Effects of Aerobic Exercise on Molecular Aspects of Asthma: Involvement of SOCS-JAK-STAT

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ABSTRACT

Background: *Aerobic training (AT) decreases airway inflammation in asthma, but the underlying cellular and molecular mechanisms are not completely understood. Thus, this study evaluated the participation of SOCS-JAK-STAT signaling in the effects of AT on airway inflammation, remodeling and hyperresponsiveness in a model of allergic airway inflammation.*

Methods: *C57Bl/6 mice were divided into Control (Co), Exercise (Ex), HDM (HDM), and HDM+Exercise (HDM+ Ex). Dermatophagoides pteronyssinus (100ug/mouse) were administered oro-tracheally on days 0, 7, 14, 21, 28, 35, 42 and 49. AT was performed in a treadmill during 4 weeks in moderate intensity, from day 24 until day 52.*

Results: *AT inhibited HDM-induced total cells ($p<0.001$), eosinophils ($p<0.01$), neutrophils ($p<0.01$) and lymphocytes ($p<0.01$) in BAL, and eosinophils ($p<0.01$), neutrophils ($p<0.01$) and lymphocytes ($p<0.01$) in peribronchial space. AT also reduced BAL levels of IL-4 ($p<0.001$), IL-5 ($p<0.001$), IL-13 ($p<0.001$), CXCL1 ($p<0.01$), IL-17 ($p<0.01$), IL-23 ($p<0.05$), IL-33 ($p<0.05$), while increased IL-10 ($p<0.05$). Airway collagen fibers ($p<0.01$), elastic fibers $p<0.01$) and mucin ($p<0.01$) were also reduced by AT. AT also inhibited HDM-induced airway hyperresponsiveness (AHR) to methacholine 6,25mg/ml ($p<0.01$), 12,5mg/mL ($p<0.01$), 25mg/mL ($p<0.01$) and 50mg/mL ($p<0.01$). Mechanistically, AT reduced the expression of STAT6 ($p<0.05$), STAT3 ($p<0.001$), STAT5 ($p<0.01$) and JAK2 ($p<0.001$), similarly by peribronchial leukocytes and by airway epithelial cells. SOCS1 expression ($p<0.001$) was upregulated in leukocytes and in epithelial cells, SOCS2 ($p<0.01$) was upregulated in leukocytes and SOCS3 down-regulated in leukocytes ($p<0.05$) and in epithelial cells ($p<0.001$).*

Conclusions: *AT reduces asthma phenotype involving SOCS-JAK-STAT signaling.*

Key words: asthma, exercise immunology, SOCS, JAK, STAT.

INTRODUCTION

Asthma has now become the most prevalent chronic disease in developed countries and affects over 10% of adults [1,2]. Asthma is a chronic inflammatory airway disease, which involves interactions of genetic and environmental factors, whose consequences leads to cough, wheezing, airway hyperresponsiveness and obstruction, caused by inflammation, mucus overproduction, angiogenesis and airway remodeling [1,2]. The chronic inflammation of respiratory tract in asthma is mediated by the increased expression of multiple inflammatory proteins, including cytokines, chemokines, adhesion molecules, products derived of arachidonic acid and receptors. Several signaling pathways have been proposed to be involved in the pathogenesis of asthma. Among those, SOCS (Suppressor of cytokine signaling protein family), JAK (Janus kinase) and STAT (Signal transducer and activator of tran-

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scription) signaling pathways seems to have a key role [8-12]. However, the results are still controversial, since that in the asthmatic context; some studies show SOCS proteins inhibiting asthmatic phenotype, while others show SOCS increasing asthmatic phenotype, through JAK/STAT modulation [8-12]. Aerobic exercise, which is the main component of pulmonary rehabilitation programs [13] and improves physical fitness, is a strong and effective therapy for pulmonary diseases including asthma [37]. Beyond that, the participation in a pulmonary rehabilitation program is associated with reduced exacerbation [47]. Aerobic exercise, indeed, at low and moderate intensity, can ameliorate lung inflammatory response in the context of different lung diseases and insults, such as emphysema and chronic obstructive pulmonary disease (COPD) [14, 20-22], pulmonary fibrosis [23,24], acute lung injury/acute respiratory distress syndrome [25-27], air pollution [28,29] and asthma [30-35]. However, specifically for asthma, the possible involvement of SOCS, JAK and STAT signaling in the beneficial effects of aerobic exercise is unknown. Aerobic exercise is capable to modulate SOCS, JAK and STAT signaling in different organs and cells, such as blood, testis, kidney, skeletal muscle, endothelial cells, heart and lungs [14-19, 48]. However, until this moment, a single study has investigated the effects of aerobic exercise on STAT3 expression in the lungs, which was done in the context of an experimental model of COPD [14].

Cellular and molecular mechanisms of asthma

Asthma is characterized by chronic inflammation of the respiratory tract. The disease is associated with acute episodes or simply, exacerbations, when the intensity of this inflammation increases [49]. The majority of asthma patients are atopic and have an allergic pattern of inflammation in their airways, which extends from the trachea down to peripheral airways [50]. Allergic inflammation is driven by CD4+ T-helper 2 (Th2) lymphocytes, which secrete interleukin(IL)-4, IL-5 and IL-13 and is referred as Type 2 (T2) asthma, whereas some asthmatic patients have different pattern of inflammation which is known as non-T2 asthma and is associated with more severe disease [51]. In T2 asthma, which represent the most prevalent form of the diseases is characterized by accumulation of eosinophils, mastocytes, CD4+ T helper cells, while non-T2 asthma, beyond these classical cells involved in the allergic process, also present high accumulation of neutrophils. Asthmatic inflammation results in airway narrowing and airway hyperressponsiveness (AHR) which represent the mains physiological abnormality of asthma [50]. The mechanisms of AHR are still unclear but are probably associated with increased release of pro-inflammatory mediators by inflammatory cells (particularly mast cells), increasing the contractility of airway smooth muscle, increasing the sensitivity of airway sensory nerves resulting in airway narrowing for geometric reasons [50].

The molecular mechanisms involved in the pathogenesis of asthma is still not fully understood, however it seems likely that the inhaled allergens activate mast cells, epithelial cells and dendritic cells to locally release several pro-inflammatory mediators, such as chemokines. Released chemokines (CCL17 and CCL22 from dendritic cells and CCL11 from epithelial cells), recruit inflammatory cells especially TH2 cells from blood to the lungs. TH2 cells have a central role in orchestrating the inflammatory response in allergy through the release of interleukin-4 (IL-4) and IL-13 (which stimulate B cells to synthesize IgE), IL-5 (which is necessary for eosinophilic inflammation) and IL-9 (which stimulates mast-cell proliferation). Mast cells release several bronchoconstrictor mediators, including mast cell tryptase, cysteinyl leukotrienes and prostaglandins. In addition, Th17 cells are also increased in asthmatic patients, preferentially in those with severe asthma. These cells may orchestrate neutrophilic inflammation by inducing the release of CXCL8 from airway epithelial cells [52-53]. Furthermore, asthmatic patients may have reduced number of regulatory T (TReg) cells, which suppress TH2 cells, suggesting further TH2-cell proliferation in asthmatic condition [50].

