

Research article

Experimental allergic airway inflammation impacts gut homeostasis in mice

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ARTICLE INFO

Keywords:

Asthma
Allergic inflammation
Lung
Gut and mucosa

ABSTRACT

Background: /Aims: Epidemiological data show that there is an important relationship between respiratory and intestinal diseases. To improve our understanding on the interconnectedness between the lung and intestinal mucosa and the overlap between respiratory and intestinal diseases, our aim was to investigate the influence of ovalbumin (OVA)-induced allergic airway inflammation on gut homeostasis.

Methods: A/J mice were sensitized and challenged with OVA. The animals were euthanized 24 h after the last challenge, lung inflammation was determined by evaluating cells in Bronchoalveolar lavage fluid, serum anti-OVA IgG titers and colon morphology, inflammation and integrity of the intestinal mucosa were investigated. IL-4 and IL-13 levels and myeloperoxidase activity were determined in the colon samples. The expression of genes involved in inflammation and mucin production at the gut mucosa was also evaluated.

Results: OVA challenge resulted not only in lung inflammation but also in macroscopic alterations in the gut such as colon shortening, increased myeloperoxidase activity and loss of integrity in the colonic mucosal. Neutral mucin intensity was lower in the OVA group, which was followed by down-regulation of transcription of *ATO1* and up-regulation of *TJP1* and *MUC2*. In addition, the OVA group had higher levels of IL-13 and IL-4 in the colon. Ova-specific IgG1 and OVA-specific IgG2a titers were higher in the serum of the OVA group than in controls.

Conclusions: Our data using the OVA experimental model suggested that challenges in the respiratory system may result not only in allergic airway inflammation but also in the loss of gut homeostasis.

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1. Introduction

Asthma and inflammatory bowel disease (IBD) are chronic inflammatory diseases of the respiratory and digestive systems, respectively [1,2]. Changes in the mucosal surfaces are believed to be responsible for the overall increase in chronic inflammatory diseases that affect the respiratory and digestive systems [3]. In the past few decades, the incidence and prevalence of asthma and IBDs have increased in Western countries.

Remarkably, evidences in the literature suggest an overlap between respiratory and intestinal diseases [4–11]. Interactions between intestinal and respiratory mucosa were reported for the first time in 1968, since patients with pulmonary fibrosis were more likely to develop inflammatory bowel diseases [4]. It is currently believed that almost half of patients with ulcerative colitis (UC) and Crohn's disease (CD) have subclinical pulmonary abnormalities, such as low-grade inflammation [5,6]. A relatively recent broad-spectrum study of patients with IBD showed that 48% of adult patients and 71% of child and adolescent patients had bronchial hyperreactivity [5,6].

Other data also corroborate the existing communication between the mucous compartments of the respiratory and digestive systems. COVID-19, a disease caused by SARS-CoV-2, has also clinical manifestations both in the lungs and intestine. Although this disease mainly affects the lungs and is transmitted via the airways; clinical evidence suggests that the intestine may be another target organ for the virus. A study also noted that the severity of COVID-19 may be associated with older adults due to decreased microbiota diversity that occurs during the aging process, which causes dysbiosis [12]. Dysbiosis in the intestinal microbiota can result in the disrupted integrity of the intestinal barrier, which can lead to the translocation of SARS-CoV-2 from the lung to the gut lumen through the circulatory and lymphatic systems [13].

Despite these evidences, the mechanisms underlying the association between inflammatory diseases of the respiratory and digestive systems are still not fully elucidated [14,15].

Furthermore, diseases of the lungs and gut are still treated individually when treatment strategies should consider both systems. Therefore, this study aimed to evaluate the influence of pulmonary allergic inflammation induced by ovalbumin on gut homeostasis.

2. Methods

2.1. Animals

Male A/J mice (17–24 g; 6–7 weeks old) were obtained from the animal facility of the Institute of Biomedical Sciences (University of São Paulo, Brazil) but were originally from Jackson Laboratories. The animals were housed in groups of five per cage in a light- and temperature-controlled room (12 h light/dark cycles and $21 \pm 2^\circ\text{C}$) and were given free access to a standard chow diet (Nuvilab CR1, Quimtia, Paraná, Brazil). We evaluated A/J mice because they are one of the strains that is most susceptible to allergic airway inflammation and experimental colitis [16–19]. All experiments involving laboratory animals were evaluated and approved by the Institutional Animal Care and Use Committee of the Federal University of São Paulo under protocol 5547290818. All procedures were in accordance with Brazilian National Law number 11.794 (Arouca Law), Decree 6.899 and normative resolutions from the Conselho Nacional de Controle de Experimentação Animal (CONCEA), the federal agency that regulates all research activities involving animal use in Brazil.

2.2. Induction of allergic lung inflammation

Mice were sensitized via the intraperitoneal (IP) injection of 50 μg of OVA grade V (Sigma Chemical Co., St. Louis, MO, USA) dissolved in 200 μl of sterile phosphate-buffered saline (PBS) and 1.6 mg of injected alum (Prod# 77,161, Thermo Scientific, Rockford, USA) on Days 0 and 7, respectively. Mice were challenged with 50 μl of OVA intratracheal (IT) dissolved in sterile PBS on Days 14 and 21 [20]. The animals were euthanized 24 h after the last challenge (i.e., Day 22). Basal animals were not treated, and control animals were sensitized with saline and injected with alum; for challenge, they received PBS and, OVA sensitization group were sensitized with OVA and alum, for challenge they received PBS. The following groups were studied: OVA non-sensitized and OVA non-challenge mice (Basal group); OVA-sensitized and PBS challenge mice (Control group); and OVA sensitized, and OVA challenged mice (OVA group).

