

Research Article

Creatine supplementation impairs airway inflammation in an experimental model of asthma involving P2 × 7 receptor

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Creatine (Cr) is a substrate for adenosine triphosphate synthesis, and it is the most used dietary supplement among professional and recreative athletes and sportsmen. Creatine supplementation may increase allergic airway response, but the cellular and molecular mechanisms are unknown. We used murine model of OVA-induced chronic asthma and showed that Cr supplementation increased total proteins, ATP level, lymphocytes, macrophages, and IL-5 levels in BALF, as well as IL-5 in the supernatant of re-stimulated mediastinal lymph nodes. IL-5 and IL-13 expression by epithelial cells and by peribronchial leukocytes were increased by Cr. Cr augmented the expression of P2 × 7 receptor by peribronchial leukocytes and by epithelial cells, and increased the accumulation of eosinophils in peribronchial space and of collagen fibers in airway wall. In human cells, while Cr induced a release of ATP, IL-6, and IL-8 from BEAS-2B cells, whole blood cells, such as eosinophils, and CD4⁺ T cells, P2 × 7 receptor inhibitor (A740003) reduced such effects, as denoted by reduced levels of ATP, IL-6, and IL-8. Therefore, Cr supplementation worsened asthma pathology due to activation of airway epithelial cells and peribronchial leukocytes, involving purinergic signaling.

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Keywords: Asthma · creatine supplementation · cytokines · epithelial cells · Th2 immune response



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Asthma is a chronic inflammatory disease compromising more than 300 million of people around the world [1]. The disease is characterized by bronchial hyperresponsiveness, wheezing, cough, partially reversible airway narrowing, which impairs the quality of life of asthmatic individuals [1]. Asthmatic airways present accumulation of eosinophils, Th type 2 (Th2) lymphocytes, mastocytes, and dendritic cells, as well as pulmonary remodeling, characterized by increased deposition of extracellular matrix proteins on the airway wall, increased mucus synthesis, and smooth muscle thickness [1].

Research has demonstrated the importance of different cellular types to asthma pathogenesis, including cells from hematopoietic system and from lung structural cells [2–4]. Among those, eosinophils and Th2 lymphocytes (i.e. CD4⁺ T lymphocytes) are cells from the hematopoietic system that exert a central role in asthma pathogenesis and progression [5, 6]. Similarly, lung structural cells, such as airway epithelial cells, also contribute to asthma pathogenesis and progression [4, 7]. Airway epithelium can facilitate the selective migration of leukocytes to airway lumen because the constant epithelial damage–repair process results in increased release of epithelial-derived growth factors involved on remodeling process [4, 7]. Asthmatic phenotype can be induced by different allergens, not only aeroallergens but also in response to food allergens [8].

Cr is the most popular dietary supplement used by sportsmen and professional athletes desiring increased muscle mass and strength [9, 10]. Beyond that, Cr presents several health benefits in face of several acute and chronic diseases [10, 11]. However, in the context of allergic airway inflammation, it has been demonstrated that Cr supplementation increases some features of asthma by activating leukocytes and airway epithelium in a model of OVA-induced chronic allergic airway inflammation [12, 13]. However, the effects of Cr on activation of human cells in the context of asthma are unknown. Thus, we combined mouse model-based analysis and in vitro assessment of human cells to investigate the effects of Cr supplementation on key cells involved in the pathogenesis of asthma.

Results

Cr supplementation increases chronic allergic airway inflammation and LN activation

Figure 2 shows that OVA and OVA + Cr groups presented increased number of total cells (A; $p < 0.001$), eosinophils

(B; $p < 0.01$), lymphocytes (C; $p < 0.001$), neutrophils (D; $p < 0.001$), and macrophages (E; $p < 0.001$) in BALF in comparison to Co and Cr groups. In addition, the results showed that Cr increased the number of lymphocytes (C; $p < 0.05$) and macrophages (E; $p < 0.05$) when OVA versus OVA + Cr were compared. Concerning the BALF levels of IL-5, the results demonstrated that OVA + Cr group presented higher levels in comparison with all other groups (F; $p < 0.001$). Related to BALF levels of IL-13, the results showed that both OVA (G; $p < 0.001$) and OVA + Cr (G; $p < 0.001$) groups presented higher levels of IL-13 when compared to Co and Cr groups. The synthesis and release of IL-4 by re-stimulated mediastinal LNs (MLN) presented higher levels in OVA (H; $p < 0.01$) and in OVA + Cr (H; $p < 0.001$) groups when compared to Co and Cr groups. For IL-5 synthesis and release by re-stimulated MLN, the results showed that OVA (I; $p < 0.001$) and OVA + Cr (I; $p < 0.001$) presented higher levels of IL-5 in comparison to Co and Cr groups. In addition, it was observed that OVA + Cr presented higher levels only in OVA group (I; $p < 0.05$). For IL-13 synthesis and release by re-stimulated MLN, the results showed that OVA (J; $p < 0.001$) and OVA + Cr (J; $p < 0.001$) groups presented higher values when compared to Co and Cr groups.

Creatine supplementation increases ATP and total proteins accumulation in the lungs

Figure 3 shows that Cr (A; $p < 0.05$) alone induced ATP accumulation in BALF. In addition, OVA (A; $p < 0.05$) and OVA + Cr (A; $p < 0.001$) presented higher levels of ATP in BALF when compared to Co group. Of note, OVA + Cr group also presented higher levels of ATP in BALF when compared to Cr (A; $p < 0.05$)

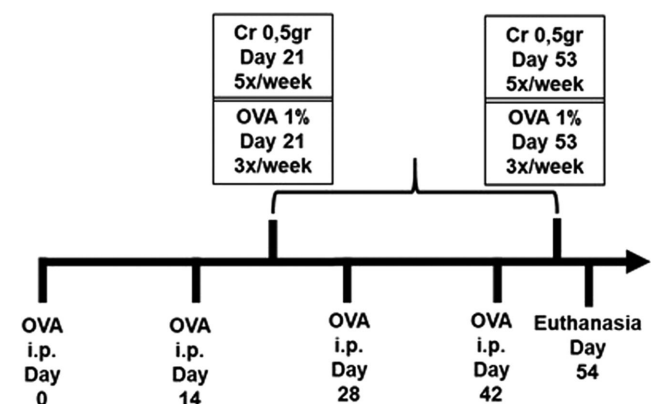


Figure 1. Timeline of experimental protocol. Cr, creatine.

