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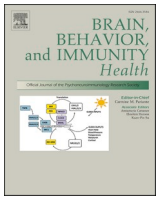

Repeated saline injections reduce the pulmonary allergic inflammatory response in rats by inducing short-term stress☆

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ABSTRACT

Purpose: Asthma is characterized by pulmonary cell infiltration and hyper-responsiveness of the airways. Short-term stress reduces airway inflammation. Thus, in the present study, we examined the effects of short-term stress induced by repeated treatment with saline injections on the pulmonary allergic inflammatory response in rats.
Methods: Adult male rats were divided into three groups: Naïve group (non-sensitized, challenged, or treated rats), Control group (rats sensitized with ovalbumin (OVA) to induce lung inflammation), and Saline group (rats treated for five days with saline before OVA sensitization). Inhalation challenges were performed one week after the booster with aerosolized OVA. On day 18, the effect of saline injections on total and differential leukocytes in bronchoalveolar lavage (BAL), femoral marrow lavage (FML), and blood was evaluated. The percentage of mucus, serum corticosterone, collagen, cytokines in lung explants, and norepinephrine levels were also measured.
Results: OVA sensitization increased the circulating leukocytes and their migration to the lung, decreasing the bone marrow leukocytes. The repeated saline injections prevented this migration by decreasing the number of leukocytes in BAL and blood in the control group. Cytokine Interleukin-4 (IL-4) was higher in the control group than in the naive and saline groups; cytokines Interleukin-6 (IL-6), Interleukin-10 (IL-10), and tumor necrosis factor-alpha (TNFα) were higher in the control and saline groups than in the naïve group; Interferon gamma (IFNγ) was higher in the saline group than in the naive and control groups; norepinephrine increased in animals sensitized with OVA and was higher only in the saline group relative to the naïve group.
Conclusions: These results suggest that short-term stress could contribute to the anti-allergic airway inflammation effects of a given treatment.

1. Introduction

Allergic asthma is a complex, heterogeneous disease characterized by reversible intermittent obstruction and chronic airway inflammation. The initial allergic response includes bronchial hyperreactivity and infiltration of lymphocytes and eosinophils into the submucosa of the airways (Ochoa-Amaya et al., 2016, 2015; Wong et al., 2001) The pulmonary allergic inflammation model has been used to study the pathophysiology of asthma in rodents (de Oliveira-Higa et al., 2015a, 2023; Wong et al., 2001)

Stress is a biological response to a noxious stimulus that induces activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) (Kiss and Aguilera, 1993). The brain is a target of stress, and the hippocampus was the first brain region besides the hypothalamus to be recognized as a target of glucocorticoids. Stress and stress hormones produce adaptive and maladaptive effects on this brain region throughout the life course (McEwen, 2007). Moreover, physiological stress activates sympathetic nerve fibers to release

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norepinephrine (NE) into the local tissue microenvironment and systemic circulation. The proximity of nerve terminals to immune cells allows the SNS to target the antigen-activated immune cells directly. A high concentration of NE needs to be released within its microenvironment shortly after an antigenic challenge to regulate the immune target cell because NE is rapidly metabolized and taken back into the nerve terminal (Padro and Sanders, 2014) NE ligation of cellular β-adrenoreceptors (AR) triggers a G-protein–coupled signaling cascade leading to cyclic AMP (cAMP) synthesis, protein kinase A phosphorylation, and transcription factor activation (Padro and Sanders, 2014; Sloan et al., 2010). It was already known that stress may influence the development or exacerbation of allergic pulmonary inflammation (Lee, 2015; Ligeiro de Oliveira et al., 2008). For instance, experimental and clinical studies indicate that stress during childhood increases allergic inflammation and causes immune alterations (Gonçalves et al., 2016). Moreover, epidemiological studies suggest that stress exacerbates asthma (Leick et al., 2012).

Considering a simplistic analysis of the mutual interactions between the immune, neuroendocrine, and behavioral systems, the stress response activates the hypothalamic-pituitary-adrenal axis and the adrenomedullary system, leading to a release of glucocorticoids and catecholamines that, in turn, could influence the immune system and the course of diseases. In this sense, several cytokines, growth factors, and lipid mediators of inflammation can influence the response of the hypothalamic-pituitary-adrenal axis, acting particularly on the locus coeruleus. The locus coeruleus (LC) is a stress-responsive brainstem and an important part of the SAM axis in regulating norepinephrine (NE) release. NE secretion and expression of tyrosine hydroxylase (TH), an enzyme involved in NE synthesis, are stimulated because of activation of the SAM axis. Modulation of the SAM axis by the HPA axis through projections from the LC to stress-related brain regions interconnected with the hypothalamus (Lee, 2015).

In addition to variations in how the cells look and move, macrophages surrounded enteric neurons. These neurons released norepinephrine, an autonomic/sympathetic nervous system neurotransmitter involved in the stress response, to 'instruct' macrophages to activate an anti-inflammatory response. Gut macrophages differentially expressed the β2-adrenergic receptors (ARs) on their surface, allowing them to respond to norepinephrine (Gabanyi et al., 2016). Macrophages are essential for local homeostasis but also play an important role in inflammation and protective immunity when they change from "peaceful regulators" to "powerful aggressors." The abundant presence of innate and adaptive lymphocytes and a rich mixture of products such as TGF-α and IL-10 are implicated as signals that control macrophage responses and the gut macrophage phenotype (Mowat and Bain, 2011).

