

ORIGINAL ARTICLE

Evaluation of Th17-related cytokines and IFN γ production from blood mononuclear cells of moderate and severe asthmatic children reveals methylprednisolone does not decrease IL-22 levels

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Abstract

Objective: The aim of this study was to correlate IL-6, IL-17A, IFN γ , and IL-22 production with asthma disease severity and to evaluate if methylprednisolone downregulated cytokine production in peripheral blood mononuclear cells (PBMCs). **Methods:** Forty-two children with chronic persistent asthma and 34 non-asthmatic children were selected. Cytokines were quantified by ELISA from serum or PBMCs supernatants, after the PMA and Ionomycin stimulation, with or without methylprednisolone at 100 μ M. **Results:** Our data showed undetectable levels of serum cytokines in most patients and controls. In the PBMCs, we have observed a higher production of IL-17A than IL-22 among asthmatics and controls, although it is not statistically significant. IL-6, IFN γ , and IL-17A levels were significantly reduced after methylprednisolone treatment ($p = 0.02$, 0.03 , and 0.03 , respectively) in Severe Persistent Asthma (SPA) and in Moderate Persistent Asthma (MPA), ($p = 0.007$, 0.01 , and 0.007 , respectively). However, IL-22 levels were unaffected (SPA, $p = 0.12$ and MPA, $p = 0.93$). **Conclusion:** Methylprednisolone downregulated IL-6, IL17A, and IFN γ , but not IL-22, in stimulated PBMCs from asthmatic children indicating that methylprednisolone has no effect on IL-22 production by PBMCs.

Keywords

Immunomodulation, interferon gamma, interleukin, interleukin 17, interleukin 22, treatment

History

Received 3 June 2014

Revised 22 August 2014

Accepted 25 August 2014

Published online 17 September 2014

Introduction

Asthma is a prevalent and serious chronic inflammatory airway disease that affects 300 million people worldwide. Nevertheless, its pathogenic mechanisms are not completely understood. The prevailing consensus states that the immunological basis of atopic sensitization is due to exacerbated T helper type 2 cytokine production (IL-4, IL-5, IL-9, and IL-13) in response to allergens [1]. The Th1/Th2 paradigm has played an important role in improving our understanding of immunological changes in asthma [2]. However, asthma is now increasingly being recognized as a heterogeneous disorder and roles for Th1, Th2, and more recently Th17, Th9, Th22, and regulatory T cells have been identified [3].

Mild-to-moderate asthma is generally characterized by chronic airway inflammation consisting of activated Th2 lymphocytes and eosinophil infiltrates in association with IgE production and airway hyperresponsiveness [4]. Severe asthma presents with different pathological features and it is characterized by a mixed Th2/Th1 phenotype with a possible contribution from Th17 cells [5,6]. Cytokines produced by these cells such as tumor necrosis factor (TNF)- α , interferon (IFN) γ , and interleukin (IL)-17 are elevated and may induce mixed granulocytic airway infiltration [7]. Severe asthma patients are generally refractory to glucocorticoid treatment and up to 35% of asthmatic patients do not improve lung function despite the use of high doses of inhaled steroids, the gold-standard anti-inflammatory therapy [8]. These patients represent 5–7% of the overall asthmatic population, and yet they account for 40–50% of health costs of asthma, and who incur significant morbidity, reduced lung function, and decreased quality of life [9].

Although many murine and human studies have contributed to clarify the role of Th17 in immune and inflammatory diseases, their role in allergy and asthma remains to be elucidated [10]. There are few reports on how

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these cells behave in the pediatric population, especially in developing countries. A full understanding of the immune components involved in asthma pathogenesis, and the main triggers involved in cellular inflammation, is critical for a successful treatment. To understand the role of cytokines in pediatric asthma, we have investigated IL-6, IL-17A, IL-22, and IFN γ levels in serum and stimulated peripheral blood mononuclear cells (PBMCs) from Moderate and Severe Persistent Asthmatic children treated with methylprednisolone. We chose these cytokine due to their central roles. IL-6 helps to induce Th17 phenotype and IL-17A and IL-22 are the most important Th17 cytokines. IFN γ has a central role on immune system and besides that, it is secreted by pathogenic Th17 cells. So, we hypothesized that IL-6, IL-17A, IL-22, and IFN γ should be higher in the severe asthma group. We have also hypothesized that methylprednisolone reduces more significantly the levels of these cytokines in patients with moderate asthma than patients with severe asthma.

