Regulatory T cells in induced sputum of asthmatic children: association with inflammatory cytokines

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ABSTRACT
Background and objective: CD4+CD25+ regulatory T (Treg) cells play an essential role in maintaining immune homeostasis. In this study, we investigated whether the induced sputum (IS) pool and the function of CD4+CD25+ Treg cells are altered in asthma pediatric patients.

Methods: Treg activity was studied in the IS of 40 asthmatic children. CD3+ cells were analyzed for the expression of FoxP3 mRNA by real time reverse transcription–polymerase chain reaction (RT–PCR). IS cells from asthmatics and controls were stained for Treg markers and analyzed by flow cytometry. We also studied the ability of Treg cells to differentiate monocytes toward alternatively activated macrophages (AAM), and to suppress proinflammatory cytokines.

Results: (i) Mild and moderate asthmatics had significantly decreased expression of FoxP3/β-actin mRNA and decreased proportions of CD4+CD25highFoxP3+ cells compared to healthy children; (ii) patients with moderate asthma had even lower proportions of FoxP3 expression compared to mild asthmatic patients; (iii) monocytes cultured with Treg cells displayed typical features of AAM, including up-regulated expression of CD206 (macrophage mannose receptor) and CD163 (hemoglobin scavenger receptor), and an increased production of chemokine ligand 18 (CCL18). In addition, Treg cells from asthmatics have a reduced capacity to suppress LPS-proinflammatory cytokine production from monocytes/macrophages (IL-1, IL-6 and TNF-α). Conclusion: Asthma pediatric patients display a decreased bronchial Treg population. The impaired bronchial Treg activity is associated with disease severity.

Keywords: Bronchial asthma, CD4+CD25highFoxP3+, induced sputum, inflammatory cytokines, regulatory T cells.

RIASSUNTO
Finalità: Le cellule T regolatorie (Treg) CD4+CD25+ svolgono un ruolo fondamentale nel mantenere l'omeostasi dell'immunità. In questo studio si è ricercato se la quantità e la funzione delle cellule Treg CD4+CD25+ risultino alterate nello sputo indotto (IS) di pazienti asmatici in età pediatrica.

Metodi: L'attività dei Treg è stata studiata prendendo in esame l'IS di 40 bambini asmatici. Le cellule CD3+ sono state analizzate per l'espressione di FoxP3 mRNA attraverso la retro trasformazione–polimerase chain reaction (RT–PCR). Le cellule dell'IS di asmatici e soggetti di controllo sono state colorate specificamente per i marker Treg e analizzate con la citometria a flusso. Si è studiata altresì la capacità delle cellule Treg di differenziare i monociti in macrofagi alterativamente attivati (AAM), e di sopprimere citochine proinfiammatorie.

Risultati: Risultati principali ottenuti: (i) Pazienti affetti da asma lieve e moderato mostravano una considerevole riduzione dell'espressione di FoxP3/β-actin mRNA e una ridotta proporzione di cellule CD4+CD25highFoxP3+ in confronto a bambini sani; (ii) Pazienti affetti da asma moderato presentavano proporzioni ancora più basse di espressione di FoxP3 rispetto ai pazienti con asma lieve; (iii) Monociti coltivati con le cellule Treg hanno mostrato i tratti caratteristici di AAM, comprese le espressioni up-regolate di CD206 (recettore macrofagico per il mannosio) e CD163 (recettore "scavenger" per l'emoglobina), e un aumento della produzione di ligando 18 delle chemocine (CCL18). Inoltre i Treg degli asmatici evidenziano una ridotta capacità di sopprimere la produzione delle citochine LPS-proinfiammatorie da monociti/macrofagi (IL-1, IL-6 e TNF-α). Conclusioni: I pazienti pediatrici con asma presentano un decremento della popolazione bronchiale di Treg. La compo-
**INTRODUCTION**

