ARQUIVOS DE ASMA, ALERGIA E IMUNOLOGIA

Original Article

Interferon-gamma signaling is downregulated in peripheral blood mononuclear cells from asthma patients

A sinalização de interferon-gama é diminuída em células mononucleares do sangue periférico de pacientes asmáticos

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ABSTRACT

Introduction: Interferon-gamma (IFN-γ) signaling is mediated by crosstalk of receptors, such as IFN-γ receptor 1 (IFN-γ R1), transcription factors, such as signal transducer and activator of transcription 1 (STAT1) and suppressors of cytokine signaling 1 (SOCS1). Here, we evaluated the role of IFN-γ signaling in peripheral blood mononuclear cells (PBMCs) from asthma patients and control individuals. **Methods:** PBMCs from adult healthy non-asthmatic controls (n = 12; male and female, 18-60 years old) and patients diagnosed with asthma (n = 18; male and female, 18-60 years old) were stimulated with IFN-γ (0.25, 0.5 and/or 1.0 ng/mL) and, after 24h, the production of CXC motif chemokine 10 (CXCL10) was evaluated (by enzyme linked immunosorbent assay) as well as the expression of IFN-γ R1, STAT1 (both by flow cytometry assay) and SOCS1 (by real-time qPCR assay). **Results:** CXCL10 production was reduced in a dose-dependent manner in PBMCs from asthma patients stimulated with IFN-γ when compared to control individuals. While IFN-γ induced an increase in IFN-γ R1 expression and phosphorylated STAT1 (pSTAT1) activation in PBMCs from the control group, a reduction in both IFN-γ R1 and pSTAT1 was observed in PBMCs from asthma patients. IFN-γ increased SOCS1 mRNA expression in PBMCs from asthma patients when compared to IFN-γ-stimulated cells from control individuals. **Conclusion:** Taken together, our results demonstrated that IFN-γ signaling is downregulated in asthma patients.

Keywords: Asthma, STAT1 transcription factor, interferon-gamma.

RESUMO

Introdução: A sinalização de interferon-gama (IFN-γ) é mediada por receptores, como o receptor 1 de IFN-gama (IFN-γR1), fatores de transcrição, como o transdutor de sinal e o ativador de transcrição 1 (STAT1) e supressores de sinalização de citocina 1 (SOCS1). Neste trabalho, avaliamos o papel da sinalização de IFN-γ em células mononucleares do sangue periférico (PBMCs) de indivíduos com asma e controle. **Métodos:** Células mononucleares do sangue periférico (PBMCs) de adultos saudáveis e não asmáticos (n = 12, homens e mulheres, 18-60 anos) e pacientes diagnosticados com asma (n = 18, homens e mulheres, 18-60 anos) foram estimuladas com IFN-γ (0,25, 0,5 e/ou 1,0 ng/mL) e após 24 horas a produção de CXCL10 foi avaliada por ensaio de imunoabsorção enzimática (ELISA), bem como o receptor 1 de IFN-γ (IFN-γ R1). Também foram avaliadas as expressões do transdutor de sinal e ativador da transcrição 1 (STAT1) (por citometria de fluxo) e supressor de expressão de sinalização de citocinas 1 (SOCS1) (por ensaio qPCR em tempo real). **Resultados:** A produção de CXCL10, uma quimiocina induzida por IFNγ, foi reduzida de maneira dependente da dose em PBMCs de pacientes com asma estimulados com IFN-γ (0,25-1,0 ng/mL) quando comparado ao grupo controle. Enquanto IFN-γ induziu um aumento da expressão de IFN-γ R1 e ativação da fosforilação de STAT1 (pSTAT1) em PBMCs do grupo controle, uma redução de ambas (IFN-γ R1 e pSTAT1) foi observada em PBMCs de pacientes com asma. O IFN-γ aumentou as PBMCs de expressão do mRNA de SOCS1 de pacientes com asma quando comparado às células estimuladas por IFN-γ do controle. **Conclusão:** Em conjunto, nossos resultados demonstraram que a sinalização de IFN-γ é sub-regulada em pacientes com asma.

Descritores: Asma, fatores de transcrição STAT1, interferon-gama.