2.3. Bronchoalveolar lavage fluid (BALF)

The trachea was cannulated after euthanasia. The lungs were washed twice with 0.8-ml aliquots of PBS injected through the cannula. The total number of cells in BALF was counted using a Countess® Automated Cell Counter (Countess, Invitrogen Life Technologies, Carlsbad, CA, USA). The cells were resuspended at 5×10^5 cells/ml. Differential cell counts were performed using cytocentrifuge analysis and prepared from aliquots of BALF (200 μl) centrifuged for 1 min using a cytocentrifuge (Fanem, São Paulo, Brazil). Cells were stained with Instant Prov (Newprov, São Paulo, Brazil), and a total of 300 cells were counted to determine the proportion of neutrophils, eosinophils, and mononuclear cells using standard morphological criteria.

2.4. Measurement of cytokine production

After euthanasia, the proximal portion of the colon and lung were collected and homogenized in 1 ml of solution (RIPA with Protease Inhibitor). The samples were centrifuged $5000 \times g$ for 10 min at 4°C . After centrifugation, the colon supernatant was plated in

a 96-well microplate and analyzed in a Bioplex (Luminex 200 - Bio-plex® 200 Array System, Bio-Rad, Hercules, CA, USA) using R&D Systems antibodies (Minneapolis, USA) and Manager Bio-Plex 4.0 software. The values were normalized by the weight of the tissues. The following colon cytokines were analyzed: IL-4, IL-13 and IL-33. After centrifugation, the lung supernatant was analyzed using a sandwich enzyme-linked immunosorbent assay (ELISA). We used antibodies and standards for IL-4, IL-5, and IL-13 from R&D Systems and 96-well microplates (Immunoplate Maxisorb; Nunc), according to the manufacturer's instructions.

2.5. Determination of OVA-specific IgE, IgG1, and IgG2a production

After euthanasia, serum was collected to determine OVA-specific IgG1, IgG2a, and IgE antibodies, the plates were coated overnight at 4 °C with ovalbumin in a coating buffer solution. The plates were washed three times with washing buffer (PBS containing 0.05% Tween-20) and blocked with assay diluent (PBS containing 10% heat inactivated fetal bovine serum (LGC Biotecnologia, Cotia, São Paulo, Brazil) for 1 h. The serum samples from the mice were diluted in the test diluent and added to the plates after three more washes. After 2 h of incubation at room temperature (RT), the plates were washed five times, and diluted *anti-IgG1*, *anti-IgG2a* and *anti-IgE* detection antibodies were added to the plate. The plates were incubated for 1 h at room temperature and washed. The Substrate Reagent Set was added, and the plates were left for 30 min in the dark. The colorimetric reaction was interrupted by the addition of 2 N H₂SO₄. The absorbance was acquired at 450 nm in a Biotek Synergy HT microplate reader and was analyzed by Gen 5 software (BioTek). The values of the absorbance samples were considered after subtracting the values from the wells incubated with only fresh mouse serum at the same dilutions. The data are shown in optical density (OD) units [21].

2.6. MPO assay

For the quantification of myeloperoxidase (MPO), 50 mg from the colon and lung, they were removed, washed, homogenized in 1.9 ml of phosphate buffer (PBS) at a concentration of 5 times and centrifuged for 10 min at 5000×g. The supernatant was discarded, and the erythrocytes were lysed. The samples were then centrifuged, the supernatant was discarded, the pellet was suspended in 1.9 ml of 0.5% hexadecyl trimethylammonium bromide (HTAB) (Sigma-Aldrich Brasil LTDA Cotia, São Paulo, Brazil), and it was frozen three times in liquid nitrogen and centrifuged at for 10 min. The supernatant was used in the enzymatic assay, and the absorbance was recorded at 490 nm. The protein concentration was determined, and the MPO activity was defined as the amount of enzyme degrading 1 μmol/ml of peroxide at 37 °C, expressed in units/mg of protein [22].

2.7. EPO assay

The evaluation of eosinophil infiltration into the lung was performed by quantifying the amount of eosinophilic peroxidase in the tissue. Briefly, 100 mg of tissue was homogenized in 1.9 ml of phosphate-buffered solution concentrated by 5-fold (PBS 5X) and centrifuged for 10 min at 12,000 rpm. The supernatant was discarded, and the erythrocytes were lysed. The samples were then centrifuged, the supernatant was discarded, and the pellet was suspended in 1.9 ml of 0.5% hexadecyltrimethylammonium bromide in PBS, frozen three times in liquid nitrogen and centrifuged at 4 °C at 12,000 rpm for 10 min. The supernatant was used in the enzymatic assay by adding an equal amount of substrate (1.5 mmol/L *o*-phenylenediamine and 6.6 mmol/L H₂O₂ in 0.075 mmol/L Tris-HCl (pH 8). The reaction was stopped with 50 μl of 1 M H₂SO₄, and the absorbance was read at 492 nm.

