

ATTENUATION OF SEPSIS-INDUCED ACUTE LUNG INJURY IN MICE WITH IGE-MEDIATED FOOD ALLERGY

Atenuação da lesão pulmonar aguda induzida por sepse em camundongos com alergia alimentar mediada por IgE

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RESUMO

Introdução: A sepse apresenta elevado índice de mortalidade apesar da terapia antimicrobiana. Conhecimento sobre o processo fisiopatológico da sepse auxiliará na terapêutica e na redução da morbimortalidade. **Objetivo:** Avaliar os efeitos da lesão pulmonar aguda (LPA)/síndrome do desconforto respiratório agudo (SDRA) induzida pela sepse em camundongos com alergia alimentar mediada pela imunoglobulina E (IgE). **Método:** Cinquenta camundongos BALB/C machos (6 semanas) foram randomizados em grupo alérgico (AL) e controle (C). A alergia alimentar foi induzida por administração subcutânea de ovalbumina (10 mg). Os animais receberam solução aquosa contendo clara de ovo (desafio oral). A alergia foi confirmada pelo teste sorológico ELISA. A SDRA foi induzida por sepse, pela cirurgia de ligadura e perfuração de cecum (AL-SEPSIS e SEPSIS), e o grupo controle foi submetido a cirurgia fictícia (sham) (AL e C). A eutanásia ocorreu 24 horas após a indução da SDRA e 48 horas após o desafio oral. Foram coletados o lavado broncoalveolar para análise das células inflamatórias e o pulmão, em capacidade residual funcional (CRF), para análise histológica e morfométrica. **Resultados:** O grupo alérgico aumentou os níveis de IgE circulante. Espessamento da parede alveolar e infiltrado de células inflamatórias foram maiores em todos os grupos comparados com o grupo C. Os grupos AL e AL-SEPSIS não diferiram entre si mas exibiram menor espessamento da parede alveolar e infiltrado de células inflamatórias em comparação com o grupo SEPSIS. Resultados similares foram observados no lavado broncoalveolar. **Conclusão:** A alergia alimentar mediada por IgE atenua o espessamento da parede alveolar e o infiltrado inflamatório pulmonar em camundongos com sepse.

Palavras-chave: Sepse; Alergia alimentar; Síndrome do desconforto respiratório agudo; Lesão pulmonar aguda.

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ABSTRACT

Introduction: Sepsis has a high mortality rate despite antimicrobial therapy. Knowledge about the pathophysiological process of sepsis will aid in therapeutics and in the reduction of morbidity and mortality. **Objective:** To evaluate the effects of sepsis-induced acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) in immunoglobulin E (IgE)-mediated food allergy. **Method:** Fifty male BALB/C mice (6 weeks) were randomized to allergic (AL) and control (C). The allergy was induced by subcutaneous administration of ovalbumin (10 mg). The animals received the aqueous solution containing egg white (oral challenge). Allergy was confirmed by the ELISA serological test. Sepsis was induced through cecal ligation and puncture surgery (AL-SEPSIS and SEPSIS groups), and the control group underwent sham surgery (AL and C groups). Euthanasia occurred 24 hours after induction of ARDS, and 48 hours after oral challenge. The bronchoalveolar lavage was collected for inflammatory cells analysis, and the lung was collected in functional residual capacity (FRC) for histological and morphometric analysis. **Results:** The allergic group increased levels of circulating IgE. Thickening of the alveolar wall and inflammatory cellular infiltration were higher in all groups compared to C group. The AL and AL-SEPSIS groups did not differ among themselves but showed lower thickening of the alveolar wall and inflammatory cellular infiltration compared to SEPSIS group. Similar results were observed in bronchoalveolar lavage. **Conclusion:** IgE-mediated food allergy attenuates alveolar wall thickening and inflammatory pulmonary infiltrate in sepsis mice.

Keywords: Sepsis; Food allergy; Acute respiratory distress syndrome, Acute lung injury.