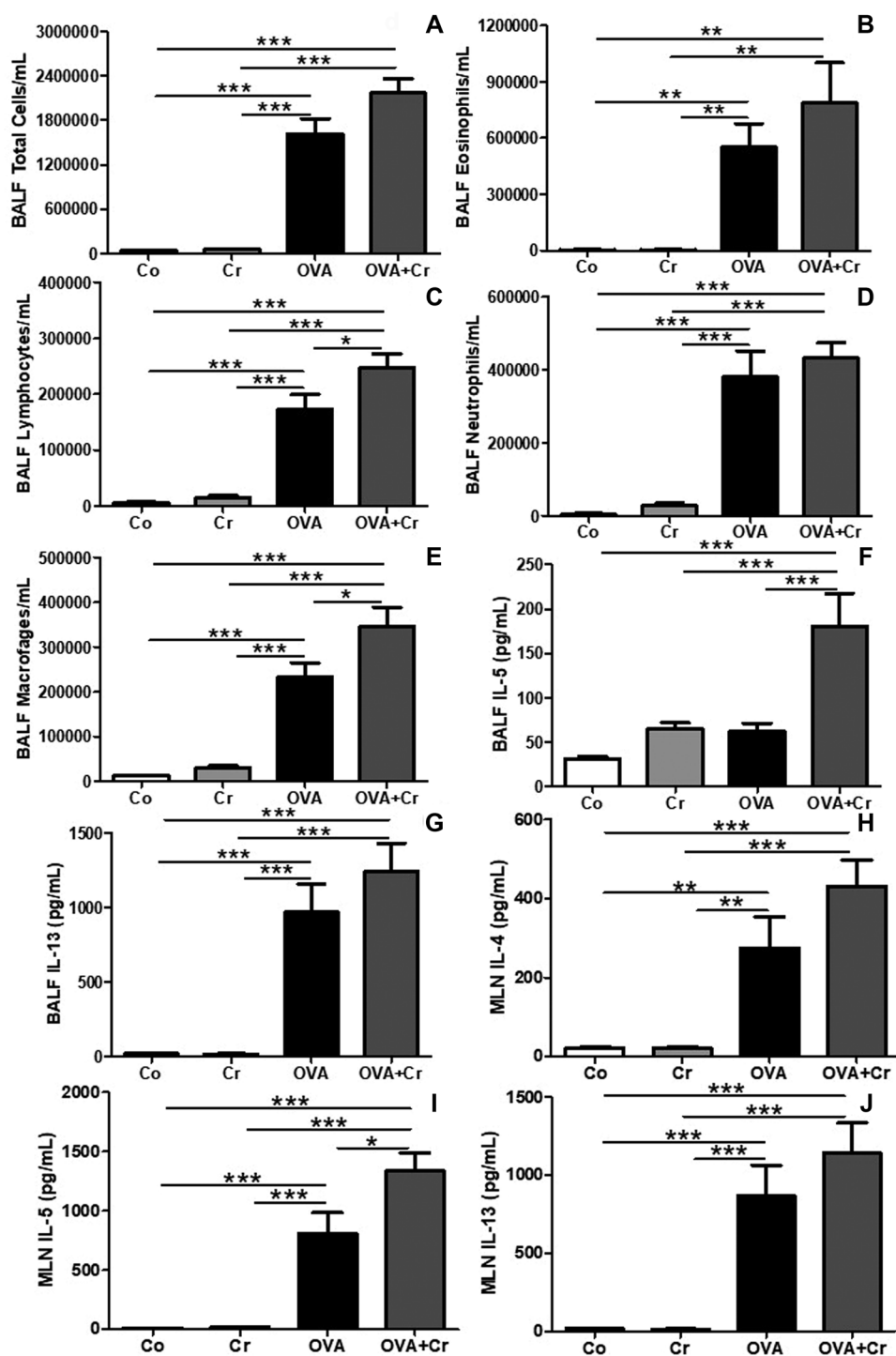


Figure 2. Cell count and cytokine levels from BALF and cell culture supernatant from mediastinal lymph nodes (MLN). Total (A) and differential cell counts (eosinophils (B); lymphocytes (C); neutrophils (D); and macrophages (E)) were performed on BALF cells from all BALB/c mice from control (Co), creatine (Cr), OVA (OVA), and OVA and creatine (OVA + Cr) groups and the results are shown as mean $\pm$ SEM of all experiments. Two independent sets of experiments were carried out using six mice, giving a total number of 12 mice per group. Cytokines levels (pg/mL) were evaluated by ELISA both in the BALF (IL-5 (F) and IL-13 (G)) and in the MLN culture (IL-4 (H); IL-5 (I) and IL-13 (J)). * p < 0.05; ** p < 0.01; and *** p < 0.001.

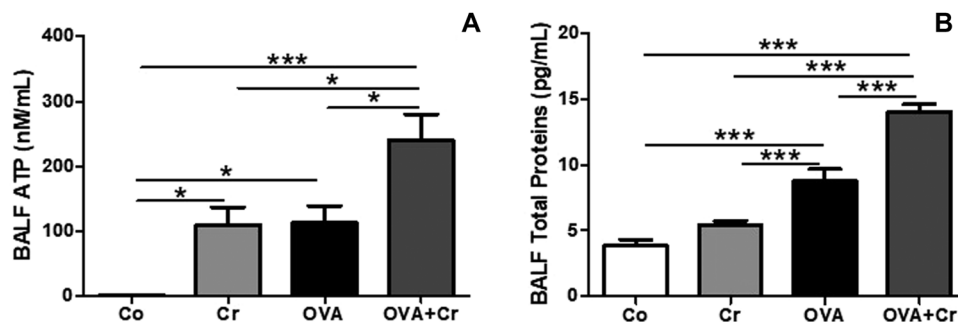


Figure 3. Evaluation of ATP and total proteins concentration in the BALF. Concentration of ATP (nM/mL; A) and total proteins (pg/mL; B) was measured on BALF obtained from all BALB/c mice from control (Co), creatine (Cr), OVA (OVA), and OVA and creatine (OVA + Cr) groups and the results are shown as mean $\pm$ SEM of all experiments. Two independent sets of experiments were carried out using six mice, giving 12 mice per group. * $p < 0.05$ and *** $p < 0.001$.

and OVA (A; $p < 0.05$) groups. Figure 3 also shows that OVA and OVA + Cr induced accumulation of total proteins in the lungs, when compared to Co and Cr groups. Furthermore, OVA + Cr group presented higher levels of total proteins in BALF when compared to Cr (B; $p < 0.001$) and OVA (B; $p < 0.001$) groups. Here, it is important to note that increased levels of total proteins in BALF means increases in the pulmonary vascular permeability, a key event for induction of pulmonary inflammation [14].