Macrophages are key in the bidirectional crosstalk between the immune and nervous systems. M1 macrophages are classically activated by interferon (IFN)-γ while M2 macrophages are alternatively activated by IL-4, IL-13, and IL-10, which are responsible for antigen presentation (Carrick and Begg, 2008). The inhibitory neurotransmitters reduce the excessive production of pro-inflammatory cytokines by macrophages and brain parenchyma activities stimulated by pro-inflammatory cytokines. Moreover, neurotransmitters control the functions of the macrophages to remove cellular debris, ingest foreign material, and induce inflammatory reactions. Cytokines stimulate neurons to modulate social interactions, stressful HPA axis responses, and autonomic nervous activities (Reyes-García and García-Tamayo, 2009).

The adaptive immune system cells (B and T cells) express β2AR exclusively. On average, $CD4^+$ and $CD8^+$ T cells express approximately 200–400 binding sites per cell. NE, adrenaline, and other ligands stimulate α- and β-AR with several affinities and on different cell types. β2AR are the most common receptors among adrenergic receptors in the immune system(Mirotti et al., 2010). The β2AR is expressed on murine $CD4^+$ naïve T and Th1 cells but not on Th2 cells(Padro and Sanders, 2014). Cytokines (IFNγ, IL-10, and IL-6) are important in inflammatory conditions like acute respiratory distress syndrome (ARDS) by modulating the recruitment and activation of leukocytes. IFNγ and IL-10 are anti-inflammatory in endotoxin-induced lung injury(Silva Ibrahim et al., 2015). Early systemic IL-10 releases following injury has beneficial effects because it inhibits pro-inflammatory cytokine synthesis and leukocyte recruitment(Kobbe et al., 2012). Type I IFNs are protective in animal models of arthritis and inflammatory bowel disease by suppressing the production and function of inflammatory cytokines (Ivashkiv and Donlin, 2014). Eosinophils express IL-12 and IFNγ (Jacobsen et al., 2007).

Previous stress (e.g., handling and placebo treatments) could cause a placebo effect that attenuates allergic responses, disease progression, and mortality in conditions such as rheumatoid arthritis, lupus (Ader et al., 1995), and asthma (Schedlowski et al., 2015). Exposure to six weeks of repeated injection resulted in an anxiety-like phenotype, decreased systemic inflammation (i.e., reduced plasma levels of TNFα and IL-4), increased corticosterone reactivity, increased microglial activation, and decreased neuronal differentiation in the dentate gyrus (Du Preez et al., 2020). In addition, studies showed that saline injection could alter thermoregulatory responses, affecting the effect of the injected drug on brain cells (Kiyatkin, 2013; Kiyatkin and Lenoir, 2011)' and depressive-like states (Izumi et al., 1997). However, during acute or chronic treatment, it is generally assumed that saline intravenous injection does not induce stress or physiological effects and serves only as a control for drug-induced effects. Thus, we decided to investigate the effects of repeated saline injections on the pulmonary allergic inflammatory response.

2. Materials and methods

2.1. Animals

Wistar rats obtained from the School of Veterinary Medicine and Animal Science of the University of São Paulo were housed with controlled ventilation, humidity, and temperature (22–23 °C) in a light/dark cycle of 12 h (lights on at 7:00 a.m.). Water and food were provided *ad libitum* for the experiment and withdrawn during the experimental observations. The FMVZ-USP Department of Pathology instructions based on the *"Committees on Care and Use of Laboratory Animal Resources"* National Research Council, USA, were applied to all animals. Every attempt has been made to reduce the number of animals involved and their suffering to a minimum.

2.2. Experimental design/allergic lung inflammation model

Male rats (n = 20) ranging from 80 to 100 days of age or weighing between 270 and 370 g were randomly divided into three groups: naïve group - rats not sensitized or treated (N group)); rats sensitized and challenged with ovalbumin (C group); and rats treated with three daily intraperitoneal (ip) injections (06h30; 14h00; 21h00) of saline solution 0.9% for five days before sensitization with ovalbumin (S group). The C and S groups were also sensitized with OVA seven days after the first sensitization (booster). The protocol for animal sensitization and booster was 10 μg of OVA (Egg Albumin Grade II Sigma-Aldrich St. Louis MO USA) plus 10 mg of aluminum hydroxide (EMS Pharmaceuticals São Paulo Brazil) dissolved in phosphate-buffered saline (PBS) at a dose of 0.1 mg kg^{-1}. On days 15, 16, and 17, all animals of the C and S groups were challenged with 10 μg of aerosolized OVA (1%) for 15 min per day in an inhalation chamber connected to an ultrasonic nebulizer (de Oliveira et al., 2011) (Fig. 1).

