

ORIGINAL ARTICLE

Evaluation of the expression of the transcription factor Yin-Yang-1: association with TGF- β in a lung allergic inflammation in a mouse model with different severity grades

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ABSTRACT

Background. Allergic asthma is one of the most prevalent childhood diseases. This disease is characterized by airway inflammation and remodeling. The mechanisms implicated in the pathogenesis of this disease remain unclear. Several studies have shown that TGF- β plays an important role in the pathogenesis of asthma. In addition, the polymorphism of the TGF- β promoter region results in the overexpression of TGF- β via regulation of the transcription factor Yin-Yang-1 (YY1). It has recently been demonstrated that YY1 may be involved in the pathogenesis of asthma by the regulation of IL-4 and IL-10. The aim of this study was to evaluate the association between the YY1 and TGF- β expression levels in a murine model of lung allergic inflammation.

Methods. In this study we used a lung allergic inflammatory murine model with different severity degrees. Tissue microarray technology and immunohistochemistry were used to evaluate YY1 and TGF- β expression. The density expression was measured by quantitative methods using specific software.

Results. Expression of both proteins correlated with the degrees of severity of lung allergic inflammation. A similar result was observed with mucus production.

Conclusions. These results corroborate the role of YY1 and TGF- β in the pathogenesis of this disease.

Key words: lung allergic inflammation, Yin-Yang-1, TGF- β , tissue microarray.

INTRODUCTION

Asthma is a complex disease physiologically characterized by varying degrees of airway obstruction as well as diverse abnormalities in epithelium, lamina propria and submucosa.¹ This is a very frequent disease that represents a severe public health problem. It has been calculated that asthma prevalence varies considerably worldwide: from <5% in Greece and Indonesia to >25% in Australia and New Zealand.² In Mexico, this entity is among the first ten

causes associated with the use of health services, especially Emergency Services and External Consultations.³

Asthma is the most prevalent disease found in a pediatric population and is associated with high costs and a deterioration in quality of life for the many patients with the disease.¹ This entity can present in patients of any age and from any gender or socioeconomic level; however, it affects mostly children with a history of atopic syndrome.² Biomedical research has contributed important advances to understand the pathogenetic mechanisms of asthma and has also produced advances in therapy with a significant impact over the management and control of the disease.^{3,4} Nevertheless, the number of asthmatic patients keeps increasing and the proportion between incidence and prevalence of this entity has not been eliminated, making this a severe and difficult to manage disease.⁵

The etiology of asthma is complex and multifactorial. It involves the interaction of genetic factors and environment stimuli. Most of the data regarding the pathophysiology of this entity (especially atypical asthma) refer to imbalance

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between phenotype Th-1 (cell-mediated immunity) and phenotype Th-2 (antibody-mediated immunity). Genetics, intrauterine environment, maternal and child nutrition, respiratory infections, daily activities and environment may all contribute to disturb this delicate balance.¹ Inflammation and changes in airways may result from the immune system's reaction to all of these factors.⁶⁻⁸ Factors that favor phenotype Th-2 ultimately lead to atopic syndrome. Use of RNA isolation techniques and improvement of mediator detection methods have provided evidence that after initial sensitivity, the increase of mastocytes and eosinophils results from the release of specific cytokines coded in chromosome 5q. These are released by Th2 lymphocytes activated by allergic, nonallergic or intrinsic asthma. These cytokines include interleukins IL-3, IL-4, IL-5, IL-6, IL-13 and granulocyte-monocyte colony stimulating factor (GM-CSF).⁷

Recently, several studies on fibrogenic growth factors associated with asthma have been carried out. Several studies have found an increased expression of insulin-like growth factor type 1 (IGF-1), epidermal growth factor (EGF) and transforming growth factor beta (TGF- β), both in bronchial biopsies and bronchoalveolar lavage (BAL).⁹⁻¹¹ TGF- β is expressed by epithelial airways cells, eosinophils, Th2 lymphocytes, macrophages and fibroblasts and may be bonded to and stored in the subepithelial matrix of airways cells.¹⁰ TGF- β is important for the development, growth, transformation and repairing of tissue as well as fibrosis and modulation of immune inflammatory response.¹¹ Expression of TGF- β has a correlation with subepithelial fibrosis level and is increased in eosinophilic infiltrates of patients with severe asthma.¹² However, this cytokine may play a protective role in mild asthma with lower fibrosis levels because it acts as an IgE production inhibitor and reduces mastocyte proliferation.¹³ Murine model studies for asthma have demonstrated TGF- β have anti-inflammatory and profibrotic effects.⁹