Methods

Subjects

Forty-two patients with chronic persistent asthma aged between 6 and 20 years and 34 controls aged between 6 and 17 years were recruited for this study (Table 1). Patient recruitment was conducted at the Division of Pediatric Allergy at Hospital das Clinicas, Federal University of Pernambuco (HC – UFPE), Recife, Brazil. The severity of asthma was assessed based on GINA guidelines [11] and lung function tests including Forced Expiratory Volume (FEV₁) (% predicted) were performed. Participants also completed the Asthma Control Test (ACT). Total IgE was measured and atopy was based on positive results of allergy tests to house dust mites (FDA Allergenic, Immunotech, Brazil).

None of the patients had been treated with systemic steroids within 1 month before the study and had never been treated with other immunosuppressive agents. Children that presented atopic dermatitis, history of food allergy, autoimmune disease, immunodeficiency, infection, and cancer

were excluded. The non-asthmatic controls had no clinical history compatible with respiratory or allergic disease. Written informed consent was obtained from all the children’s parents. This study was approved by the Research human ethical committee of the Health Science Center (CCS/UFPE) by number 479/10.

Cytokine titration

Cytokines were quantified in serum from all recruited asthmatic children and controls, by sandwich enzyme-linked immunosorbent assay (ELISA) following manufacturer’s instructions. ELISAs used were IL-6 (BD Biosciences, San Jose, CA), IL-17A (eBiosciences, San Diego, CA), IL-22 (eBiosciences, San Diego, CA), and IFN γ (BD Biosciences, San Jose, CA). Cytokines were measured and their lower detection limits were 9.37 pg/ml, 15.625 pg/ml, 31.25 pg/ml, and 7.81 pg/ml. These kits were also used for the cytokine measurement of PBMC culture supernatants of 18 patients.

Peripheral blood mononuclear cells

PBMCs were obtained by centrifugation on Ficoll Paque™ Plus (density 1.077vg/ml – GE Healthcare Bio-Sciences, Ahmedabad, India) and resuspended in RPMI 1640 medium (Gibco, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (Gibco, Carlsbad, CA), 10mM HEPES (Gibco, Carlsbad, CA), and 200 U/ml penicillin/streptomycin (Gibco, Carlsbad, CA). Cell viability was determined by trypan blue exclusion and the samples were only used when viability was >98%. For assays, 18 patients were selected: nine with severe persistent asthma and nine with moderate persistent asthma. PBMCs (1 × 10⁶ cells/ml) were stimulated with PMA/ionomycin (PI) and treated with methylprednisolone (100 μM) or untreated, and incubated for 48 h. We chose not to do the culture of healthy volunteers because the pattern of response of IL-6, IL17A, IL22, and IFN- γ levels by methylprednisolone had already been previously published in two studies of our group [12,13].

Statistical analysis

Wilcoxon’s signed rank test was used to compare differences in cytokine production of PBMCs. For serum cytokine analysis, Mann–Whitney *U*-test was performed. For all tests, *p* values <0.05 were considered significant. Results are expressed as means ± standard deviation (SD) and all data were plotted and analyzed using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA).

Results

General characteristics of subjects

According to the GINA guidelines, 42 patients with allergic asthma could be divided into two subgroups: 27 with moderate persistent asthma (MPA) and 15 with severe persistent asthma (SPA) (Table 1). There was no significant difference in age or gender among the two groups of patients with asthma and the normal control group (Table 1). Total IgE levels were significantly higher among patients with MPA (1513 UI/ml) compared with SPA (988 UI/

Table 1. Demographic, clinical and laboratory data of asthma patients.

	Asthma	Controls
Number of patients	42	34
Age (years)	12.7	11.7
Mean (range)	8–21	6–17
Sex		
Female/male	17/25	16/18
Classification		
SPA	15	
MPA	27	
FEV1 % predicted	SA: 75.3 MPA: 88.0	
IgE level	1347	
Allergen-specific IgE (positive/negative)	29/13	
ACT score	SA: 15.57 MPA: 12	

FEV, forced expiratory volume; ACT, asthma control test; SPA, severe persistent asthma; MPA, moderate persistent asthma.

ml). In accordance with asthma severity, mean scores for ACT were also different among these groups (MPA = 15.6 and SPA = 12).

Th17-related cytokines and IFN γ in serum

We did not detect serum IL-6, IL-17A, IL-22, and IFN γ in most patients. Only two controls had detectable levels of (26.5 and 28.5 pg/ml). Among asthmatics, only three had detectable IFN γ levels (13, 10, 9 and 11 pg/ml). A single control patient had detectable levels of IL-22 (114.59 pg/ml) and all asthmatic patients had levels above the detection limit. All 34 controls and 42 asthmatics evaluated had undetectable levels of serum IL-17A (<15.625 pg/ml). Two controls had detectable levels of IL-6 (6.71 and 10.28 pg/ml). Among asthmatics, serum IL-6 was detected in six patients (mean = 98.41 $\pm$ 111.24 pg/ml).