2.3. Dosage of corticosterone and total counting of circulating leukocytes

On day 18, 24 h after the challenge with OVA, the animals were deeply anesthetized with xylazine 2% (5 mg kg^{-1}) and ketamine 5% (30 mg kg^{-1}). The peritoneal cavity of the rats was opened to collect two aliquots of blood samples from the abdominal aorta. These samples were

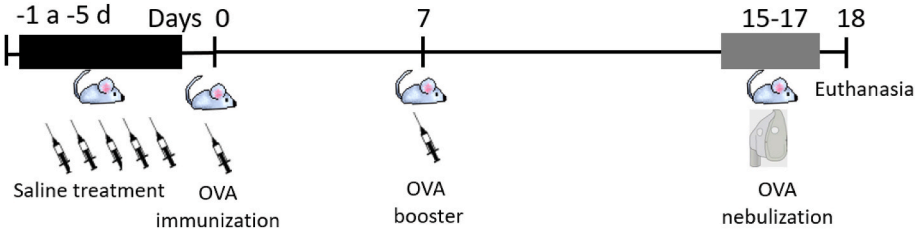

Fig. 1. Experimental design of saline injection treatment before sensitization on ovalbumin-induced pulmonary allergic inflammatory response.
Timeline illustrating treatment protocol of 5 days of saline injections prior to OVA immunization, OVA boost 7 days later and OVA nebulizations from day 15 to day 17.

diluted 1:20 in Turk's liquid (3% acetic acid). In the first blood collection for total counting of circulating leukocytes, plastic syringes containing 50 µl of 8% EDTA were used. A blood smear stained with May-Grunwald-Giemsa made a differential count of cells in the blood under a light microscope (de Oliveira et al., 2011). Serum blood aliquots taken in the second collection were used to determine corticosterone and stored at −80 °C. Serum corticosterone was determined by commercial kits "enzyme-linked immunoassay (ELISA) kit (Arbor Assays #K014)" according to the manufacturer's instructions with a serum dilution of 1:300. Samples of blood were taken at the same time (9–12 m) to decrease variability in the data.

2.4. Bronchoalveolar lavage (BAL) and leukocyte counts

Bronchoalveolar lavage (BAL) was performed according to DE LIMA, SIROIS, and JANCAR(de Lima et al., 1992). The lungs were washed with 4.0 × 5.0 ml heparinized PBS or RPMI-1640 (20 ml) through a polyethylene cannula (1 mm internal diameter) inserted by tracheotomy. The fluid collected from the pleural cavity was used to evaluate the volume of exudate (volume of fluid withdrawn - the volume of injected PBS) and for the count of inflammatory cells contained in the pleural exudate. For this, it was centrifuged at 170×*g* for 10 min at 20 °C in a clinical centrifuge and the cell pellet was resuspended in RPMI-1640 (1 ml). The supernatant was discarded. Aliquots of the cell suspension were diluted 1:20 with Turk's solution, and the total leukocyte count was done in Neubauer's chamber. Differential leukocyte count was done on smears stained by the Giemsa method.

2.5. Femoral marrow lavage (FML) and counting of leukocytes

The total number of bone marrow cells was quantified in the femoral marrow lavage (FML) obtained immediately after BAL, and to make the peripheral blood collections, the femurs of the rats were removed. A needle attached to a plastic syringe containing PBS (5 ml) was inserted into the femoral medulla to allow cell lavage. The FML was centrifuged (170 g × 10 min). The remaining cellular pellet was resuspended, processed, and analyzed for total leukocyte count as described for BAL.

2.6. Collection of samples

To investigate whether changes in the lungs on cell migration were related to the effect of the treatment on the generation of cytokines IL-4, IL-6, IL-10, TNFα, and IFNγ in allergic rats, these cytokines concentrations were measured in supernatants from lung explants with 24 h culture and corrected for lung tissue weight. The cytokines in lung explants and BAL were determined using ELISA kits. For histopathology, the samples were dipped in 10% formaldehyde to assess the cellular infiltration to the lung, the evaluation with the acid (PAS) to detect mucus production, and the picrosirius technique for detecting collagen.

2.7. Measurement of plasma catecholamines

We applied a previously described method (Foti et al., 1987) with relevant changes for our sample volumes and equipment. The measurement of catecholamines was performed using high-performance liquid chromatography (HPLC) combined with electrochemical detection (HPLC-ED; Shimadzu Model 20A) in reverse phase with ion pairing (heptane sulfonic acid). The HPLC-ED automatically injects 70 µl per sample. The system maintained an isocratic flow of 1 ml min^{-1}, and the oven temperature was maintained at 50 °C. We used a 15 × 4.6 mm column and five µm particle size (C-18 Shimpack). The electrical potential in the electrochemical detector (Altec-Decade) was 800 mV. The mobile phase consisted of phosphate-citrate buffer (3 mM heptane-sulfonic acid, 20 mM anhydrous sodium phosphate, 23 mM sodium citrate, 0.1 mM EDTA, 3% methanol [v/v], and 1% acetonitrile [v/v]) pH 3.18. The mobile phase was filtered via a membrane with 0.45 µm porosity and deaerated with an ultrasonic bath. For the extraction of plasma catecholamines, we used a liquid-liquid extraction technique and 34-dihydroxy benzylamine (DHBA) as the internal standard. Thus, 500 µl of plasma was transferred to a 2-ml microtube with 300 µl of 0.2 M perchloric acid solution, 30 mg aluminum oxide, and 1 ng ml^{-1} of the internal standard. This solution was subjected to 30 min of agitation on an orbital platform and centrifuged under refrigeration at 2000×*g* for 10 min. The supernatant was removed, and the remainder was washed three times with 1 ml of ice-cold deionized water each time and centrifuged for at 2000×*g* for 10 min. After washing, analytes were eluted with 150 µl of the perchloric acid solution, vortexed for 5 min, and subjected to the same centrifugation procedure. The supernatant was recovered and analyzed.

