

Research Article

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Influence of Oral Tolerance on Lung Cytokines Expression and Oxidative Stress Activation in Guinea Pigs with Chronic Inflammation

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Abstract

Objective: We had previously demonstrated that oral induced tolerance attenuated lung tissue hyperresponsiveness, eosinophil inflammation and extracellular matrix remodelling in a model of chronic inflammation in guinea pigs. In the present study, we evaluated if these responses were associated to alterations on Th1/Th2 cell expression on airways and distal lung.

Methods: Animals received seven inhalations of ovalbumin (1-5 mg/mL; OVA group) or saline (SAL group) during 4 wk. Oral tolerance (OT) was induced by offering *ad libitum* ovalbumin 2% in sterile drinking water starting with the 1st inhalation (OT1 group) or after the 4th (OT2 group). After the last inhalation, lungs were removed for the histological analysis using morphometry. We assessed IL-2, IL-4, IL-13, IFN- γ and iNOS both in airways and distal lung.

Results: There was an increase in IL-2, IL-4, IL-13, IFN- γ and iNOS positive cells both in airways and alveolar septa in ovalbumin-exposed guinea pigs compared with controls ($P < 0.05$). Both in airways and in lung tissue there was a decrease in IL-4, IL-13 and iNOS positive cells in OT1 and OT2 compared to OVA ($P < 0.05$). Considering IL-2 expression, there was an increase in OT1 and OT2 compared to OVA ($P < 0.05$). We observed positive correlations among the functional responses and some inflammation and oxidative stress pathway activation markers evaluated, especially in alveolar wall.

Conclusion: Oral tolerance induces a shift in Th1/Th2 and influences oxidative stress activation both in airways and distal lung of animals with chronic pulmonary allergic inflammation. These results may clarify the mechanisms involved in the attenuation of mechanical responsiveness, inflammation and remodelling of airways and distal lung by oral tolerance, as previously shown in this animal model.

Keywords: Allergic sensitization; Asthma; Cytokines; Guinea pigs; Lung inflammation; Oral tolerance; Ovalbumin

Introduction

Asthma is a chronic and inflammatory pulmonary disease characterized not only by alterations in the airways but also in the distal lung parenchyma [1-3]. Inflammation and oxidative stress are important pathophysiological features of asthma that can be verified both in airways and lung parenchyma [4-10].

Asthma results from an allergen response in which T helper (Th)2 cytokines, including interleukin (IL)-4 and IL-13, have important effects that contribute to the persistence of inflammation and to the irreversibility of lung function alterations [11-14].

Nitric oxide (NO), in turn, is an important modulator of the inflammation in asthma [10,15-17]. NO is synthesized from L-arginine by NO synthase (NOS), whose inducible form (iNOS) is stimulated by many proinflammatory cytokines [18]. The iNOS-derived NO contributes to bronchoconstriction, airway inflammation and remodeling [10,19]. In addition, NO can interact with superoxides, resulting in the formation of the potent oxidant peroxynitrite, leading to lipid peroxidation and consequent generation of isoprostanes, which are markers for activation of the oxidative stress pathway [20,21].

Oral tolerance (OT), classically defined as prior administration of the antigen by the oral route, has been recognized as an alternative treatment to autoimmune and allergic diseases [22-25]. In atopic patients, oral, sublingual, or inhaled administration of antigens leads to a reduction in symptoms and decreased local inflammation, as well as a

reduction in dyspnea and airway hyperresponsiveness.

Experimental oral tolerance studies have shown that airway remodeling is effectively inhibited in animals with chronic allergic inflammatory that have become tolerant. This effect was associated to a reduction in airway eosinophilic inflammation, eosinophilopoiesis, peribronchial edema formation and hyperresponsiveness, mainly in distal airways [7]. Specifically in distal lung parenchyma, previously studies had shown that oral tolerance attenuates lung tissue hyperresponsiveness, which is associated with attenuation in eosinophil inflammatory and extracellular matrix remodeling, mainly related to collagen and elastic fibers deposition [9].

Other studies related to oral tolerance have shown suppression of Th2 allergic response, with decrease of the number of eosinophils

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in the lung and bone marrow, reduction of the production of specific antibodies, mucus and Th2 cytokines in the lung, as well as bronchial hyperresponsiveness [26-29].

Anyway, since oral tolerance is a physiologic and non-toxic therapy, its use for the treatment of allergic and autoimmune inflammatory diseases becomes clinically completely feasible, acting primarily in the suppression of the inflammation through the induction of regulatory T cells [30].

It is very important to understand the mechanisms of oral tolerance in allergic diseases using an appropriated animal model of chronic pulmonary allergic inflammation, even for the future testing of pharmacological approaches. In the present study, we evaluated the influence of oral tolerance on the alterations of Th1/Th2 cell expression and oxidative stress activation in airways and distal lung of guinea pigs with chronic pulmonary allergic inflammation, and correlated the responses with pulmonary mechanics.

Materials and Methods

All animals received humane care in compliance with National Institutes of Health *Guide for Care and Use of Laboratory Animals*, and all protocols and procedures described in this study were approved by the Institutional Review Board of The University of São Paulo (São Paulo, SP, Brazil).