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Funding source: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (no. 475349/2010-5), Fundação de Apoio a Pesquisa do Estado de Minas Gerais (FAPEMIG; APQ 01631/11 e APQ-01873-14), Rede de Pesquisa em Doenças Infecciosas Humanas e Animais do Estado de Minas Gerais, and Universidade Federal do Triângulo Mineiro (UFTM), Brazil. This study was partly financed by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

Submitted: 5/14/2020, accepted: 5/30/2020.

Arq Asma Alerg Imunol. 2020;4(2):198-204.

http://dx.doi.org/10.5935/2526-5393.20200027

Introduction

Asthma is an inflammatory disease of the airways characterized by migration and accumulation of leukocytes, mucus hypersecretion, and airway hyperresponsiveness.[1] Based on the presence or absence of eosinophilic inflammation, asthma can be mainly divided into T helper 2 (Th2) asthma, the most common phenotype, and non-Th2 asthma, associated with obesity and smoking and driven by neutrophils.[2] Most patients with asthma have persistent symptoms that are readily controllable by standard asthma therapies.[1,2]

Interferon-gamma (IFN-γ) could counter-regulate Th2 immune responses.[3] A shift in the Th1/Th2 cytokine balance toward a type 1 profile may contribute to protection against asthma and allergy.[4] The concentration of IFN-γ was reduced in peripheral blood monocytes stimulated with *Dermatophagoides pteronyssinus* in children and adults with asthma when compared to controls.[5] The reduced production of IFN-g is associated with increased risk of recurrent virus-induced wheezing.[6] Conversely, IFN-γ plays a significant role in severe asthma pathology (non-Th2 asthma). Increased IFN-γ expression in bronchial biopsy specimens and higher IFN-γ protein levels in bronchoalveolar lavage fluid (BALF) were found in severe asthma patients when compared to controls and individuals with mild to moderate asthma.[7] IFN-γ exerts its downstream effects by binding to IFN-γ receptors (IFN-γR)1 and IFN-γR2,[8] leading to recruitment and activation of a transcription factor known as signal transducer and activator of transcription 1 (STAT1),[9] whose activation is modulated by suppressor of cytokine signaling 1 (SOCS1).[10]

In this study, we selected allergic asthma patients and evaluated IFN-γ signaling in peripheral blood mononuclear cells (PBMCs) from these patients and from control individuals by assessing the production of CXC motif chemokine 10 (CXCL10), also known as interferon gamma-induced protein-10 (IP-10), and the expression of IFN-γR1, STAT1 and SOCS1.

Materials and Methods

Patients

The present study was approved by the Universidade Federal do Triangulo Mineiro Human Research Ethics Committee (number 1377). All participants were informed about the survey and signed a consent form. Patients diagnosed with asthma (n=18; male and female, 18-60 years old) were pre-selected from the university hospital according to specific inclusion criteria for enrolled patients that included either a physician's diagnosis of stable asthma or current clinically defined persistent asthma symptoms,[11] or both. Adult healthy non-asthmatic controls (n=12; male and female, 18-60 years old) were recruited based on the absence of asthma clinical criteria. Exclusion criteria were the presence of any inflammatory diseases, chronic diseases, history of recurrent infections, viral hepatitis, autoimmune diseases, chronic obstructive pulmonary diseases, recent use of systemic corticosteroids or immunotherapy, and incomplete follow-up, and negative skin prick test. Healthy individuals were selected based on the absence of a history of asthma or clinical symptoms. Exclusion criteria for the control group were the same as those for the asthma group, except for negative skin prick test.

Skin prick test

All individuals underwent skin prick tests with the following commercial allergen extracts: dust mite (*Blomia tropicalis*, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), extract of cockroach (*Blattella germanica* and *Periplaneta americana*), mold (*Alternaria alternata*), and pet dander (*Felis domesticus* and *Canis familiaris*) obtained from Anthygenus (Brazil). A mean wheal diameter of 3 mm or larger than that of negative healthy individuals was considered to be positive. Asthma patients with a positive skin prick test and controls with a negative skin prick test were selected for blood collection.

Sample collection

Peripheral blood was collected by venipuncture in a heparinized tube. From each patient, 20-25 mL of venous blood was collected. PBMCs were isolated by density-gradient centrifugation over Histopaque 1077 (Sigma Aldrich), and only those with results above 95% purity were used.