Multiple mechanisms are associated with TGF- β activity. An important example are transcription mechanisms regulated by inflammatory cytokines, nitric oxide and reactive oxygen species, which are found in airways of asthmatic patients. There are reports where polymorphism of C-509T (a TGF- β promoter) has an impact over the expression of this cytokine. The Yin-Yang-1 (YY1) transcription factor expresses everywhere with

zinc fingers and may work as transcription activators or inhibitors.¹⁴ Affinity of YY1 in patients with allele T at position 509 of TGF- β promoter is increased ~30% compared with patients with allele C. This study suggests such polymorphism is decisive for asthma susceptibility because patients with allele T present increased TGF- β base levels in airways.¹⁵ On the other hand, it has been described that YY1 plays an important role in the pathophysiology of asthma because heterozygote mice for YY1 [yy1(+/-)] gene present a significant reduction of the allergic pulmonary inflammation response associated with inhibition of cytokines Th-2 and IL-4.¹⁶ Therefore, it is reasonable to propose that a higher YY1 expression corresponds to an increased TGF- β expression, showing a direct relationship between the transcription factor and the cytokine. At the same time, a high expression of both proteins will be directly associated with more severe asthma cases. At the present time, no study has been found showing the relationship between YY1 transcription factor expression and TGF- β expression and whether such a relationship contributes to asthma pathophysiology. Therefore, we undertook this study using tissue microarray techniques and immunohistochemical stain in a murine model to determine pulmonary allergic inflammation with different severity levels.

MATERIALS AND METHODS

Models of Pulmonary Allergic Inflammation

Groups of six BALB/c male mice, 6-8 weeks of age, were used and kept under sterile containers with a 0.22- μ m filter, with access to food and water and housed at a 21-24°C temperature before sacrifice. Animal handling was carried out according to the protocol from the animal facility of the National Institute of Nutrition and Medical Sciences "Salvador Zubirán." A pulmonary allergic inflammatory model has been described previously.^{17,18} We used the following sensitization and challenge schemes for each severity model.

Model 1: Mild Pulmonary Allergic Inflammation

On days 0 and 5, mice received 100 μ L i.p. of a suspension with 10 μ g ovalbumin (OVA) in 1 mg alum (SUPERFOS, Taastrup, Denmark). On day 12, mice received 0.75% OVA directly in the trachea. Allergic response was evaluated on day 16.

Model 2: Moderate Pulmonary Allergic Inflammation

On days 0 and 5, mice received 100 μ L i.p. of a suspension with 10 μ g OVA in 1 mg alum. On days 12 and 24, mice received 0.75% OVA directly in the trachea. Allergic response was evaluated on day 28.

Model 3. Severe Pulmonary Allergic Inflammation

On days 0 and 5, mice received 100 μ L i.p. of a suspension with 10 μ g OVA in 1 mg alum. On days 12, 22 and 32, mice received 0.75% OVA directly in the trachea. Allergic response was evaluated on day 36.

Control

Each control group with six mice received only saline solution (SS) instead of OVA and the procedure was carried out as previously described according to the model.

Mice were sacrificed on the day specified according to each model. They were first anesthetized in a chloroform chamber. Blood was then collected by axillary incision and mice were sacrificed to obtain lungs. Lungs received absolute ethanol perfusion and were placed in 10 mL absolute ethanol tubes until ready to be included in paraffin and dissected. Sections were stained using hematoxylin-eosin (H&E) to mark pulmonary tissue with perivascular or peribronchial inflammation in order to build tissue microarrays.

Tissue Microarray

Tissue microarray was built according to what was previously reported.¹⁹ After identifying the region of interest (four regions per each lung tissue sample), which precisely represented the lesion to be analyzed (a perivascular or peribronchial inflammatory infiltrate), we proceeded to build the microarray using the semiautomatic Advanced Tissue Arrayer (ATA 100) from Chemicon. We used a 0.5-mm needle to mark 3-mm deposits in a “blank” 2.5 \times 2.5 cm paraffin block and delimited a 5 \times 5 matrix, regarding it as the receiving block. Once the matrix was done, tissue cylinders from each sample were extracted from the donor block using a 0.4-mm needle (H&E section was used as guide). Each of the cores was implanted in the receiving block, completing a 168 tissue matrix and filling with a small amount of liquid paraffin over the array to even core height. Afterwards, the array was incubated at 60°C for 15 min to homogenize cores with paraffin or receiving block and eliminate spaces between cores and block. Finally, the array was cooled for 5 min on ice and 4- μ m

sections were obtained using a rotary microtome (Leica Microsystems, Heerbrugg, Switzerland).