Sensitization protocol

The protocol used to induce chronic airway inflammation was the same as previously described [31-33]. Male guinea pigs (GP), initially weighing 300-350 g, were individually placed in a plexiglas box (30 cm×15 cm×20 cm) coupled to an ultrasonic nebulizer (Soniclear, São Paulo, Brazil). A solution of ovalbumin (Sigma Chemical, St. Louis, MO) diluted in 0.9% NaCl sterile solution (saline) was offered by aerosol. To induce chronic airway inflammation, the animals were submitted to seven inhalations during 4 weeks, twice a week, with increasing concentration of ovalbumin (1-5 mg/mL) to counteract toleration. The solution was aerosolized for 15 min or until respiratory distress occurred (onset of sneezing, coryza, cough, and/or indrawing of thoracic wall). The observer who made the decision to withdraw the GP from the inhalation box was blinded of the treatment status of the animal. The time that GP were in contact to aerosol was denominated inhalation time. Control animals (SAL group) received aerosolized saline.

Induction of oral tolerance

Oral tolerance (OT) was induced as previously described by offering to the animals 2% of ovalbumin diluted in sterile drinking water (final dilution: 0.02 g/ml) *ad libitum* for 4 weeks beginning concomitantly with the 1st inhalation (OT1 group) or beginning 24 h after the 4th inhalation (OT2 group) [7,9]. The water volume that was drunk by the animals was measured every day, and the median value was 80 mL of ovalbumin solution during a period of 24 h. Both OVA and SAL groups (see *Experimental groups*) consumed the same sterile water volume. The process of tolerization was maintained until the end of the experimental protocol.

Experimental groups

Animals received: 1) inhalations with saline and sterile water for drinking during 4 wk (SAL, *n*=11); 2) aerosolized OVA and sterile water for drinking during 4 wk (OVA, *n*=11); 3) aerosolized OVA and *ad libitum* 2% OVA in sterile water for 4 wk beginning with the 1st

inhalation (OT1 group, *n*=11); and 4) aerosolized OVA and *ad libitum* 2% OVA in sterile water for 2 wk starting after the 4th inhalation (OT2 group, *n*=11).

Histology and morphometry

Seventy-two hours after the 7th inhalation, the guinea pigs were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneal injection) and after thoracotomy they were exsanguinated. The heart and lungs were excised *en bloc*. The right lung was removed, fixed with buffered 4% paraformaldehyde for 24 h, and then transferred to 70% ethanol. Sections were processed for paraffin-embedding and slices of 5 µm in thickness were obtained. After that, immunohistochemistry was performed as previously described [34]. Histological sections were submitted to morphometric analysis, performed with a microscope with an integrating eyepiece with 100 points and 50 lines, using a point-counting technique [35]. To measure the amount of airways positive cells, we selected three transversely sectioned non-cartilaginous airway that were magnified ×1000; three fields were analyzed for each airway sections. For lung tissue analysis, a total of 10-15 fields were analyzed per lung at a magnification of ×1000. The positive cells were expressed in cells/unit area (10⁴ µm²) [10,36].

Evaluation of cytokine expression cell

Sections were deparafinated and washed 3 times for 10 minutes with H₂O₂ 10volumes (V) 3% to inhibit endogenous peroxidase activity. Antigen retrieval was performed with citrate solution for 30 minutes. Sections were incubated with anti-IL-2, anti-IL-4 and anti-IL-13 (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and left overnight at 48°C. An ABC Vectastain Kit (Vector Laboratories, Burlingame, CA, USA) was used as secondary antibody and 3,3 Diaminobenzidine (DakoCytomation, Carpinteria, CA, USA) was used as chromogen. The sections were counterstained with Harris hematoxylin (Merck, Darmstadt, Germany).

Evaluation of IFN-γ

The immunohistochemistry for research of IFN-gamma was performed by the LSAB method. The silanized sections were deparafinated and hydrated with alcohol baths. Then the sections were washed in flowing water followed by endogenous peroxidase blocking with 3% H₂O₂ 10V 7 times 5 minutes each. After that, the sections were washed in flowing water, deionized water and PBS. Antigen retrieval was achieved by high temperature (95°C). After this period, the sections were washed in flowing water, deionized water and PBS. After blocking, IFN-gamma primary monoclonal antibodies were applied over the sections and incubated overnight. Then the sections were washed in PBS and incubated. After this step, the sections were washed in PBS and revealed by 3,3 DAB chromogen (DakoCytomation, Carpinteria, CA, USA). Finally, the sections were washed in flowing water and counterstained with Harris hematoxylin (Merck, Darmstadt, Germany).

Evaluation of iNOS positive cells

For iNOS detection we used the same sections employed for the morphometric evaluation of inflammatory cells. Immunohistochemistry was performed as previously described [19]. Subsequently, the sections were incubated seven times 5-min at room temperature with a blocking solution containing 3% hydrogen peroxide. Polyclonal rabbit antisera against iNOS (LabVision, NeoMarkers, Fremont CA, RB-9242-P) were used as primary antisera [incubation overnight in a humid chamber (refrigerator) at 4-8°C, 1:400 dilution in BSA]. After three 5-min washes in phosphate-buffered saline (PBS), sections were incubated

with a secondary antibody (Vector ABC Elite, HRP, PK-6101-Vector Laboratories, INC, Burlingame, CA) at 37°C in a humidity chamber. Slides were given three more 5 min washes in PBS and revealed with 3,3-diaminobenzidine (DAB) (DakoCytomation, Carpinteria, CA, EUA, K3468). Slides were given abundant washes in tap water. This was followed by Harrys hematoxylin counterstaining for 1 min.