INTRODUCTION

In more recently consensus, sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. The hyperactivity of the host can generate organic dysfunction and even a modest degree of organ dysfunction is associated with a 10% increase in mortality¹. Septic shock is a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality. For this reason, the quick Sequential [Sepsis-related] Organ Failure Assessment (qSOFA) was created in 2016 to help clinicians to identify patients with increased risk of death. High death risk patients can be promptly identified at the bedside with qSOFA, ie, alteration in mental status, systolic blood pressure ≤ 100 mm Hg, or respiratory rate ≥ 22 /min¹.

Macrophages play an important role in regulating the inflammatory response to injury by exerting cellular and intercellular effects and releasing a number of cytokines (TNF- α , IL-1, IL-6, IL-8, and IL-10). Macrophages also activate neutrophils, monocytes, microvascular endothelial cells and neuroendocrine reflexes, which are important factors to the onset and progression of acute lung^{2,3}. Pulmonary microvascular leakage is one of the characteristics of blood-air barrier dysfunction in septic acute lung injury (ALI)/acute respiratory distress syndrome (ARDS). ALI/ARDS is characterized by non-cardiogenic pulmonary edema with diffuse bilateral infiltrates, decreased pulmonary compliance and hypoxemia refractory to high concentrations of oxygen. Precipitating factors for the development of ARDS are classified as primary (direct injury to the lung) and secondary (indirect injury to the lung). In the latter case, lung injury is caused by extrapulmonary conditions and the subsequent activation of

systemic inflammation. Sepsis is considered a major risk factor for the development of ARDS^{2,4}.

A number of different animal models of sepsis have been developed in order to study the complex pathophysiology of this syndrome. Cecal ligation and puncture (CLP) surgery is the model that most closely resembles sepsis in humans resulting from trauma with perforation of the bowel, colitis and postoperative peritonitis⁵.

Experimental studies demonstrate that bacterial infection may suppress or modulate allergic responses^{6,7,8}. In these studies, mice allergic to ovalbumin and inoculated with lipopolysaccharide (LPS) intranasally exhibited significantly lower numbers of macrophages, lymphocytes, neutrophils, and eosinophils in the bronchoalveolar lavage fluid when compared with control and allergy groups, showing a decrease in airway responsiveness and pulmonary inflammation. A food allergy triggers reactions that can affect one or more target organs, such as the skin, respiratory tract, gastrointestinal tract and cardiovascular system. IgE-mediated food allergy affects approximately 2% of the population worldwide. The main cytokines involved in this type of allergy are the interleukins IL-4, IL-5, and IL-13. IL-4, which is considered an anti-inflammatory interleukin, inhibits macrophage function by increasing the production of the IL-1 receptor antagonist and inhibiting the production of IL-1^{9,10}. Moreover, IL-4 is considered the main regulator of IgE¹¹. IgE antibody production is induced by Th2 cells activated by IL-4 and IL-13 and is regulated by Th1-related cytokines, such as IFN- γ and IL-12. However, the Th1/Th2 shift, favoring an imbalance toward Th2, is considered the key factor for the development of food allergy^{7,12}.

Although a number of studies in the literature demonstrate the modulation of

bacterial infusion by allergies, the effect of IgE-mediated food allergy on sepsis has not been studied. Given the direct relationship between levels of IL-4 and IgE¹³, we postulate that IgE-dependent food allergy could attenuate sepsis-induced ARDS. The aim of the study was to evaluate the effects of sepsis-induced ARDS in mice with IgE-mediated food allergy.

METHOD

Animals and experimental groups

All experimental protocols were performed in accordance with guidelines for the humane use of laboratory animals of the institution at which the experiments were carried out and received approval from the Ethic Committee for Animal Experimentation of the *Universidade Federal de Minas Gerais* (Brazil)[protocol # 227/08, (CETEA)] in accordance of Resolução 466/12, of Conselho Nacional de Saúde. The mice were kept eight per cage at a constant temperature ($22\pm 2^\circ\text{C}$) with a 12-h dark/light cycle. Fifty male BALB/C mice (6 weeks of age) were randomly divided into two groups: allergic (AL) and non-allergic controls (C). Subsequently, part of these animals either underwent CLP surgery [AL-SEPSIS (n = 15) and SEPSIS (n = 15)] or were sham-operated [AL (n = 10) and C (n = 10)].

