, respectively; Fig. 5A–B).

Compared with the healthy controls, the level of the Th2-associated cytokine IL-5 was slightly increased in patients with severe asthma ($p < 0.001$), whereas it was dramatically increased in patients with mild and moderate asthma ($p < 0.001$; Fig. 5C), suggesting Th2 inflammation is predominant in these asthma cases. Therefore, our results indicate that the plasma cytokine milieu in patients with severe asthma is skewed toward Th1 and Th17 predominant phenotypes, whereas that in patients with mild and moderate asthma is skewed toward Th2 inflammation.

To further explore the suppressive function in asthma patients, the Treg-generated immunosuppressive cytokine TGF- β_1 was also assessed. The level of TGF- β_1 was significantly lower in patients with severe asthma than in those with mild and moderate asthma ($p < 0.01$ and $p < 0.01$, respectively), although it was still higher than that in healthy controls ($p < 0.05$; Fig. 5D). This suggests a defective immunosuppressive function in severe asthma patients compared with that in mild and moderate asthma patients.



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Fig. 4. Flow cytometric detection of the production of intracellular IL-17 and IFN- γ by CD4⁺Foxp3⁺ T cells in PBMCs after 6 h of stimulation with phorbol myristate acetate/ionomycin. (A and C) Representative figures of intracellular staining of FoxP3 versus IL-17A or IFN- γ in CD4⁺ Tregs. (B and D) Proportions of Foxp3⁺IL-17⁺ and Foxp3⁺IFN- γ ⁺ among CD4⁺ T cells in the four groups. (E) Proportions of IL-17-secreting T-regulatory cells (Foxp3⁺IL-17⁺) correlated with Th17 cells (Foxp3⁺IL17⁺). (F) Proportions of IFN- γ -secreting T-regulatory cells (Foxp3⁺IFN- γ ⁺) correlated with Th1 cells (Foxp3⁺IFN- γ ⁺). (G and H) Frequencies of Th17 and Th1 cells in healthy controls and asthma patients of different severities.

4. Discussion

CD4⁺FoxP3⁺ regulatory T cells play a central role in dampening immunological inflammation and the induction of allergen tolerance. Theoretically, Treg cells can modify the susceptibility to allergy and asthma and interfere with the development of these diseases at different stages. However, previous studies identifying the Treg population as a

whole reported conflicting results on the roles of Treg cells in disease progression in asthma patients. In addition to the heterogeneity of the asthma populations studied, the most likely explanation for this inconsistency is that human Treg cells are phenotypically and functionally heterogeneous. It has been demonstrated that the human CD4⁺FoxP3⁺ compartment contains suppressive rTreg and aTreg cells, as well as a non-Treg subpopulation that secretes abundant pro-