Data analysis

Statistical analysis was performed using *Sigma Stat* software (SPSS, Chicago, IL). The data were examined using ANOVA when the distribution was normal and the variances were homogeneous. We also obtained the Pearson correlation coefficient (R) to assess the associations of the respiratory system resistance (Rrs) and elastance (Ers) or tecidual resistance (Rt) and elastance (Et) scores with the markers for IL-2, IL-4, IL-13, IFN-γ and iNOS. Values were expressed as mean and standard deviation (SD) and with vertical bar graphics. A P value of less than 0.05 was considered significant.

Results

Measurement Th1/Th2 cell expression

Table 1 show values of IL-2, IL-4 and IL-13 in the airway analysis, respectively. There was an increase in IL-2, IL-4 and IL-13 positive cells in OVA compared to SAL ($P<0.05$) in the airways. There was a decrease in IL-4 and IL-13 positive cells in OT1 ($1.59 \pm 0.19/10^4 \mu\text{m}^2$ and $3.9 \pm 0.3/10^4 \mu\text{m}^2$, respectively) and OT2 ($1.11 \pm 0.21/10^4 \mu\text{m}^2$ and $4.9 \pm 0.8/10^4 \mu\text{m}^2$, respectively) compared to OVA group ($14.45 \pm 1.17/10^4 \mu\text{m}^2$ and $13.4 \pm 1.8/10^4 \mu\text{m}^2$, respectively; $P<0.05$). Regarding IL-2, there was an increase in OT1 ($7.11 \pm 0.62/10^4 \mu\text{m}^2$) and OT2 ($6.61 \pm 0.99/10^4 \mu\text{m}^2$) compared to SAL group ($2.68 \pm 0.72/10^4 \mu\text{m}^2$; $P<0.001$). There was no statistically significant difference between OT1 and OT2 animals in OVA group (OVA group: $7.07 \pm 0.29/10^4 \mu\text{m}^2$).

For the lung tissue analysis, there was an increase in IL-2, IL-and

Th1/Th2 cell expression	SAL	OVA	OT1	OT2
IL-2	2.68±0.72	7.07±0.29 [#]	7.11±0.62 [#]	6.61±0.99 [#]
IL-4	3.48±1.31	14.45±1.17 ^{**}	1.59±0.19	1.11±0.21
IL-13	8.41±1.28	13.4±1.8 ^{**}	3.9±0.3	4.9±0.8
IFN-γ	16.3±1.4	32.1±1.5 [*]	17.9±4.0	20.9±2.0
Oxidative Stress Marker	SAL	OVA	OT1	OT2
iNOS	8.5±0.9	14.7.4±1.1 [*]	7.2±1.8	8.3±2.6

The IL-2, IL-4, IL-13, IFN-γ and iNOS are expressed in positive cells/ $10^4 \mu\text{m}^2$. [#] $P<0.001$, compared with the SAL group; ^{**} $P<0.001$, compared with the SAL, OT1 and OT2 groups. ^{*} $P<0.05$, compared with the SAL, OT1 and OT2 groups.

Table 1: Absolute values of the morphometric analysis for inflammatory and oxidative stress markers, in the airway wall.

Th1/Th2 cell expression	SAL	OVA	OT1	OT2
IL-2	1.86±0.59	2.1±0.3	5.0±0.5 [*]	5.2±0.2 [*]
IL-4	0.78±0.61	5.8±0.7 ^{**}	4.2±0.2	3.4±0.2
IL-13	2.35±1.18	5.4±0.5 ^{**}	1.03±0.13	0.7±0.2
IFN-γ	1.29 ± 0.22	4.96 ± 0.72 ^{**}	1.99 ± 0.43	1.86 ± 0.29
Oxidative Stress Marker	SAL	OVA	OT1	OT2
iNOS	17.7±1.67	20.7±1.0 ^{**}	12.8± 1.9	14.3±2.2

The IL-2, IL-4, IL-13, IFN-γ and iNOS are expressed in positive cells/ $10^4 \mu\text{m}^2$. ^{*} $P<0.05$, compared with the SAL and OVA groups; ^{**} $P<0.05$, compared with the SAL, OT1 and OT2 groups.

Table 2: Absolute values of the morphometric analysis for inflammatory and oxidative stress markers, in the lung tissue.

IL-13 (Table 2) positive cells in OVA compared to SAL ($P<0.05$). There was a decrease in IL-4 and IL-13 positive cells in OT1 ($4.2 \pm 0.2/10^4 \mu\text{m}^2$ and $1.03 \pm 0.13/10^4 \mu\text{m}^2$, respectively) and OT2 ($3.4 \pm 0.2/10^4 \mu\text{m}^2$ and $0.7 \pm 0.2/10^4 \mu\text{m}^2$) compared to OVA ($5.8 \pm 0.7/10^4 \mu\text{m}^2$ and $5.4 \pm 0.5/10^4 \mu\text{m}^2$, respectively; $P<0.05$). Considering IL-2 expression, there was an increase in OT1 ($5.0 \pm 0.5/10^4 \mu\text{m}^2$) and OT2 ($5.2 \pm 0.2/10^4 \mu\text{m}^2$) compared to OVA ($2.1 \pm 0.3/10^4 \mu\text{m}^2$, $P<0.05$).

Measurement of IFN-γ

We observed an increase in IFN-γ expression in the airways of the ovalbumin-exposed guinea pigs ($32.1 \pm 1.5/10^4 \mu\text{m}^2$) compared to normal saline-exposed ones ($16.3 \pm 1.4/10^4 \mu\text{m}^2$). The oral tolerance treatment reduced this response in ovalbumin-exposed animals (OT1 group: $17.9 \pm 4.0/10^4 \mu\text{m}^2$ and OT2 group: $20.9 \pm 2.0/10^4 \mu\text{m}^2$; $P<0.05$). There was no statistically significant difference between tolerance and saline groups (Table 1).