2.8. Statistical analysis

The Kolmogorov-Smirnov test was employed to verify the distribution of the data. All the date showed a normal distribution, therefore, ANOVA (Analysis of Variance) and the Student-Newman-Keuls test were used to analyze the results since all the data showed a normal distribution. GraphPad Instat 5.01 was employed for statistical analysis. Data were reported as means ± SEM, and significant differences were considered for $p \leq 0.05$.

3. Results

In the BAL cell counts, the control group exhibited a higher number of total cells than the naive and saline groups (ANOVA $p = 0.0083$; $F = 6.446$) (Fig. 2A), suggesting that treatment with saline decreased total leukocytes in BAL relative to the control group. Additionally, ANOVA revealed significant differences ($p = 0.0464$; $F = 3.741$) in the differential BAL cell counts (Fig. 2B). The control group revealed a higher total number of macrophages ($p = 0.0464$; $F = 3.741$) and eosinophils ($p < 0.0001$; $F = 22.52$) than the naïve and saline groups. No statistical differences were detected between the three groups in lymphocyte and neutrophil counts. Therefore, OVA sensitization increases the total cell count and the number of macrophages and eosinophils in the BAL, while prophylactic treatment with saline prevented this migration of leukocytes to the lung.

Similarly, in the total cellular blood leukocyte count, the control

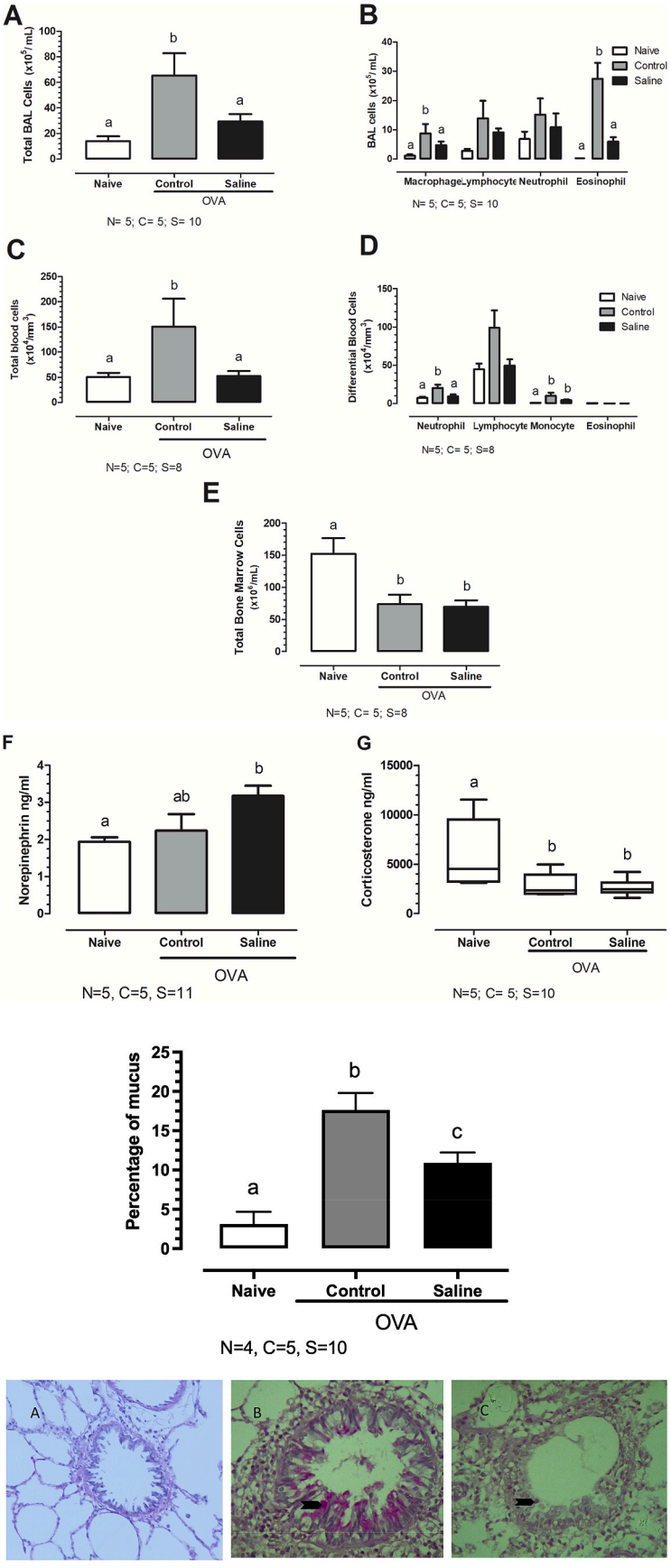

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Fig. 2. Effects of five days of saline treatment prior to OVA-immunization on the number of cells present in BAL (A, B), blood (C, D), and bone marrow cells (E), norepinephrine (F), corticosterone (G) plasma levels, and effects on the percentage of mucus (H) in the pulmonary allergic response. N = naive; C = control allergic rats; S = saline allergic rats treated with saline for five days. Mucus staining by PAS staining in rat lungs. (A) naive group. (B) control group. (C) vehicle group. Different letters above the columns indicate statistical differences. Results are reported as mean ± SEM. $p < 0.05$ (ANOVA and Newman-Keuls Multiple Comparison Test).

Column graphs displaying lower total BAL cell count, eosinophil and macrophage counts and total blood cell counts in the saline group compared to control group. Both asthmatics groups present lower total bone marrow cells and higher norepinephrine than naive group. Histopathological PAS images show few goblet staining in saline group. Marking for mucus by dyeing PAS in rat lungs. (A) naïve group. (B) control group. (C) saline group.

group presented the highest number of circulating cells relative to the other two groups (ANOVA $p = 0.0452$; $F = 3.835$). No statistical differences were observed between the naïve and saline groups in this parameter (Fig. 2C). Regarding the differential blood cell count, the control group exhibited more neutrophils than the naive and saline groups (ANOVA $p = 0.0387$; $F = 3.963$). The naive group was statistically lower for monocyte counts than the control and saline groups (ANOVA $p = 0.0388$; $F = 3.959$). No difference in circulating blood lymphocytes and eosinophil counts between groups was detected ($p > 0.05$) (Fig. 2D). These findings indicate that OVA sensitization increases the total cellular blood leukocyte count and the number of neutrophils and monocytes. Furthermore, the prophylactic saline treatment prevented the increase in allergic animals circulating leukocytes and neutrophils.