Stimulus

PBMCs (1×10^6 cell/mL) were cultivated in 96-well plates and were stimulated with IFN-γ (0.25, 0.5 and/or 1.0 ng/mL) in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin.[12]

CXC motif chemokine 10 (CXCL10)

The supernatant was collected 24h after IFN-γ stimulation, and CXCL10 concentration was determined by ELISA according to the manufacturer's instructions (BD Biosciences Pharmingen).

Phosphorylated STAT1 (pSTAT1) and IFN-γ receptor expression on PBMCs

The pSTAT1 expression was assessed by cytometry according to de Oliveira et al.[13] Briefly, 24h after IFN-γ stimulation, PBMCs were fixed with pre-warmed BD Cytofix Buffer (4% paraformaldehyde) for 10 min at 37 °C. After centrifugation, the cells were permeabilized in ice-cold methanol for 30 min and then stained with mouse monoclonal antibodies against pSTAT1 (BD Biosciences Pharmingen) or their corresponding mouse IgG2a isotype (BD Biosciences Pharmingen) for 60 min followed by PE-conjugated goat anti-mouse IgG2a secondary antibody for another 45 min at 10 °C in the dark. Isotype control from nonstimulated and stimulated cells were used to establish gates. The cells were then washed, resuspended, and subjected to analysis.

The IFN-γR1 (CD119) expression was assessed according to Lee et al.[14] Briefly, 24h after IFN-γ stimulation, cells recovered from culture were washed twice in PBS and stained at 4°C for 40 minutes with mouse monoclonal antibodies against IFN-γR1 (CD119) (BD Biosciences Pharmingen) or their corresponding mouse IgG2a isotype (BD Biosciences Pharmingen) for 60 min followed by PE-conjugated goat anti-mouse IgG2a secondary antibody for another 45 min at 10 °C in the dark. Isotype control from nonstimulated and stimulated cells were used to establish gates. The cells were then washed, resuspended, and subjected to analysis.

The expression of pSTAT1 and IFN-γR1 in 50,000 viable cells was analyzed by flow cytometry (FACS Calibur; BD Biosciences).

SOCS1 expression

At 30 min after IFN-γ stimulation, total RNA was extracted from cells using Pure Linkr RNA Mini Kit (Life Technologies). cDNA was synthesized by reverse transcription (RT) from total RNA with SuperScript VILO MasterMix (Invitrogen) according to the manufacturer's instructions. Duplicate qPCR reactions were performed with primers for SOCS1 (forward: 5'-TTTT TCGCCCTTAGCGTGA-3', reverse: 5'-AGCAGCTCGAAGAGGCAGTC-3') and control GAPDH (forward: 5'-CCACCCATGGCAAATTCC-3', reverse: 5'-TCGCTCCTGGAAGATGGTG-3') using cDNA-specific TaqMan Gene Expression Assays with a StepOnePlusTM Real-Time PCR System. In each 5 µL TaqMan reaction, cDNA (corresponding to 100 ng reverse transcribed RNA) was mixed with 0.25 µL TaqMan Gene Expression Assay, 2.5 µL TaqMan Universal PCR Master Mix, and 1.25 µL H_2O. The polymerase chain reaction (PCR) conditions were 95 °C for 20 s, followed by 50 cycles at 95 °C for 3 s, and 60 °C for 30 s.

The Ct (cycle threshold) values for SOCS1 mRNA were normalized to GAPDH to provide the delta Ct values. The relative mRNA expression was determined using the Livak method (the $2^{-\Delta\Delta CT}$ method for real-time PCR)[13].

Statistical analysis

The results were expressed as mean ± standard deviation (SD). An evaluation of the results was performed by an analysis of variance (ANOVA) followed by a Tukey post-test among the means using Graph Pad PRISM. P values less than 0.05 were considered statistically significant.