Cr supplementation increases airway eosinophilic inflammation and airway remodeling

Figure 4 shows that Cr (A; $p < 0.05$) alone induces accumulation of eosinophils in the peribronchial space, similarly to OVA (A; $p < 0.05$) and to OVA + Cr (A; $p < 0.001$). However, OVA + Cr presented even higher density of eosinophils in airway wall when compared to Cr (A; $p < 0.05$) and OVA (A; $p < 0.05$) groups. Importantly, Cr supplementation alone (B; $p < 0.05$) induces collagen fibers accumulation in airway walls, similarly to OVA (B; $p < 0.05$) and OVA + Cr (B; $p < 0.01$). In addition, the Figure 4C–F shows representative photomicrographs of picrosirius stained slides of Co, Cr, OVA, and OVA + Cr groups, respectively.

Cr supplementation increases IL-5 and IL-13 expression in the airways

Figure 5 shows that OVA (A; $p < 0.001$) and OVA + Cr (A; $p < 0.001$) groups presented increased expression of IL-5 by airway epithelium when compared to Co and Cr groups. However, OVA + Cr presented higher expression of IL-5 by airway epithelium when compared to Cr (A; $p < 0.001$) and OVA (A; $p < 0.001$) groups. The results also showed that OVA (B; $p < 0.001$) and OVA + Cr (B; $p < 0.001$) groups presented increased expression of IL-5 by peribronchial leukocytes when compared to Co and Cr groups. Similarly to epithelial expression, OVA + Cr group presented higher expression IL-5 by peribronchial leukocytes when compared to Cr (B; $p < 0.001$) and OVA (B; $p < 0.001$) groups.

Regarding IL-13 expression by airway epithelium, the results demonstrated that Cr alone (C; $p < 0.01$) increased its expression, as well as OVA (C; $p < 0.001$) and OVA + Cr (C; $p < 0.001$) groups in comparison to Co group. In addition, OVA + Cr group presented higher IL-13 expression by airway epithelium when compared to Cr (C; $p < 0.01$) group. When the IL-13 expression by peribronchial leukocytes was evaluated, the results showed that Cr alone (D; $p < 0.01$) increased its expression, as well as OVA (D; $p < 0.001$) and OVA + Cr (D; $p < 0.001$) groups in comparison to Co group. Once again, Cr exacerbated the pro-inflammatory response, since it increased the expression of IL-13 even when compared to Cr (D; $p < 0.001$) and OVA (D; $p < 0.001$) groups. In addition, the Figure 5E–H shows representative photomicrographs of IL-5 stained slides of Co, Cr, OVA, and OVA + Cr groups, respectively.

Cr supplementation increases the expression of P2 $\times$ 7 receptor in the airways

Figure 6 shows that Cr alone (A; $p < 0.01$) increases P2 $\times$ 7 receptor expression by peribronchial leukocytes, similar to OVA (A; $p < 0.001$) and OVA + Cr (A; $p < 0.001$) groups. However, it is important to note that OVA + Cr group presented even higher expression of P2 $\times$ 7 receptor by peribronchial leukocytes when compared to Cr (A; $p < 0.001$) and OVA (A; $p < 0.001$) groups. In the same way, Cr alone (B; $p < 0.05$) increases P2 $\times$ 7 receptor expression by airway epithelium, similar to OVA (B; $p < 0.01$) and OVA + Cr (B; $p < 0.001$) groups. Once again, Cr supplementation exacerbated the expression of P2 $\times$ 7 receptor, since OVA + Cr group presented higher expression of P2 $\times$ 7 receptor by airway epithelium when compared to Cr (B; $p < 0.001$) and OVA (B; $p < 0.001$) groups.

Cr supplementation induces activation of human hematopoietic and airway epithelial cells

Figure 7 shows that under Cr stimuli (10 mM) for 4 h, human whole blood cells responded to increased production of IL-6

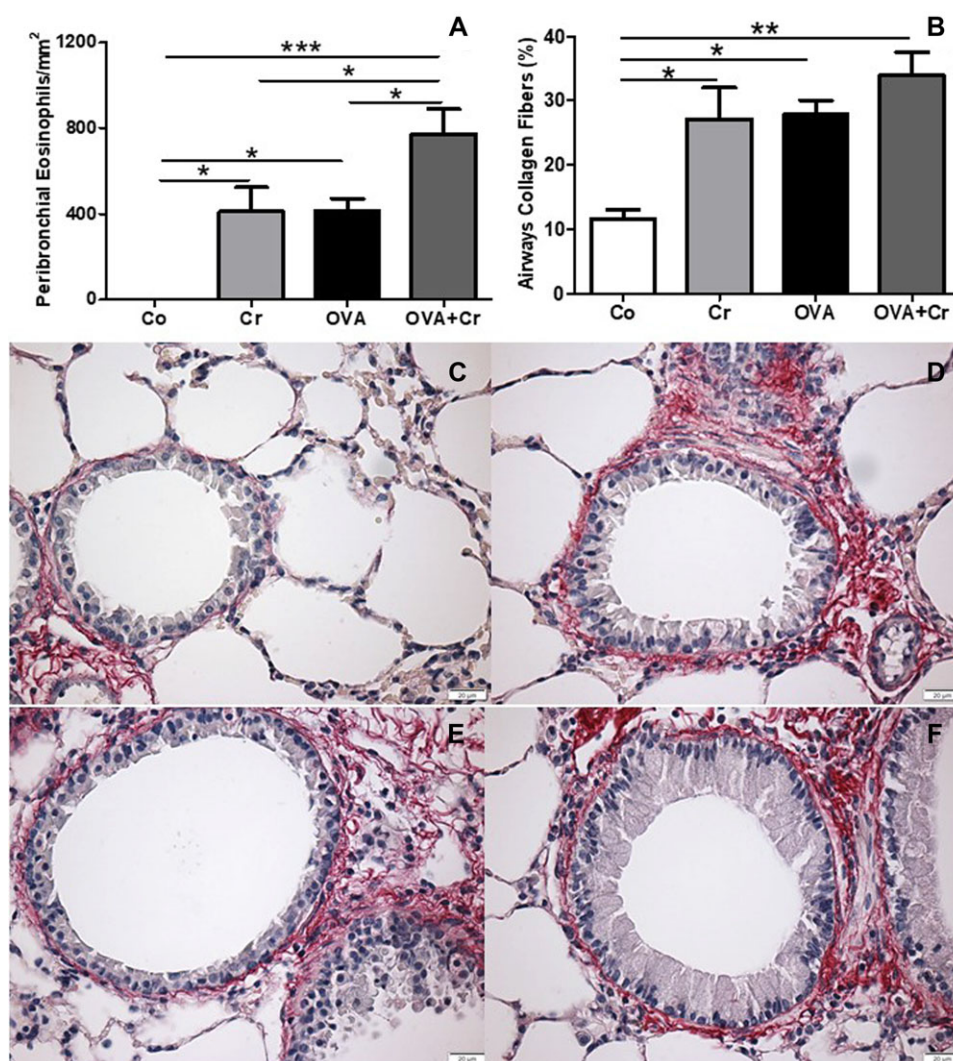


Figure 4. Quantification of eosinophils and of collagen fibers in airways wall. The density of eosinophils in the peribronchial space (eosinophils/mm²; A) from slides stained with hematoxylin H&E and the collagen fibers accumulation in airway walls (airway collagen fibers (%); B) from slides stained with picosirius. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. (C) and (D) are representative photomicrographs of slides stained with picosirius from Control (C); Cr (D); OVA (E); and OVA + Cr (F) groups. Scale bar 20 μ m. All analysis was performed in the pulmonary tissue obtained from all mice from control (Co), creatine (Cr), OVA (OVA), and OVA and creatine (OVA+Cr) groups and the results are shown as mean $\pm$ SEM of all experiments. Two independent sets of experiments were carried out using six mice, giving a total of 12 mice per group.