2.8. Histopathological analysis

A portion of the proximal colon was removed immediately after euthanasia and fixed in 10% buffered formaldehyde (Synth®, Diadema, São Paulo, Brazil). After a minimum fixation time (48 h), this tissue was subjected to dehydration, xylol clearing, bathing and paraffin embedding. Then, 3-μm sections were stained with periodic acid-Schiff (PAS) counterstained with hematoxylin, hematoxylin-eosin (H&E) and Alcian Blue (AB) counterstained with hematoxylin. Two sections per animal were analyzed under an optical microscope, and the choice of the photographed fields was performed at random. The sections stained with H&E were used to measure the thickness of the muscular and mucous layers of the colon. Three random fields were photographed per section (objective 10 ×) and three measures were obtained for each field [23]. For morphometric analysis, we used the ZEN 2.6 (blue edition) program (ZEISS, Jena, Thuringia, Germany). For the analysis of the intensity of the staining of PAS and Alcian Blue (pH 2.5), positive goblet cells Image-Pro Plus Version 4.5.0.29 software (Media Cybernetics) were used, in which the entire area of intact tissue in the representative photos of the intestinal colon was selected and the percentage by area of cells with high staining intensity and low staining intensity obtained. A ratio was then made between these values to obtain the data on the degree of intensity of the types of mucus produced. The quantification of goblet cell abundance was performed. Briefly, 1,500 consecutive cells, including PAS/AB positive or negative, were counted in each specimen. The number of the goblet cells was represented by the number of PAS/AB positive cells by the total number of quantified cells. Histological slides were also used to perform a qualitative analysis of the integrity of the intestinal mucosa.

2.9. Colon length measurement

On the day of euthanasia, the intestinal colons of the mice were removed, and the length was established using a conventional ruler.

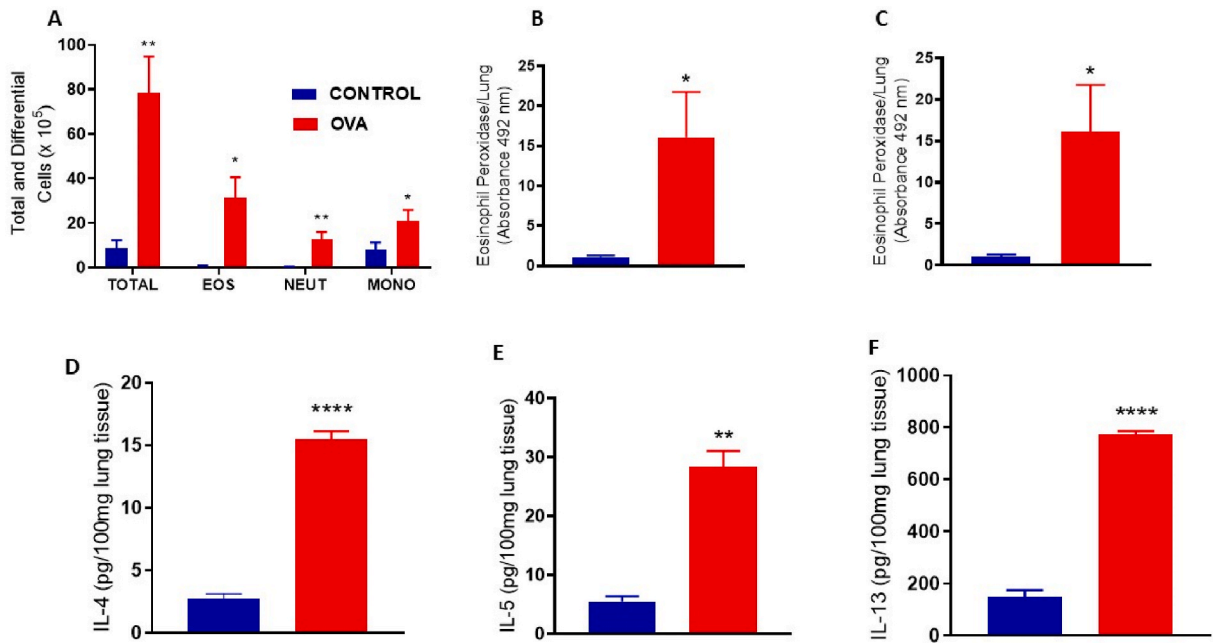


Fig. 1. Allergic airway inflammation analysis in mice. (A) The total number of cells and differential (Eos: eosinophils; Neut: neutrophils; Mono: monocytes) number of cells in bronchoalveolar lavage (BALF). (B) Myeloperoxidase (MPO) and Eosinophil Peroxidase (EPO). (C) Interleukin 4 (IL-4), (D) Interleukin 5 (IL-5) and (E) Interleukin 13 (IL-13). Groups: Control = Animals sensitized with saline and aluminum hydroxide and challenged with saline; OVA = Animals sensitized with OVA and aluminum hydroxide and challenged with OVA. The statistical significance between two experimental groups was assessed using an unpaired *t*-test * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.0001$ Control versus OVA. Bars represent the mean $\pm$ SEM. Data are representative of two independent experiments ($n = 6$ mice/per group).