Chronic mucosal inflammation plays an essential role in the pathogenesis of asthma. Pathological pathways of asthma are observed early in childhood, bronchial inflammation being observed in infants and remodelling in younger children [1,2]. Interactions between dendritic cells, monocytes/macrophages and lymphocytes induce, amplify or modulate the ongoing inflammation [3]. Recent studies have also investigated regulatory T (Treg) cells in asthma [4,5], and it is possible that the recruitment of Treg cells into the airways suppresses allergic airway inflammation [6]. A recent study examining pediatric asthmatic patients found a low percentage of CD4+CD25high T cells (Tregs) in the bronchoalveolar lavage (BAL) compared to healthy controls or children treated with corticosteroids [7]. Additional studies in animal models of allergic airway inflammation have provided more insight into the role of Tregs in asthma [6,8]. During inflammation, the interactions between CD4+CD25+ Treg cells and antigen presenting cells (APC) are likely to involve not only dendritic cells (DC) but also monocytes/macrophages which play a critical role in both innate and adaptive immunity. Indeed, these cells are able to recognize pathogens and/or “danger signals” via Toll-like receptors (TLRs) and other pattern-recognition receptors and produce a wide array of cytokines and chemokines. The initial inflammatory response is carried out by macrophages that produce high amounts of proinflammatory cytokines. These macrophages with a higher phagocytic capacity produce anti-inflammatory cytokines and are characterized by an increased expression of the mannose receptor CD206 and/or the hemoglobin scavenger receptor CD163. These cells are often referred to as M2 or alternatively activated macrophages (AAM) [9-10]. Given the pivotal role of CD4+CD25+ Treg cells in maintaining self-tolerance, we here investigated whether the pool and the function of CD4+CD25+ Treg cells are altered in induced sputum from bronchial asthma patients. Then, we assessed a previously uncharacterized function of CD4+CD25+FoxP3+ Treg cells, namely their ability to directly promote the alternative action of monocytes/macrophages.

**MATERIAL AND METHODS**

**Study groups**

Subject characteristics are shown in Table I. Patients were under consultation at the Department of Respiratory Pediatrics of A. Mami Hospital. The diagnosis of asthma was based on a history of episodic wheezing and dyspnea. Asthma severity was classified according to GINA (Global Initiative for Asthma) criteria [11]. All patients from asthmatic groups were atopic as defined by at least two positive skin prick tests to common allergens. Asthma was classified as mild in twenty cases. These patients had no regular treatment with inhaled steroids. Eighteen other patients were suffering from moderate persistent asthma. They were treated with inhaled steroids (400–500 µg of beclomethasone daily). At sampling time, a good control of asthma was reached in all cases and the patients had no evidence of respiratory infection. Subjects with a history of respiratory infection during the previous four weeks were excluded from the study. Blood and induced sputum samples were collected from subjects during their visits to the outpatient clinics. Informed consent was obtained from all subjects’ parents or guardians, and approval was obtained from our institutional review board.

**Sputum induction and processing**

Induced sputum was obtained as we recently reported [12]. Before sputum induction all patients inhaled salbutamol (200 µg) via a metered dose inhaler. Baseline peak expiratory flow (PEF) was measured and this was repeated following salbutamol inhalation and after each 5-minute inhalation of nebulized hypertonic saline. The procedure was stopped if the PEF fell by > 10% following saline or by > 20% at any time during the induction procedure. Solid sputum material was separated from saliva before processing as we have recently reported [12]. Briefly, selected sputum was weighed and 0.1% dithiothreitol (DTT) (Sigma-Aldrich, Poole, UK) in phosphate-buffered saline (PBS) was added at a ratio of 4 ml to 1 g sputum. The sputum was incubated with DTT at room temperature for 15 minutes on a rolling mixer. The same volume (4 ml to 1 g sputum) of PBS was added to the sputum and then filtered through 48 µm nylon gauze. The filtrate was centrifuged at 400 g (Sorvall RT6000D, Kendro, Bishop’s Stortford, UK) for 10 minutes at 4°C to pellet cells. The cells were resuspended in PBS containing 0.1% bovine serum albumin (BSA). The viability of the sample was determined by trypan blue exclusion staining (Sigma-Aldrich) in a Neubauer hemocytometer (Merck Eurolabs, Lutterworth, UK). Induced sputum cells were resuspended (2 × 10⁵ cells/ml) in PBS and cytospin slides were prepared (Shandon cytospin; 400 rpm, 5 min), stained (Shandon Diffquick), and coded, and 500 cells were counted and expressed as % of non squamous epithelial cells. Absolute counts were generated from the differential and total counts.

**Cell isolation from peripheral blood**

Peripheral blood was obtained from 10 patients with moderate bronchial asthma (the same patients studied for Treg cells induced sputum) and from 7 healthy children, following informed consent. Peripheral blood mononuclear cells (PBMC) were
isolated by using density gradient centrifugation (lymphocyte separation media, PAA Laboratories). Monocytes (> 95% purity) were isolated by using anti-CD14 microbeads or by depleting nonmonocytes (Miltenyi Biotec). CD4+ T cells (> 95% purity) were purified by using a T cell isolation kit, and CD4+CD25+ T cells were enriched with anti-CD25 microbeads (Miltenyi Biotec) resulting in > 90% CD4+CD25+ T cells with > 87% expressing FoxP3+ cells. In addition, separation of CD4+CD25high and CD4+CD25− T cells from total CD4+ T cells was performed by using MoFlo®.