Regarding FML, the control and saline groups showed fewer bone marrow cells than the naive group (ANOVA $p = 0.0042$; $F = 8.060$). No statistical difference was detected between the saline and control groups in FML (Fig. 2E). Regarding plasma neurochemistry, the saline group demonstrated a higher level of NE than the naïve group (ANOVA $p = 0.0241$; $F = 4.613$) (Fig. 2F). No statistically significant differences in adrenaline were detected between groups ($p > 0.05$; $F = 0.1588$) (unshown data). The naive group exhibited higher corticosterone levels than the control and saline groups ($p = 0.0138$; $F = 5.572$) (Fig. 2G).

The control and saline groups exhibited statistically more mucus than the naive group (ANOVA $p = 0.0004$ $F = 13.44$). In contrast, the saline group presented less mucus than the control group ($p < 0.05$) (Fig. 2H and photo 1). No statistically significant differences in collagen were detected between groups ($p > 0.05$) (unshown data). No statistical differences between groups were detected in the adrenal gland weight (Naïve 132.0 ± 13.93; Control 122.2 ± 8.55; Saline 106.8 ± 8.58 mg) and in the gland weight/body weight ratio (Naive 0.34 ± 0.03; Control 0.35 ± 0.01; Saline 0.30 ± 0.02 mg. g-1) (ANOVA; $p > 0.05$; $F = 2.612$).

The IL-4 concentration was statistically higher in the control group than in the naive and saline groups (ANOVA $p = 0.0008$; $F = 11.48$). The saline group exhibited statistically less IL-4 than the control group (ANOVA $p = 0.0008$; $F = 11.48$) (Fig. 3A). The allergic groups (saline and control groups) had higher concentrations of cytokines IL-6 (ANOVA $p < 0.0001$; $F = 170.0$) (Fig. 3B), IL-10 (ANOVA $p = 0.0011$; $F = 10.45$) (Fig. 3C), and TNFα (ANOVA $p = 0.0479$; $F = 3.697$) (Fig. 3E) than the naive group. IFNγ concentration in cultures of lung explants was higher in the saline group than in the control and naive groups (ANOVA $p = 0.0279$; $F = 4.452$) (Fig. 3D).

4. Discussion

In inflammatory and autoimmune diseases, a deregulated response of activated and differentiated TCD4+ lymphocytes occur. This phenomenon was evidenced in the experiment described here. Epithelial cells of the airways express abundant proliferation of immune-modulating cytokines/chemokines eliciting the proliferation, recruitment, and activation of all pro-inflammatory leukocytes (Jacobsen et al., 2007). Concerning differential BAL, the saline group presented lower macrophage and eosinophil cell migration than the control group. The control group had a higher requirement of bone marrow cells because of the highest cellular migration to the lung. However, the results in the present study and from works previously published by our group show that part of these anti-inflammatory effects may be due to the increase in NE to produce bronchodilation, increase respirations (Ochoa-Amaya et al., 2016), and reduce blood flow to the lung and, in this way, the arrival of inflammatory cells to the lung in the stressed Saline group.