Results

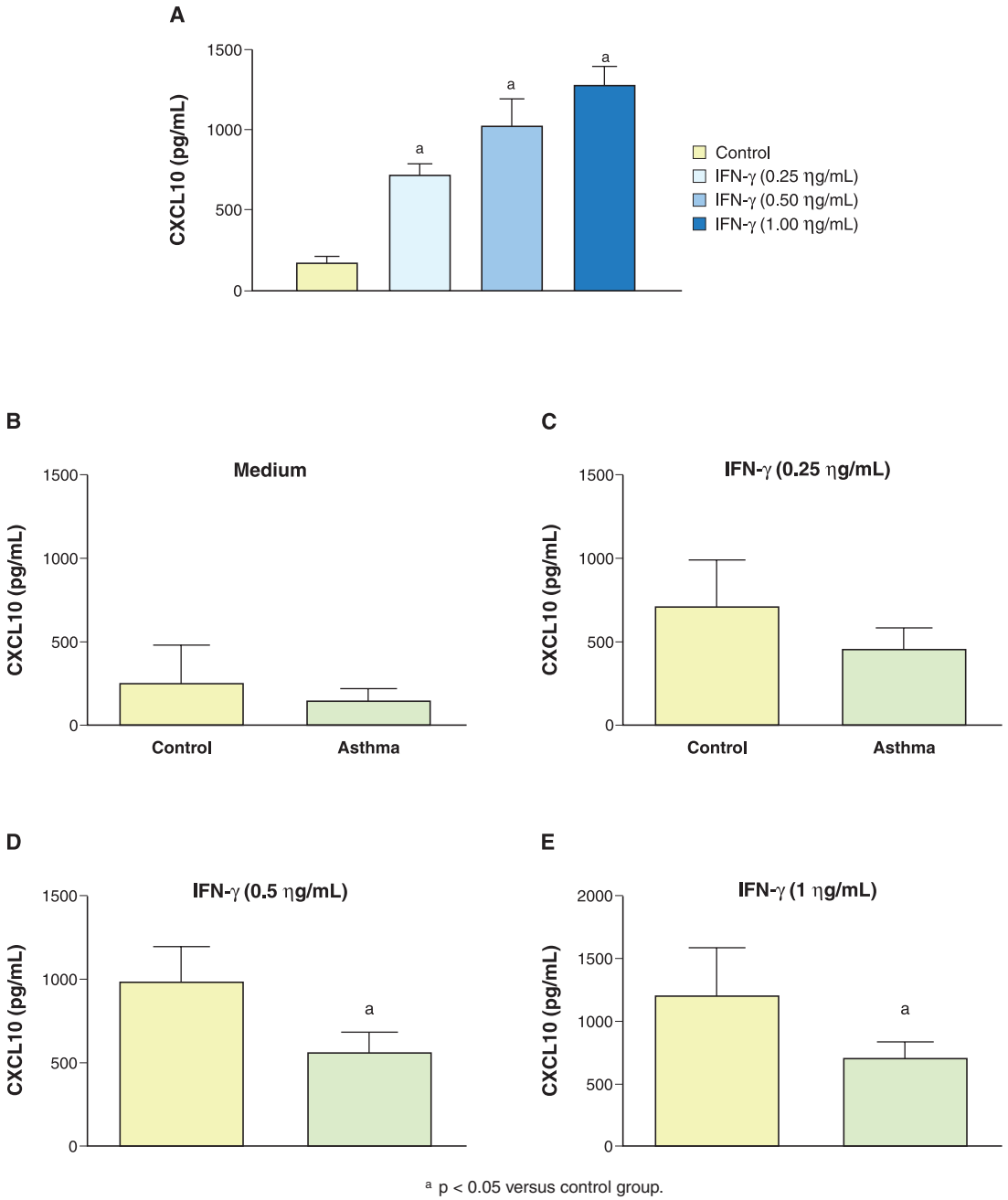
Participant characteristics

We enrolled 12 healthy non-asthmatic controls, of which (58.3% female, 42.7% male; mean age 30 [20-35] years old). Blood eosinophil counts were lower than 230/mm^3 in the control group with negative skin prick tests. We also enrolled 18 patients with allergic asthma (44.4% female, 55.6% male; mean age 35 [27-42] years old). All asthmatic subjects were positive to skin prick test – *D. pteronyssinus* (94.5%), *D. farina* (84.3%), *B. tropicalis* (50.0%), *B. germanica* (11.1%), *P. americana* (11.1%), *A. alternate* (11.1%), *F. domesticus* (66.7%), and *C. familiaris* (38.9%) –, with percentage of predicted forced expiratory volume in first second (FEV_1) value > 71% and blood eosinophil counts greater than 300 cells/mm^3. Asthma patients were classified as having moderate asthma and were treated with inhaled corticosteroids.