Flow cytometry for Treg cells expression in induced sputum
Further separation of CD3+ T cells (negative selection by monodisperse), induced sputum (IS) cells were stained with FITC-conjugated anti-CD4, PE-conjugated anti-FoxP3 and allophycocyanin (APC)-conjugated anti-CD25 antibodies with appropriate isotype controls (all from eBiosciences, San Diego, CA, USA). Flow cytometry was performed using a fluorescence activated cell sorter (FACS) Calibur instrument with CellQuest software (Becton Dickinson, San Diego, CA, USA). List mode files were collected for 200,000 cells from each sample. CD25high cells were defined as described elsewhere [13-14].

PBMC co-cultures of T cells and monocytes
Monocytes and T cells (2:1 ratio) were co-cultured in Roswell Park Memorial Institute (RPMI) medium 1640, supplemented with 1% penicillin/streptomycin, 1% glutamin, and 10% heat-inactivated FCS. Monocytes (5 × 105/ml) were cultured in either 200- or 500-µl cultures without T cells (monocytes), with CD4+CD25− T cells or CD4+CD25+ T cells (CD25+) for 40 h in the presence of 50 ng/ml anti-CD3 mAb (OKT3, Ortho Biotech), after which the different cultures were stimulated for 24 h with Lipopolysaccharide (LPS) 50 ng/ml (Sigma); this was found to be the optimal concentration out of a dose range (0–100 ng/ml) to stimulate monocytes for interleukin (IL)-6 and tumor necrosis factor (TNF)-α production.

Detection of cytokines and chemokines
IL-6, TNF-α (Invitrogen), CCL18 (R & D Systems), were measured by ELISA, and IL-1β, was measured by Luminex (Upstate) by using a Luminex 100 system, according to the manufacturer’s instructions.

Statistical analysis
Statistical analysis was performed with GraphPad Prism 4.03 software by using Wilcoxon matched paired tests or impaired t-test. P values < 0.05 were considered significant.

RESULTS
The proportion of Treg in induced sputum from bronchial asthma
As shown in Figure 1A, patients with bronchial asthma (mild asthma and moderate asthma) had significantly decreased gene expression of the transcriptional factor FoxP3 in CD3+ cells compared to healthy children (mean ± SD: 5.40 ± 1.70; range: 3.0 – 9.0). Significant differences were observed in the FoxP3 expression between mild (2.61 ± 0.98; range: 1.0 – 5.0) and moderate bronchial asthma (1.16 ± 0.60; range: 0.3 – 2.0) (p = 0.0001). This reduction of FoxP3 was extended and confirmed by flow cytometry, showing decreased proportions of CD4+CD25highFoxP3+ cells in the CD4+ cell population of the mild (0.85 ± 0.48; range: 0.2 – 2.0) and moderate (0.80 ± 0.39; range: 0.4 – 1.7) bronchial asthma patients compared to healthy children (2.33 ± 0.83; range: 0.98 – 3.9) (Figures 1B and 1C).

Values of CD4+CD25highFoxP3+ cells in the CD4+ cell population were also expressed in absolute number [(mean ± SD) x106/mL]. The decreased CD4+CD25highFoxP3+ cells in mild asthmatics (0.047 ± 0.023) and in moderate asthmatics (0.044 ± 0.021) were maintained when values were expressed as absolute values compared to healthy controls (0.13 ± 0.046). No significant differences were observed between mild and moderate asthmatics when values were expressed as absolute values.

Alternative activation in monocytes/macrophages induced by Treg cells
A previously uncharacterized function of Treg cells, namely their ability to steer monocyte differentia-
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Figure 2A and 2B show that, relative to control monocytes, Treg-treated monocytes displayed a strong up-regulation of the typical AAM markers CD206 (n = 15, p < 0.05) and CD163 (n = 7, p < 0.02). The increased expression of CD206 and CD163 was greater in moderate asthmatics than in healthy controls, when monocytes were co-cultured with CD4+CD25+ T cells (Figures 2A, 2B). We also determined the presence of CCL18, a chemokine that is specifically overexpressed by AAM [15], and found this to be significantly enhanced in monocyte/Treg co-cultures in asthmatics when compared to healthy controls (Figure 2C).