We also observed (Table 2) an increase in the number of cells positive for IFN-γ in the animals exposed to inhalations of ovalbumin (OVA group: 4.96 ± 0.72 cells/ $10^4 \mu\text{m}^2$) compared with the saline-exposed group (SAL: 1.29 ± 0.22 cells/ $10^4 \mu\text{m}^2$). The oral tolerance treatment reduced this response in ovalbumin-exposed animals (OT1 group: $1.99 \pm 0.43/10^4 \mu\text{m}^2$ and OT2 group: $1.86 \pm 0.29/10^4 \mu\text{m}^2$; $P<0.05$). There was no statistically significant difference between tolerance and saline groups.

iNOS-positive cells

In the airways, there was an increase in iNOS expression (Table 1) in OVA compared to SAL ($P<0.05$). There was a decrease in OT1 ($7.2 \pm 1.8/10^4 \mu\text{m}^2$) and OT2 ($8.3 \pm 2.6/10^4 \mu\text{m}^2$) iNOS positive cells compared to OVA group ($14.7.4 \pm 1.1/10^4 \mu\text{m}^2$; $P<0.05$). There was no statistically significant difference between tolerance and control groups (SAL: $8.5 \pm 0.9/10^4 \mu\text{m}^2$).

The iNOS expression in alveolar septa (Table 2) of the guinea pigs showed an increase in iNOS-positive cells ($20.7 \pm 1.0/10^4 \mu\text{m}^2$) in OVA group ($P<0.05$) compared to normal saline-exposed animals ($17.7 \pm 1.67/10^4 \mu\text{m}^2$). The oral tolerance treatment group reduced the iNOS positive cells in OT1 group ($12.8 \pm 1.9/10^4 \mu\text{m}^2$) and OT2 ($14.3 \pm 2.2/10^4 \mu\text{m}^2$) compared to OVA ($P<0.05$). There was no difference in saline-exposed animals.

Representative photomicrographs of immunohistochemical staining for IL-2, IL-4, IL-13, IFN-γ and iNOS in the airways of the four animal groups are shown in Figure 1. The guinea pigs exposed to ovalbumin inhalation (OVA group) presented prominent increases in the numbers of all types of analyzed cells (Figures 1B, 1E, 1J, 1N, 1R) in comparison with the non-sensitized animals (SAL group: Figures 1A, 1E, 1I, 1M, 1Q). There was a decrease in IL-4, IL-13, IFN-γ and iNOS positive cells for both groups of oral induced tolerized animals (OT1, Figures 1G, 1K, 1O, 1S; OT2, Figures 1H, 1L, 1P). There was increase in the IL-2 expression in both groups of oral induced tolerized guinea pigs (OT1, Figure 1C; OT2, Figure 1D).

Figure 2 presents photomicrographs of lung tissue strips from guinea pigs in the four experimental groups submitted to immunohistochemical staining for IL-2, IL-4, IL-13, IFN-γ and iNOS-positive cells detection. The animals exposed to ovalbumin (OVA group) presented increased numbers of IL-2- (Figure 2B), IL-4- (Figure 2F), IL-13- (Figure 2J), IFN-γ- (Figure 2N) and iNOS- (Figure 2R) positive cells compared with the saline-exposed animals (SAL group; Figures 2A, 2E, 2I, 2M, 2Q). There was a decrease in IL-4, IL-13, IFN-γ

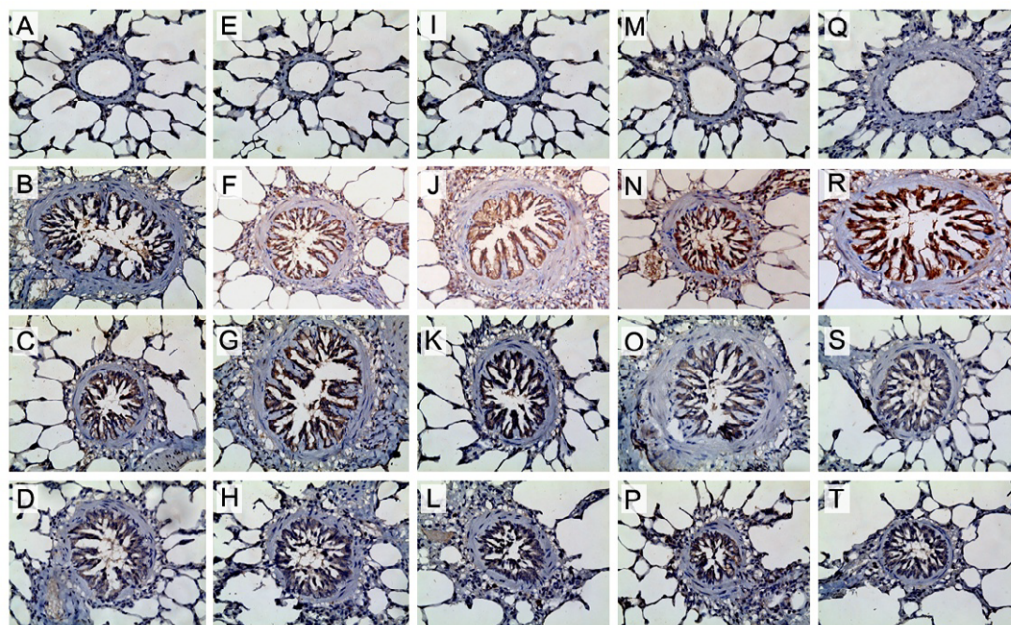


Figure 1: Photomicrographs of the results of immunohistochemical analyses of airway walls to detect IL-2 (panels A–D, 400X), IL-4 (panels E–H, 400X), IL-13 (panels I–L; 400X), IFN- γ (panels M–P, 400X) and iNOS (panels Q–T, 400x) positive cells from guinea pigs exposed only to saline (panels A, E, I, M and Q) or ovalbumin (panels B, F, J, N and R). The guinea pigs from OT1 (panels C, G, K, O and S) and OT2 (panels D, H, L, P and T) groups are also represented.