Immunohistochemical Technique

We measured the reaction for each marker at the same time on the different microarrays in order to reduce variations among experiments. Sections were mounted on special adhesive slides (Instrumedics Inc., Richmond, IL). They were kept in a bacteriological incubator overnight to remove paraffin as follows: samples were hydrated using three xylene baths (8 min each), 2X 100% ethanol baths, 1X 90% ethanol bath, 1X 70% ethanol bath and 1 distilled water bath (5 min each). Antigen was retrieved using boiling sodium citrate for 20 min. Slides were washed to eliminate citrate excess. Endogenous peroxidase activity was eliminated using methanol and 3% hydrogen peroxide three times for 15 min. Nonimmunological bonding of antibodies and tissue was blocked by submerging samples for 4 h in 2% normal swine serum. Afterwards, sections were incubated overnight at room temperature in humid chambers with anti-YY1 antibodies 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-TGF- β antibodies 1:2000 (Santa Cruz Biotechnology). They were then incubated with a conjugated second antibody [anti-rabbit IgG with streptavidin-horseradish peroxidase (HRP) conjugate](DAKO, Carpinteria, CA). Finally, staining was achieved adding diaminobenzidine substrate (DAB) for 3 min; reaction was stopped with running water and contrast was obtained using hematoxylin for 10 sec. Finally, tissue was dehydrated using 5-min baths for each of the following: distilled water, 70% ethanol, 90% ethanol, 100% ethanol and xylene. Preparations were covered with resin and left to dry at room temperature.

Densimometric Analysis

Slides were analyzed on a microscope (Olympus BX-40, Olympus, Tokyo, Japan) and expression density was measured in four areas with perivascular infiltrate and four areas with peribronchial infiltrate for each subject (six mice per group) in a constant 200 μ m² area using the image analysis software Image-Pro Plus (Media Cybernetics, Silver Spring, MD).

Statistical Analysis

Collected data were used to create a database, which was processed using the Prism statistic analysis software

(GraphPad Software, Inc., San Diego, CA). Data were presented using averages per group and standard deviation. We used one-way ANOVA to evaluate the difference in expression density of immunohistochemical reactions. Tukey multiple comparison test was used to identify differences between groups. We carried out a Pearson correlation analysis; $p \leq 0.05$ was considered statistically significant.

RESULTS

Figure 1 shows allergic response induced by OVA. Using H&E staining (a-h), we were able to evaluate perivascular inflammatory infiltrate (a-d) as well as peribronchial inflammatory infiltrate (e-h) on different levels of pulmonary allergic inflammation: mild (b and f), moderate (c and g) and severe (d-h). There is a clear increment of inflammatory infiltrate as the severity level advances. The three models show a significant increase of inflammatory infiltrate at perivascular (a) and peribronchial (e) levels when compared with control group that received only saline solution (a and d). Another important characteristic of pulmonary allergic inflammation is mucus secretion; therefore, we evaluated the presence of mucus at each severity model using PAS stain (periodic acid Schiff), which stains mucin in mucus (i-l). We observed a significant increase of mucus in mice that received OVA (j-l) compared to control subjects (i). We also observed a gradual increase in mucus quantity associated with severity level. These results demonstrate that we were able to reproduce three levels of pulmonary allergic inflammation.

Figure 2 shows how tissue microarrays were made. Microarray 1 included sections of pulmonary tissue containing perivascular areas of animals with different OVA-induced inflammation levels and control subjects (this microarray contained ~100 samples). Microarray 2 included pulmonary samples with peribronchial areas from animals with different OVA-induced inflammation levels and control animals (this microarray also contained also ~100 samples). After microarray blocks were obtained, we carried out special sections in slides for YY1 and TGF- β immunohistochemical tests.

Figure 3 shows a representative microphotograph of YY1 stain in perivascular (a-d) and peribronchial (e-h) infiltrates. Staining was obtained mainly at nuclear and cytoplasm levels both for inflammatory infiltrate cells as well as bronchial epithelium cells. We observed a clear

increase in YY1 expression in the group of subjects with severe pulmonary allergic inflammation (d and h) compared with the moderate (c and g) and mild (b and f) allergic inflammation groups. However, the three inflammation models showed a higher YY1 expression when compared with the control group (a and e). We obtained similar results on TGF- β immunostaining (Figure 4). Expression of this cytokine was observed at perivascular (a-d) and peribronchial (e-h) levels, chiefly on cytoplasm of both inflammatory infiltrate cells and bronchial epithelium cells. The higher expression was observed in the severe pulmonary allergic inflammation group (d and h) compared with moderate (c and g) and mild (b and f) groups. The control group (a and e) showed minimal expression.