(A; $p < 0.001$) and IL-8 (B; $p < 0.001$), as well as eosinophils IL-6 (C; $p < 0.001$) and IL-8 (D; $p < 0.001$), and CD4⁺ T lymphocytes IL-6 (E; $p < 0.01$) and IL-8 (F; $p < 0.01$). Similar stimulatory effects were observed when human airway epithelial cells (BEAS-2B) were stimulated overnight with Cr, responding to increased production of IL-6 (G; $p < 0.001$) and IL-8 (H; $p < 0.001$).

Cr supplementation increases ATP release

Figure 8 shows that under Cr stimuli (10 mM) for 4 h, human whole blood cells (A; $p < 0.001$) responded to increased release of ATP, as well as eosinophils (B; $p < 0.001$) and CD4⁺ T lymphocytes (C; $p < 0.001$). Likewise, in hematopoietic cells, under Cr stimuli

overnight, human airway epithelial cells BEAS-2B responded to increased release of ATP (D; $p < 0.001$).

Discussion

This study showed for the first time that the exacerbatory effects of Cr supplementation in a model of asthma involve the purinergic signaling, as demonstrated by Cr-increased ATP accumulation in the lungs, as well as increased expression of P2 $\times$ 7 receptor by peribronchial leukocytes and by airway epithelium. In addition, Cr-induced ATP and pro-inflammatory cytokines release by human whole blood cells, eosinophils, and CD4⁺ T lymphocytes.

Cr supplementation is widely used by athletes and sportsmen to increase muscle mass and strength [9, 10], but its use is not

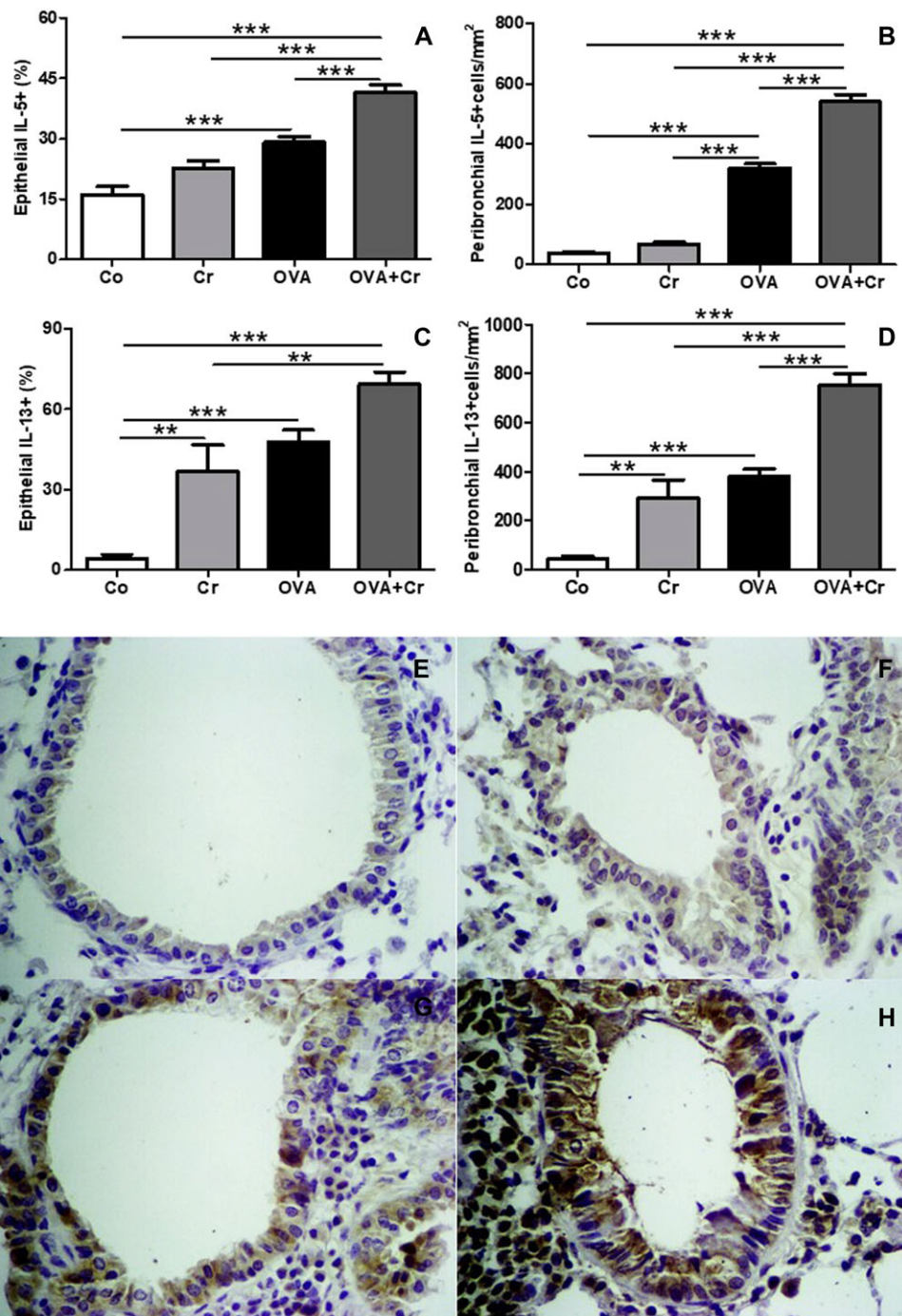


Figure 5. Quantification of IL-5 and IL-13 expression by airway epithelium and peribronchial leukocytes. IL-5 receptor expression by airway epithelium was quantified and presented as percentage of positive epithelium area (A) and the peribronchial leukocytes positive for IL-5 were quantified and presented as cells/mm² (B). IL-13 expression by airway epithelium was quantified and presented as percentage of positive epithelium area (C) and the peribronchial leukocytes positive for IL-13 receptor were quantified and presented as cells/mm² (D). ***p* < 0.01 and ****p* < 0.001. All analyses were performed in all mice from control (Co), creatine (Cr), OVA (OVA), and OVA and creatine (OVA+Cr) groups and the results are shown as mean ± SEM of all experiments. Two independent sets of experiments were carried out using six mice, giving a total of 12 mice per group. (E–H) are representative photomicrographs from Co, Cr, OVA, and OVA + Cr groups, respectively, stained for IL-5. Scale bar 20 μm.