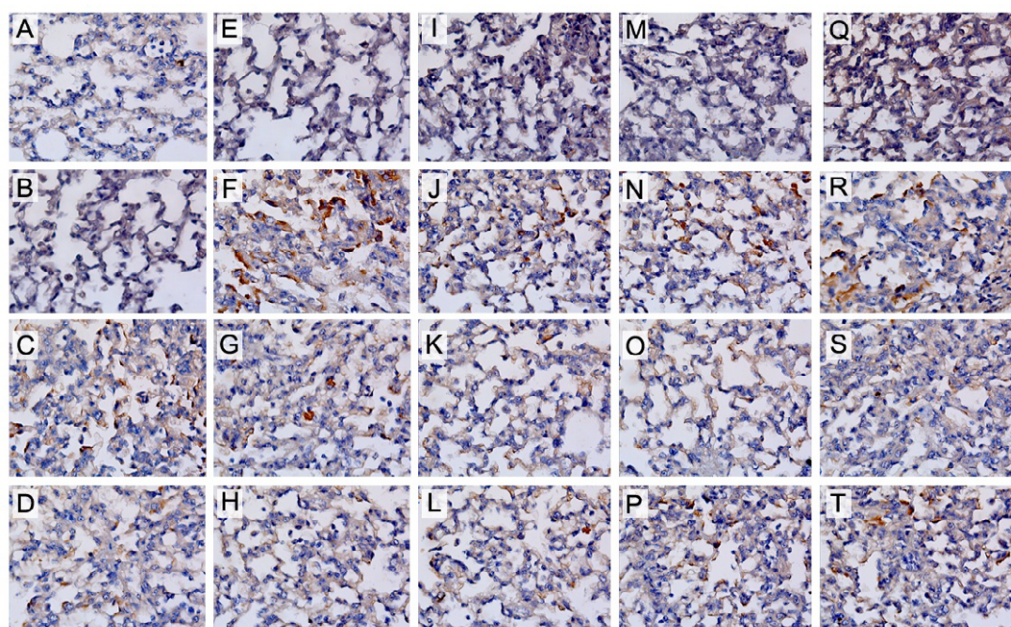


Figure 2: Photomicrographs of the results of immunohistochemical analyses of lung tissue to detect IL-2 (panels A–D, 400X), IL-4 (panels E–H, 400X), IL-13 (panels I–L; 400X) IFN- γ (panels M–P, 400X) and iNOS (panels Q–T, 400x) positive cells from guinea pigs exposed only to saline (panels A, E, I, M and Q) or ovalbumin (panels B, F, J, N and R). The guinea pigs from OT1 (panels C, G, K, O and S) and OT2 (panels D, H, L, P and T) groups are also represented.

and iNOS-positive cells for both groups of oral induced tolerized animals (OT1, Figures 2G, 2K, 2O and S; OT2, Figures 2H, 2L, 2P). There was an increase in the IL-2 expression in both groups of induced oral tolerized guinea pigs (OT1, Figure 2C; OT2, Figure 2D).

Correlations

Tables 3 a and b present the correlations of Rrs and Ers with the number of positive cells for IL-2, IL-4, IL-13, IFN- γ and iNOS in airways walls, respectively. We observed significant correlations just

	Rrs	Ers
	<i>r value</i>	<i>r value</i>
	<i>p value</i>	<i>p value</i>
IL-2	0.449	0.642
	0.0244	0.000537
IL-4	0.302	0.474
	0.142	0.0166
IL-13	0.241	0.404
	0.292	0.0620
IFN- γ	0.150	0.447
	0.505	0.0323
iNOS	0.176	0.374
	0.458	0.105

Table 3: Pearson correlations of Rrs and Ers with the measures for IL-2, IL-4, IL-13, IFN- γ and iNOS.

	Rrs	Ers
	<i>r value</i>	<i>r value</i>
	<i>p value</i>	<i>p value</i>
IL-2	0.542	0.549
	0.00761	0.00665
IL-4	0.341	0.335
	0.112	0.118
IL-13	0.731	0.734
	0.0000751	0.0000677
IFN- γ	0.752	0.746
	0.0000349	0.0000432
iNOS	0.563	0.542
	0.00511	0.00755

Table 4: Pearson correlations of Rt and Et with the measures for IL-2, IL-4, IL-13, IFN- γ and iNOS.

among Rrs and IL-2, as well as among Ers and IL-2, IL-4 and IFN- γ . There were no significant correlations among all the other parameters evaluated.

Tables 4 a and b present the correlations of Rt and Et with the number of positive cells for IL-2, IL-4, IL-13, IFN- γ and iNOS in alveolar wall, respectively. We observed a significant correlation among Rt and Et for IL-2, IL-13, IFN- γ and iNOS, but not for IL-14.