We subsequently measured the YY1 and TGF- β expression densities. Figure 5 shows YY1 expression density graph where bars represent the average YY1 expression in four peribronchial infiltrate areas and four perivascular infiltrate areas from each animal of each group (six subjects per group), with a total of 24 vessels and 24 bronchi. Results show a statistically significant increase of pulmonary allergic inflammation according to severity level, both in perivascular ($p = 0.001$, ANOVA) and peribronchial infiltrates ($p = 0.01$, ANOVA). All groups that received OVA presented an increased YY1 expression when compared with control groups.

Figure 6 shows measurement of TGF- β expression density at perivascular and peribronchial levels. There is a significant increase of expression in subjects that received OVA compared with subjects that received SS ($p = 0.001$, ANOVA).

With data obtained for YY1 and TGF- β expressions, we carried out a Pearson's statistical test, which shows a significant correlation with a 95% confidence interval ($p = 0.029$, $r = 0.633$). This means that YY1 expression is directly proportional to TGF- β expression (Figure 7).

DISCUSSION

Recent studies have demonstrated that YY1 transcription factor plays an important role in asthma pathophysiology through the regulation of certain cytokines such as IL-4.¹⁶ On the other hand, it has been reported that YY1 positively regulates TGF- β expression in cells from BAL and pulmonary tissue from asthmatic patients. We know that the polymorphism in TGF- β promoter has been associated

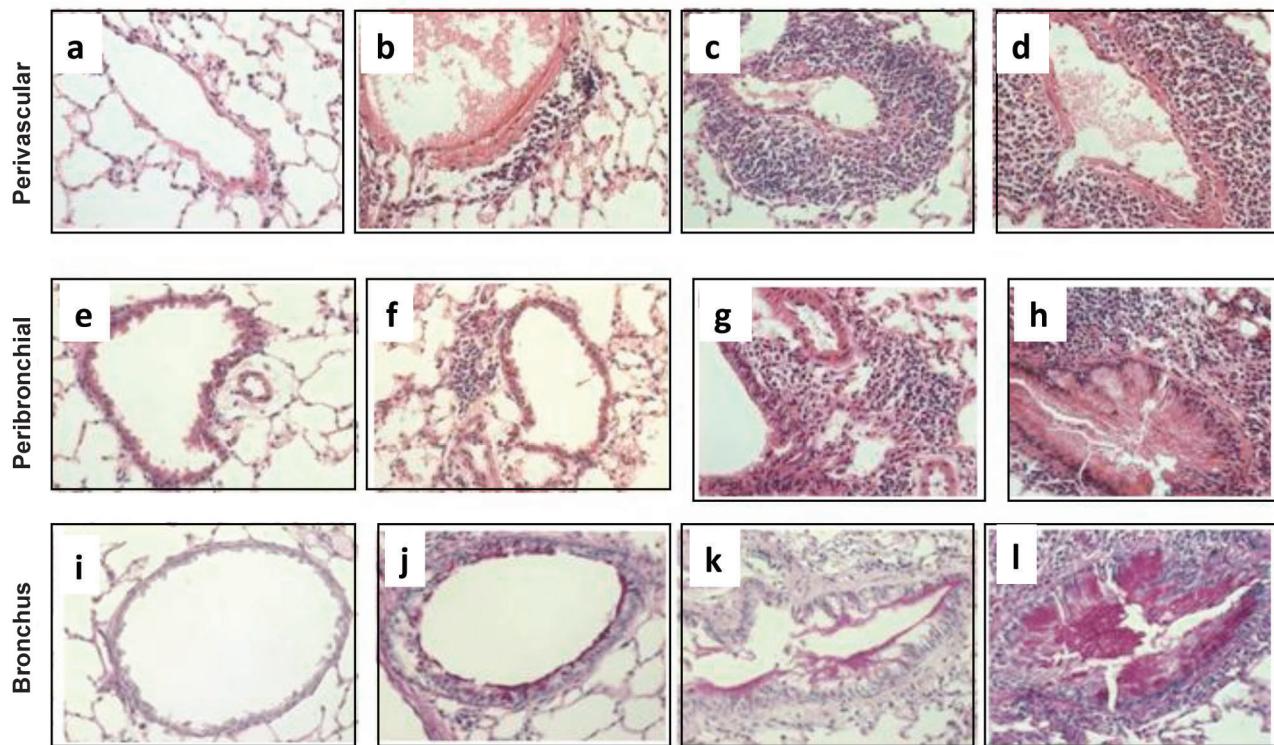


Figure 1. Evaluation of allergic response induced by OVA challenge. H&E stain (a-h) and PAS stain (i-j) to determine evolution of inflammatory infiltrate and mucus production, respectively. Pulmonary allergic inflammation model at perivascular (a-d) or peribronchial (e-h) levels; mild (b,f,i), moderate (c,g,j) and severe (d,h,k). Magnification x20.