Exercise-induced asthma

Exercise is one of the most common triggers of bronchospasm in persons with and without asthma. Exercise-induced bronchoconstriction (EIB) is defined as transient, reversible bronchoconstriction that develops after strenuous exercise [54]. It is a heterogeneous syndrome occurring in a variety of settings, ranging from the asymptomatic military recruit (whose condition is detected by diagnostic exercise challenge) to the leisure-time athlete with known asthma to the elite athlete for whom EIB may represent an overuse or injury syndrome [55]. Airway obstruction following exercise was first observed among individuals with underlying asthma from which the term exercise-induced asthma (EIA) was derived. Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, and it is associated with bronchial (or airway) hyperresponsiveness. Similar post-exercise asthma-like symptoms have been observed in persons without the presence of co-existing asthma, particularly in athletes. In this population the phenomenon has been referred to as exercise-induced bronchoconstriction (EIB) [56]. According to work group report of American Academy of Allergy, Asthma and Immunology, EIA represents a distinct clinical category of asthma [56]. In fact, most if not all patients with asthma develop symptoms of asthma after a suitable exercise challenge [56]. Moreover, even cases of asthma in which exercise appears to be the only trigger of bronchial obstruction (pure EIA) may be manifestations of chronic inflammation of the airways [56].

Exercise as an anti-inflammatory therapy for asthma

Recent studies strongly support the notion that exercise intervention improve asthma control in adults [57, 58]. These includes improvements in measures such as lung function and quality of life [59], breathlessness [60], and controller therapy [61] while animal models have shown improvements in airway inflammation [62, 63, 64].

Yet asthma is a chronic inflammatory disease, it is highly suggested that effective anti-inflammatory treatments for asthma should reduce diseases progression and the risk of exacerbations [65]. Although there are now effective medications for controlling asthma, it remains poorly controlled in the community with frequent symptoms and exacerbations [66]. It is clear, therefore, that the development of alternative and effective anti-inflammatory therapies for the treatment of asthma is probably the greatest unmet therapeutic need at present. In health, a large body of evidence demonstrates that

lack of physical activity or fitness is associated with an increased risk of cardiovascular disease, stroke, cancer, diabetes and many other chronic diseases [67, 68]. The studies by our group and the others demonstrate that exercise, specifically, chronic aerobic exercise, at low and moderate intensity, can ameliorate lung inflammatory response in asthma condition [30-35, 69]. In a very recent study, our team demonstrated that aerobic exercise attenuated asthma phenotype through modulation of inflammatory cytokines (e.g. IL-5 and IL-13) as well as Leukotriene pathway (LTA4H, CysLT1 receptor, CysLT2 receptor, LTC4 synthase, and BLT2) in an ovalbumin (OVA) model of asthma (30). LTs are potent pro-inflammatory mediators, involved in several aspects of asthma pathophysiology, including bronchoconstriction, edema formation, mucus hypersecretion, as well as inflammatory cell proliferation, activation, and survival [70-72]. In addition, aerobic exercise attenuates dendritic cell and lymphocyte activation in Ovalbumin (OVA) model of allergic airway inflammation [31]. Dendritic cells are the major antigen-presenting cells in the airways and play critical role in initiation and progression of asthma. These cells release several chemokines (e.g,. CCL17, CCL22) to attract Th2 cells into the airways, serving as major regulators of Th2 immune response in asthma [73]. As mentioned previously, Th2 cells have a central role in orchestrating the inflammatory response in asthma [49]. Aerobic exercise also attenuates lung inflammation through attenuation of OVA-specific IgE and IgG1 titers [32]. Furthermore, exercise increases the concentration and expression of several anti-inflammatory mediators such as IL-10 and IL-1ra in the lungs to attenuate lung inflammation [30-35]. Based on these evidences it can be concluded that aerobic exercise decreases airway inflammation and remodeling in, at least, a murine model of asthma. In addition, the anti-inflammatory effects of aerobic exercise on asthmatic airway inflammation has been demonstrated not only in murine models of asthma, but also in asthmatic individuals [37].

SOCS-JAK-STAT pathway in asthma and the role of exercise

Inflammation is etiologically linked to the pathogenesis of all of most of the chronic diseases, and chronic low-grade systemic inflammation is correlated with disease severity in many [70-73]. Cytokines play a pivotal role in the initiation and development of asthma by regulating the expansion of Th2 cells and by mediating many of the Th2 effector functions that underlie the pathogenic events of an asthmatic response [70-73]. Several studies have recently been performed to elucidate the signaling pathways used by cytokines to mediate their actions. These studies have revealed that cytokine-mediated signals are primarily transduced by the JAK-STAT signaling cascade [8-12, 74-77]. The Janus kinase (JAK)–signal transducer of activators of transcription (STAT) pathway, is now recognized as an evolutionarily conserved signaling pathway employed by diverse cytokines, interferons, growth factors, and related molecules and therefore, involved in inflammatory processes. Jaks (tyrosine kinases) engage with cytokine receptors and mediate tyrosine phosphorylation of their associated receptors and recruited proteins, including STATs [78]. Tyrosine phosphorylated STATs are released from the receptors and form homodimers, which translocate to the nucleus where they bind canonical sequences and modulate transcription [79]. In addition to tyrosine phosphorylation, STATs are serine phosphorylated within their transcriptional activation domain, influencing their transcriptional activation function, stability, and non-canonical functions [78]. STAT proteins are critical mediators of immunity to pathogens. Indeed, inflammation was one of the earliest biological functions associated with STAT proteins, from the anti-viral functions of STAT1, to the polarized T helper cell responses that required STAT4 and STAT6. STATs are also acetylated, methylated, sumoylated, and ubiquitylated, which alters their stability, dimerization, nuclear localization, transcriptional activation function, and association with histone acetyltransferases and histone deacetylases [78]. Importantly, Jak/Stat activation is tightly regulated through the expression of positive (cytokines, receptors, tyrosine kinases) and negative regulators (tyrosine phosphatases, protein inhibitors of activated Stat, suppressor of cytokine signaling [SOCS] proteins) [78].

In this signaling pathway, binding of a cytokines such as IL-4 or IL-12 to their receptors leads to the activation of members of the JAK family of receptor-associated kinases. These kinases subsequently activate, via tyrosine phosphorylation, preexistent cytoplasmic factors termed STATs. Tyrosine phosphorylation allows the STAT proteins to dimerize and translocate to the nucleus, where they mediate changes in gene expression by binding specific DNA elements. The SOCS (suppressors of cytokine signaling) family proteins (in particular SOCS3) are the best understood negative regulators of the JAK-STAT pathway [10, 80-82], and are composed of eight proteins, i.e. SOCS1–7 and SH2 cytokine-inducible protein (CIS) [80-82]. The regulatory function of SOCS proteins is critical to the normal functioning and cessation of the primary cytokine signal and it is achieved at many levels in the intracellular biochemical cascade [80-82]. For example, SOCS1 is thought to inhibit the catalytic activity of JAKs by binding to the activation loop of the catalytic domain through both its kinase inhibitory region (KIR) and SH2 domain. Binding of SOCS1 to JAK kinases therefore blocks further signaling in a negative feedback loop [80-82]. Exercise has been shown to modulate SOCS-JAK-STAT signaling pathways in different cells and tissues including blood, testis, kidney, skeletal muscle, endothelial cells, heart and lungs [14-19, 48]. However, no information is available concerning the potential involvement of SOCS, JAK and STAT signaling in the beneficial effects of aerobic exercise in asthma. We have recently investigated the effects of aerobic exercise on STAT3 expression in the lungs of smoked-exposed animals [14]. We demonstrated that aerobic exercise reduced smoke-induced STAT3 expression and phosphorylation in airway epithelial cells, peribronchial leukocytes, and parenchymal leukocytes. The reduction in STAT3 expression and phosphorylation was accompanied by reduction in smoked-induced inflammatory cytokines (IL-1β, IL-17, TNF-α) and induction of IL-10 levels in BALF and serum in the mice.