CXCL10 concentration is reduced in PBMCs from asthma patients after IFN-γ stimulation

In the first step, we evaluated the effect of IFN-γ (0.25-1 ng/mL) on CXCL10 production in PBMCs from the control group. As observed in Figure 1A, IFN-γ increased CXCL10 concentration in a dose-dependent manner when compared to nonstimulated cells.

Next, we evaluated the modulation of IFN-γ in CXCL10 production in the asthma group. No significant difference in CXCL10 concentration was observed in nonstimulated PBMCs from the control and asthma groups (basal profile) (Figure 1B). A similar result was also observed in cells stimulated with a low concentration of IFN-γ (0.25 ng/mL) (Figure 1C). However, at higher concentrations of IFN-γ (0.5 and 1 ng/mL), CXCL10 production was lower in PBMCs from asthma patients when compared to control individuals (Figure 1D and 1E, respectively). CXCL10 production was reduced by ~40% from



[a] p < 0.05 versus control group.

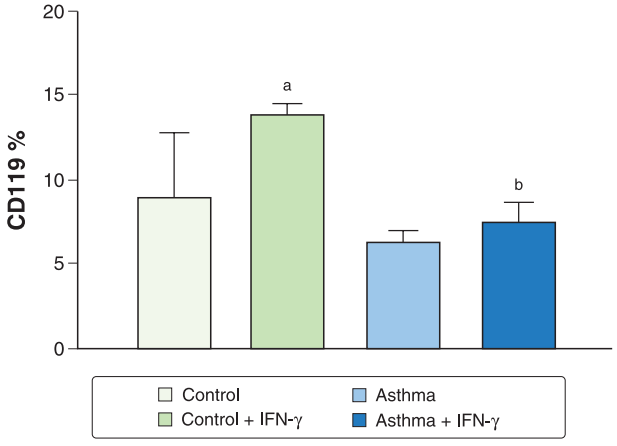
Figure 1

CXCL10 production is reduced in PBMCs from asthma patients after IFN-γ stimulation. Dose response effect of IFN-γ (0.25-1.0 ng/mL) in PBMCs from the control group (A). PBMCs from controls and asthma patients were not stimulated (B; basal) or stimulated with IFN-γ at a dose of 0.25 ng/mL (C), 0.5 ng/mL (D), and 1 ng/mL (E). After 24 h, the culture supernatants were collected and CXCL10 concentrations were measured by ELISA kit. The data are reported as mean ± SD. All samples were tested in triplicate

975.23 ± 64.09 pg/mL (Control + IFN-γ at 0.5 ng/mL) to 594.36 ± 77.65 pg/mL (Asthma + IFN-γ at 0.5 ng/mL) and from 1,205.87 ± 105.90 pg/mL (Control + IFN-γ at 1 ng/mL) to 718.64 ± 77.50 pg/mL (Asthma + IFN-γ at 1 ng/mL) (mean ± SD; $p < 0.05$). The dose of 1 ng/mL of IFN-γ was chosen for the next set of experiments.

IFN-γ R1 expression is reduced in PBMCs from asthma patients

IFN-γR1 expression was increased in IFN-γ-stimulated PBMCs from control individuals when compared to nonstimulated PBMCs from control individuals. No significant difference was observed in IFN-γR1 expression between nonstimulated cells from control individuals and asthma patients. There was a reduction of expression of IFN-γR1 in IFN-γ-stimulated PBMCs from asthma patients when compared to IFN-γ-stimulated PBMCs from control individuals (Figure 2). The IFN-γR1 expression was reduced by ~47% from 13.76 ± 1.06 % (Control + IFN-γ) to 7.34 ± 1.12% (Asthma + IFN-γ) (mean ± SD; $p < 0.05$).