2.10. Gene expression

RNA was extracted from intestinal colon samples using TRIzol LS Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The resulting RNA was treated with deoxyribonuclease Ambion DNase I (RNase-free) (Life Technologies, Carlsbad, CA, USA), and the enzyme was inactivated at 65 °C for 10 min cDNA synthesis was performed using the SuperScript™ Vilo™ Synthesis Kit for RT-PCR (Invitrogen Life Technologies). Controls without RNA and without enzyme mix were used. Quantitative PCR was performed in a StepOne Plus System thermocycler (Applied Biosystems, Foster City, CA, USA) using the primers MUC2: 5'-ACTGCACATTCTTCAGCTGC-3' and 5'-ATTCATGAGGACGGTCTTGG-3' and ATOH1: 5'-GCCTCTGGTCTGGGTTTCAC-3' and 5'-GCAAGGATGAAGTCCCAAGGT-3' and TJP1: 5'-ATCCCTCAACAAGGGCCATTC-3' and 5'-CACTTGTTTGGCCAGGTTTA-3' [23]. The reactions comprised 50 ng of cDNA, 0.25 μ L of each primer (25 pM), and 5 μ L of Power Sybr Green® (Applied Biosystems, Foster City, CA, USA) in a total of 10 μ L. Amplification comprised forty cycles at 95 °C/15P, 60 °C/1', 95 °C/15P, followed by two steps at 95 °C/15P and 60 °C/1' and a final step at 0.3 °C–95 °C/15P. Fold-changes in gene expression were calculated by the comparative cycle threshold (CT) method. The expression of target genes was normalized to a reference gene [Δ CT = CT (target)–CT (reference)]. Relative expression of the target genes was performed using the $\Delta\Delta$ CT method, using β -actin as an endogenous control [24]. The fold-change in gene expression was calculated as $2^{-\Delta\Delta$ CT, where $\Delta\Delta$ CT = Δ CT (sample) – Δ CT (calibrator) and where the mean Δ CT of samples from control mice was used as a calibrator.

2.11. Statistical analysis

The data obtained were analyzed statistically by Student's *t*-test and one-way ANOVA followed by Tukey's multiple comparison test. The differences were considered statistically significant when $p < 0.05$. Statistical analyses and graphs were prepared using GraphPad Prism 4.0 software.

3. Results

3.1. Experimental asthma model

The asthma protocol used was able to induce allergic pulmonary inflammation, mainly characterized by a high number of eosinophils 24 h after the last challenge. OVA mice group showed an increase in total cells including eosinophils, neutrophils and mononuclear cells in the airways (Fig. 1A–F), while control versus basal groups ($p = 0.1089$), and control versus OVA sensitization are

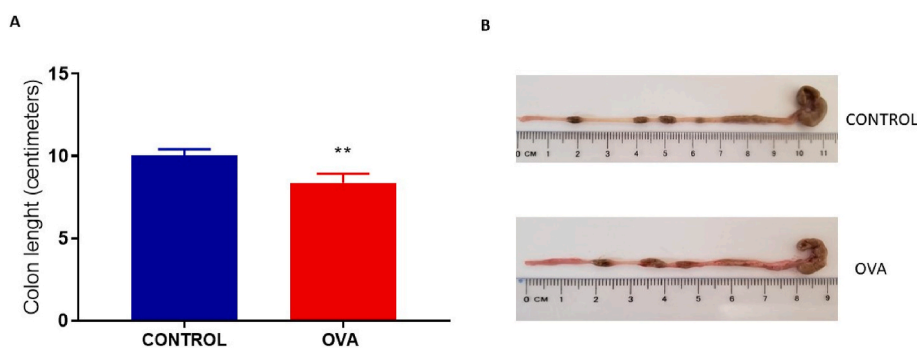


Fig. 2. Allergic airway inflammation affects colon length. Twenty-four hours after the last challenge, the mice were euthanized, and their colonies were removed. Colon length was measured as an indirect marker of inflammation. (A) The mean colon length is presented. (B) Representative photographs of the colon length of the Control and OVA groups. The statistical significance between two experimental groups was assessed using unpaired *t*-test ** = $p < 0.01$, Control versus OVA. Bars represent the mean $\pm$ SEM. Data are representative of two independent experiments ($n = 6$ mice/per group).

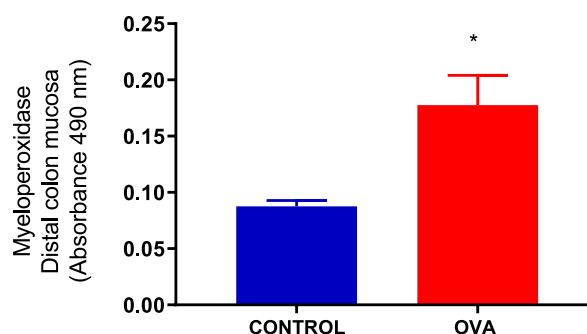


Fig. 3. Allergic airway inflammation induces colon mucosal changes, as evaluated by myeloperoxidase enzyme (MPO). Twenty-four hours after the last challenge, the mice were euthanized, and their colons were removed to analyze MPO as a marker of neutrophilic inflammation. Unpaired *t*-test ** = $p < 0.01$, Control versus OVA. Bars represent the mean $\pm$ SEM. Data are representative of two independent experiments ($n = 6$ mice/per group).

not significantly different ($p = 0.1149$). Because the basal, control and OVA sensitization groups have the same total cellularity in the airways, further analyses were conducted using only the control group as a negative control.

3.2. Allergic airway inflammation affects the colon length and increases colon inflammation

The lengths of the large intestines were measured, and the animals in the OVA group showed a significant reduction in the length of the intestine compared to the animals in the control group (Fig. 2 A and B). The nasoanal lengths of these animals were also measured, which indicated that there was no significant difference between the size of the animals in each group.

The MPO test was performed in the mucosal layer of the colon. We found a significant increase in MPO in the OVA group compared with the control group (Fig. 3). There were no differences among groups in the EPO analysis (data not shown).

3.3. Neutral mucin intensity, MUC 2, TJP1 and ATOH 1 expression in the colon are affected by allergic airway inflammation

Goblet cells have been implicated as players in promoting intestinal homeostasis and host defense [25]. We observed that there were no significant differences in the number of goblet cells or in the intensity of acid mucin production between the control and OVA groups (Fig. 4 A, C). Interestingly, we observed that there was a decrease in the intensity of the neutral mucin in the colons of animals in the OVA group compared to the control group (Fig. 4B). Furthermore, histological sections stained with PAS and Alcian Blue showed that the colonic mucosal barrier of the animals in the control group had greater integrity than the colonic mucosal barrier of the animals in the OVA group. Considering the six animals that were analyzed in the control group, five had a preserved mucosal barrier, whereas in the OVA group, only one had a preserved mucosal barrier (Fig. 4D and E). Also, we evaluated the thickness of the intestinal mucosa and smooth muscle. There were no statistically significant differences in these parameters (Supplementary Figure 1).