Moreover, the control and saline groups (allergic pulmonary inflammation groups) had fewer total cells in the bone marrow than the naive group. The reduced bone marrow cells in the saline group might be caused by the suppressed cell proliferation and differentiation from stimulation of β2AR. Besides, it could be related to the lower number of cells in the bloodstream and the BAL. The βAR is predominantly expressed in bone marrow during the early stages of activation and the αAR during later stages. Stimulating the β2AR suppresses bone marrow cell proliferation and differentiation (Padro and Sanders, 2014). Furthermore, certain leukocyte generation in bone marrow is impacted by SNS via β-ARs, leading to the preferential generation of populations of pro-inflammatory leukocytes (Pongratz and Straub, 2014).

In the context of short-term stress to which the animals in the Saline group were subjected, it can result in deregulation or suppression of immune function. Both the protocol of converting the animals into pulmonary allergies and the frequency of three injections per day of saline solution for five days before sensitization to the antigen decreased corticosterone. It increased the activity of the sympathetic-adrenal-medullary axis (SAM), which could be involved in the secretion of IL-4 in the stress response. Both allergic groups presented higher levels of IL-4 during allergic pulmonary inflammation than the naive group to differentiate naïve Th0 cells into Th2 cells (Kang and Weaver, 2010). IL-4 and IL-10 are anti-inflammatory cytokines (Wong et al., 2011); those cytokines increase during allergic pulmonary inflammation to reduce inflammation (Ochoa-Amaya et al., 2016; Ochoa-Amaya et al., 2015).

IL-6 is synthesized by Th2 and antigen-presenting cells and is implicated in the inflammatory response. When IL-6 increases, ACTH secretion is stimulated since IL-6 activates the HPA axis. Elevated IL-6 concentrations have also been recorded in response to physical and psychological stressors. Therefore, elevated IL-6 concentrations in control and saline groups could have influenced the corticosterone concentrations in these animals. However, after exposure to a chronic stressor, it is common for animals to become habituated to it, resulting in a decreased glucocorticoid response. (Hohlbaum et al., 2018; Sutherland et al., 2009) showed that a single anesthesia injection increases fecal corticosterone metabolites in male mice, reflecting high acute stress levels. Otherwise, mice habituate to the chronic stressor after repeated anesthesia injections, and no differences in corticosterone are detected. Similar results were observed in the current study, where the naïve group (one single injection) showed higher serum corticosterone levels than saline and control groups (multiple injections). Therefore, handling procedures and repeated injections required to convert the control and saline animals into allergic decreased the allergic response intensity since corticosterone levels are lower in saline and control groups than in the naïve group. Such approaches are based on classic conditioning of physiologic responses and alternatively two-way communication between the immune and central nervous systems (Lückemann et al., 2020).

The inflammatory response is balanced by anti-inflammatory factors, including IL-10 and TNF receptors (Pavlov et al., 2003). The Th2 immunity pattern is characterized by the release of IL-4, IL-6, and IL-10 and is associated with a humoral immune response or antibody production (Sternberg, 2001), as observed in the current investigation. Both allergic