Therefore, we hypothesized that the anti-inflammatory effects of aerobic exercise in an experimental model of asthma, would be due to its effects on inflammatory cytokines, involving SOCS-JAK-STAT signaling pathway.

Therefore, we aimed to perform an original study to investigate the potential role of SOCS-JAK-STAT signaling pathway in the effects of aerobic exercise training on airway inflammation, remodeling and hyperresponsiveness in a

model of house dust mite-induced allergic airway inflammation. Of note, this is the first study testing the effects of aerobic exercise in face a real life allergen (house dust mite – *dermatophagoides pteronyssinus*) inducing an asthma phenotype, through a direct contact with the respiratory mucosa, like happens in humans.

MATERIALS AND METHODS

Animals and study design

The experimental protocol was approved by the ethical committee of the Nove de Julho University. All animal care and experimental procedures followed the international recommendations of the Helsinki convention for the use and care of animals.

120 male C57Bl/6 mice (aged 8 weeks and weighing 20g approximately) were maintained under standard conditions with controlled temperature (22°C - 25°C) and relative humidity (50%-60%) on a 12 h light/dark cycle. They were provided with food and water *ad libitum*. The animals were randomly distributed into the following experimental groups (n = 3 x 10 animals in each group): 1. Control (Con - not sensitized and untrained), 2. Exercise (Exe - not sensitized and trained), 3. HDM (HDM - sensitized to HDM and untrained), 4. HDM + Exe (HDM + Exe - sensitized with HDM and trained).

Protocol of chronic allergic lung inflammation

Under anesthesia using ketamine (100 mg/kg) and xylazine (10 mg/kg), HDM groups received *Dermatophagoides pteronyssinus* extract (HDM 100µg/mouse) (Greer Laboratories, Lenoir, NC) diluted in 50µl of phosphate buffered saline (PBS), orotracheally administered, on days 0, 7, 14, 21, 28, 35 e 42 [36].

Physical test and exercise training protocol

On days 14 to 16 mice were placed on the treadmill (Inbramed, Brazil) for 15 min at a speed of 0.5 km/h and a 15% incline for adaptation to avoid stress induction [34,35]. On days 17 and 42 the maximal exercise test was performed as previously described [34,35]. The treadmill training occurred to 50% of maximal exercise capacity reached in the physical exercise test, which correspond to the moderate intensity [34,35]. It has begun on day 18 and was performed over 4 weeks, 60 min per session for five days a week.

Total and differential cell counting in bronchoalveolar lavage fluid (BALF)

The lungs were carefully washed with 1.5 ml of saline (3×0.5 ml) via tracheal cannula. The samples were centrifuged (900 ×g for 7 min at 4°C), and the resulting cell pellet was re-suspended in PBS (1 ml). BALF total cell count were performed under staining using trypan blue using a hematocytometer (Neubauer chamber) [34]. The differential cell count was carried out after cytocentrifuge preparations (Cytospin®, Fanem, Brazil) stained with May–Grünwald–Giemsa solution [34].

Cytokine measurements in BALF

The BALF levels of IL-4 (DY404; assay range: 15.6 - 1,000 pg/mL), IL-5 (DY405; assay range: 31.2 - 2,000 pg/mL), IL-13 (DY413; assay range: 62.5 - 4,000 pg/mL), CXCL1 (DY453; assay range: 15.6 - 1,000 pg/mL), IL-17 (DY421; assay range: 15.6 - 1,000 pg/mL), IL-23 (DY1887; assay range: 39.1 - 2,500 pg/mL), IL-33 (DY3626; assay range: 15.6 - 1,000 pg/mL) and IL-10 (DY417; assay range: 31.2 - 2,000 pg/mL) were determined by ELISA, using R&D Systems Duo Set kits (MN, USA), according to the manufacturer's recommendations. All reads were done in a SpectraMax i3 microplate reader (Molecular Devices, CA, USA).

Airway inflammation and remodeling

The lungs were removed in bloc and perfused and fixed under positive pressure of 20 cmH_2O with 4% paraformaldehyde solution for 24 hours. The lungs were embedded in paraffin and sectioned in 4 µm slices. The staining was performed with hematoxylin and eosin (HE) for quantification of eosinophils, lymphocytes, neutrophils and macrophages in the peribronchial space, with Picrossirius, for quantification of collagen fibers and with Weigert's resorcin–fuchsin with oxidation for quantification of elastic fibers in airways wall [34]. Periodic Schiff acid plus blue alcian was used for quantification of mucus production in airway epithelium [30]. Five airways of each mouse were used for the analysis [30,34]. All images were taken using a camera QColor5 (Olympus, PA, USA) attached to a microscope Olympus BX40 (Olympus, PA, USA), while the image analysis was done using CellSens software (Olympus, PA, USA) [30].

Immunolocalization and quantification of SOCS1, SOCS2, SOCS3, STAT3, STAT5, STAT6 and JAK2

Immunohistochemistry was performed in 4 µm slices, which were incubated overnight at 4°C with the following primary antibodies: anti-SOCS1 (sc-7006; 1:2.000), anti-SOCS2 (sc-7008; 1:2.000), anti-SOCS3 (sc-7010; 1:2.000), anti-JAK2 (sc-278; 1:20.000), anti-STAT3 (sc-482; 1:20.000), anti-STAT5 (sc-835; 1:20.000) and anti-STAT6 (sc-981; 1:20.000) (Santa Cruz Biotechnology, CA, USA). The reaction was followed by incubation with proper secondary antibodies conjugated with biotin-streptavidin-peroxidase and counter-stained with Harris' hematoxylin, as previously described [20,21,26,29,30,34,35]. Since the immunoreaction was observed in airway epithelium and in peribronchial leukocytes, the quantitative analysis of the expression of each protein was done as follow, in five airways of each mouse:

Positive peribronchial leukocytes: the number of positive peribronchial leukocytes in the peribronchial space (area comprehended between airway basal membrane and airway adventitia) was counted, and the results expressed as number of positive cells per square millimeter [30,35].

Positive area of airway epithelium: the total area of airway epithelium was measured, and the positive area of airway epithelium for each protein was quantified. Then the results were expressed as percentage of airway epithelium positive for each protein [30,34].

Evaluation of airway hyperresponsiveness (AHR)

AHR was evaluated in conscious mice using whole body plethysmograph (Buxco Europe, Winchester, UK) to growing doses (Basal, PBS, 6,25 mg/mL, 12,5 mg/mL, 25 mg/mL and 50 mg/mL) of methacholine (MCh), by using the enhanced

pause (Penh), which correspond to the level of airway obstruction [30].

Statistical analysis

All data were analyzed, and the graphs were built using the software GraphPad Prism 5.0 (CA, USA). Since all data presented parametric distribution, statistical analysis was performed by one-way analysis of variance (ANOVA ONE-WAY) and by Student-Newman-Keuls as post-hoc test. P <0.05 was considered significant. All graphs were presented as mean and standard deviation.