CD4+CD25+ Tregs from moderate asthma did not inhibit the proinflammatory response of monocytes/macrophages to LPS

Next, we determined the ability of Treg-treated monocytes/macrophages from moderate asthmatics to respond to LPS. We investigated 10 healthy controls and 7 moderate asthmatics.
controls and 7 moderate asthmatics. As shown in Figures 3A, 3B and 3C monocytes cultured in the presence of CD4+CD25- effector T cells displayed increased production of proinflammatory cytokines. To exclude possible contributions of T cell-derived cytokines in these assays, we repeated these experiments twice with monocytes that were repurified after Treg co-culture and obtained similar results.

Following co-culture of monocytes from healthy controls with autologous CD4+ CD25+ FoxP3+ Treg cells, there was significant suppression in their capacity to produce proinflammatory cytokines IL-1β (Figure 3A), TNF-α (Figure 3B) and IL-6 (Figure 3C) compared to monocytes cultured alone. In patients with moderate asthma, monocytes cultured alone produced more IL-1β, TNF-α and IL-6 than monocytes from healthy controls. Co-culture of monocytes from moderate asthmatic CD4+ CD25+ FoxP3+ Treg cells failed to suppress TNF-α, IL-6 and IL-1β production.

### Increased expression of Treg cells in induced sputum in patients with moderate asthma after treatment with inhaled corticosteroid

Eight patients with moderate asthma were investigated for Treg expression before and after 6 weeks of treatment with inhaled corticosteroids.
They received a stepwise treatment after enrollment, including inhaled corticosteroid (budesonide) and β2-agonists, as recommended by GINA [11]. Six weeks later, the patients were revisited. They were completely controlled regarding asthmatic symptoms after treatment according to the Asthma Control Test (www.asthmacontrol.com). Induced sputum was collected again. Changes of CD25^high FoxP3^+ cells in the CD4^+ cell population were measured and compared to values obtained before the treatment. CD4^+CD25^high T cell percentages were increased as reported in Figure 4.

**DISCUSSION**

In the present study we found decreased proportions of CD4^+CD25^{high}FoxP3^+ cells in induced sputum of the bronchial asthmatic groups, particularly in patients with moderate asthma, indicating low numbers of Treg cells compared to healthy children. We believe that using the combination of CD4^+CD25^{high} with FoxP3 as a marker of Treg cells will reflect more accurately the true numbers of Treg cells in induced sputum from asthmatics. In this study, the decreased proportions of CD4^+CD25^{high}FoxP3^+ cells in mild and moderate asthma patients compared to healthy controls is
supported by low expression of FoxP3 mRNA in CD3+ cells in the same patients. We also provide evidence for a previously uncharacterized role of human CD4+CD25+FoxP3+Treg cells, their ability to steer differentiation of monocytes toward AAMs. The expression of CD206, CD163 and CCL18 production was increased in moderate asthmatics than in healthy controls, when monocytes were co-cultured with CD4+CD25+.

To confirm the concept that treatment with inhaled corticosteroid could increase the frequency of peripheral CD4+CD25high T cells, we compared the quantitative change of this cell subset in 8 patients with moderate asthma before and after 6 weeks of inhaled corticosteroids. Although the increase after the treatment was slight, it was statistically significant. We speculate that dosage and course of the treatment may influence the final results. Further investigations are therefore needed to clarify this issue in asthma. Recently Zhang et al. [16] reported a slight increase of Treg cells after 4 weeks of inhaled budesonide treatment, but the difference observed before and after treatment was not statistically significant.

The frequency and phenotypic characteristics of CD4+, FoxP3+ T cells as well as their capacity to suppress inflammatory cytokine production and proliferation of CD4+, CD25+ T cells (target cells) were comparable in young (age ≤ 40 years) and elderly (age ≥ 65 years) individuals. However, when CD4+, FoxP3+ Treg and CD4+, CD25+ T cells were co-cultured, the production of anti-inflammatory cytokine IL-10 from CD4+, CD25+ T cells was more potently suppressed in the elderly than in the young. This finding was not due to changes in cytotoxic T lymphocyte antigen (CTLA)-4 expression or apoptosis of CD4+, FoxP3+ Treg and CD4+, CD25+ T cells. Taken together, these data [17] suggested that aging may affect the capacity of CD4+, FoxP3+ T cells to regulate IL-10 production from target CD4+ T cells in humans although their other cellular characteristics remain unchanged. We can speculate that no significant differences are observed between young and adult asthmatics in their peripheral blood circulation. However, no data were found concerning values of Treg cells in adult induced sputum. Homeostasis of Treg is controlled by several factors, among them the circulation and possibly their redistribution to extra-vascular tissue [18]. We can only speculate which factors contribute to decreased percentages of Tregs in induced sputum of asthmatics.