Because neutral gut mucin is affected by lung inflammation, we analyzed the expression of some genes involved in mucus production. We investigated the genes TJP1, MUC2 and ATOH1. Our analyses revealed that ATOH1 was down regulated whereas MUC2 and TJP1 were upregulated in the OVA group compared to the control group. ATOH1 is an essential factor that controls goblet cells

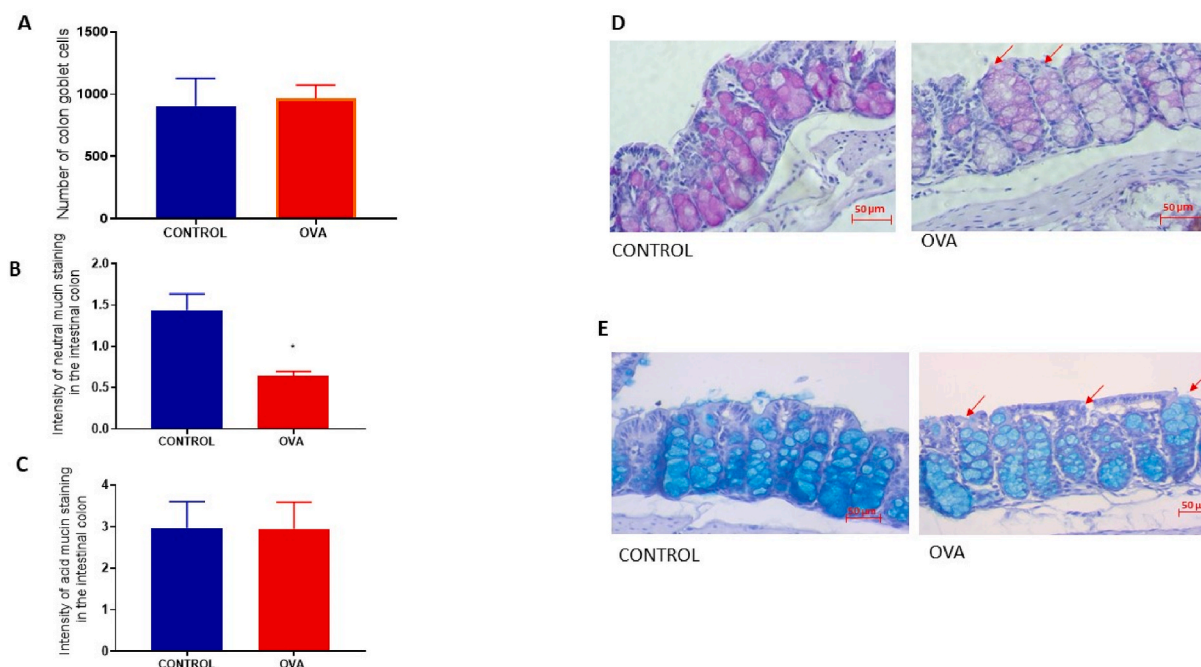


Fig. 4. Allergic airway inflammation affects neutral mucin intensity in the colon. Twenty-four hours after the last challenge, the mice were euthanized, and their colons were removed to analyze mucus production. (A) Total number of goblet cells found for every 1500 colonocytes. (B) Analysis of neutral mucin production in the intestinal colon. (C) Analysis of acid mucin production in the intestinal colon. (D) Photomicrograph representing the mucosal layer of the colon stained with PAS and HE. The pink-stained cells are goblet cells, and the blue cells superior to the goblet cells are colonocytes. (E) Representative photomicrograph of the intestinal colon stained with Alcian Blue. The larger, blue-stained cells are goblet cells, and the smaller dark blue cells superior to the goblet cells are colonocytes. The arrows indicate regions where there is a decrease in the integrity of the mucous layer. Microscope: Axio Imager, 40×. The statistical significance between two experimental groups was assessed using an unpaired *t*-test * = $p < 0.05$, ** = $p < 0.01$, Control versus OVA. Bars represent the mean $\pm$ SEM. Data are representative of two independent experiments ($n = 6$ mice/per group).

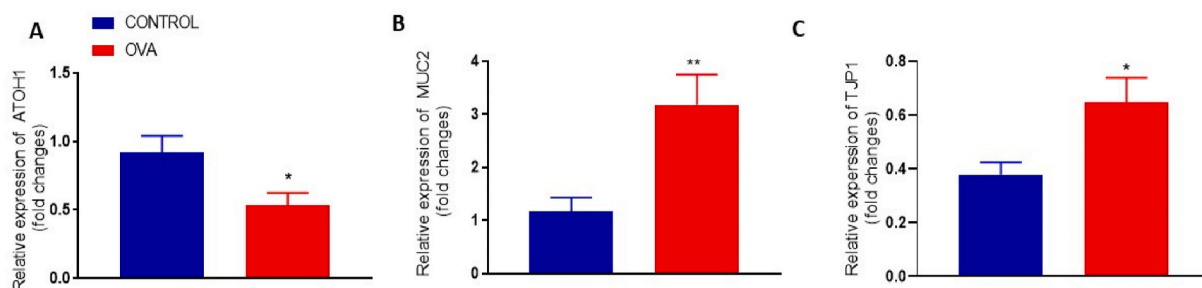


Fig. 5. Allergic airway inflammation affects the transcription of genes in colon tissue. Relative transcription of (A) ATOH1, (B) MUC2 and (C) TJP1 was determined by RT-qPCR in colon tissue of mice euthanized 24 h after the last challenge. The statistical significance between two experimental groups was assessed using an unpaired *t*-test * = $p < 0.05$, Control versus OVA. Bars represent the mean $\pm$ SEM. Data are representative of two independent experiments ($n = 6$ mice/per group).