with a poor prognosis for this disease. This is because the site where this polymorphism is found is a bond recognition sequence for YY1. Patients with this polymorphism present a cytosine (C) instead of a thymine (T) in the consensus site for YY1; therefore, this factor bonds with greater affinity and produces a high TGF- β expression.¹⁵

Several studies have reported that TGF- β plays an important role in asthma pathogenesis because patients with severe asthma present a high expression of this cytokine, whereas patients with mild or moderate symptom onset present a less significant expression.^{9-12,20,21}

It is reasonable to propose that there is a direct relationship between YY1 expression and TGF- β expression and this relationship is associated with asthma severity, which has previously not been described. This study describes, for the first time, a direct relationship between YY1 expression and TGF- β expression in a murine model of pulmonary allergic inflammation (a characteristic clinical manifestation of asthma) with different severity levels

using tissue microarrays. This technique has been used widely to study the expression of proteins. The advantage of this technology is that a single paraffin block contains an orderly set of 100-300 different tissues; therefore, when sections are obtained from this block, it is possible to analyze the expression of a certain protein at the same time with the advantage of reducing variations between essays and reducing the cost and time required to carry out the experiment. This allows obtaining a more reliable and significant result where more powerful statistical tests can be carried out because there is reduced data dispersion for each analyzed group.

Figure 3 shows that a mild YY1 expression is present in the control group that received only SS. This can be explained because YY1 presents a constitutive protein expression. Another explanation may be that when subjects are inoculated directly in trachea, tissue receives a mechanically induced stress that generates certain cellular response inducing YY1 expression. It would be neces-

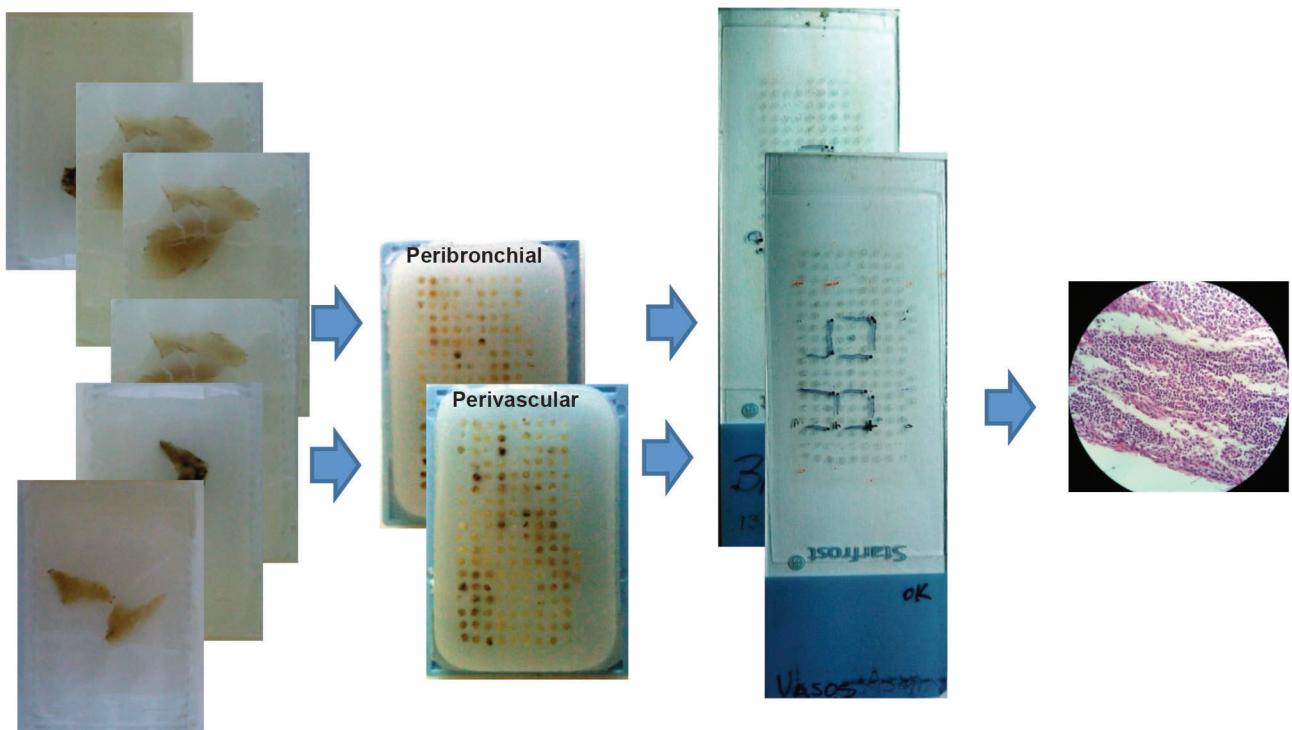


Figure 2. Tissue microarray schema.