Sensitization protocol

Food allergy was induced by ovalbumin¹⁴. On Day 0, the mice received a subcutaneous (sc) injection of 0.2 ml of saline containing 10 μg of ovalbumin (Sigma, St. Louis, MO, USA) plus 1 mg of $\text{Al}(\text{OH})_3$ as the adjuvant. Secondary sensitization consisted of a sc injection of 10 μg of soluble ovalbumin, 14 days after the primary sensitization (Day 14). The control group received a corresponding volume of saline solution. Seven days after the secondary sensitization (Day 21), the bottle containing tap water was replaced

with gauze-filtered 20% egg white solution for 48 hours (Days 21 to 23). This solution contained approximately 10 mg of ovalbumin/ml (Figure 1).

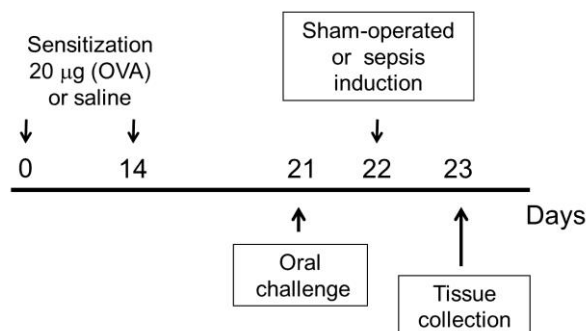


Figure 1. Sensitization experimental protocol. On 0 and 14 days, the mice received a subcutaneous (sc) injection of ovalbumin or saline. Seven days after the secondary sensitization (Day 21), the bottle containing tap water was replaced with gauze-filtered 20% egg white solution for 48 hours (Days 21 to 23). The animals were sacrificed on Day 23.

Serum antibodies

Serum samples were obtained from the groups following oral antigen exposure for the measurement of anti-ovalbumin IgE. Anti-ovalbumin IgE antibodies were measured by capture-ELISA using plates coated with rat anti-mouse IgE, 50 μl of total serum and biotinylated ovalbumin, as described elsewhere¹⁴. The results are reported as arbitrary units using a positive reference serum assigned to be 1000 units.

Sepsis induction

The animals were anesthetized with an intraperitoneal injection of a solution containing xylazine ($0.43\text{ mg}\cdot\text{Kg}^{-1}$) and ketamine ($0.5\text{ mg}\cdot\text{Kg}^{-1}$). A midline abdominal incision was made on shaved mice. The cecum was exposed and clamped in order to produce semioclusion to intestinal flux. The cecum was perforated twice on one side and through the cecal wall on the opposite side with a large 18-gauge needle. After this procedure, the region of exposure was carefully stretched to release the intestinal content into the

peritoneal cavity. Sham animals underwent the same procedures, but without the CLP^{5,15}. The mice were sacrificed 24 h following sepsis induction, which was 48 h after the oral challenge (Day 23 of the experiment).

Bronchoalveolar lavage protocol

Half of the animals in each group were subjected to bronchoalveolar lavage. Under anesthesia, the animals were tracheotomized and placed with the head elevated 30° from the horizontal. Subsequently, the lungs were filled with 1.0 ml of sterile saline at room temperature in two aliquots of 0.5 ml through the tracheotomy tube. After a short massage of the thorax, the fluid was aspirated, stored in Eppendorf vials and processed within three hours. The samples were subjected to cell count (Coulter counter) and checked against the Neubauer chamber. The count was conducted by two examiners who were unaware of the project.

Histological and morphometric analysis of lung

The histological and morphometric analysis of the lung was performed based on Rodrigues-Machado et al.¹⁶. The left lung was stored in 10% PBS form and embedded in paraffin. Sections of 5 µm were prepared and stained with hematoxylin-eosin. Analysis of the thickening of the alveolar wall on each slice was conducted to produce 10 randomly captured images on each region (upper, middle and lower) of the left lung, which was visualized using a 40× objective and digitized using a micro camera (JVC TK-1270/RGB), totaling approximately 1.6×10^6 µm² of tissue analyzed. The area of the thickening of the alveolar wall for analysis was determined using the KS300 program contained in the Carl Zeiss Image Analyzer. The pixels of the alveolar walls were selected from the real image, with the

subsequent creation of a binary image and obtainment of the area in µm².