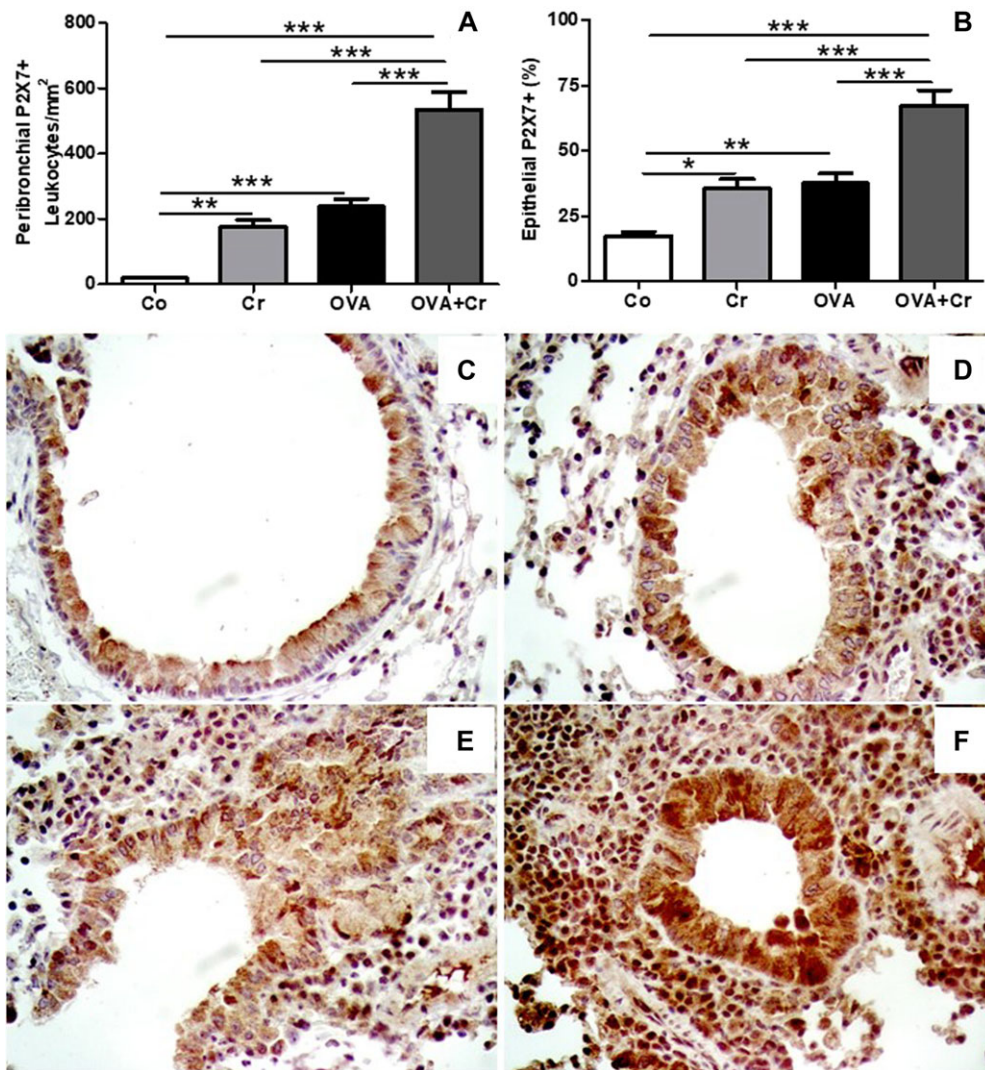


Figure 6. Quantification of P2 × 7 receptor expression by peribronchial leukocytes and airway epithelium. Peribronchial leukocytes positive for P2 × 7 receptor were quantified and presented as cells/mm² (A) and the P2 × 7 receptor expression by airway epithelium was quantified and presented as percentage of positive epithelium area (B). **p* < 0.05; ***p* < 0.01; and ****p* < 0.001. All analyses were performed in the pulmonary tissue obtained from all mice from control (Co), creatine (Cr), OVA (OVA), and OVA and creatine (OVA+Cr) groups and the results are shown as mean ± SEM of all experiments. Two independent sets of experiments were carried out using six mice, giving a total of 12 mice per group. (C–F) are representative photomicrographs from Co, Cr, OVA, and OVA + Cr groups, respectively, stained for P2 × 7 receptor. Scale bar 20 μm.

restricted to sports medicine, but also for prevention and as adjuvant for treatment of several diseases [10, 11]. Some years ago, the effects of Cr supplementation were tested in an experimental model of asthma, but its effects were not protective as expected, but on contrary, it has exacerbated asthma phenotype [12, 13]. So far, a single study has demonstrated that Cr supplementation activates airway epithelium in vivo, in a model of OVA-induced asthma [13]. In the present study, it was confirmed that Cr supplementation activates airway epithelium, as demonstrated by increased expression of IL-5 and IL-13 by epithelial cells in an OVA model. In addition, the present study came out demonstrating for the first time that Cr supplementation is also able to activate human epithelial cells (BEAS-2B), since Cr supplementation induced IL-6 and IL-8 release, beyond ATP. Such effects of Cr sup-

plementation activating airway epithelial cells are very important, especially in the asthmatic context, since airway epithelium are not a simple physical barrier of respiratory system, but actively contributes to asthma development, synthesizing and releasing several pro-inflammatory and pro-fibrotic mediators under different stimuli [13].