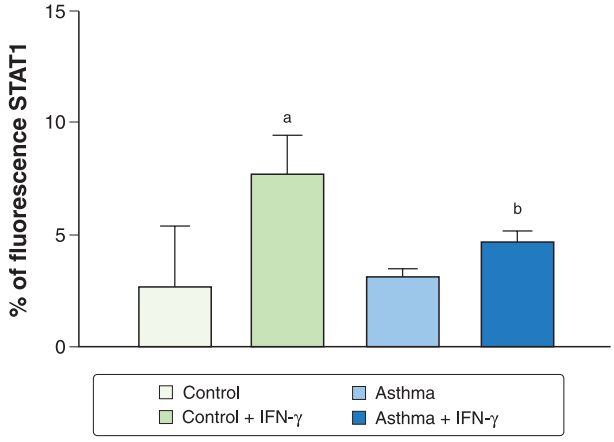


[a] $p < 0.05$ versus control group.
[b] $p < 0.05$ versus control + IFN-γ group.

Figure 2
IFN-γR1 expression is reduced in asthma patients. After 24h, cells were recovered and analyzed for the percentage of fluorescence IFN-γR1 (CD119) in 50,000 cells by cytometry. The data are reported as mean ± SD. All samples were tested in duplicate

STAT1 activation is reduced in PBMCs from asthma patients after IFN-γ stimulation

IFN-γ significantly increased STAT1 phosphorylation in PBMCs from the control group when compared to nonstimulated cells. It was observed a reduction of pSTAT1 activation in PBMCs from asthma patients stimulated with IFN-γ when compared to control cells stimulated with IFN-γ. The STAT1 phosphorylation was reduced by ~38% from 7.62 ± 1.77 % (Control + IFN-γ) to 4.66 ± 0.38 % (Asthma + IFN-γ) (mean ± SD; $p < 0.05$). No significant difference was observed in STAT1 phosphorylation in PBMCs from asthma patients stimulated or not stimulated with IFN-γ. No significant difference was observed in STAT1 phosphorylation in nonstimulated PBMCs from asthma patients and control individuals (Figure 3).



[a] $p < 0.05$ versus control group.
[b] $p < 0.05$ versus control + IFN-γ group.

Figure 3
STAT1 activation is upregulated in PBMC from asthma patients. PBMCs from controls and asthma patients were stimulated or not with IFN-γ (1 ng/mL). After 24h, cells were recovered and analyzed the percentage of pSTAT1 fluorescence of in 50,000 cells by cytometry. Data are reported as mean ± SD. All samples were tested in duplicate

SOCS1 mRNA expression is upregulated in PBMCs of asthma patients after IFN-γ stimulation

Once SOCS1 was able to inhibit STAT1 pathway, we next evaluated SOCS1 expression in PBMCs. No significant alteration of SOCS1 mRNA expression was observed in PBMCs from the control group stimulated with IFN-γ when compared to nonstimulated cells. No significant alteration of SOCS1 mRNA expression was observed in nonstimulated PBMCs from control and asthma patients. However, SOCS1 mRNA expression was increased in PBMCs from asthma patients stimulated with IFN-γ (~2.4 fold) when compared to nonstimulated cells (Figure 4).

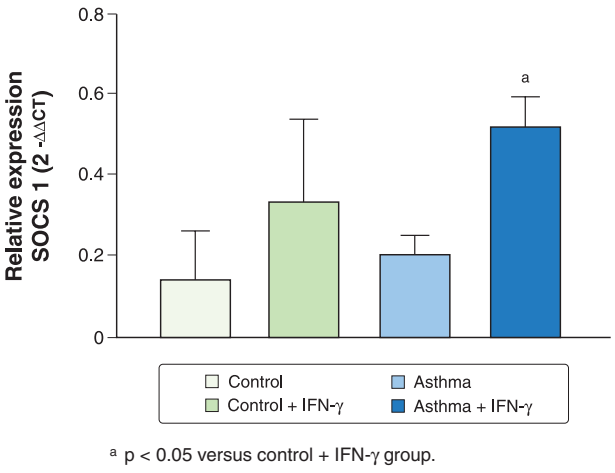


Figure 4
SOCS1 mRNA expression is upregulated in PBMCs from asthma patients after IFN-γ stimulation. PBMCs from controls and asthma patients were stimulated or not with IFN-γ (1 ng/mL). At 30 min after stimulation, SOCS1 mRNA expression was quantified by qPCR. The data are reported as mean ± SD (n = 4-5). All samples were tested in duplicate. The results are expressed as mean ± SD. +p < 0.05 versus asthma group