RESULTS

Effects of aerobic exercise on physical capacity and on body weight

The results showed that comparing the initial with final physical test in terms of time (minutes) the Control (2.4±2.04 min; p>0.05) and HDM (3.25±3.95 min; p>0.05) groups did not present significant increases in physical capacity. On the other hand, Exercise (13.9±5.27 min; p<0.05) and HDM+Exercise (11.91±3.23 min; p<0.05) presented significant improvements. When the body weight was analyzed (final body weight minus the initial body weight), the results showed that no significant differences were found (p<0.05).

Aerobic exercise reduces pulmonary inflammation

Figure 2 shows that the HDM model of chronic allergic airway inflammation significantly increases the number of total cells (Figure 2A; p<0.05), eosinophils (Figure 2B; p<0.01), neutrophils (Figure 2B; p<0.01) and lymphocytes (Figure 2B; p<0.05) in BALF. On other way, aerobic exercise reduces HDM-induce increases the number of total cells (Figure 2A; p<0.05), eosinophils (Figure 2B; p<0.05), neutrophils (Figure 2B; p<0.05) and lymphocytes (Figure 2B; p<0.05) in BALF.

Complementarily, quantitative histological analysis revealed that HDM model of chronic allergic airway inflammation significantly increases the number of eosinophils (Figure 3A; p<0.001), neutrophils (Figure 3B; p<0.001) and lymphocytes (Figure 3C; p<0.01) in the peribronchial space. Again, aerobic exercise was able to reduce HDM-induce increases the number of eosinophils (Figure 3A; p<0.001), neutrophils (Figure 3B; p<0.001) and lymphocytes (Figure

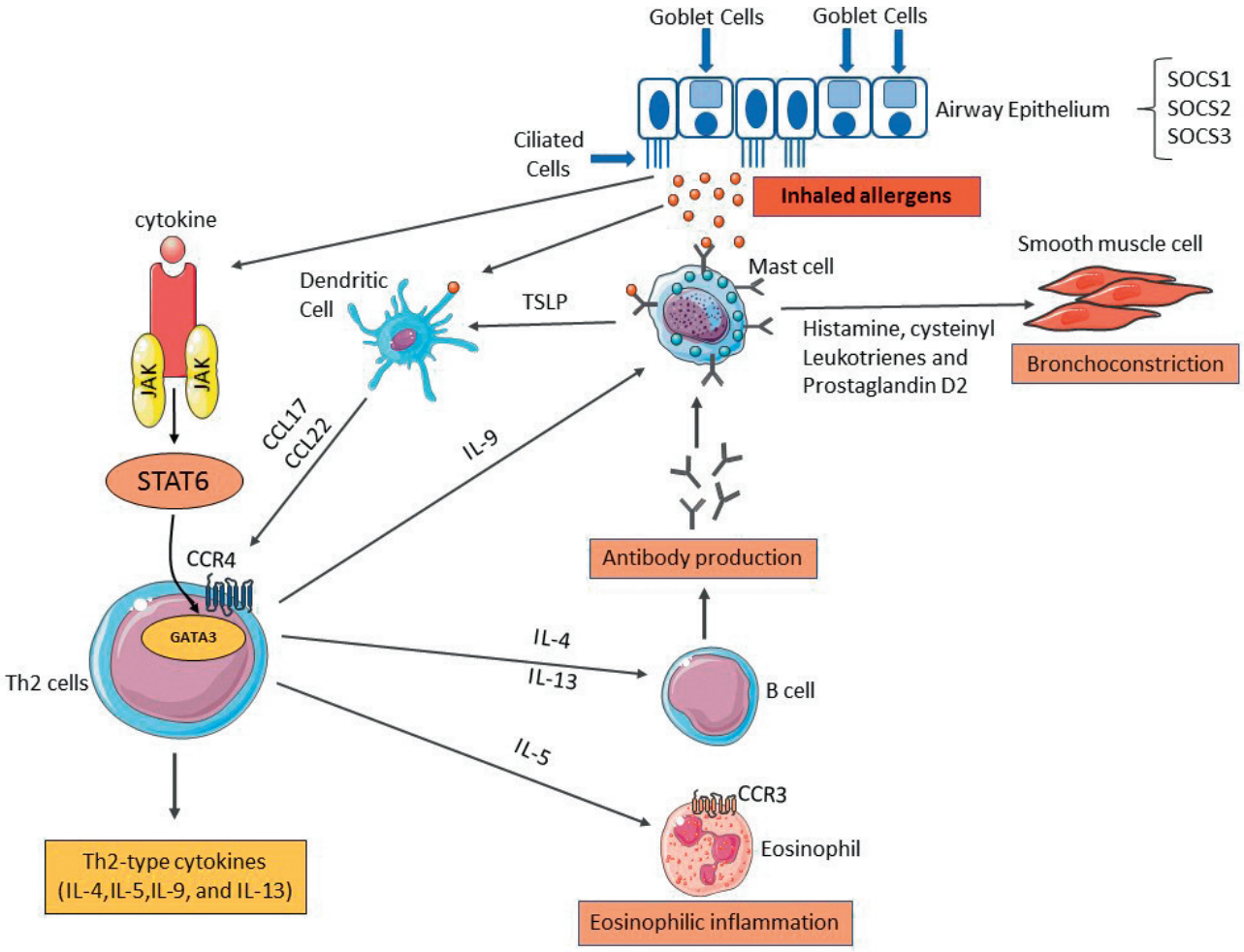

Figure 1. Inflammatory and immune cells involvement in asthma. Inhaled allergens activate lung Dendritic cells and Mast cells to release several chemotactic factors. Activated dendritic cells release the chemokines CCL17 and CCL22, which act on Chemokine-receptor 4 (CCR4) to recruit T helper 2 (TH2) cells. Activation of the transcription factor GATA3 in TH2 cells leads to secretion of the cytokines IL-4 and IL-13 (which stimulate B cells to synthesize IgE), IL 5 (which is necessary for eosinophilic inflammation) and IL 9 (which stimulates mast-cell proliferation). Activated mast cells release several bronchoconstriction mediators including cysteinyl leukotrienes and prostaglandin D2. TSLP: thymic stromal lymphopoietin., CCL17: CC chemokine ligand 17., CCL22: CC chemokine ligand 22., CCR3: CC- chemokine receptor 3., CCR4: CC-chemokine receptor 4., IL-4: interleukin 4, IL-5: interleukin 5, IL-9: interleukin 9., IL-13: interleukin 13., GATA3: GATA binding protein 3.