The morphometric analysis of pulmonary inflammatory infiltrate was carried out on the images selected for the assessment of the thickening of the alveolar wall. All cells contained in each image were quantified using the KS300 program (Carl Zeiss Image Analyzer). The nuclei of leukocytes and other cell types usually present in lung tissue were counted through nuclear pixel selection from the real image and subsequently transformed into a binary image for subsequent analysis. For the final analysis, the images captured on different planes were grouped and the results in the left lung were shown. Pathological changes were assessed by a pathologist blinded to the allocation of the animals to the different groups.

Statistical analysis

The data were analyzed using one-way analysis of variance, followed by either the Newman-Keuls comparison test or Student's t-test when appropriate. Comparisons between the allergic and non-allergic groups were carried out using a non-paired test. The level of significance was set at $p < 0.05$. The data are reported as mean ± SEM.

RESULTS

IgE increased significantly in the allergic group in comparison to the control group (Figure 2). Average daily water consumption was similar among both sensitized and non-sensitized mice (4.7 ± 0.2 ml). However, average daily egg white solution consumption was higher in non-sensitized mice (8.3 ± 0.8 ml) in comparison to sensitized mice (4.1 ± 0.4 ml). All groups exhibited the same food intake (data not shown). Animals in the allergic group exhibited lower body weight gain between the first sensitization (Day 0) and the oral challenge (Day 21) in comparison to the

control group (Figure 3). The average weight gain in the control and allergic groups was 0.97 ± 0.19 g and 0.31 ± 0.16 g, respectively.

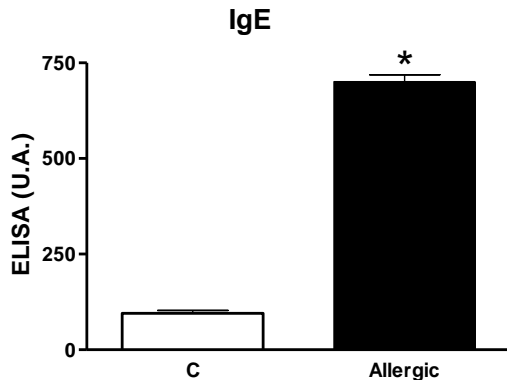


Figure 2. IgE level in BALB/c mice following sensitization and oral challenge. U:A: Arbitrary unit. * $p < 0.05$ in relation to control group (C). Data expressed as mean \pm SE.

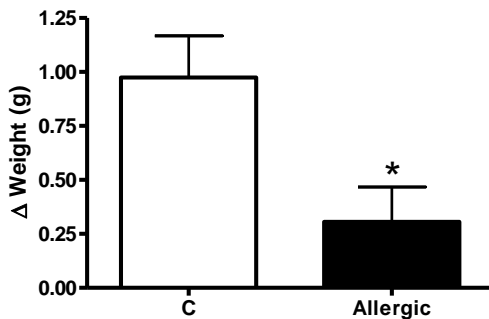


Figure 3. Effects of sensitization and oral challenge on body weight of BALB/c mice. * $p < 0.05$ in relation to control group (C). Data expressed as mean \pm SE.

On Day 22, the allergic (AL) and non-allergic control (C) animals underwent either CLP surgery [AL-SEPSIS (n = 15) and SEPSIS (n = 15)] or were sham-operated [(AL (n = 10) and C (n = 10)). Twenty-four hours after sepsis induction (Day 23), the animals were euthanized. Thickening of the alveolar wall was higher in all groups compared to C group. However, alveolar wall thickening in AL and AL-SEPSIS groups was lower in SEPSIS group (Figure 4A). Similar results were observed in the pulmonary inflammatory cell infiltrate evaluation. The

number of total cells increased significantly in all groups in relation to C group and AL and AL-SEPSIS groups exhibited significantly lower levels of the inflammatory cell infiltrate than the SEPSIS group (Figure 4B).