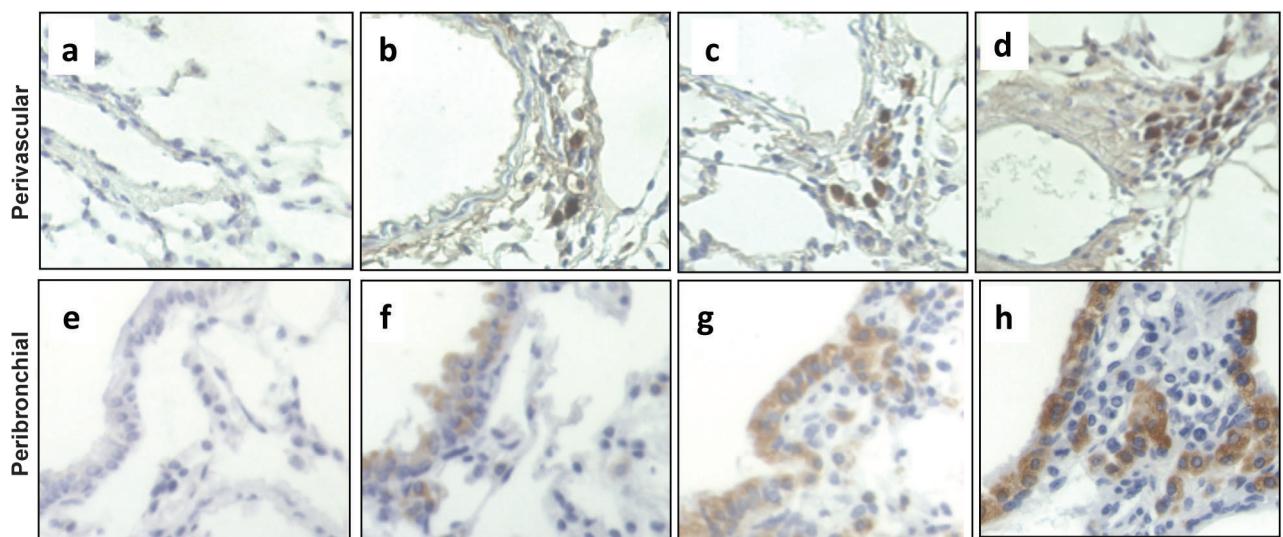


Figure 3. Immunohistochemical tests to identify YY1 in pulmonary tissue. Perivascular inflammatory infiltrate (a-d) and peribronchial inflammatory infiltrate (e-h) with different pulmonary allergic inflammation levels: mild (b,f), moderate (c,g) severe (d,h) and control group (a,e). Magnification x40.

sary to include a group of mice that were not subjected to tracheal administration to rule out or to confirm this hypothesis.

We observed that there is a higher YY1 expression in perivascular inflammatory infiltrates (Figures 3a-d and Figure 5A) than in peribronchial infiltrates (Figures 3e-h and Figure 5B). This may be associated with inflammatory cell migration towards tissues through blood vessels. The perivascular region has the first contact with inflammatory cells that then migrate to other zones, e.g., to peribronchial or interstitial levels.

The severe pulmonary allergic inflammation model shows that there is a higher YY1 expression compared with the mild and moderate models. This may be explained because during the inflammatory process several pro-inflammatory cytokines are released in pulmonary tissue that may stimulate YY1 expression. At the same time, when this transcription factor is overexpressed, it regulates the expression of other cytokines important in asthma pathogenesis such as IL-4 and IL-10.¹⁶ We know that the expression of nuclear factor kappa-B (NF-κB) can be regulated by pro-inflammatory cytokines. On the other hand, recent studies have demonstrated that NF-κB positively regulates YY1 expression.^{22,23}

Figures 4 and 6 show an important difference in the expression of TGF-β between groups treated with OVA and control groups. This is consistent with several reports

that have demonstrated that TGF-β plays an important role in asthma pathophysiology and that a higher expression of this cytokine is associated with a more severe onset of the disease.^{9-11,13,20,21} TGF-β is an anti-inflammatory cytokine; however, in environments with a high amount of Th-2 lymphocytes, this cytokine acts chiefly in the development, growth and repair of tissues and fibrosis as well as modulation of immune inflammatory response.¹⁰ The SS group shows a discreet expression of this cytokine and can be explained because TGF-β is a constitutively expressed protein. As previously mentioned, it has been demonstrated that the TGF-β code gene promoter presents a polymorphism at C-509T locus, which induces a higher affinity YY1 bonding to this promoter and activates overexpression of this cytokine.¹⁵ Until now, there is no study that directly associates YY1 with TGF-β expression.