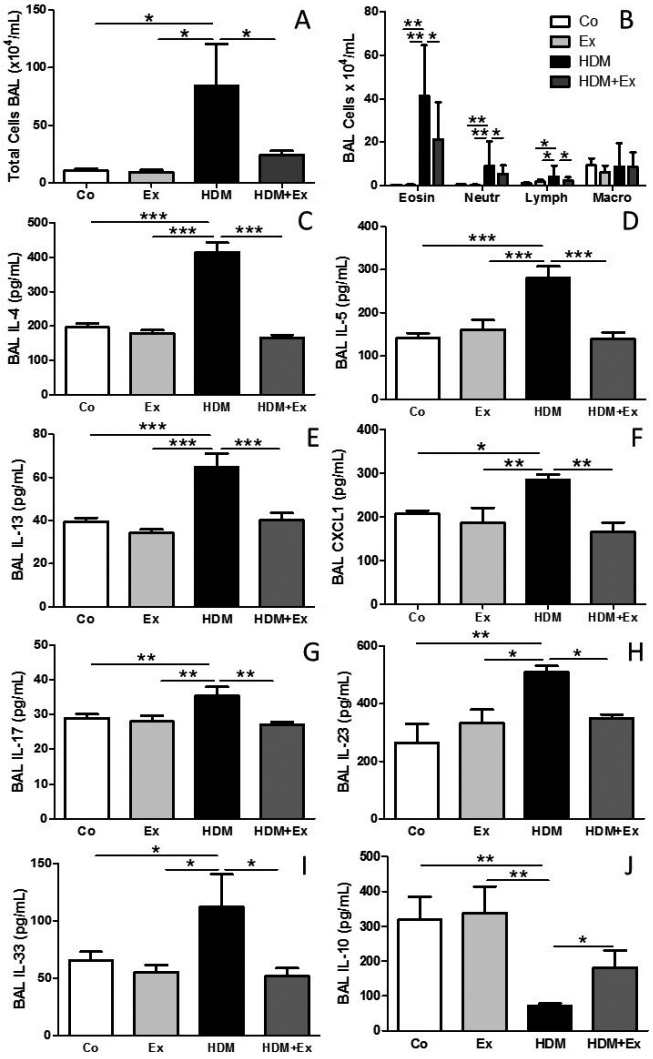

Figure 2. Figure 2 shows the number of total cells in BAL (Figure 2A), eosinophils, neutrophils, lymphocytes and macrophages in BAL (Figure 2B), IL-4 levels in BAL (Figure 2C), IL-5 levels in BAL (Figure 2D), IL-13 levels in BAL (Figure 2E), CXCL1 levels in BAL (Figure 2F), IL-17 levels in BAL (Figure 2G), IL-23 levels in BAL (Figure 2H), IL-33 levels in BAL (Figure 2I) and IL-10 in BAL (Figure 2J). * p<0.05, ** p<0.01 and *** p<0.001.

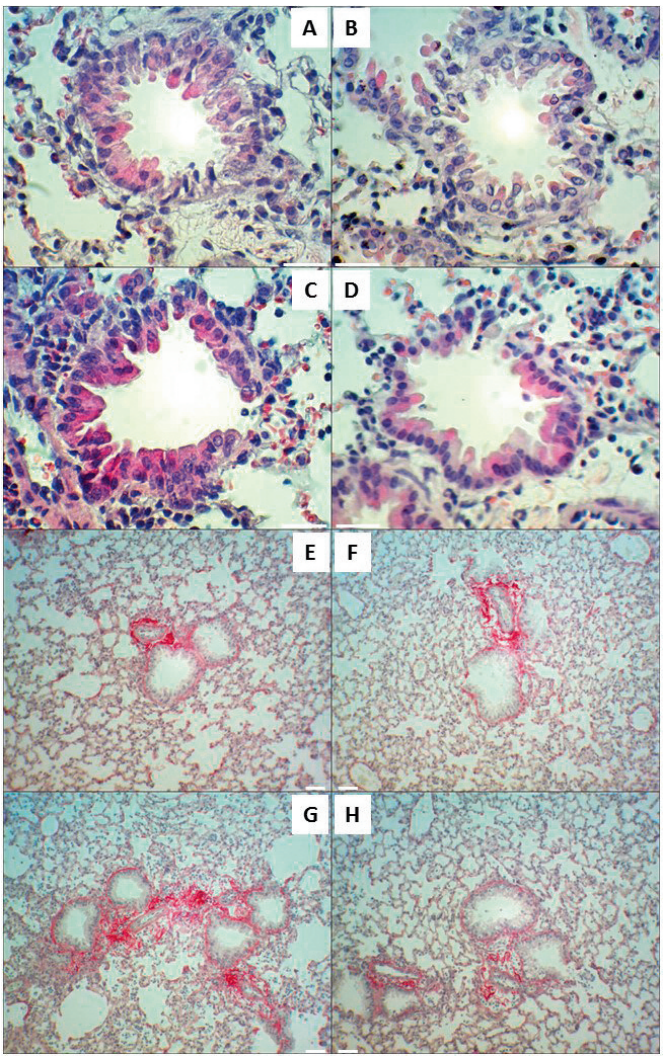

Figure 3. Figure 3 shows the density of eosinophils (Figure 3A), neutrophils (Figure 3A) and lymphocytes (Figure 3C) in airways wall, and of collagen fibers (Figure 3D), elastic fibers (Figure 3E) and mucin (Figure 3F) in the airways. * p<0.05, ** p<0.01 and *** p<0.001.

3C; p<0.001) in the peribronchial space. Figure 4A-D shows representative photomicrographs taken from HE staining, through which the peribronchial inflammation analysis were done.

Aerobic exercise reduces pro-inflammatory cytokines and increases IL-10

Figure 2 shows that the HDM model of chronic allergic airway inflammation significantly increases the BALF levels of Th2 cytokines IL-4 (Figure 2C; p<0.001), IL-5 (Figure 2D; p<0.001), IL-13 (Figure 2E; p<0.001), Th17 cytokine IL-17 (Figure 2G; p<0.01), IL-23 (Figure 2H; p<0.01), IL-33 (Figure 2I; p<0.05) and CXCL1 (Figure 2F; p<0.05), while reduces the levels of IL-10 (Figure 2J; p<0.01). On contrary, aerobic exercise reduces HDM-induce increases in the levels of Th2 cytokines IL-4 (Figure 2C; p<0.001), IL-5 (Figure 2D; p<0.001), IL-13 (Figure 2E; p<0.001), Th17 cytokine IL-17 (Figure 2G; p<0.01), IL-23 (Figure 2H; p<0.05), IL-33 (Figure 2I; p<0.05) and CXCL1 (Figure 2F; p<0.01), while increases the levels of IL-10 (Figure 2J; p<0.05).

Aerobic exercise reduces airway remodeling

Figure 3 shows that the HDM model of chronic allergic airway inflammation significantly increases airway remodeling, notably through accumulation of collagen fibers in airways wall (Figure 3D; p<0.05), elastic fibers in airways wall (Figure 3E; p<0.001), and mucin production by airway epithelium (Figure 3F; p<0.001). Importantly, aerobic exercise was able to reduces HDM-induce increases in airway remodeling, as noted through accumulation of collagen fibers in airways wall (Figure 3D; p<0.05), elastic fibers in airways wall (Figure 3E; p<0.01), and mucin production by airway epithelium (Figure 3F; p<0.01). Figure 4E-G shows representative photomicrographs taken from Picrossirius staining, through which the collagen fibers accumulation in airways wall analysis were done.

Aerobic exercise reduces airway hyperresponsiveness (AHR)

Figure 5 shows that the HDM model of chronic allergic airway inflammation significantly increases AHR, as demon-

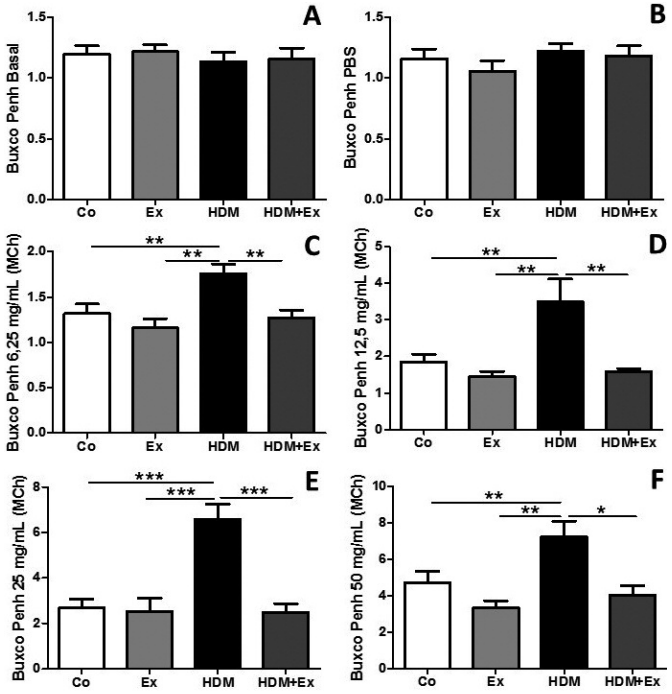

Figure 4. Figure 4 shows representative photomicrographs of quantitative histological analysis, being stained with hematoxylin and eosin for analysis of airway inflammation (Figure 4A; 400x magnification) and with picrosirius for analysis of collagen accumulation in the airways (Figure 4B; 200x magnification). Scale bar are 25 µm and 50 µm, respectively.