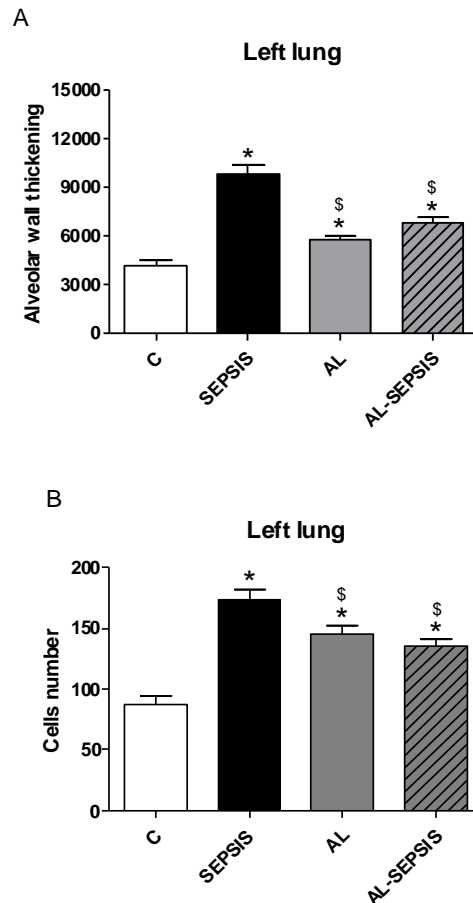


Figure 4. Histological and morphometric analysis of lung parenchyma. A: Alveolar wall thickening (μm^2). 4B: Inflammatory infiltrate. * $p < 0.05$ in relation to C group. \$ $p < 0.05$ in relation to SEPSIS group. Data expressed as mean \pm SEM.

Figure 5 shows histological images of the left lung representative of one animal from each group, demonstrating that IgE-dependent food allergy prevented sepsis-induced changes. Figure 5A shows normal parenchymal architecture. Figure 5B shows parenchymal architecture similar with control. SEPSIS group presented marked thickening and inflammatory cell

infiltration of the alveolar wall (5C). AL-SEPSIS group presented reduced thickening

and inflammatory cell infiltration of the interalveolar space.