Cr is the main substrate for ATP synthesis, which is an essential molecule for energy generation and it is present intracellularly at higher concentrations (5–10 mM) than in the extracellular milieu (1–50 nM) [15]. While physiological concentrations of ATP in the extracellular milieu does not activate leukocytes, higher concentrations of ATP (>250 nM < 1 mM) can activate, for instance, CD4⁺ T lymphocytes [15]. Increased ATP concentrations in the extracellular milieu bind and activate purinergic

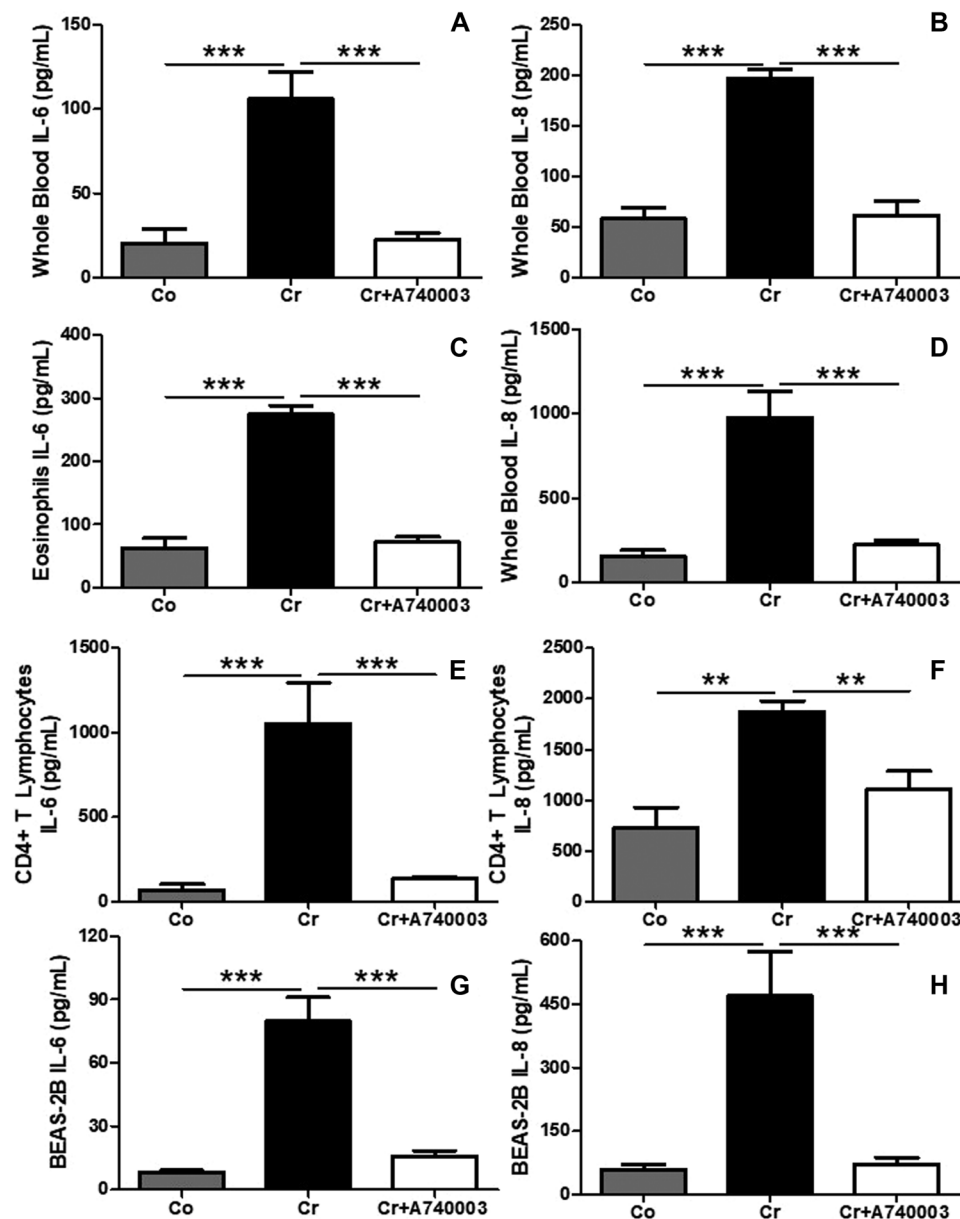


Figure 7. Evaluation of pro-inflammatory cytokines (IL-6 and IL-8) in immune and airways epithelial cells treated with creatine in vitro. Whole blood cells and isolated T CD4+ lymphocytes and eosinophils obtained from three nonallergic volunteers were incubated with or without 10 mM creatine + 25nM A740003 (a P2 × 7 receptor inhibitor) for 4 h, while BEAS-2B cells (a lineage of human airway epithelial cells) were incubated or not with the same concentration of creatine (10 mM) + 25nM A740003 (a P2 × 7 receptor inhibitor) overnight. Three sets of the experiments were done in triplicate and the results represent the mean ± SEM of all experiments. After the incubation period, the supernatants were used to evaluate the concentration of pro-inflammatory cytokines (IL-6 and IL-8; pg/mL). Whole blood cells (A and B), eosinophils (C and D), T CD4+ lymphocytes (E and F), and BEAS-2B cells (G and H) incubated with creatine significantly increased the release of IL-6 (A, C, E, and G) and IL-8 (B, D, F, and H) in comparison to the values observed in the control cells (not incubated with creatine). ***p* < 0.01 and ****p* < 0.001.

receptors, especially P2 × 7 receptor [16, 17], although other purinergic receptors, such as P2Y2 [18] and P2Y6 [4] can also be bound and activated by increased ATP concentrations. In the present study, it was demonstrated for the first time that Cr supplementation increases ATP release from epithelial cells, but also from human whole blood cells, CD4+ T lymphocytes, and eosinophils, reinforcing the pro-allergic effects of Cr supplementation.

Indeed, it has been demonstrated that increased ATP concentration drives asthma phenotype through P2 × 7 receptor activation, such as pulmonary eosinophilic inflammation, exacerbated Th2 immune response, airway remodeling, and Airway hyperresponsiveness (AHR) [16, 17, 20]. In this way, the present study showed that Cr supplementation also increases airway eosinophilic inflammation, pulmonary levels of Th2 cytokines (IL-4, IL-5, and IL-13), and airway remodeling. In addition,