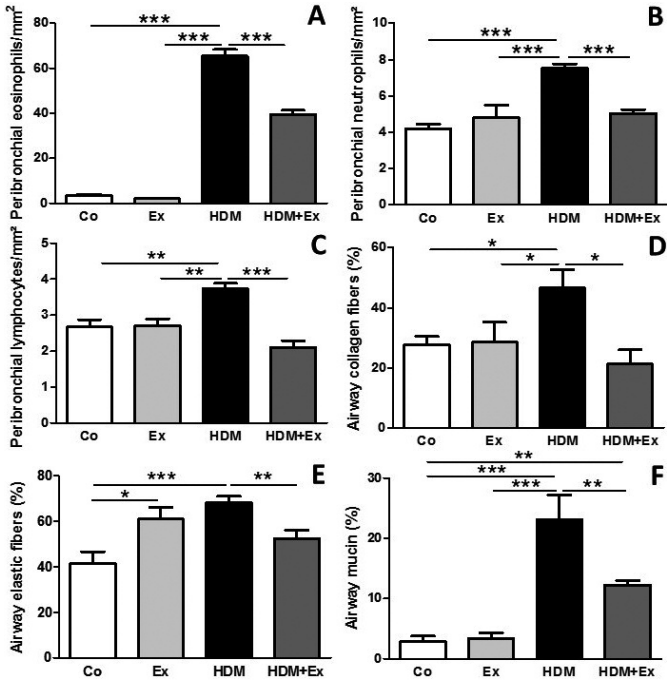

Figure 5. Figure 5 shows airway hyperresponsiveness (AHR) for growing doses of methacholine (Mch), measured using whole body plethysmography. The results are demonstrated as enhanced pause (Penh). * p<0.05, ** p<0.01 and *** p<0.001.

strated through methacholine (MCh) aerosol challenge: 6,25 mg/mL (Figure 5C; p<0.01), 12,5 mg/mL (Figure 5D; p<0.01), 25 mg/mL (Figure 5E; p<0.001) and 50 mg/mL (Figure 5F; p<0.01). Of note, aerobic exercise significantly reduces HDM-induce increases in AHR, as demonstrated through methacholine (MCh) aerosol challenge: 6,25 mg/mL (Figure 5C; p<0.01), 12,5 mg/mL (Figure 5D; p<0.01), 25 mg/mL (Figure 45E; p<0.001) and 50 mg/mL (Figure 5F; p<0.05).

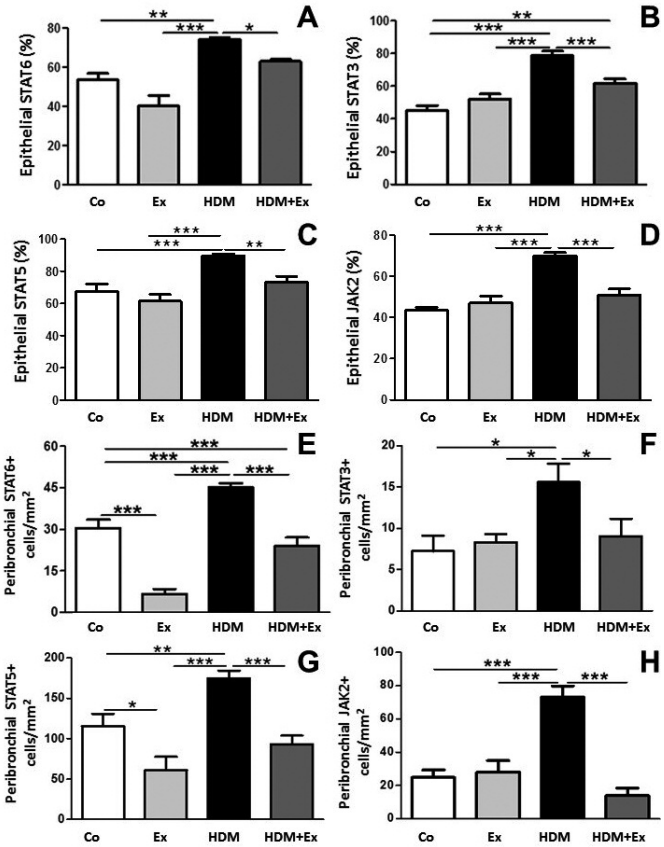

Figure 6. Figure 6 shows the epithelial expression of STAT6 (Figure 6A), STAT3 (Figure 6B), STAT5 (Figure 6C) and JAK2 (Figure 6D) and of STAT6 (Figure 56E), STAT5 (Figure 6F), STAT3 (Figure 6G) and JAK2 (Figure 6H) by peribronchial leukocytes. * p<0.05, ** p<0.01 and *** p<0.001.

Aerobic exercise reduces STAT6, STAT3 and STAT5 and JAK2 expression

Figure 6 shows that the HDM model of chronic allergic airway inflammation significantly increases epithelial expression of STAT6 (Figure 6A; p<0.01), STAT3 (Figure 6B; p<0.001), STAT5 (Figure 6C; p<0.001) and JAK2 (Figure 6D; p<0.001). In addition, HDM model of chronic allergic airway inflammation significantly increases the expression STAT6 (Figure 6E; p<0.001), STAT3 (Figure 6F; p<0.05), STAT5 (Figure 6G; p<0.01) and JAK2 (Figure 6H; p<0.001) by peribronchial leukocytes. Contrarily, aerobic exercise significantly reduces HDM-induce increases in epithelial expression of STAT6 (Figure 6A; p<0.05), STAT3 (Figure 6B; p<0.001), STAT5 (Figure 6C; p<0.01) and JAK2 (Figure 6D; p<0.001). In addition, aerobic exercise also reduced HDM-induce increases the expression STAT6 (Figure 6E; p<0.001), STAT3 (Figure 6F; p<0.05), STAT5 (Figure 6G; p<0.001) and JAK2 (Figure 6H; p<0.001) by peribronchial leukocytes. Figure 7 shows representative photomicrographs taken from STAT6 (Figure 7 A-D) and from STAT3 (Figure 7 E-H) immunostaining.