(Fig. 5A–C) [26].

3.4. Allergic airway inflammation increases IL-4 and IL-13 in the colon and increases OVA-specific IgG1 antibody and OVA-specific IgG2a antibody in the blood

The cytokines IL-4, IL-13, IL-33, TNF- α , CCL-5, CCL-2 and IL-10 and were measured in the intestinal colon through a multianalytical ELISA test (Multiplex). There was a significant difference only for two cytokines, IL-13 (Fig. 6A) and IL-4 (Fig. 6B), in the colon of animals in the OVA group compared to the control group. IL-33 levels (Fig. 6C) were not different between groups (Fig. 6). OVA-specific IgG1 (Fig. 6D), IgG2a (Fig. 6E) and IgE (Fig. 6F) antibodies were measured as serum markers of Th2 and Th1 responses, respectively. An increase in OVA-specific IgG1 and OVA-specific IgG2a was then identified in the animals in the OVA group compared

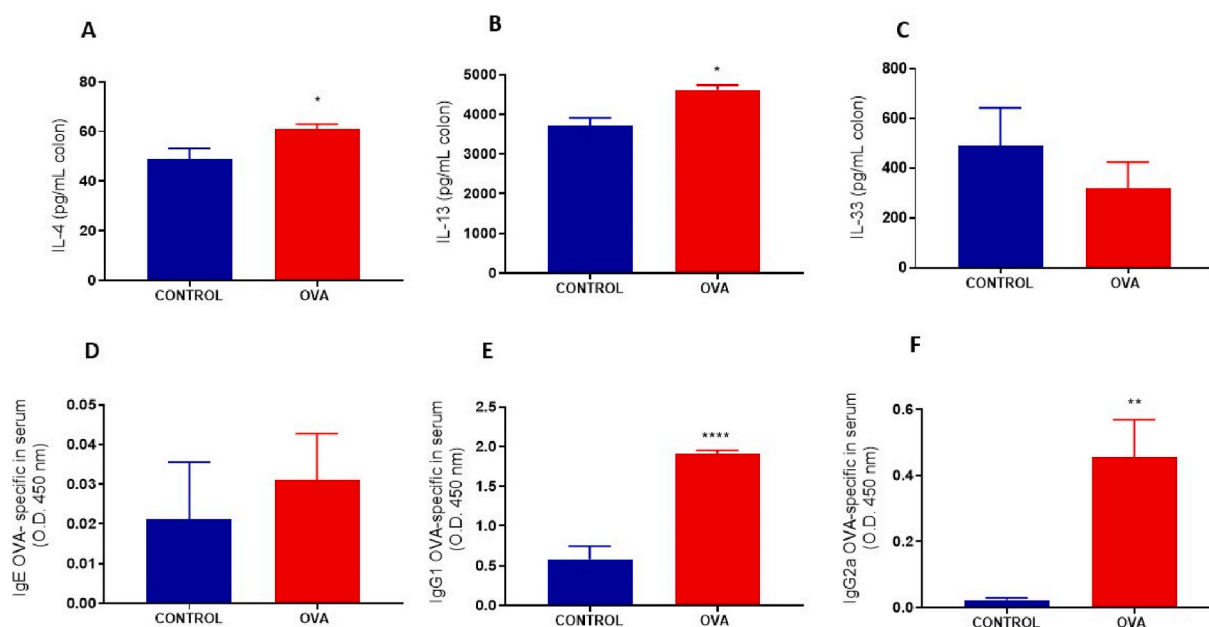


Fig. 6. Allergic airway inflammation affects colon cytokine and immunoglobulin levels in the serum. IL-4 (A), IL-13 (B), and IL-33 (C) in the colon tissue homogenate of mice euthanized 24 h after the last challenge. OVA-specific IgE (D), OVA-specific IgG1 (E) and OVA-specific IgG2a (F) in serum. Of mice euthanized 24 h after the last challenged unpaired *t*-test * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$ Control versus OVA. Bars represent the mean $\pm$ SEM. Data are representative of two independent experiments ($n = 6$ mice/per group).

to the animals in the control group (Fig. 6). The concentrations of TNF- α , CCL-5, CCL-2 and IL-10 were not significantly different in different groups (Supplementary Figure 1).

4. Discussion

There is evidence indicating that lung disease may affect the gut, but studies investigating the effects of allergic inflammation in the colon are rare [4–11]. Here, we investigated the effects of allergic airway inflammation on colon homeostasis in an experimental model. First, we induced experimental allergic inflammation in the airways with OVA to induce inflammatory cell influx, such as eosinophils, neutrophils, and mononuclear cells, in the airways. Our results showed that inflammation of the airways led to morphological changes in the colon, which can be confirmed by the reduction in colon length in the OVA group compared to the control group. The reduction in the length of the colon is a marker of gut damage and a phenotypic characteristic that has already been used in the literature to characterize gut inflammation [27–29]. Mice treated with 1% sodium dextran sulfate (SSD) to induce IBD showed colon shortening, and when treated with a diet that mimics fasting, this change was reversed, and there was improvement in other parameters of gut inflammation [27]. Colon shortening is also an observable characteristic that affects people with UC and CD [28,29], which suggests that our experimental model may have induced a pathological condition similar to these diseases. However, studies reporting on the length of the colon as a result of allergic lung inflammation remain limited.