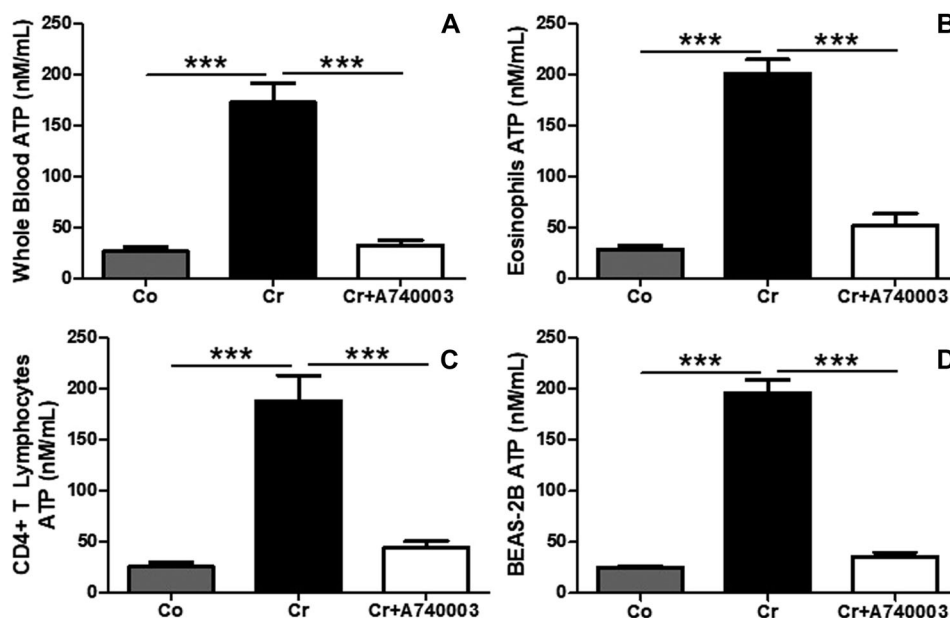


Figure 8. Evaluation of ATP concentration in immune and airways epithelial cells treated with creatine in vitro. Whole blood cells and isolated T CD4⁺ lymphocytes and eosinophils obtained from three nonallergic volunteers were incubated with or without 10 mM creatine + 25 nM A740003 (a P2 × 7 receptor inhibitor) for 4 h, while BEAS-2B cells (a lineage of human airway epithelial cells) were incubated or not with the same concentration of creatine (10 mM) + 25 nM A740003 (a P2 × 7 receptor inhibitor) overnight. Three sets of the experiments were done in triplicate and the results represent the mean ± SEM of all experiments. After the incubation period, the supernatants were used to evaluate ATP concentration (nM/mL). Whole blood cells (A), eosinophils (B), T CD4⁺ lymphocytes (C), and BEAS-2B cells (D) incubated with creatine significantly increased the release of ATP in comparison to the values observed in the control cells (not incubated with creatine). ****p* < 0.001.

the present study also showed for the first time that Cr supplementation increases IL-5 and IL-13 synthesis and release by re-stimulated MLNs, once again reinforcing the pro-allergic effects of Cr supplementation. The importance of P2 × 7 receptor in the pro-allergic response is reinforced since P2 × 7 receptor activation also has been demonstrated to increase T cells activation, especially in the context of allergic inflammation [17]. In fact, this study shows for the first time that Cr supplementation activates purinergic signaling, since in addition to inducing ATP release by epithelial cells (BEAS-2B), whole blood cells, CD4⁺ T Lymphocytes, and eosinophils, Cr supplementation also induces ATP accumulation in the lungs, as measured in BALF and beyond to increase the expression of P2 × 7 receptor by airway epithelium and by peribronchial leukocytes in an OVA model of asthma.

Cr-induced activation of peribronchial leukocytes is also of importance, especially in the context of asthmatic inflammation, since several leukocytes play a central role in asthma pathogenesis, progression, and severity [1, 2, 5, 6, 17, 18, 20–22]. Here, it was demonstrated that Cr supplementation increased the accumulation of leukocytes in BALF cells, beyond to induce the expression of IL-5 and IL-13 by peribronchial leukocytes. Of note, a previous study has showed that Cr supplementation induced IL-5 and IL-13 expression by airway epithelium, but in such study the effect of Cr on IL-5 and IL-13 expression by peribronchial leukocytes was not investigated [13]. The translation of these findings to human beings can be denoted since under Cr stimuli, human whole blood cells, CD4⁺ T lymphocytes, and eosinophils responded to

increased release of ATP and pro-inflammatory cytokines. Furthermore, activation of pulmonary leukocytes in the context of asthmatic inflammation also plays a central role in the airway remodeling process, through the release of growth factors [14]. In this context, Cr-induced ATP release and the activation of peribronchial leukocytes were followed by increased airway remodeling, as demonstrated by accumulation of collagen fibers in airway wall. As part of the explanation for such effects, it has been demonstrated that Cr supplementation induces the expression of several growth factors by airway epithelium, such as TGF-β, vascular endothelial growth factor, IGF-1, and EGF receptor [13].

In conclusion, this study shows that Cr supplementation exacerbates allergic airway inflammation and remodeling, involving the activation purinergic signaling and airway epithelial cells and leukocytes.

Materials and Methods

Animal study

Experimental protocol

The experiments performed in this study were approved by the review board for human and animal studies of the School of

Medicine of the University of Sao Paulo, under protocol number 503/05.

Figure 1 is a schematic representation of the experimental protocol. Forty-eight male BALB/c mice (20–25 g) were divided into four groups ($n = 2 \times 6$): Control, Cr, OVA, and OVA + Cr. Sensitization to OVA (Salto[®], Sao Paulo, Brazil) occurred by using i.p. injection of OVA (10 μ g/mouse) absorbed in aluminium hydroxide on days 0, 14, 28, and 42, as previously described [12, 13]. A solution of 1% OVA in PBS was used for aerosol challenge (30 min per session; thrice per week), beginning on 21st day until 53rd day [12, 13]. Cr supplementation (0.5 g/kg/d) was administered by oral gavage from 21st day until 53rd day [12, 13]. This dose of Cr in mice corresponds to 3 g/kg/day in humans and is enough to increase the total creatine concentration by 260–500% in lungs according to a previous study [23]. Twenty-four hours after the last aerosol and Cr supplementation, animals were anesthetized using i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), tracheostomized, and cannulated for the collection of BALF, which was performed by washing thrice with 1.0 mL PBS containing 0.1 mM EDTA.

Analysis of BALF total and differential cell count by flow cytometry

Total cells were counted using a hemacytometer (Neubauer chamber) and differential cells were analyzed by flow cytometry as previously described [3, 4, 16, 24] in all mice ($2 \times 6 = 12$ mice per group). Briefly, after counting and washing, BALF cells were stained for 30 min with anti-IAd/I-Ed FITC (macrophages/DCs), anti-Siglec F PE (eosinophils), anti-CD3 and anti-CD19 cy-chrome (lymphocytes), and anti-CD11c APC (macrophages/DCs) in PBS containing 0.5% BSA and 0.01% sodium azide [3, 4, 16, 24]. Analysis was performed on a FACS Calibur flow cytometer, using Cellquest and FlowJo software. BALF supernatant was stored at -80°C for cytokine analysis.

ATP, total protein, and cytokine levels in BALF

ATP measuring in BALF was performed in fresh BALF supernatants, by chemiluminescence, according to manufacturer's recommendations (ATPlite Assay; PerkinElmer, Wellesley, MA) in all mice ($2 \times 6 = 12$ mice per group) [16]. Total protein measurement was performed using Thermo Scientific Pierce BCA Protein Assay Kit, as previously described [11]. IL-5 and IL-13 levels were determined by ELISA [16].