Th17-related cytokines and IFN γ in PBMC cultures

As we could not assess cytokine production in serum, we quantified IL-6, IL-17A, IL-22, and IFN γ from cultured PBMCs from patients. IL-6 production was 1083.27 $\pm$ 635.69 pg/ml in SPA and 1021.77 $\pm$ 667.29 pg/ml in MPA patients ($p = 0.57$) (Figure 1A). IL-22 production was 452.39 $\pm$ 623.03 pg/ml in SPA and 155.54 $\pm$ 170.03 pg/ml in MPA ($p = 0.43$) (Figure 1B). IFN γ levels were 29 840.00 $\pm$ 35 230.83 pg/ml in SPA and 20 975.36 $\pm$ 25 235.54 pg/ml in MPA ($p = 0.84$) (Figure 1C). IL-17A in the stimulated cultures was 1160.10 $\pm$ 1581.29 pg/ml in SPA and 202.98 $\pm$ 128.67 pg/ml in the MPA group ($p = 1.00$) (Figure 1D).

There was no statistically significant difference in cytokine production among the severe and moderate persistent asthma groups.

The effect of methylprednisolone on the production of Th17-related cytokines and IFN γ by cultured PBMCs

Following treatment of PBMCs with methylprednisolone at 100 μ M, IL-6 mean production was 502.60 $\pm$ 392.11 pg/ml in SPA ($p = 0.02$) and 679.00 $\pm$ 488.78 pg/ml in the MPA group ($p = 0.007$) showing significantly reduced levels compared with PI stimulated cells (Figure 1A). IL-22 production after methylprednisolone treatment was 409.85 $\pm$ 603.33 pg/ml in SPA ($p = 0.12$) and 134.35 $\pm$ 91.33 pg/ml in MPA ($p = 0.93$) (Figure 1B). IFN γ production was 19 336.19 $\pm$ 28 593.42 pg/ml in SPA after methylprednisolone treatment ($p = 0.03$) and 3429.02 $\pm$ 4008.29 pg/ml in MPA ($p = 0.007$) (Figure 1C). IL-17A production was significantly reduced in the SPA group (559.73 $\pm$ 858.73 pg/ml, $p = 0.03$) and the MPA group (90.59 $\pm$ 67.65 pg/ml, $p = 0.01$) compared with PI stimulated cells (Figure 1D).

Discussion

We demonstrated that serum IL-6, IL-17A, IL-22, and IFN γ levels were mostly undetectable in asthmatic and control patients. To explain the undetectable serum cytokines, we have to consider that all asthmatic patients were being treated with high doses of inhaled steroids in association with long-action bronchodilators (LABA). The mean doses of inhaled budesonide of our patients were 411 mcg/d in the MPA group