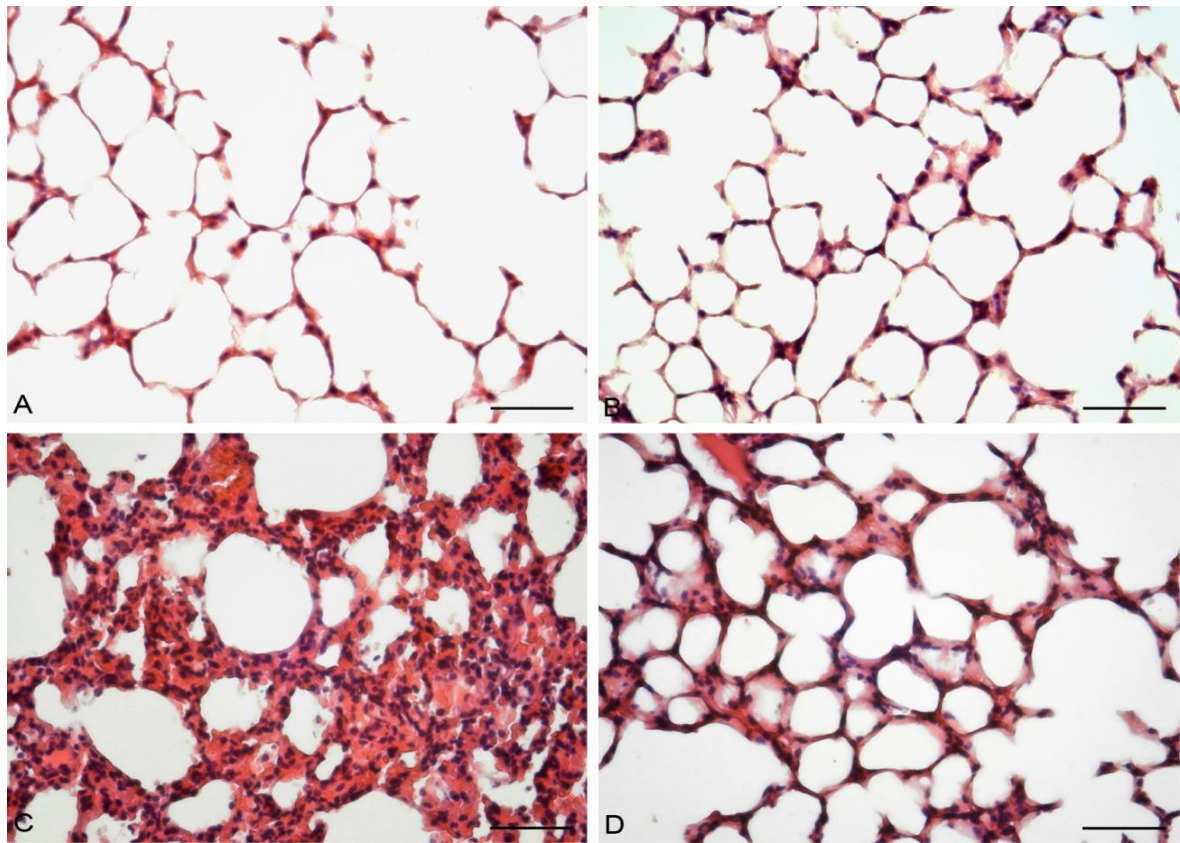


Figure 5. Photomicrographs of mouse lung parenchyma; pulmonary histological sections stained with hematoxylin-eosin; A) C group: no major histological abnormalities are observed; B) AL group: similarly to C group, no major histological abnormalities are observed; C) SEPSIS group: greater thickening of alveolar walls and greater amount of inflammatory cells contrasting with the sham-operated groups; D) AL-SEPSIS group shows important attenuation of alterations observed in SEPSIS group. Bar=20 μ m.

Bronchoalveolar lavage fluid was performed on 24 animals. The volume of fluid recovered was 0.76 ± 0.02 ml. Total cells, lymphocytes, neutrophils, and macrophages in the SEPSIS group

increased significantly in relation to the other groups (Figure 6). Inflammatory cells in the AL and AL-SEPSIS groups were similar and did not differ significantly from the C group.