Discussion

In the present study, it was verified that the oral tolerance attenuates cell expression of Th2 cytokines and iNOS-positive cells in an experimental model of chronic allergic pulmonary inflammation, suggesting that this therapeutic strategy could prevent airways and distal lung functional and structural changes induced by chronic allergic inflammation.

Some authors have previously evaluated the effects of oral tolerance in experimental models of airway disease [27,29,37]. They observed a reduction in airway inflammation, airway hyperresponsiveness, and mucous secretion induced by chronic inflammation. Although these previous investigators showed a beneficial effect of oral tolerance in airways responses, to our knowledge, the present study has provided the first evidence on the effects of oral tolerance in lung tissue responses in an experimental model of chronic lung inflammation.

The role of lung tissue in asthma physiopathology has also been intensively investigated in the last years. The lung tissue strips have been used to study the mechanical and pharmacological properties of the lung periphery [38,39]. We have recently demonstrated that in

this experimental model of chronic allergic inflammation, the lung tissue is involved not only in the early but also in the late inflammatory response [40]. Furthermore, from previous studies we have observed that this experimental model presents some important features of asthma pathophysiology, as intense bronchoconstriction, airways eosinophilic and lymphocytic inflammation, and airway and bronchial vessels remodeling [10,16,31]. Allergic asthmatics have established antigen-responsive CD4⁺ T cells in the airways and local lymphoid tissue. Children predisposed to develop asthma often have antibodies to common allergens, indicating that their immune system is already primed to the allergen. Based on animal models of asthma, one might speculate that feeding allergens to allergic asthmatics would increase inflammation, which can be dangerous. Thus, animal models might indicate that attempts to stimulate mucosal tolerance should be used to prevent or treat asthma [41].

We used two different types of oral-inducing tolerance. In the first protocol we began the tolerance together with the sensitization protocol. The aim of this protocol is related to a preventive strategy. After the fourth inhalation, guinea pigs have already showed expression of cytokines in pulmonary cells. For this reason, we also evaluated if oral induced tolerance may be used as a therapeutic approach, reverting the inflammatory and remodeling processes. Then, in the second protocol, the animals received the oral antigen beginning twenty four hours after the fourth ovalbumin inhalation. We had previously demonstrated that at this moment (after the fourth ovalbumin inhalation) the animals had an increase in the IgG1 antibody titres (1:320) [32].

The chronic asthmatic response is tightly regulated by a network of cytokines and chemokines, such as IL-4, IL-5 and IL-13 [42,43]. Short and long-term stressors engage differences in the regulation of the Th1/Th2 cytokine profile [43,44]. In the present study we observed that animals submitted to chronic inflammatory stimuli have an increase in the airways and lung tissue cytokine-positive cells. The tolerization process induced a significant decrease in Th2 cytokines, represented by IL-4 and IL-13 positive cells, both in airways and lung tissue. On the other hand, oral tolerance did not reduce the expression of the IL-2 positive cells, a TH1 cytokine. Asthmatic responses in humans are mainly orchestrated by Th2 cells and their cytokines, particularly IL-4, IL-13, and IL-5 [45]. Therefore, the inhibition of Th2 cytokines is an important outcome of oral tolerance.

On the other hand, IFN- γ , another Th1 cytokine, suppresses airway hyperresponsiveness and reinforces the principle that the pathogenesis of asthma involves a co-operative interaction between Th2 and Th1 cytokines [46]. Although the role of IFN- γ in asthma is controversial, this cytokine seems to play a role both in acute and chronic asthma. The attenuation of IFN-gamma positive cells achieved with oral tolerance is also indicative of the potential beneficial effects of this form of treatment for asthma.

We also showed in the present study that iNOS-positive cells were reduced among animals submitted to oral tolerance in relation to the animals not submitted to oral tolerance in both airways and distal lung tissue. Several authors have discussed that NO released by nitric oxide synthases activation contribute to the oxidative stress, amplifying the deleterious and harmful effects on lungs [16]. The potent oxidant peroxynitrite is formed by NO and superoxide interaction by a rapid iso-stoichiometric reaction [10,47]. Haddad et al. suggested that peroxynitrite may contribute to the injury of pulmonary surfactant, increasing the alveolar collapse. Once iNOS positive cells are reduced by oral tolerance, beneficial effects are expected in the lungs [48].

The positive correlation between attenuation of respiratory mechanics data and the number of cells positive for different markers reinforces the importance of oral tolerance on the inflammatory responses of chronic allergic lung disease. In a recent study, Kim et al. (2013), investigating the preventive effects of OT on allergic inflammation in the upper and lower airways in a mouse model of allergy sensitized with OVA, verified that oral tolerance reduced the IFN- γ and IL-13 levels in nasal and bronchoalveolar lavage fluids compared with the allergy group. These reductions were also accompanied by attenuation of airway hyperresponsiveness [49]. Similarly, Birmingham et al. (2013) found that feeding mice with antigen induced oral tolerance, which was accompanied by attenuation of eosinophil numbers, cytokine production and airway hyperresponsiveness. An interesting aspect of this study was to compare the efficacy of oral tolerance between younger and aged mice. It was found that despite aged mice also developed oral tolerance to the antigen the effect was greater in younger mice [50].

In conclusion, oral tolerance induces a shift Th1/Th2 and oxidative stress activation in airways and distal lung of animals with chronic pulmonary allergic inflammation. These results may clarify the mechanisms involved in the attenuation of mechanical responsiveness, inflammation and remodeling of airways and distal lung by oral tolerance, as previously shown in this animal model.