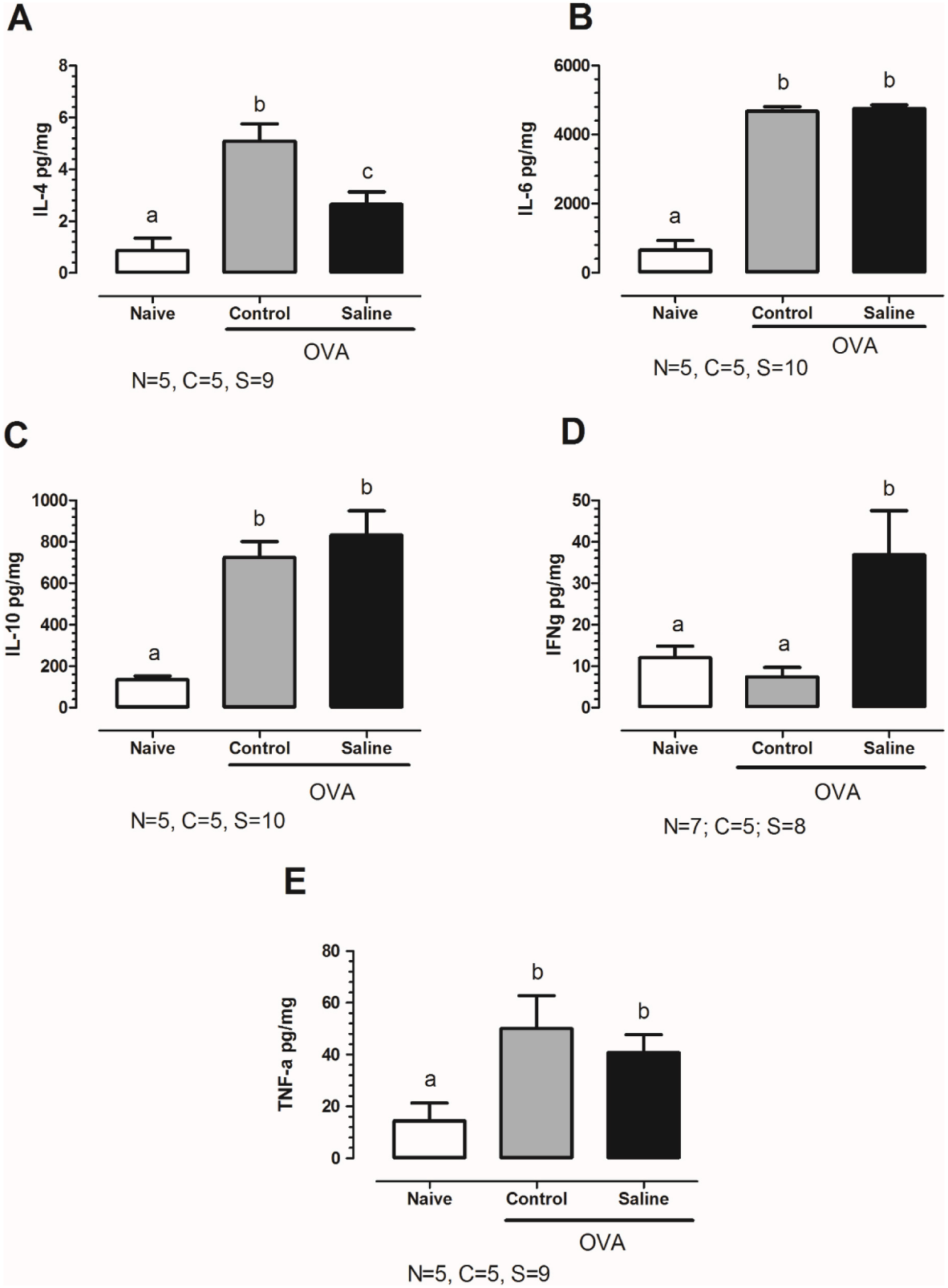

Fig. 3. Effects of saline treatment (5 days) before OVA-immunization on IL-4, IL-6, IL-10, TNFα, and IFNγ in allergic rats. Cytokines IL-4 (A), IL-6 (B), IL-10 (C), TNF-α (D), and IFNγ (E) are released by lung explant culture. N = naive; C = control allergic rats; S = saline allergic rats treated with saline for five days. Different letters above the columns indicate statistical differences. Data are reported as mean ± SEM. $p < 0.05$ (ANOVA and Newman-Keuls Multiple Comparison Test).
Column graphs displaying low IL-4 and high IFNγ levels in the saline group compared to controls. In both allergic groups the IL-6, Il-10 and TNF-α levels are higher than naive group.

pulmonary inflammation groups showed higher NE, IL-6, and IL-10 levels than the naive group. B lymphocytes, Th2 cells, and macrophages produce IL-10. This cytokine acts on antigen-presenting cells by suppressing their function. It is a negative regulator of the cellular immune response and in allergic disease is involved in suppressing mast cell and neutrophil activity. In addition, IL-10 is linked to the reduction of IgE synthesis in allergic responses involving the participation of mast cells (De Oliveira et al., 2011).

Pro-inflammatory cytokines such as TNF-α possess significant systemic effects, including the ability to activate the HPA axis. TNF-α is essential to normal inflammatory/immune homeostasis (Swanson et al., 2001). In this experiment, lung explant culture for 24 h presented higher TNF-α production differences in the allergic pulmonary inflammation groups than in the naive group. Thus, these results evidenced a possible effect of allergic pulmonary inflammation on the production and action of endogenous NE on TNF-α concentration because the most inflamed subjects had higher NE production. The primary targets of NE action in macrophages are cytokine genes. Production of pro-inflammatory TNF increased following exposure to either a 1AR or 2AR agonist. These findings imply that monocytes and macrophages express various AR subtypes and that the stimulation of 2ARs inhibits the production of inflammatory cytokines. Nevertheless, the functional impact on macrophages may depend on the NE concentration. Low levels of AR stimulation reduced macrophage cytokine production and phagocytic activity (Padro and Sanders, 2014).

The saline group had a higher concentration of IFNγ, differing statistically from the naive and control groups, probably because of the immunomodulatory action of NE. Catecholamines affect the Th1 and Th2 patterns of the cytokine production profile (Sternberg, 2001). High catecholamine concentrations stimulate anti-inflammatory β-ARs preferentially (Pongratz and Straub, 2014). β2AR simulation enhances the anti-inflammatory response. In vitro exposure to NE or a selective β2AR agonist reduced the production of IL-2 by murine $CD4^+$ naïve T cells and under Th1-promoting culture conditions induced the differentiation of naïve T cells into Th1 cells, which released higher IFNγ levels upon re-stimulation with no effect on the number of Th1 cells that developed. These findings suggest a positive role for NE and β2AR stimulation in regulating the extent of $CD4^+$ naïve T- and Th1 cell-mediated responses in vitro and in vivo through regulation of secreted IFNγ levels. To bind NE, Naïve $CD4^+$ T cells only expressed the β2AR subtype, with stimulation of this receptor generating Th1 cells, which released 2–4 times more IFN-γ (Swanson et al., 2001). Given that NE enhanced Th1 differentiation, the mechanism through which this happened could be due to an enhanced number of IFN-γ-producing Th1 cells after further stimulation (Swanson et al., 2004).