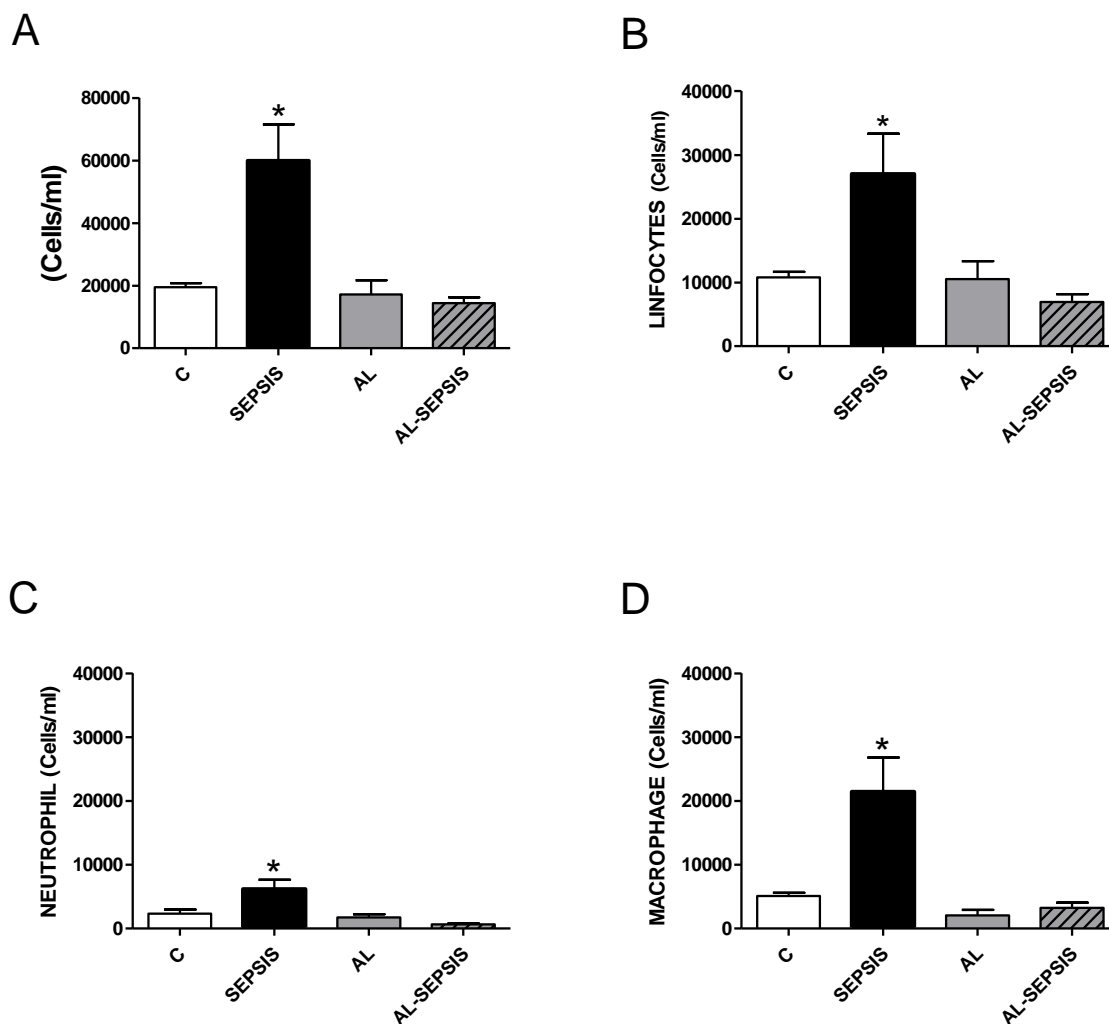


Figure 6. Bronchoalveolar lavage fluid; A-Total cells; B-lymphocytes; C-Neutrophils; D-Macrophages. * $p < 0.05$ in comparison to other groups. Data expressed as mean \pm SEM and presented as total cells per mL.

DISCUSSION

The present study demonstrates, for the first time, that IgE-dependent food allergy attenuates the cyto-histopathological changes in sepsis-induced ARDS, as evidenced by the reductions in inflammatory cell infiltrate and alveolar wall thickening as well as the reduced inflammatory response observed in the bronchoalveolar lavage fluid.

The CLP-induced sepsis model is established in the literature for the induction of ARDS and reflects the signs of sepsis found in humans resulting from trauma with

perforation of the bowel, colitis or post-operative peritonitis. This model may induce a more severe sepsis than the model that only uses the injection of endotoxins, as the changes observed following CLP are caused by endotoxins and tissue necrosis, which increase the levels and types of cytokines and inflammatory cells⁵. The mechanisms of bacterial translocation from the lumen of the intestine to other sites are not yet fully determined. However, the cascade of cytokines associated with sepsis, trauma, and inflammation is involved in the modulation of intestinal epithelial permeability^{1,17,18}. Hess *et al.*¹⁹ found that

IL-4 decreased bacterial transmigration through the intestinal epithelium.

A number of studies demonstrate the importance of cytokines in the modulation of Th1 and Th2 pathways^{8,20,21}. Wu *et al*⁸ found that the intranasal administration of recombinant *Lactococcus lactis* bacterium capable of secreting IL-12 in asthmatic mice allergic to ovalbumin was able to inhibit pulmonary inflammation and reduce anaphylactic manifestations. According to the authors, the increased levels of IFN- γ and reduced levels of IL-4 in these animals promoted Th1 induction and Th2 suppression responses, resulting in decreased airway hyperresponsiveness and reduced migration of inflammatory cells. The changing profile of cytochemical Th2 to Th1 is a good marker of the efficacy of immunotherapy⁸. According to Fenton *et al.*⁹, IL-4 is a reciprocal inhibitor of IL-12. Th2 cells may function as physiological regulators of the immune response by inhibiting the potentially harmful Th1 responses. Therefore, we postulate that elevated levels of IL-4 could attenuate the release of IL-12 and modulate the shift from Th1 to Th2 response and thus decrease the degree of pulmonary abnormalities observed in sepsis.