According to the obtained results, we were able to confirm reports where YY1 expresses significantly in pulmonary tissue of mice subjected to pulmonary allergic reaction. However, for the first time, we reported that YY1 expression increases directly according to disease severity level. Also, for the first time we demonstrated that there is a direct relationship between YY1 expression and TGF-β. This expression is supported by findings of Silverman et al. who observed that YY1 regulates TGF-β through transcription.¹⁵

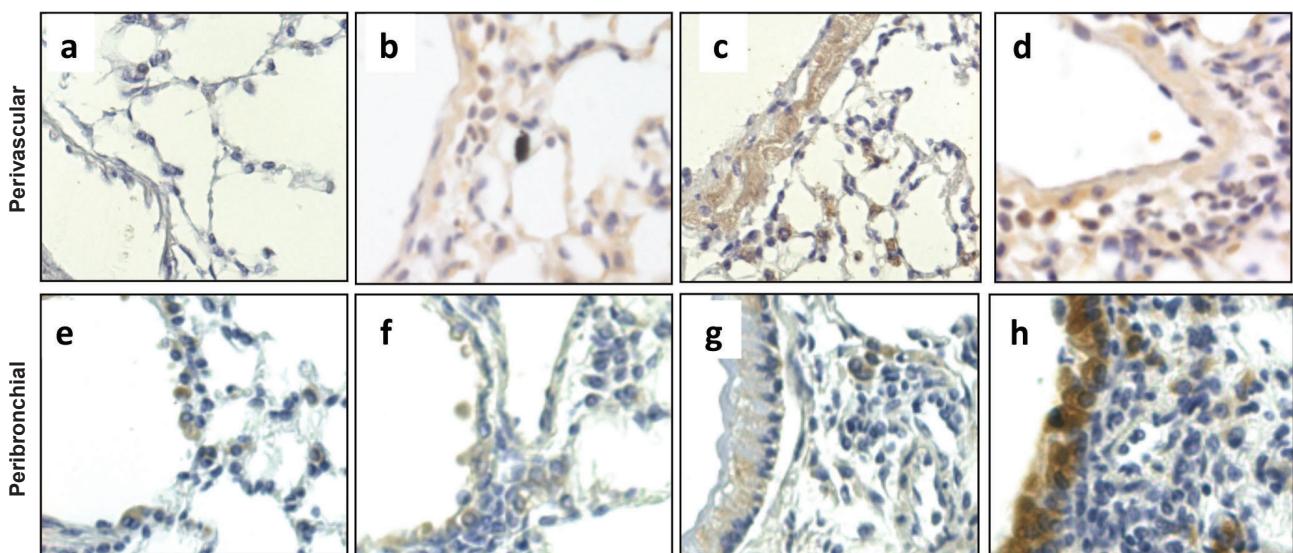


Figure 4. Immunohistochemical test to identify TGF-β in pulmonary tissue. Perivascular inflammatory infiltrate (a-d) and peribronchial inflammatory infiltrate (e-h) with different pulmonary allergic inflammation levels: mild (b,f), moderate (c,g) severe (d,h) and control group (a,e). Magnification $\times 40$.