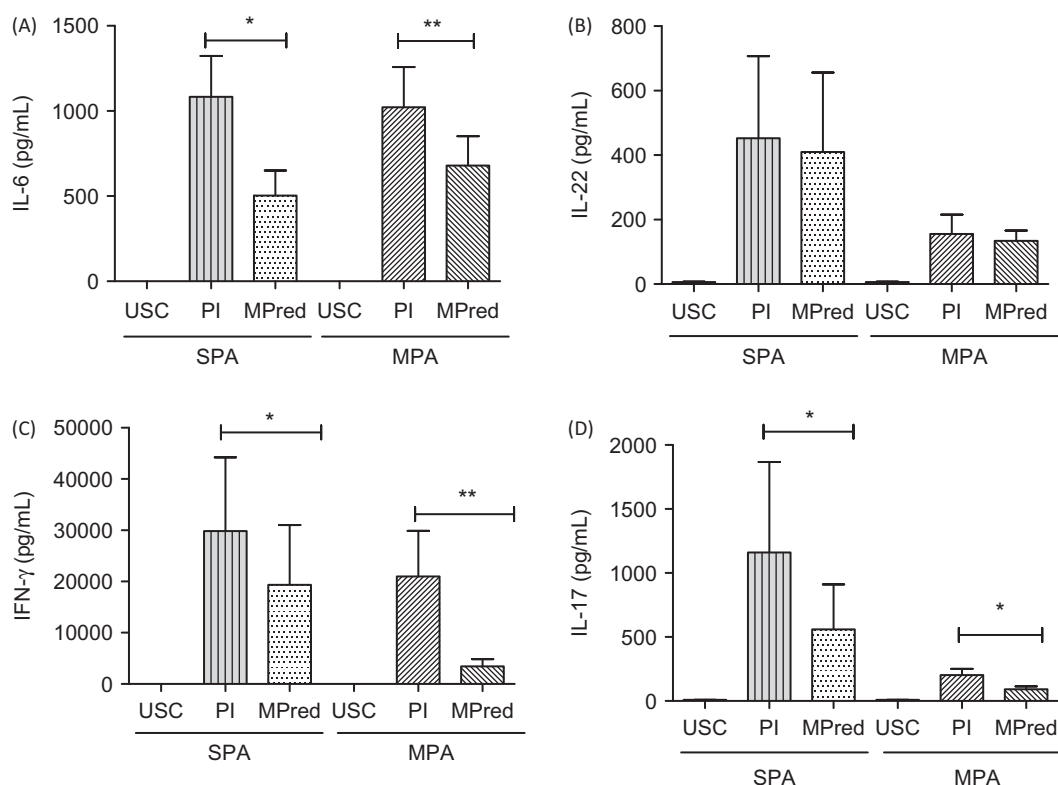


Figure 1. Evaluation of cytokine release in PBMC cultures from asthmatic patients. (A) Methylprednisolone (MPred) significantly decreased IL-6 levels in both groups but not IL-22 (B). The same pattern was observed for IFN- γ (C) and IL-17A (D). * $p < 0.05$, ** $p < 0.01$. SPA, severe persistent asthma; MPA, moderate persistent asthma; PI, PMA and Ionomycin; USC, unstimulated cells.

and 506 mcg/d in the SA group. Furthermore, cytokines have their own microenvironment and characteristically have a short half-life [14].

We also demonstrated that IL-6, IL-17A, IL-22, and IFN γ production in PMA and ionomycin stimulated PBMCs were similar among severe and moderate persistent asthmatics. This could be related to ubiquitous cytokine behavior; for example, IL-6 is considered as an innate cytokine, and together with TGF- β , it plays an important role in Th17 differentiation. However, IL-6 appears to have a more functional role through the promotion of Th2 and suppression of Th1 differentiation, as well as determining adaptive immune responses [2].

In a mouse model of chronic asthma, anti-IFN γ treatment substantially reduced the influx of chronic inflammatory cells into the airways, inhibiting AHR, but did not alter eosinophil influx or airway remodeling [15]. In humans, IFN γ is increased in asthmatic airways and IFN γ -producing sputum T cells are observed in severe asthma [16]. In contrast to these findings, we did not observe higher IFN γ concentrations among severe asthmatics ($p=0.84$). However, the aforementioned paper used sputum cells directly reflects the asthmatic microenvironment.

In Th17 cells, the coproduction of IFN γ and IL-17 is common [10]. Increased levels of IL-17A and IL-17F in the airways are correlated with the severity of asthma, suggesting the involvement of Th17 cell-derived cytokines in the pathogenesis of severe asthma [17,18]. Multiple evidence suggests the involvement of IL-17A and IL-17F in neutrophil infiltration in animal models of asthma and in asthmatic humans, correlating with disease severity [19]. IL-17A also stimulates fibroblasts in the bronchial epithelium to induce the expression of several cytokines and chemokines that are important for neutrophilic recruitment and granulopoiesis, which are related to disease severity, often with steroid-resistant behavior [20–22]. Our data demonstrated increased IL-17A and IL-22 production in the SPA group compared with the MPA group, although this was not statistically significant. Sample size could perhaps explain these results, although we have to consider the high total IgE levels observed, as explained previously. Furthermore, our results indicate that despite the clear indications in the literature for a role of IL-17 role in asthma physiopathology, this cytokine is not a good systemic marker for disease activity.

IL-6, IL-17A, and IFN γ were reduced after methylprednisolone treatment, but a greater reduction was observed among the MPA group. Steroid-resistant behavior observed in severe asthma patients might explain this result. In addition, high levels of Th17 cells might be insensitive to glucocorticosteroid treatment in allergic airway disease in mice, as IL-17 and IL-22 production is not affected by steroid treatment [18]. Interestingly, IL-22 levels were not reduced compared with the other cytokines tested in this study following methylprednisolone treatment. This may be due to the involvement IL-22 in steroid-resistant asthmatic profiles.

Conclusion

We assessed IL-17A, IL-22, IFN γ , and IL-6 levels in serum and PBMCs from severe persistent asthmatic patients and

moderate persistent asthmatic patients. We demonstrated these cytokines were mostly undetectable in our population serum. We also showed that methylprednisolone down-regulated IL-6, IL-17A, and IFN γ production, but not IL-22, indicating a potential role for this cytokine in steroid-resistant asthma.

Declaration of interest

The authors report that they have no conflicts of interest to declare. This study was supported by Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (Facepe), Instituto de Ciência e tecnologia – Inovação Farmacêutica (INCT>If), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de pessoal de Nível Superior (CAPES).

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