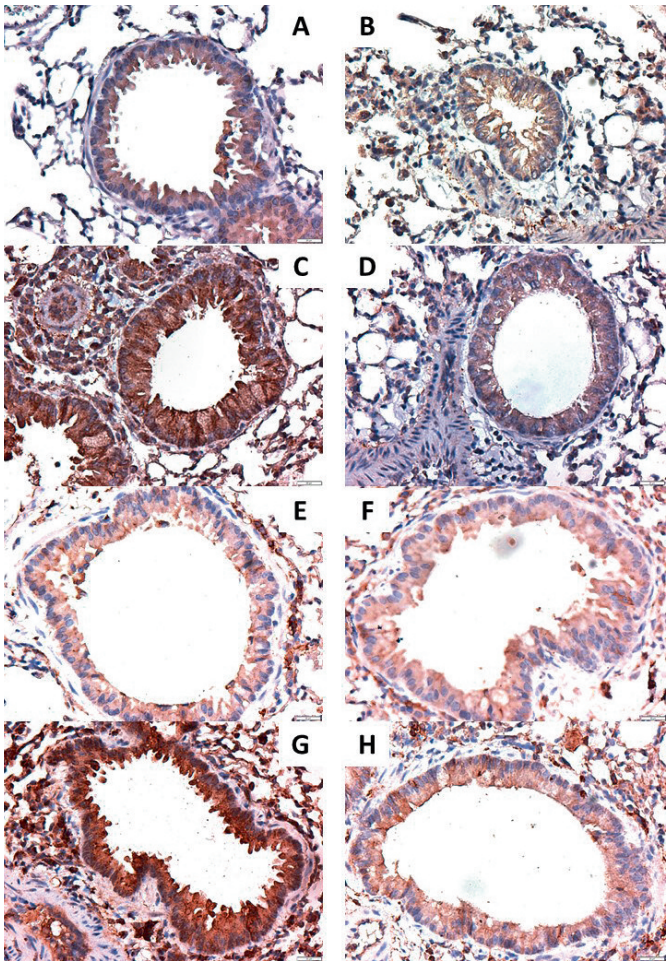

Figure 7. Figure 7 shows representative photomicrographs of quantitative immunohistochemistry analysis of the expression of STAT6 (Figure 7A-D; Co, Ex, HDM and HDM+Ex groups, respectively) and STAT3 (Figure 7E-H; Co, Ex, HDM and HDM+Ex groups, respectively). Images are at 400x magnification. Scale bar are 25 μm.

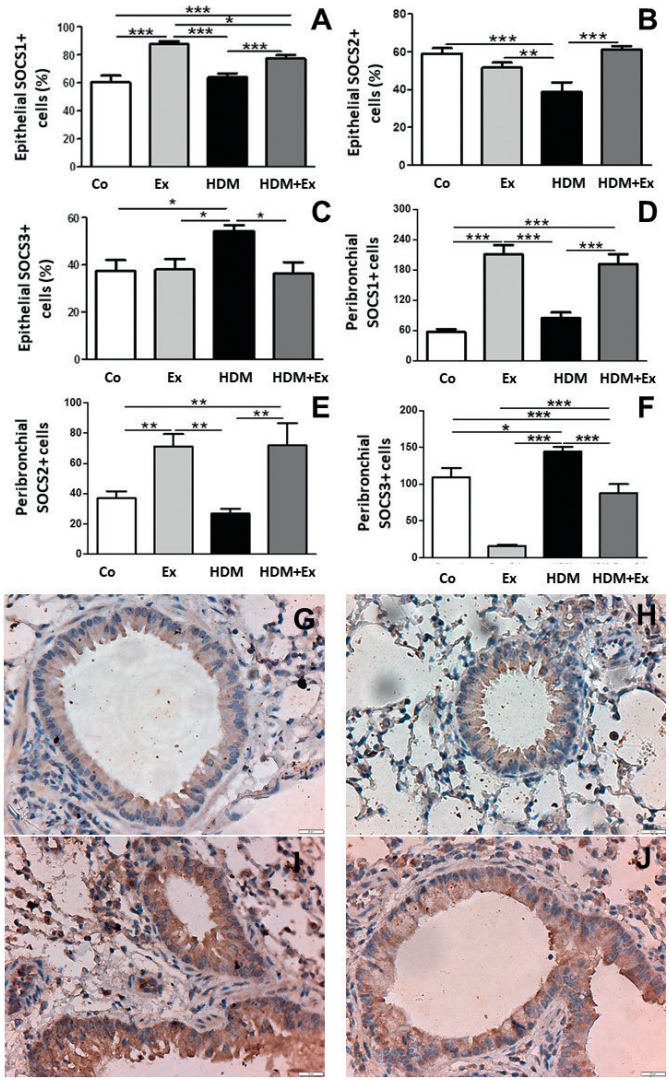

Figure 8. Figure 8 shows the quantitative analysis of the expression of SOCS1 (Figure 8A), SOCS2 (Figure 8B), SOCS3 (Figure 8C) by airway epithelium and of SOCS1 (Figure 8D), SOCS2 (Figure 8E), SOCS3 (Figure 8F) by peribronchial leukocytes. * p<0.05, ** p<0.01 and *** p<0.001. Figure 8G-J shows representative photomicrograph of quantitative immunohistochemistry analysis of the expression of SOCS3, of Co, Ex, HDM and HDM+Ex groups, respectively. Images are at 400x magnification. Scale bar are 25 μm.

Aerobic exercise modulates SOCS1, SOCS2 and SOCS3 expression

Figure 8 shows that the HDM model of chronic allergic airway inflammation significantly does not change epithelial expression of SOCS1 (Figure 8A; p>0.05), but reduces epithelial expression of SOCS2 (Figure 8B; p<0.001), while increases epithelial expression of SOCS3 (Figure 8C; p<0.05). In addition, HDM model of chronic allergic airway inflammation does not change the expression of SOCS1 (Figure 8D; p>0.05) and SOCS2 (Figure 8D; p>0.05), by peribronchial leukocytes, but increases the expression of SOCS3 by peribronchial leukocytes (Figure 8F; p<0.05). Of importance, aerobic exercise in non-sensitized (Ex) and in sensitized (HDM+Ex) mice significantly increases the epithelial expression of SOCS1 (Figure 8A; p<0.001). Aerobic exercise also restores the epithelial expression of SOCS2 (Figure 8B; p<0.001), while reduces HDM-induce epithelial expression of SOCS3 (Figure 8C; p<0.05). Regarding the expression SOCS1 (Figure 8D; p<0.001) and SOCS2 (Figure 8E; p<0.01) by peribronchial leukocytes, aerobic exercise increases their expression in non-sensitized and in sensitized mice. Furthermore, aerobic exercise reduces HDM-induce the expression of SOCS3 by peribronchial leukocytes (Figure 8F; p<0.001). Figure 8 shows representative photomicrographs taken from SOCS3 (Figure 8 G-J) immunostaining.

DISCUSSION

The present study shows for the first time the involvement of SOCS-JAK-STAT signaling on the beneficial effects of regular aerobic exercise at low intensity reducing asthma phenotype, denoted as reduced eosinophilic inflammation, Th2 immune response, airway remodeling and AHR. In addition, this is the first study performing a complete description of several SOCS-JAK-STAT proteins in a model of HDM-induced asthma.

Asthmatic airway inflammation is characterized by accumulation of several cell types in airways wall, including mast cells, dendritic cells, Th2 lymphocytes and eosinophils [2,3]. However, eosinophilic inflammation is considered a hallmark of asthmatic airway inflammation and its levels is correlated

to asthma severity and risk of exacerbations [37]. The literature already has shown that aerobic exercise can reduces eosinophilic inflammation in asthmatics patients [38] and in experimental models of asthma induced by ovalbumin [30-35], but this is the first study showing that aerobic exercise can reduces eosinophilic inflammation in an experimental model of HDM-induced asthma phenotype. Thus, using a more physiological model of asthma using HDM, in which sensitization occurs through a direct contact activating with airways mucosa, i.e. airway epithelium [36,39], reinforce the importance of aerobic exercise, the main component of a program of pulmonary rehabilitation, in the control of eosinophilic inflammation for asthma.