Next, we analyzed MPO in the colon mucosa as an inflammatory parameter. The data show that the OVA group presented increased levels of MPO compared to the control group. These data also support the hypothesis that inflammation of the lung increases susceptibility to developing IBD since the presence of neutrophils is rare in healthy individuals, but their numbers increase dramatically in cases of inflammatory disease or infection [30]. The influx of neutrophils into the inflamed mucosa is one of the most prominent features of UC [31]. Neutrophils are also known to be effector cells that cooperate with lymphocytes in chronic gut inflammation, encouraging the transition to colon cancer [32–34]. In contrast, the levels of eosinophil peroxidase were not altered in the colon mucosa, suggesting that eosinophils do not participate in the breakdown of intestinal homeostasis in this experimental protocol.

In addition to inflammation, we analyzed mucus production. The colon is the organ with the thickest mucus layer, measuring approximately 830 μm , and, in contrast to the small intestine, it is composed of an inner stratified layer that is mostly sterile and an outer loose layer that is a habitat for bacteria [35,36]. This structuring is critical for preventing pathogens from invading the mucosa to cause intestinal inflammation. The physiologic relevance of distinct mucin subtypes is not well understood. The analysis of colon photomicrographs showed that the PAS staining intensity in the OVA group was decreased compared to that in the control group, which revealed a decrease in the production of neutral mucins in the OVA group. Neutral mucin is denser than acidic mucin and has a greater capacity to protect against mechanical damage and greater lubrication capacity when mucous cells are exposed to abrasive and irritating agents [37]. Substantiating the role of neutral mucin in maintaining mucosal integrity, our qualitative analysis of the integrity of the intestinal mucosal layer through photomicrographs indicates that the barrier function of animals in the OVA group was affected. An impaired barrier function makes the individual more likely to develop IBD [38–42]. Similar results have been shown in

previous studies, where it was observed that pneumonia induces intestinal injury [43] and decreases intestinal epithelial proliferation [44]. There were no significant differences between the groups regarding the intensity of staining with Alcian Blue, a dye with great specificity for staining acidic mucins [45]. Acid mucin acts to protect against bacterial translocation, as it is less degradable by bacterial glycosidases and host proteases [46,47]. Several factors can affect the differentiation and maturation of goblet cells and the secretion of mucin. The abnormal proliferation and differentiation of goblet cells, as well as the deficient synthesis and secretion of mucins, results in intestinal mucosal barrier dysfunction. We analyzed some genes involved in the gut mucosal barrier, such as MUC2, TJP1 and ATOH1. ATOH1 is negatively affected by airway allergic inflammation, while MUC2 and TJP1 are positively affected. ATOH1 is a proneural basic helix-loop-helix transcription factor that is required in a variety of developmental contexts and is an essential factor that controls the differentiation of goblet cells [48,49]. The reduced ATOH1 expression in the colon suggests that airway inflammation can affect the differentiation of intestinal secretory cells and can cause long-term damage to the integrity of the mucosal barrier. Previous studies showed that 70% of a sample of patients diagnosed with colorectal cancer have significantly decreased expression of ATOH1 compared to tissue-matched normal LI samples and revealed that ATOH1 is a tumor suppressor *in vitro* and *in vivo* [50]. *Muc2* gene in the mouse encodes the major intestinal mucin MUC2, eliminates the mucus barrier resulting in physical contact between intestinal bacteria and the mucosa. Although ATOH1 contributes to increases in MUC2 expression, several other transcription factors can drive MUC2 expression and can be involved in this pathway, such as CDX2 [51], GATA4 [52] and FOXA1 and FOXA2 [53], which can explain the increased MUC2 expression in mice that also showed a decrease in ATOH1 expression; however, these transcription factors were not evaluated in this study. Moreover, immunological responses can regulate the expression of MUC2. A murine enteroid culture system demonstrates that IL-33 indirectly induces goblet cell differentiation through IL-13 predominantly by ILC2, increasing MUC2 and ATOH1 expression [54]. Furthermore, colonic goblet cells express several TLR subtypes [55], suggesting inherent TLR-mediated mucin regulation in goblet cells, since pathogen-associated molecular patterns (PAMPs) are ligands for these receptors [56], as shown in an elegant study, wherein upon stimulation by ligands such as LPS in TLRs, PAMPs promoted the downstream activation of NOD-like receptor family pyridin domain containing 6 (NLRP6) and induced MUC2 secretion from goblet cells [56]. In this context, possible changes in the microbiota composition induced by airway inflammation can contribute to the increase in MUC2 expression. In this study, we also identify tight junction protein 1 (TJP1) as a putative mediator of glucosylceramide-induced protection and/or stabilization of the colonoids [57,58]. While we mainly focused on TJP1 during this study as marker for changes in cellular tight junctions, there are many other proteins involved in the assembly, maintenance, and function of tight junctions. Some other examples include claudin-3, and occluding. Thus, the TJP1 gene is related to intestinal permeability. Increased permeability is implicated in the pathogenesis of some intestinal disease. *In vitro* and *in vivo* studies have linked down-regulation of the scaffolding protein ZO-1, encoded by the TJP1 gene, to increased tight junction permeability [59]. The decreased TJP1 expression level observed in inflamed IBD and UC mucosa, compared to non-inflamed mucosa, is consistent with a loss of ZO-1 protein (encoded by TJP1) in dextran sulfate sodium induced colitis in mice [60]. Also, they observed an association between loss of ZO-1 and increased paracellular permeability [60].