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References

- Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM (2000) Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 161: 1720-1745.
- Kraft M (1999) The distal airways: are they important in asthma? *Eur Respir J* 14: 1403-1417.
- Mauad T, Silva LF, Santos MA, Grinberg L, Bernardi FD, et al. (2004) Abnormal alveolar attachments with decreased elastic fiber content in distal lung in fatal asthma. *Am J Respir Crit Care Med* 170: 857-862.
- Marques RH, Reis FG, Starling CM, Cabido C, de Almeida-Reis R, et al. (2012) Inducible nitric oxide synthase inhibition attenuates physical stress-induced lung hyper-responsiveness and oxidative stress in animals with lung inflammation. *Neuroimmunomodulation* 19: 158-170.
- Reis FG, Marques RH, Starling CM, Almeida-Reis R, Vieira RP, et al. (2012) Stress amplifies lung tissue mechanics, inflammation and oxidative stress induced by chronic inflammation. *Exp Lung Res* 38: 344-354.
- Starling CM, Prado CM, Leick-Maldonado EA, Lanças T, Reis FG, et al. (2009) Inducible nitric oxide synthase inhibition attenuates lung tissue responsiveness and remodeling in a model of chronic pulmonary inflammation in guinea pigs. *Respir Physiol Neurobiol* 165: 185-194.
- Ruiz Schütz VC, Drewiacki T, Nakashima AS, Arantes-Costa FM, Prado CM, et al. (2009) Oral tolerance attenuates airway inflammation and remodeling in a model of chronic pulmonary allergic inflammation. *Respir Physiol Neurobiol* 165: 13-21.
- Angeli P, Prado CM, Xisto DG, Silva PL, Passaro CP, et al. (2008) Effects of chronic L-NAME treatment lung tissue mechanics, eosinophilic and extracellular matrix responses induced by chronic pulmonary inflammation. *Am J Physiol Lung Cell Mol Physiol* 294: L1197-1205.
- Nakashima AS, Prado CM, Lanças T, Ruiz VC, Kasahara DI, et al. (2008) Oral tolerance attenuates changes in in vitro lung tissue mechanics and extracellular matrix remodeling induced by chronic allergic inflammation in guinea pigs. *J Appl Physiol* (1985) 104: 1778-1785.
- Prado CM, Leick-Maldonado EA, Yano L, Leme AS, Capelozzi VL, et al. (2006) Effects of nitric oxide synthases in chronic allergic airway inflammation and remodeling. *Am J Respir Cell Mol Biol* 35: 457-465.
- Southam DS, Ellis R, Wattie J, Inman MD (2007) Components of airway hyperresponsiveness and their associations with inflammation and remodeling in mice. *J Allergy Clin Immunol* 119: 848-854.
- Brown RH, Pearce DB, Pyrgos G, Liu MC, Togias A, et al. (2006) The structural basis of airways hyperresponsiveness in asthma. *J Appl Physiol* (1985) 101: 30-39.
- Yamauchi K (2006) Airway remodeling in asthma and its influence on clinical pathophysiology. *Tohoku J Exp Med* 209: 75-87.
- Finkelman FD, Urban JF Jr (2001) The other side of the coin: the protective role of the Th2 cytokines. *J Allergy Clin Immunol* 107: 772-780.
- Ricciardolo FL, Nijkamp FP, Folkerts G (2006) Nitric oxide synthase (NOS) as therapeutic target for asthma and chronic obstructive pulmonary disease. *Curr Drug Targets* 7: 721-735.
- Prado CM, Leick-Maldonado EA, Kasahara DI, Capelozzi VL, Martins MA, et al. (2005) Effects of acute and chronic nitric oxide inhibition in an experimental model of chronic pulmonary allergic inflammation in guinea pigs. *Am J Physiol Lung Cell Mol Physiol* 289: L677-683.
- Meurs H, McKay S, Maarsingh H, Hamer MA, Macic L, et al. (2002) Increased arginase activity underlies allergen-induced deficiency of c-NOS- derived nitric oxide and airway hyperresponsiveness. *Br J Pharmacol* 136: 391-398.
- Ricciardolo FL, Sterk PJ, Gaston B, Folkerts G (2004) Nitric oxide in health and disease of the respiratory system. *Physiol Rev* 84: 731-765.
- Prado CM, Yano L, Rocha G, Starling CM, Capelozzi VL, et al. (2011) Effects of inducible nitric oxide synthase inhibition in bronchial vascular remodeling-induced by chronic allergic pulmonary inflammation. *Exp Lung Res* 37: 259-268.
- Muijsers RB, Folkerts G, Henricks PA, Sadeghi-Hashjin G, Nijkamp FP (1997) Peroxynitrite: a two-faced metabolite of nitric oxide. *Life Sci* 60: 1833-1845.
- Pryor WA, Squadrito GL (1995) The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *Am J Physiol* 268: L699-722.
- Faria AM, Weiner HL (2005) Oral tolerance. *Immunol Rev* 206: 232-259.
- Karlsson MR, Kahu H, Hanson LA, Telemo E, Dahlgren UI (2000) Tolerance and bystander suppression, with involvement of CD25-positive cells, is induced in rats receiving serum from ovalbumin-fed donors. *Immunology* 100: 326-333.
- Smith KM, Eaton AD, Finlayson LM, Garside P (2000) Oral tolerance. *Am J Respir Crit Care Med* 162: S175-178.
- Weiner HL (1997) Oral tolerance: immune mechanisms and treatment of autoimmune diseases. *Immunol Today* 18: 335-343.
- Russo M1, Jancar S, Pereira de Siqueira AL, Mengel J, Gomes E, et al. (1998) Prevention of lung eosinophilic inflammation by oral tolerance. *Immunol Lett* 61: 15-23.
- Russo M, Nahori MA, Lefort J, Gomes E, de Castro Keller A, et al. (2001) Suppression of asthma-like responses in different mouse strains by oral tolerance. *Am J Respir Cell Mol Biol* 24: 518-526.
- Chung Y, Cho J, Chang YS, Cho SH, Kang CY (2002) Preventive and therapeutic effects of oral tolerance in a murine model of asthma. *Immunobiology* 206: 408-423.
- Keller AC, Mucida D, Gomes E, Faquim-Mauro E, Faria AM, et al. (2006) Hierarchical suppression of asthma-like responses by mucosal tolerance. *J Allergy Clin Immunol* 117: 283-290.
- Weiner HL, da Cunha AP, Quintana F, Wu H (2011) Oral tolerance. *Immunol Rev* 241: 241-259.
- Tibério IF, Turco GM, Leick-Maldonado EA, Sakae RS, Paiva SO, et al. (1997) Effects of neurokinin depletion on airway inflammation induced by chronic antigen exposure. *Am J Respir Crit Care Med* 155: 1739-1747.
- Leick-Maldonado EA, Kay FU, Leonhardt MC, Kasahara DI, Prado CM,