Cytokines levels in MLNs

MLN cells from all mice ($2 \times 6 = 12$ mice per group) were seeded on a 96-well plate (1×10^6 cells/mL) and re-stimulated with OVA (10 μ g/mL) for 5 days. The levels of IL-4, IL-5, and IL-13 in cell supernatant were determined by ELISA [16].

Histology — collagen fiber accumulation in airway wall

Lungs from all mice ($2 \times 6 = 12$ mice per group) were inflated with 0.5 mL of Tissue-Tek (Sakura[®], Zoeterwoude, NL) and immediately frozen in liquid nitrogen and further transferred to -80°C . Five-micrometer sections were performed in a Leica cryostat CM 3050 (Wetzlar, GE). Five airways per mouse were used to quantify the amount of collagen fiber deposition in the airway wall and the number of eosinophils in the peribronchial space. The slides were stained using picrossírius for quantification of collagen fibers in airway wall [4, 12]. The percentage of collagen fibers in the airway wall was evaluated by measuring the percentage of red staining area (corresponding to collagen fibers) in the area between the airway epithelial basement membrane until airway adventitia. Image Pro Plus 4.0 was used for quantification of collagen fibers deposition on airway wall [4, 12]. In addition, H&E staining was performed for quantification of the density of peribronchial eosinophils [4, 12]. The density of eosinophils (eosinophils/ mm^2) was determined according to the number of eosinophils into the peribronchial area, comprehended between the airway epithelial basement membrane until airway adventitia.

Immunohistochemistry

By using silanized slides, first, tissue sections from all mice ($2 \times 6 = 12$ mice per group) were fixed by using acetone for 10 min, followed by incubation for 10 min in 3% H_2O_2 solution, and for 30 min in 3% casein solution to block the unspecific antibody binding as well as with avidin/biotin blocking system. Sections were incubated with primary polyclonal antibody (IL-5, 1:250; IL-13, 1:200; P2 $\times$ 7 receptor, 1:500, Santa Cruz, CA, USA) overnight. An ABC Vectastain Kit (Vector Elite PK-6105; Vector Laboratories, Burlingame, CA) was used as a secondary antibody and 3,3-Diaminobenzidine (Sigma Chemical Co., St. Louis, MO) was used as chromogen. The sections were counterstained with Harris hematoxylin (Merck, Darmstadt, Germany). Primary antibody with isotype control serum was used as negative control. Image Pro Plus 4.0 was used for quantification of protein expressions by airway epithelium as well as by peribronchial leukocytes, as previously described [4, 12]. Supporting Information Figure 1 shows the isotype serum control staining.

In vitro studies

For all blood cell culture (whole blood cell culture, CD4^+ T lymphocytes and eosinophils), venous blood was collected from three nonallergic volunteers (men, 32 ± 4 years old) using vacuum endotoxin-free K3-EDTA tubes (Vacuette, Greiner bio-one-Frickenhäusen, Germany). The whole blood test analysis was performed by using automatic hematology analyzer Sysmex XS-800i (Kobe, Japan) to exclude any possibility of infection or eosinophilic inflammation. In addition, a skin prick test was also performed to

confirm that the volunteers were not allergic. All in vitro studies described below were performed in triplicate using the samples from all three nonallergic volunteers. For CD4⁺ T lymphocytes and eosinophils isolation, first the blood was diluted 1:2 in sterile PBS and mononuclear cells were separated from polymorphonuclear cells by using Ficoll gradient separation. After that, the mononuclear cells (for CD4⁺ T lymphocytes) and polymorphonuclear cells (for eosinophils) were subjected to specific cell isolation using the kits described below.

Whole blood cell culture

Four milliliters of whole blood were placed in a six-well plate and stimulated with 10 mM of Cr $\pm$ A740003 (a P2 $\times$ 7 receptor inhibitor; 25 nM) for 4 h, at 37°C, 5% CO₂ at humid atmosphere [17]. The supernatant was collected 4 h after incubation with Cr and it was used for cytokines measurements (IL-6 and IL-8) as marker of cell activation.

Blood CD4⁺ T lymphocytes isolation and culture

Mononuclear cells were incubated using EasySep™ Human CD4⁺ T Cell Enrichment Kit (Stemcell, Cod 19052, Cambridge, MA, USA) for CD4⁺ T cell isolation. Then, 10 $\times$ 10⁴/cells per 300 μ L were seeded in a 96-well plate and stimulated with Cr 10 mM $\pm$ A740003 (a P2 $\times$ 7 receptor inhibitor; 25 nM) for 4 h, at 37°C, 5% CO₂ at humid atmosphere [19]. The supernatant was recovered 4 h after incubation with Cr and it was used for cytokines measurements (IL-6 and IL-8) as marker of cell activation.

Blood eosinophils isolation and culture

Polymorphonuclear cells were incubated using EasySep™ Human Eosinophils Enrichment Kit (Stemcell, Cod 19256, Cambridge, MA, USA) for eosinophils isolation. Then, 10 $\times$ 10⁴/cells per 300 μ L were seeded in a 96-well plate and stimulated with 10 mM of Cr $\pm$ A740003 (a P2 $\times$ 7 receptor inhibitor; 25 nM) for 4 h, at 37°C, 5% CO₂ at humid atmosphere [17]. The supernatant was recovered 4 h after incubation with Cr and it was used for cytokine measurements (IL-6 and IL-8) as marker of cell activation.

Epithelial cells

Human epithelial cells BEAS-2B were seeded in a 24-well plate (5 $\times$ 10⁶ cells/well) and incubated using 10 mM of Cr $\pm$ A740003 (a P2 $\times$ 7 receptor inhibitor; 25 nM) overnight. Three independent experiments were done in triplicate. The supernatant was used for cytokines measurements (IL-6, IL-8) as marker of cells activation.

Statistical analysis

Graph Pad Prism 5.0 was used to perform statistical analysis and to build the graphs. One-way ANOVA followed by Bonferroni's test was used for data presenting parametrical distribution, while ANOVA on Ranks followed by Dunn's test was used for data presenting nonparametrical distribution. $p < 0.05$ was considered significant.

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Abbreviations: Cr: creatine · MLN: mediastinal LN

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**Creatine supplementation impairs airway inflammation in an experimental model
of asthma involving P2 × 7 receptor**

ONLINE SUPPLEMENT

Creatine Supplementation Impairs Airway Inflammation in an Experimental Model of Asthma

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Running head: Creatine supplementation drives Th2 phenotype.

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Statement of Conflict of Interest

All authors declare that they have no conflict of interest related to this publication.

Figure 1 shows the isotype serum control staining in the lung sections of Control (A), Creatine (B), OVA (C) and OVA+Cr (D).