The Th1 cytokine pattern is distinguished by the production of IL2 and IFNγ, cell type Th1 response as evidenced by the higher IFNγ production of the saline group. There was a dramatic reduction in IFNγ produced by Th1 cells in the naive and control groups. Our results are consistent with those reported by other authors. Asthmatic mice exposed to chronic repeated stress showed overall shifts toward Th1 predominance (Kang and Weaver, 2010; Sternberg, 2001). The saline group had higher levels of IFNγ than the other two groups, which could be thought to be one of the reasons for having lower plasma levels of corticosterone since IFNγ can inhibit corticotropin-releasing hormone in the anterior pituitary (Gonçalves et al., 2016) and IFNγ could modulate the Th1/Th2 cytokine ratio (Barnes, 2001; Ochoa-Amaya et al., 2016; Ochoa-Amaya et al., 2015) in the saline group.

These results should involve a network of neuroendocrine pathways and neuroimmunomodulatory interactions, such as the SNS and the HPA axis, for controlling inflammation as an anti-inflammatory balancing mechanism. The host thereby mobilizes the immunomodulatory resources of the endocrine and nervous systems to regulate inflammation (Ader et al., 1995; Gabanyi et al., 2016; Pavlov et al., 2003). Therefore, the stress might have activated the sympathoadrenomedullary system and the HPA axis, increasing NE, adrenaline, enkephalin, and β-endorphins secretion. These multiple pathways modulated the function of lymphocytes, natural killer (NK) cells, macrophages, and mast cells (Avital et al., 2006). Lymphopoiesis, hematopoiesis, lymphocyte homing, mature immune cell function, and immune cell surface phenotype are all regulated by stimulating ARs expressed on immune cells (Padro and Sanders, 2014).

Similarly, stress responses that modulate SNS activity significantly impact inflammation (Pongratz and Straub, 2014). NE, a neurotransmitter secreted by sympathetic nerve terminals (92–98%) (Jänig, 2014) that end next to CD4T cells (Swanson et al., 2004) within the first 24 h following Ag exposure, is another endogenous component of this microenvironment. Therefore, NE is produced and available in the immediate microenvironment of naïve CD4T cells throughout the crucial T cell activation and differentiation process. The NE production in the saline group was higher than in the naïve group. NE modulates immune function context-dependently (Pongratz and Straub, 2014). The liberation of increased amounts of NE is a mechanism in the pulmonary allergic inflammatory response since endogenous NE increases as a compensatory physiological adjustment. Also, there is evidence from several clinical findings that the neuroimmune system is involved in the pathogenesis of inflammatory conditions like rheumatoid arthritis (RA), in which systemic IFNγ levels are high. This discovery proposes a possible role for an SNS-induced β2AR-mediated effect in this pathological process, possibly impacting the differentiation of naive $CD4^+$ T cells into Th1 cells, which produce increased levels of IFNγ (Padro and Sanders, 2014), which may have occurred with allergic pulmonary inflammation in the model studied.

Thus, this experiment evidenced the participation of NE in the allergic responses of animals with lung inflammation, supporting the hypothesis of the participation of the SNS. These results suggest that a likely source of neurotransmitters to drive inflammation progression is the local release of NE from SNS nerve terminals. This reduces the allergic response observed in the saline group where NE in plasma only reached nanomolar levels (mean: 3.2 ng/ml under stress conditions). Increased inflammatory responses have also been linked to catecholamines. According to (Gabanyi et al., 2016), β-adrenergic signaling suppresses adaptive and innate immunity, while α-adrenergic signaling increases inflammation. The extensive sympathetic innervation in peripheral lymph nodes of this pathway, combined with β2AR expression in immune cells, is thought to underlie some of the general anti-inflammatory effects.

Pulmonary allergy is evident in the control group and diminished in the saline group. Saline injections also decreased the quantity of mucus relative to the control group. The phenomenon is consistent with the other variables measured in this experiment.

The results of the present experiment confirm the hypothesis that previous stress, such as saline injection before immunization, decreases the pulmonary allergic inflammatory response. These effects are through the secretion of NE by sympathetic nerve fibers in the pulmonary microenvironment, which activates the B2ARs of TH1 and eosinophil immune cells, increasing the production of IFN gamma. Based on neurobiology studies of placebo responses, immunosuppression is mediated via β-adrenoreceptors; the SNS can cause immunosuppression through β-AR. Thus, the data show a correlation with SNS activity.

5. Conclusions

The short-term stress induced by saline injections before immunization evidenced an anti-allergic airway inflammation effect. These results suggest that short-term stress could contribute to the anti-allergic airway inflammation effects of a given treatment.

CRediT authorship contribution statement

Julieta Esperanza Ochoa-Amaya: Writing – original draft, Methodology, Investigation. **Ligeiro de Oliveira Ana Paula:** Supervision,

Methodology, Investigation. **Freitas Felicio Luciano:** Writing – review & editing, Validation, Project administration, Data curation. **Maria Martha Bernardi:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

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