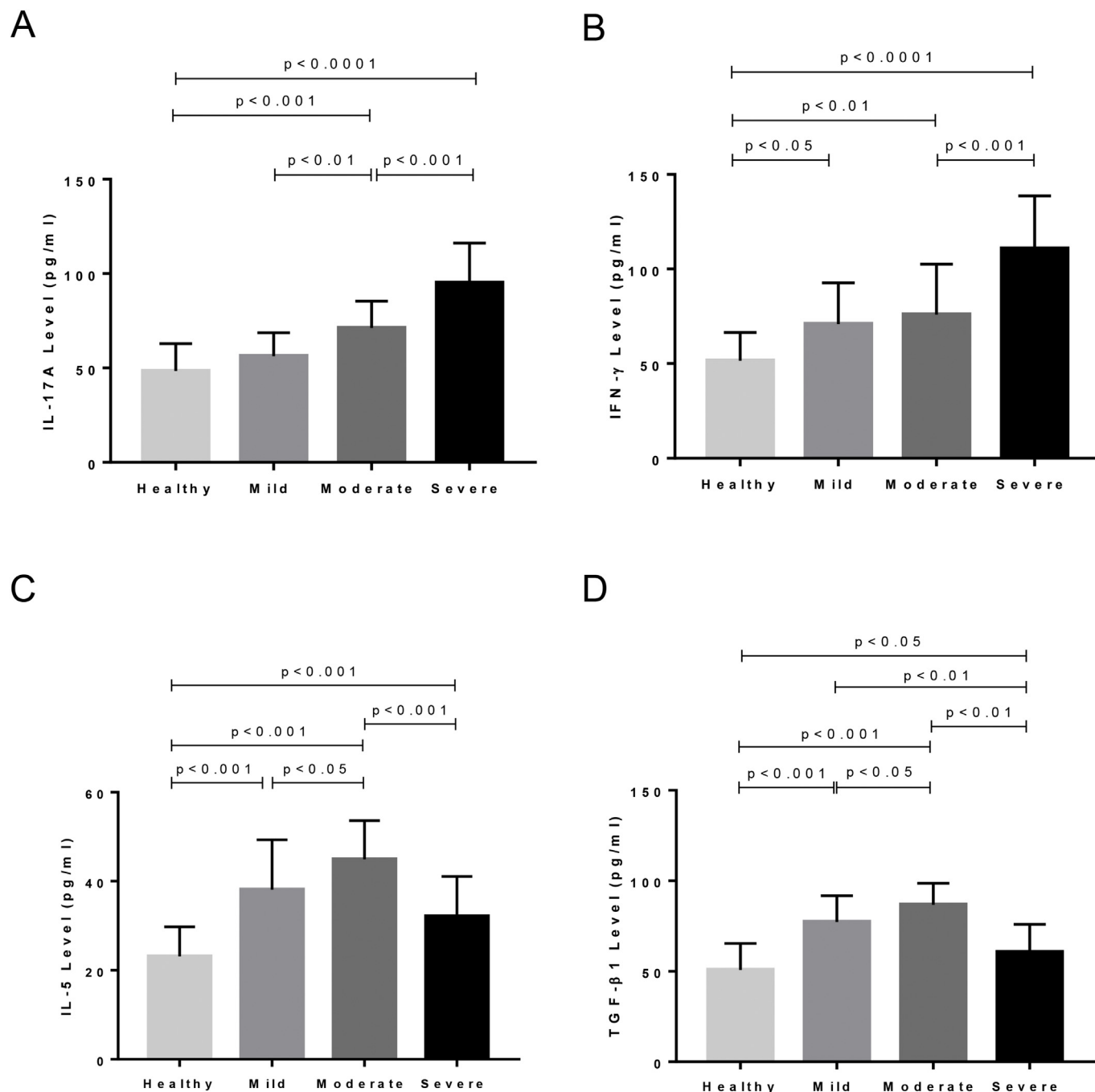


Fig. 5. Elevated levels of pro-inflammatory and immunosuppressive cytokines in patients with asthma. The levels of circulating (A) IL-5, (B) IFN- γ , (C) IL-17A and (D) transforming growth factor (TGF)- β 1 in plasma were determined by ELISA.

inflammatory cytokines [9]. The classification of CD4⁺FoxP3⁺ cells into functionally and phenotypically different subsets enables the re-evaluation of the dynamic changes of Treg cells in asthma.

CD45RA⁺ rTreg cells and CD45RA[−] aTreg cells represent a distinct differentiation status of the same Treg cells. Once activated through TCR, CD45RA⁺ rTreg cells rapidly proliferate and convert into CD45RA[−] aTreg cells, which suppress effector T cells and which promptly die [9]. Although their relationship remains unclear, naïve Ag-inexperienced CD45RA⁺ rTreg cells and memory Ag-primed CD45RA[−] aTreg cells were reported [9] to have comparable functions but different suppressive mechanisms. The significantly increased proportions of rTreg and aTreg cells in mild and moderate asthma patients suggest enhanced anti-inflammatory responses probably due to Ag exposure, whereas a strikingly increased pro-inflammatory cytokine-producing non-Treg subset and decreased proportions of rTregs detected in severe asthma patients may reflect exhaustion of anti-inflammatory responses during disease progression. Importantly, we found that the pro-inflammatory Treg subpopulation was significantly correlated with enhanced Th1 and Th17 cells in moderate and severe asthma patients, suggesting this Treg subpopulation may be relevant to the differentiation of Th1 and Th17 cells during asthma inflammation.

Treg cells contribute to the control of allergen-specific immune responses by regulating the activation and proliferation of specific Th and T cytotoxic (Tc) cells. Compromised regulation may provide an explanation for hyper-reactivity to allergens and auto antigens. However, conflicting data on the number of circulating Treg cells in asthma have been reported [13,14]. Most previous studies investigated the Treg population as a whole, whereas the emerging novel subpopulations may have antithetical actions. In the present study, mild and moderate asthma were characterized by significantly increased rTreg and aTreg cells, whereas severe asthma showed dramatically increased pro-inflammatory cytokine-secreting subpopulation. These findings led to the hypothesis that a decrease in the immunosuppressive Treg populations, together with an enhanced pro-inflammatory subpopulation, leads to compromised regulatory capacity and persistent airway inflammation in patients with severe asthma. In line with earlier studies [24–26] showing that CD4⁺CD25⁺Treg cells of asthma patients are not as effective in their suppression of Th2 as of Th1 responses, the present *in vitro* data confirmed the hypothesis that the immunosuppressive function of Treg cells in severe asthma is compromised in comparison with that in mild/moderate asthma.