The role of IL-10 and transforming growth factor (TGF) has been suggested in the Treg-mediated regulation of T cell responses in vivo [19-20]. Treg cells produce IL-10 or TGF-β or both, by which they attain most of their suppressive activity. IL-10 production suppresses exaggerated Th2 reactions. It inhibits directly and indirectly the activity of mast cells, basophils and eosinophils [21]. Peripheral blood analysis in asthmatics failed to reveal modification in absolute numbers of CD4+CD25+ T cells. However, bronchoalveolar lavage fluid of asthmatic children possessed significantly diminished levels of CD4+CD25+ T cells, IL-10 and TGF-β mRNA expression [22].

After administration of IL-2-anti-IL-2 complexes before and after allergenic stimulation, a remarkable increase in Treg counts was observed along with an increase in mRNA and IL-10 levels in lung
tissue; in addition, subjects displayed decreased pulmonary pathologic findings (mucus secretion, airway inflammation and hyper-responsiveness [23]. Recent data from Matsumoto et al. [24] raised the possibility that lung CD4+CD25-FoxP3- Treg cells may regulate asthma phenotype. The frequency of CD4+CD25-FoxP3- Treg cells among CD4+CD25+ T cells in peripheral blood of asthmatics and in murine asthma model was significantly lower than that of healthy subjects [24].

Our data in asthmatics show that CD206, CD163 and CCL18 were increased compared to healthy controls. These findings indicate that Treg-mediated up-regulation of the macrophage mannose receptor may involve a novel pathway that depends on cell contact in controls and more so in asthmatics. Our data in healthy controls were in accordance with those reported by Tiemessen et al. [10]. Our data suggest that CD4+CD25+ Treg cells from moderate asthmatics exhibit a loss of suppression inducing production of high levels of proinflammatory cytokines (IL-1α, IL-6 and TNF-α), contrasting with data obtained in healthy controls. According to all the reported data, we can speculate that the low suppressive activity of Treg cells in moderate asthma could be due in part to dysregulated cytokine production [21].

AAM are cells with strong anti-inflammatory potential involved in immune regulation and tissue remodeling [23]. Co-culture of Treg cells with monocytes/macrophages displays typical features of AAM, including up-regulated expression of CD206 (macrophage mannose receptor) and CD163 (hemoglobin scavenger receptor), an increased production of CCL18, and an enhanced phagocytic capacity. In addition, the monocytes/macrophages have reduced expression of HLA-DR and a strongly reduced capacity to respond to LPS in terms of proinflammatory mediator production (IL-1, IL-8, IL-6, IL-8, MIP-1α, TNF-α), nuclear factor (NF)-κB activation, and tyrosine phosphorylation [10,25].

Mechanistic studies reveal that CD4+CD25+FoxP3+ Tregs produce IL-10, IL-4, and IL-13 and that these cytokines are the critical factors involved in the suppression of the proinflammatory cytokine response [10,25]. CD4+CD25+ Tregs down-regulate proinflammatory cytokine production (IFN-γ and TNF-α), and directly inhibit IL-2 mRNA transcription. In vitro, the mechanism behind T cell suppression appears to be independent of IL-10 and TGF-β and based on cell contact between Tregs and responder T cells. Treg-mediated suppression in vivo involves multicellular clusters consisting of responder T cells, APC, and regulatory T cells, and during these cellular interactions membrane-bound and/or soluble inhibitory molecules contribute to suppression [10,25].

The present study is, to our knowledge, the first demonstration of decreased proportions of Treg cells in induced sputum from mild and moderate asthmatic children. Our findings in the present study, showing markedly decreased proportions of CD4+CD25+FoxP3+ cells in young asthmatic patients, suggest that deficient immune regulation is an early event in this pathology. However, further studies are needed to clarify (i): how this Treg abnormality contributes to asthma severity, (ii): the strength correlation between monocytes/macrophages and Treg cells, (iii): the production of IL-10 and TGF-β by bronchial Treg cells. The role of T cells in asthma is complex and probably tightly regulated by the microenvironment.

CONFLICT OF INTEREST STATEMENT: None of the authors has any conflict of interest to declare in relation to the subject matter of this manuscript.

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