After sensitization and oral antigen exposure, the animals exhibited an attenuation of weight gain in comparison to the non-sensitized group. One explanation for this finding would be aversion to the solution containing ovalbumin by sensitized mice. In a model similar to that used in the present study, Dourado *et al.*²² found that the increase in IL-4 was associated with increased IgE, which is an important factor for the development of the characteristics of this food allergy model that leads to an aversion to antigen ingestion and subsequent weight loss; mice deficient in IL-4 did not produce IgE and exhibited no weight loss with the oral challenge^{14,22}. Previous studies on human subjects and mice have demonstrated that IL-4 plays a

central role in the pathogenesis of gastrointestinal allergic responses^{23,24}, such as diarrhea, lose weight and changes in the intestinal wall.

Sepsis is the most important cause of ARDS of an extrapulmonary origin^{2,4}. In such cases, lung injury originates from the action of pro-inflammatory mediators released from an extra-pulmonary focal point within the systemic circulation. These mediators increase rapidly and reach a peak around four hours following exposure to toxic products. The cytokines TNF- α , IL-1 β , IL-6 and IL-8 alter the pulmonary vascular endothelium, resulting in the expression of leukocyte adhesion molecules and a reduction in cell function²⁵. In the current study, we observed a significant increase in alveolar wall thickening in the SEPSIS group and the food allergy attenuated this response. These results suggest that food allergy prevented polymicrobial sepsis changes induced by CLP. Similar results were observed in relation to the cell infiltrate in the lung parenchyma. The number of total cells increased significantly in all groups in relation to control group. However, the pulmonary inflammatory infiltrate in AL and AL-SEPSIS groups exhibited significantly lower levels of cell infiltrate than the SEPSIS group. A likely mechanism for this response may be an increase in IL-4 induced by the IgE-dependent allergy. In an animal model of pulmonary inflammation induced by hypoxia, Ozturk *et al.*²⁶ found that IL-4 reduced intra-alveolar hemorrhage, capillary congestion, interstitial edema and hypercellularity in the lung parenchyma.

The activation of neutrophils plays an important role in the mediation of tissue injury in ARDS. IL-8 is described as the main chemotactic factor for neutrophils in the blood and bronchoalveolar lavage fluid in patients with ARDS. In the present study, greater numbers of neutrophils were found, along with leukocytes and macrophages in

the bronchoalveolar lavage fluid of the SEPSIS group in comparison to other groups. Studies show that the IgG3 and IgG4 prevent and decrease the binding affinity of IL-8 with the CXC chemokine receptor, thereby reducing neutrophil chemotaxis. In contrast, the complex promotes ARDS when IgG is injected directly into the respiratory system²⁷. The main limitation of the study is the lack of analysis of inflammatory cytokines of bronchoalveolar lavage which restricted the immunological discussion of our results.

CONCLUSION

The results of the present study demonstrate for the first time that IgE-mediated food allergy was able to attenuate alveolar wall thickening, cell infiltration in the lungs and bronchoalveolar lavage fluid. The mechanisms involved in this response need to be determined. The high levels of IL-4 induced by this food allergy model may have contributed to such a response.

Author contributions statement

G.C. Silva performed the induction of food allergy, cecal ligation surgery, histological and morphometric studies, and drafted the first manuscript; L.S. Augusto performed the bronchoalveolar lavage, oral challenge animals, and drafted the manuscript; G.S. Magalhães performed the blood collection and IgE analysis and drafted the manuscript; D.L. Gargiulo and D.C. Cara determined and analyzed the IgE levels, M.V. Caliarì contributed with histologic and morphometric analysis tools, F.A. Botoni contributed with data analysis and drafted the manuscript; J.C. Serufo contributed with BALF analysis tools and drafted the manuscript, and M.G. Rodrigues-Machado conceived the study; designed and supervised experiments; reviewed the data; performed statistical analysis; and drafted, and reviewed the

manuscript. All the authors critically revised the paper and approved the final manuscript for submission.

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