- et al. (2004) Comparison of glucocorticoid and cysteinyl leukotriene receptor antagonist treatments in an experimental model of chronic airway inflammation in guinea-pigs. *Clin Exp Allergy* 34: 145-152.
33. Prado CM, Leick-Maldonado EA, Yano L, Leme AS, Capelozzi VL, et al. (2006) Effects of nitric oxide synthases in chronic allergic airway inflammation and remodeling. *Am J Respir Cell Mol Biol* 35: 457-465.
 34. Coers W, Timens W, Kempinga C, Klok PA, Moshage H (1998) Specificity of antibodies to nitric oxide synthase isoforms in human, guinea pig, rat, and mouse tissues. *J Histochem Cytochem* 46: 1385-1392.
 35. Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, et al. (1988) The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *APMIS* 96: 857-881.
 36. Almeida-Reis R, Toledo AC, Reis FG, Marques RH, Prado CM, et al. (2010) Repeated stress reduces mucociliary clearance in animals with chronic allergic airway inflammation. *Respir Physiol Neurobiol* 173: 79-85.
 37. Sakai K, Yokoyama A, Kohno N, Hamada H, Hiwada K (2001) Prolonged antigen exposure ameliorates airway inflammation but not remodeling in a mouse model of bronchial asthma. *Int Arch Allergy Immunol* 26: 126-134.
 38. Ludwig MS, Dallaire MJ (1994) Structural composition of lung parenchymal strip and mechanical behavior during sinusoidal oscillation. *J Appl Physiol* (1985) 77: 2029-2035.
 39. Tari MG, Mancino M, Monti G (1990) Efficacy of sublingual immunotherapy in patients with rhinitis and asthma due to house dust mite. A double-blind study. *Allergol Immunopathol (Madr)* 18: 277-284.
 40. Lanças T, Kasahara DI, Prado CM, Tibério IF, Martins MA, et al. (2006) Comparison of early and late responses to antigen of sensitized guinea pig parenchymal lung strips. *J Appl Physiol* (1985) 100: 1610-1616.
 41. Cohn L (2001) Food for thought: can immunological tolerance be induced to treat asthma? *Am J Respir Cell Mol Biol* 24: 509-512.
 42. Chen E, Miller GE (2007) Stress and inflammation in exacerbations of asthma. *Brain Behav Immun* 21: 993-999.
 43. Okuyama K, Ohwada K, Sakurada S, Sato N, Sora I, et al. (2007) The distinctive effects of acute and chronic psychological stress on airway inflammation in a murine model of allergic asthma. *Allergol Int* 56: 29-35.
 44. Forsythe P, Ebeling C, Gordon JR, Befus AD, Vliagoftis H (2004) Opposing effects of short- and long-term stress on airway inflammation. *Am J Respir Crit Care Med* 169: 220-226.
 45. Colavita AM, Reinach AJ, Peters SP (2000) Contributing factors to the pathobiology of asthma. The Th1/Th2 paradigm. *Clin Chest Med* 21: 263-277, viii.
 46. Kumar RK, Webb DC, Herbert C, Foster PS (2006) Interferon-gamma as a possible target in chronic asthma. *Inflamm Allergy Drug Targets* 5: 253-256.
 47. Romero PV, Zin WA, Lopez-Aguilar J (2001) Frequency characteristics of lung tissue strip during passive stretch and induced pneumoconstriction. *J Appl Physiol* (1985) 91: 882-890.
 48. Haddad IY, Ischiropoulos H, Holm BA, Beckman JS, Baker JR, et al. (1993) Mechanisms of peroxynitrite-induced injury to pulmonary surfactants. *Am J Physiol* 265: L555-L564.
 49. Kim SJ, Shin JH, Kim SC, Park CK, Kim SW (2013) Preventive effects of oral tolerance on allergic inflammation and airway remodeling in a murine model. *Am J Rhinol Allergy* 27: e11-e16.
 50. Birmingham JM, Patil S, Li XM, Busse PJ (2013) The effect of oral tolerance on the allergic airway response in younger and aged mice. *J Asthma* 50: 122-132.

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