Th2 cytokines, such as IL-4, IL-5 and IL-13 present an essential role in asthma pathogenesis and progression [4,8,30-36,39]. Previous literature shows that aerobic exercise inhibits accumulation of Th2 in BALF in ovalbumin models of asthma [30-35]. Such inhibitory effects of AE are of importance, considering that Th2 cytokines are also involved not only in the asthmatic inflammatory response, but also in the remodeling and in the AHR [4,8,10]. Furthermore, IL-17 a Th17- and epithelial-derived cytokine is thought to be involved in the proliferation and activation of fibroblast, airway smooth muscle and also in IL-8/CXCL-1 chemokine release, contributing to impairment of airway remodeling, AHR and to the inflammatory response, attracting neutrophils to the airways [5,40]. In addition, the main inflammatory consequence of IL-17 inducing the release of CXCL-1 attracting neutrophils to the airways, was observed in the present study, since the HDM model used in this study also induced increases in the levels of CXCL-1 and in the number of neutrophils in the lungs. In contrast, the present study showed for the first time that AE was able to inhibits HDM-induced IL-17 accumulation in the lungs, as demonstrated by reduced levels of IL-17 in BALF, effects that were followed by reduced levels of BALF CXLC-1 and by reduced numbers of neutrophils in BAL. Such effects of AE suggest that perhaps, AE can inhibit not only eosinophilic asthma, but also difficult to treat asthma, which is characterized by increased number of neutrophils in the airways [5,40].

IL-23 has been recently described as an important cytokine involved in asthma pathophysiology, mainly produced by dendritic cells, macrophages and airway epithelial cells [5,41]. It is involved primarily in the control of IL-17 synthesis, but also with the sensitization process, beyond to contribute to recruitment of both eosinophils and neutrophils to the airways [5,41]. IL-23 also seems to induce IL-33 synthesis and release, a cytokine involved in several aspects of asthma pathogenesis and maintenance, as in inflammation, remodeling and even in AHR [42]. IL-33 drives Th2 cells recruitment, activation, polarization through NF-kB activation, beyond to increase dendritic cells maturation and activation, which are cell types presenting a key role for asthma development [42]. Here, we demonstrated for the first time that AE was able to reduce IL-17, IL-23 and IL-33 in BALF of HDM-stimulated mice, demonstrating an extensive anti-inflammatory role of AE in the context of asthma. Of note, part of these anti-inflammatory effects can be attributed to AE-induced IL-10 release, which is an anti-inflammatory cytokine, that positively contributes to the anti-inflammatory effects of AE, as previously demonstrated [14,20,21,23,24,26,27].

Beyond exacerbated airway inflammation, airway remodeling is a hallmark of asthma, which is characterized by increased sub-epithelial deposition of extracellular matrix proteins (i.e. collagen and elastic fibers, proteoglycans and laminins), hypertrophy and hyperplasia of airway smooth muscle and epithelium and basal membrane thickness [6,7]. These structural changes in the airways remains as main challenge for treatment of asthma and are closely related to severity of the disease, to airway obstruction, breathlessness and to AHR [6,7]. In the present study, it was observed for the first time in a model of HDM-induced airway remodeling that AE reduced collagen and elastic fibers accumulation in the airways wall as well as reduced mucus production by airway epithelium, reinforcing the anti-fibrotic effects of AE, which has been already demonstrated in models of ovalbumin-induced asthma [30-35], COPD [14,20-22] and pulmonary fibrosis [23,24]. In addition, the importance of the anti-fibrotic effects of AE, which was observed in the present study, could be, at least in part correlated to the inhibitory effects of AE on AHR, which occurs in response to several factors, such as airway inflammation and remodeling [7]. Also, this inhibitory effect on AHR is particularly important, displaying that the anti-inflammatory and anti-fibrotic effects of AE result in improvement of functional response of the lungs.

Cytokine signaling depends of activation of intracellular molecules, such JAK and STATs [8-12], while JAK and STATs activation can be inhibited by SOCS proteins [8-12]. However, the literature is not unanimous concerning the inhibitory effects of SOCS proteins, since SOCS1, for instance, is upregulated in nasal epithelial cells of asthmatics and correlates with asthma severity [8]. On the other hand, it has been demonstrated that absence of SOCS1 resulted in increased asthma phenotype in a model of ovalbumin-induced asthma [43]. In the present study, which was performed using a HDM model of asthma, no changes in the expression of SOCS1 by peribronchial leukocytes or by airway epithelium were found. However, AE resulted in increased expression of SOCS1 by peribronchial leukocytes and by airway epithelium in non-sensitized and in sensitized mice groups, effect that can be involved in the inhibitory effects of AE on asthma phenotype, since that it has been demonstrated that SOCS1 suppress IL-13-dependent STAT6 activation, which constitute a central pathway for asthma development [44]. This effect of AE increasing SOCS1 expression can be reinforced, since AE not only reduced HDM-induced asthma phenotype, but also reduced the expression of STAT6, STAT5, STAT3 and JAK2 by peribronchial leukocytes and by airway epithelium. However, a direct causal effects cannot be definitively proved in the present study.

Concerning SOCS2, it was observed that HDM administration significantly reduced epithelial expression of SOCS2, while only a slight reduction in SOCS2 expression by peribronchial leukocytes was observed. In addition, these effects were followed by enhanced expression of STAT6, STAT5, STAT3 and JAK2 by peribronchial leukocytes and by airway epithelium. These findings are in partial agreement with a study from Knosp et al 2011, where the authors demonstrated exacerbated asthmatic phenotype and increased activation of STAT6 and STAT5 in SOCS2 ko mice [10]. On the other hand, AE was able to restore epithelial SOCS2 expression and to increase significantly SOCS2 expression by peribronchial

leukocytes, suggesting a possible mechanism underlying the effects of AE on asthma. However, whether the anti-asthmatic effects of AE are dependent of SOCS2 remain to be further investigated.

Increased expression of SOCS3 have been described in T-cells of asthmatic patients correlating to onset and maintenance of Th2 immune response and increased IgE levels [8]. In addition, another study showed that SOCS3 expression is increased in eosinophils of asthmatic patients and is functionally involved in eosinophil migration, adhesion and degranulation involving STAT3 activation [45]. Furthermore, it has been demonstrated that ovalbumin model of asthma results in increased expression of SOCS3 in the lungs, and that silencing of SOCS3 abrogates asthma phenotype [46]. In line with the current literature, the present study found that HDM increases SOCS3 and also STAT3 expression by airway epithelium and by peribronchial leukocytes. Such effects were significantly inhibited by AE, reinforcing the potential immunomodulatory role of AE on asthma involving SOCS-JAK-STAT signaling.

In conclusion, aerobic exercise inhibits house dust mite induce asthma phenotype, involving the modulation of SOCS-JAK-STAT signaling in airway epithelium and in peribronchial leukocytes. In addition, these experimental results point out a possible immunological and molecular mechanism underlying the beneficial effects of aerobic exercise on asthma phenotype, which should be urgently investigated in a clinical study in asthmatic individuals.

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