Discussion

Although asthma was originally defined as a type 2 (T2) immune-mediated condition, non-T2 cytokines, such as IFN-γ and IL-17A, have been implicated in its pathogenesis, particularly in patients with severe disease.[6] In those with mild to moderate asthma, IFN-γ expression in bronchial biopsy specimens and IFN-γ protein levels in the BALF are reduced compared to severe asthma patients. CXCL10 is a chemokine with chemoattractant activity toward Th1 CD4+ T cells and neutrophils, being a marker of Th1 activity.[15,16] CXCL10 transgenic mice develop a Th2 inflammatory response[16], and CXCL10 concentration is elevated in the BALF of patients with stable asthma.[17] We observed that CXCL10 production was significantly reduced in PBMCs from patients with allergic asthma after administration of IFN-γ, in a dose-response manner. However, the opposite effect was observed in PBMCs from the control group. These results suggest that chemotactic activity from activated Th1 lymphocytes is reduced in PBMCs from asthma patients.

The reduction in CXCL10 levels after IFN-γ stimulation in PBMCs from asthma patients could be associated with a reduction in IFN-γ receptor expression in cells and/or downregulation of signaling pathways. IFN-γR1 expression was reduced in IFN-γ-stimulated PBMCs from patients with allergic asthma when compared to IFN-γ-stimulated cells from control groups. Mice with gene knockouts at the IFN-γ receptor ($IFNGR^{-/-}$) have impaired ability to inhibit and resolve the allergic immune response.[18] Polymorphisms in the genes encoding IFN-γ receptor chains are associated with asthma diseases.[19,20] The lower expression of IFN-γR1 is in agreement with the decrease in CXCL10 production found in asthma patients after IFN-γ stimulation.

The STAT signaling cascade has a critical role in the IFN-γ function.[21] Mice deficient in STAT1 exhibit defective responses to IFN-γ and are more susceptible to microbial and viral infections.[22] pSTAT1 activation by IFN-γ was decreased in PBMCs from patients with allergic asthma when compared to cells from control individuals. pSTAT1 pathway inhibition is effective in abrogating CXCL10 release.[23] This result is in synergism with reduced IFN-γR1 expression and reinforces the lower CXCL10 concentration in PBMCs from asthma patients after IFN-γ stimulation.

SOCS proteins can inhibit STAT pathways,[13] and SOCS1 is a negative regulator of STAT1 in IFN-γ-stimulated cells.[24] In vitro cell cultures and in vivo animal models have demonstrated that SOCS1 is a negative regulator of Th2-dependent pathways[25]. SOCS1 gene expression in the airways in mild/moderate asthmatics was increased when compared to that of severe asthmatics.[15,16] In an ovalbumin-induced airway inflammation model, mice with gene knockouts at the SOCS1 and IFN-γ demonstrated increased eosinophilic infiltration in the lungs and elevated Th2 cytokines when compared to mice with deletion of IFN-γ.[15] SOCS1 promoter polymorphism in asthma patients leads to interferon inhibition IFNs, which could increase susceptibility to virus-induced asthma exacerbations.[26] We observed that IFN-γ increased SOCS1 expression in PBMCs from patients with allergic asthma. These results are associated with reduced CXL10 and pSTAT1 activation and, in combination with reduced IFN-γR1 expression, demonstrate a downregulation of IFN-γ signaling in asthma patients.

Conclusion

Taken together, our results indicate that IFN-γ signaling is reduced in PBMCs from asthma patients, suggesting correlation with asthma disease.

Acknowledgements

This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (no. 475349/2010-5), Fundação de Apoio a Pesquisa do Estado de Minas Gerais (FAPEMIG; APQ 01631/11 e APQ-01873-14), Rede de Pesquisa em Doenças Infecciosas Humanas e Animais do Estado de Minas Gerais, and Universidade Federal do Triângulo Mineiro (UFTM), Brazil. This study was partly funded by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

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No conflicts of interest declared concerning the publication of this article.

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