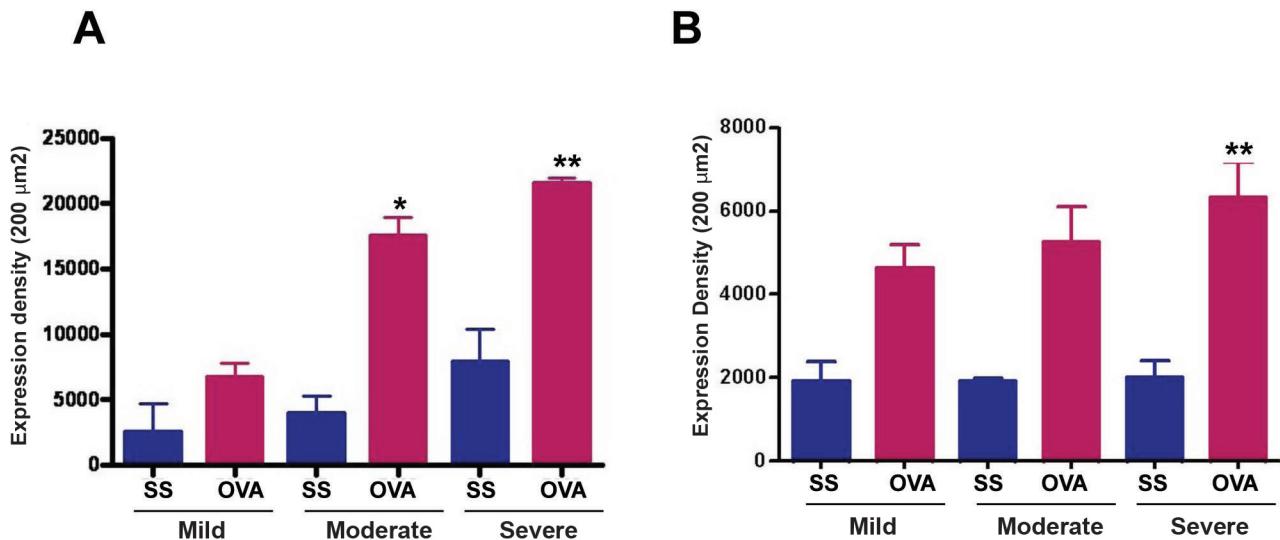


Figure 5. Analysis of YY1 expression area on perivascular (A) and peribronchial (B) inflammatory infiltrates. * $p = 0.01$; ** $p = 0.001$ (ANOVA); OVA, ovalbumin; SS, saline solution.

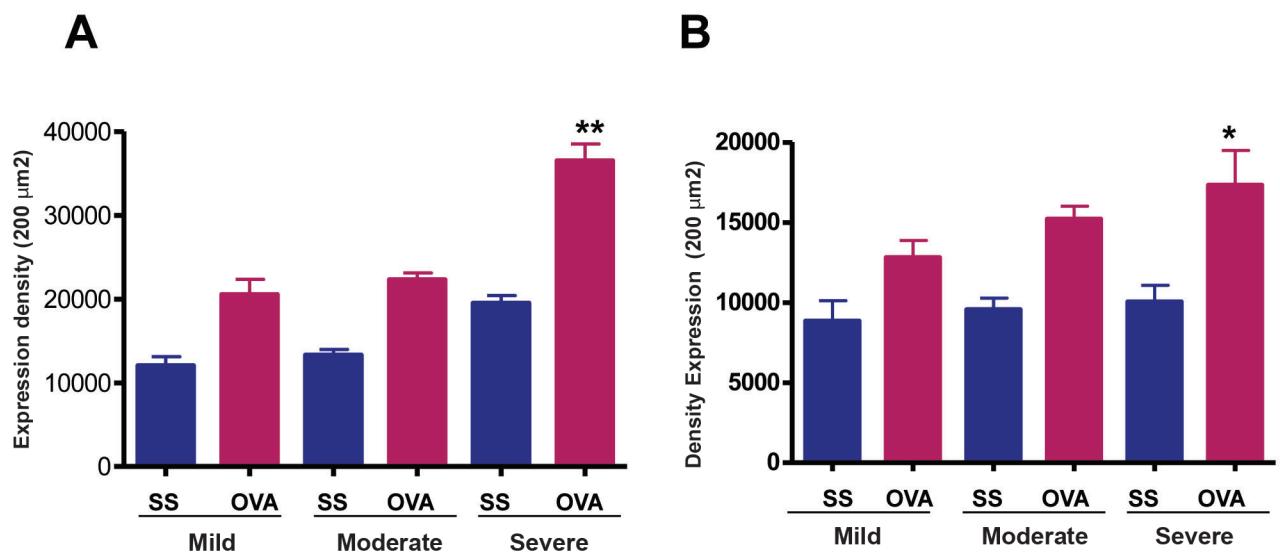


Figure 6. Analysis of TGF- β expression area on perivascular (A) and peribronchial (B) inflammatory infiltrates. * $p = 0.01$; ** $p = 0.001$ (ANOVA); OVA, ovalbumin; SS, saline solution.

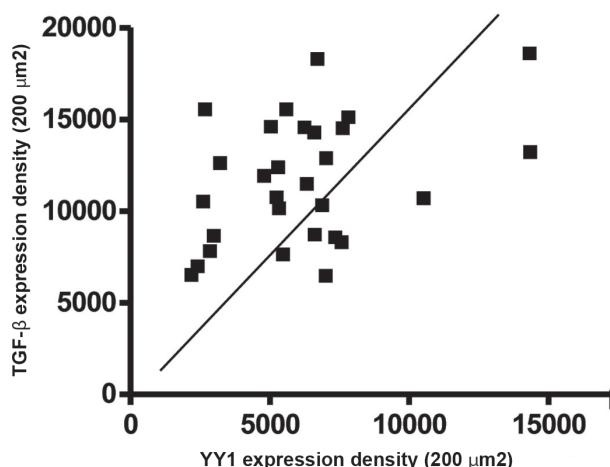


Figure 7. Correlation of TGF- β and YY1 expressions on inflammatory infiltrate of perivascular pulmonary tissue with severe allergic inflammation. 95% CI, $p = 0.029$ and $r = 0.633$ (Pearson).

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