We also measured type 2 cytokines, such as IL-4 and IL-13 in the colon to better understand mucus changes in this study. IL-4 and IL-13 were increased in the colon, they are important inducers of goblet cell metaplasia in experimental animals and have been shown to induce mucin gene expression both *in vitro* and *in vivo* [61–63]. Moreover, these cytokines also up-regulate MUC2 transcription in human colonic cancer cells through NF- κ B activation mediated by MAPK (mitogen-activated protein kinase) [64]. IL-13 is responsible for initiating inflammation in the colon during colitis pathogenesis [65]. In ulcerative colitis, IL-13 has been described as a key effector cytokine acting on epithelial cell function and initiating apoptosis [66]. Previous experiments have already shown that murine models of ulcerative colitis have excessive production of IL-13 [67,68]. A model of oxazolone colitis (CO), similar to human ulcerative colitis, showed that the production of IL-13 is a significant pathological factor in oxazolone-induced colitis, since colitis could be prevented by the administration of IL-13R2 α -Fc, a soluble receptor of IL-13 that blocks the interaction of IL-13 with its signaling receptor [67]. We also observed increased IL-4 in the colon. A previous study showed that IL-4 production in mesenteric lymph nodes was associated with inflammation of the intestinal tract in a model of food allergy and that treatment with an *anti*-IL-4 mAb reduced gut inflammation [69]. Another study showed that mice with a deficiency in IL-4 production have less neutrophil degranulation in the gut [70]. Considering that in our study there was an increase in IL-4 in the colon tissue, it can be suggested that IL-4 stimulated neutrophils, which we measured by the MPO assay. We did not find differences between groups in IL-33, perhaps because we measured all cytokines and inflammatory parameters 24 h after the last OVA challenge, and this cytokine could be affected at an earlier stage.

Related to the intestinal barrier, we can summarize that some data such as neutral mucin intensity, ATOH1 expression and epithelial barrier are affected in airway allergy. On the other hand, there was an increase in MUC2 and TJP1 expression that is positively related to an improvement in the intestinal barrier [60]. Cytokines IL-13 and IL-4 are increased in intestinal diseases and parasitic infections and are also associated with an increase in MUC2. In this study we do not have all the tools to link all these genes and cytokines, the literature is also scarce on this subject. We can hypothesize that allergic pulmonary inflammation damages the intestinal barrier epithelium but there are some compensatory mechanisms, the increase in IL-13 increases MUC2 which may improves mucus barrier [64], may be explain the normal acidic mucus production. In addition, the literature is unclear about the importance of the genes investigated in this study and their relationship with the two different mucins, acidic and basic. Future studies of our laboratory will investigate this issue.

Increased levels of IgG1 and IgG2a specific for OVA in serum were observed in the OVA group, as expected because of the lung allergy process. These antibodies represent a marker of the inflammation profile, where OVA-specific IgG1 is a response marker with a Th2 profile, while OVA-specific IgG2a is a response marker for Th1 cells [71,72]. The increase in both antibodies in the serum may be involved in the communication between the lungs and gut, but more studies are needed to reveal the role of these antibodies in the lung-gut axis.

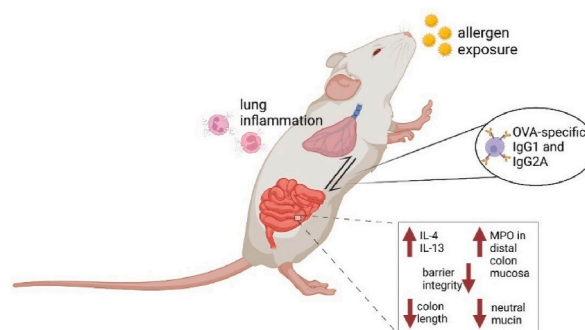


Fig. 7. Schematic representation of the allergic airway inflammation effect on the colon of mice.

In summary (Fig. 7), our data using the OVA experimental model show that lung inflammation induced some colon changes, such as colon shortening, increased MPO, IL-13, and IL-4 levels; decreased ATOH1 and increased MUC2 and TJP1 expression; and reduced neutral mucin intensity. Most of these parameters are related to predispositions for colon disease. The mechanism involved in the process of the lung-gut axis is not clear, but it is possible that antibodies and cells involved in the inflammation of the lung enter the gut through the circulatory system, affecting cells and other structures at the gut barrier. More studies are necessary to understand whether gut treatment is needed for patients with asthma, considering that the gut of these patients may be altered, increasing their susceptibility to developing gut inflammation or cancer.

5. Ethics approval and consent to participate

The Institutional Animal Care and Use Committee of Federal University of São Paulo approved all the procedures under protocol #5547290818.

Author contribution statement

Carolina Martins Nascimento, Mateus Campos Casaro, Evelyn Roxana Perez, Willian Rodrigues Ribeiro, Marcia Pinto Alves Mayer, Karin Hitomi Ishikawa, Adriana Lino-dos-Santos-Franco, Joice Naiara Bertaglia Pereira, Caroline Marcantonio Ferreira: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed with reagents, materials, and analysis data; Wrote the paper.

Funding statement

Caroline Marcantonio Ferreira was supported by grant # 2019/12324-1 and # 2021/06751-4, São Paulo Research Foundation (FAPESP).

Carolina Martins Nascimento was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (001).

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.heliyon.2023.e16429>.

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