Thunberg et al. [27] observed that inhaled allergen challenge in mild asthma increased BAL Foxp3 mRNA expression and Treg numbers, suggesting the induction of regulatory responses during active asthmatic reactions. In this regard, the increased rTreg and aTreg cells among CD4⁺ T cells in mild and moderate asthma patients and pro-inflammatory non-Tregs in moderate/severe asthma is suggestive of an induction of Foxp3 caused by allergen challenge and increased airway inflammation.

Defining the pro-inflammatory cytokine-secreting subpopulation (Fr III) is one of the striking findings of studies on human CD4⁺FoxP3⁺ Treg heterogeneity. This subpopulation contains FoxP3-RORγ double-positive cells with a Th17 cell potential, in addition to IL-2- and/or IFN-γ-producing cells [9]. Given the role of IL-17 and IFN-γ in regulating neutrophilic and macrophage inflammation in the lung, which is related to severe and steroid-insensitive asthma and COPD [28,29], these pro-inflammatory functions of the cytokine-producing T-cell population (Fr III) should be considered in the pathogenesis of inflammatory diseases. Accumulating evidence shows that IL-17A producing CD4⁺Foxp3⁺ Treg cells are also involved in the pathogenesis of various diseases [30]. The present *in vitro* stimulation experiments confirmed that this population produced IL-17 and IFN-γ and was increased in moderate/severe asthma patients. This pro-inflammatory cytokine producing subpopulation was strongly correlated with enhanced Th17 and Th1 cells in asthma patients. Along with the elevated level of Th1 and Th17 corresponding cytokines in severe asthma, our

study suggests that the pro-inflammatory cytokine producing Treg subpopulation plays an important role in promoting the shift to a Th1 and Th17 mixed pattern in severe asthma.

How could regulatory T cells be modulated by routine therapy to control or prevent asthma? Glucocorticosteroids are widely used in the treatment of allergic asthma to inhibit T-cell activation and Th2 cytokine production. Corticosteroids can induce FOXP3 expression [22,31,32] and enhance the functional regulatory capacity of Treg cells [31,33]. *In vitro*, glucocorticosteroids inhibit the induction of GATA-3 and T-bet transcription [34,35] and induce FOXP3 transcription in peripheral blood CD4⁺ T cells [22]. Taken together, these studies suggest that glucocorticosteroids act by regulating the balance between the anti-inflammatory transcriptome (FOXP3) and pro-inflammatory transcriptome (GATA-3, T-bet, and RORc2). The present data suggest that glucocorticosteroids modulate subpopulations of Treg cells by upregulating rTreg and aTreg, whereas the pro-inflammatory cytokine-secreting Treg subpopulation shows no response to corticosteroids. The dramatic increase in the pro-inflammatory Treg subpopulation with Th17 potential in patients with severe asthma suggests the possibility that this population is associated with steroid resistance. However, a definite conclusion can only be made by conducting a prospective study to specifically explore the effect of corticosteroids on Treg subpopulations.

The present study had several limitations. First, only peripheral blood samples were studied. It would be interesting to further characterize Tregs on bronchial biopsies or in BAL, because circulating Tregs and local Tregs at the inflammation site may differ markedly [36]. Secondly, the results of the current cross-sectional study only indirectly suggest an effect of glucocorticosteroid treatment on heterogeneous subpopulations of Tregs; therefore, solid conclusions cannot be made. Prospective studies are still needed to assess the effects of both inhaled and systemic glucocorticosteroids on the regulatory and pro-inflammatory subsets of CD4⁺FoxP3⁺ T cells.

5. Conclusions

In conclusion, the present study demonstrates that disturbed subpopulations of Treg cells exist in the peripheral blood of asthma patients. The accumulation of pro-inflammatory cytokine-producing Treg cells correlated well with Th17 and Th1 differentiation, reflecting more advanced disease in asthma. Our data link the disturbed Treg cell subpopulations to the differentiation of helper T-cell subpopulations, which may facilitate a better understanding of the underlying pathogenesis of inflammation in asthma.

Funding

This work was supported by the National Natural Science Foundation of China (81360008) and the Ningxia Natural Science Foundation (2018AAC03152).

Availability of data and materials

Data and materials are available upon request by contacting the correspondence author Jia Hou (hjou@live.com).

Authors' contributions

Lei Xin was responsible for preparation and analysis of blood-samples, statistical analyses, and manuscript preparation. Junjie Gao contributed with laboratory-based assays. Xiahui Ge contributed with scientific expertise and manuscript preparation. Weirong Ma, Zhigang Tian and Xiwei Zheng took part in subject recruitment. Jia Hou contributed as a primary investigator and was responsible for designing the study and analyzing the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Research ethics approval was obtained from Ethics Committee of General Hospital of Ningxia Medical University. All subjects provided written informed consent.

Consent for publication

Not applicable.

Conflicts of interest

The authors declare that they have no conflict of interests.

Acknowledgements

Not Applicable.

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