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Oral feeding of *Lactobacillus bulgaricus* N45.10 inhibits the lung inflammation and airway remodeling in murine allergic asthma: Relevance to the Th1/Th2 cytokines and STAT6/T-bet

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ABSTRACT

Asthma is a chronic disease with impacts on public health. It affects the airways causing pulmonary inflammation mediated by CD4 T cells type Th2, eosinophilia, mucus hypersecretion, and elevated IgE. The unbalance between cytokines and transcription factors is an important feature in asthma. Probiotics has gaining highlight as a therapy for chronic diseases. Thus, we investigate the *Lactobacillus bulgaricus* (Lb) effect in murine allergic asthma. BALB/c-mice were sensitized to ovalbumin (OA) on days 0 and 7 and were challenged from day 14–28 with OA. Mice received Lb seven days prior to sensitization and it was kept until day 28. The Lb attenuated the eosinophils infiltration, mucus and collagen secretion, IgE production, pro-inflammatory cytokines, TLR4 expression, GATA3, STAT6 and RORγt in lung. Otherwise, Lb increased the anti-inflammatory cytokines, the T-bet and foxp3. Finally, Lb attenuated the allergic asthma-induced inflammation and airway remodeling by interfering on Th1/Th2 cytokines and STAT6/T-bet transcription factors.

1. Introduction

Allergic diseases such as asthma have become an important public health problem. Asthma is a chronic inflammatory disease, characterized by inflammation and extensive airways contraction, bronchoconstriction, mucus production, and airflow limitation, which can be triggered by various stimuli such as environmental allergens or viral infections [1,2]. The exposure to the allergen leads to migration of inflammatory Th2 cells, B cells which produce immunoglobulin E (IgE), eosinophils, and mast cells in lung tissue, leading to bronchoconstriction and mucus production that together are the main cause of the airflow obstruction [3,4].

Recent studies suggest that during the disease, along with the increased Th2 response, imbalances occur in the Th17/T regulatory pattern of immune responses with high levels of this IL-17 and decreased IL-10 cytokine [5]. Corroborating studies indicate that Th17 cells, producers of IL-17, can recruit neutrophils to the lung contributing to the disease aggravation [6]. Among the IL-10 producing cells are regulatory T cells (Tregs), which are important to balance of immune responses and to maintain immunological tolerance to antigens, including allergens. An immune response imbalance is characterized by specific transcription factors [7]. The *T*-bet expression is associated with the differentiation of the Th1-type cytokines producing cells; GATA-3 and STAT-6 are associated with the Th2-type cytokines; RORγt is associated with the Th17-type cytokines; and foxp3 is associated with the Tregs-type cytokines [7]. Alterations in the expression or function of these transcription factors may be associated with the pathogenesis of asthma [8].

Due to the high morbidity and the limitations of existing asthma treatments [9,10], innovative action is needed against allergic responses for better control the disease. One effective treatment for asthma may be to improve Th1 immune response and simultaneously inhibit Th2/Th17 immune response to restore Th1/Th2 balance. Probiotics are promising and seem to be able to prevent and even control allergic diseases [11].

The mechanisms responsible for the development of asthma have not been fully elucidated. However, studies have observed that the use of antibiotics, a diet rich in fat, and changes in lifestyle with an

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excessive hygiene may contribute to disease incidences [12,13].

Diverse authors have evidenced that probiotics exhibit a variety of therapeutic effects, and they can to stimulate the immune system compounds secreted or present in the cellular barrier [14–17]. Thus, their use is considered a nonspecific adjuvant of the innate immune response, helping the body's defenses to promote a suitable immune response depending on pathological condition and restore the balance between pro- and anti-inflammatory cytokines secreted by activated immune cells [18]. For this reason, the probiotics have a beneficial effect for the treatment of a variety of chronic diseases, among them, allergic asthma [19,20].

It is likely that the anti-inflammatory efficacy of probiotic results from a combination of signaling pathways activated due to a specific pattern of microbe-derived ligands interacting with the corresponding receptors on host cells [21]. Probiotics could be used to control chronic disorders because they are able to orchestrate the immune response of the host [22]. Little is known, however, concerning the nature of the probiotic-host cell interactions, or how these interactions could be manipulated to obtain stronger regulatory responses [23].

In this sense, the sensors of innate immunity are target of probiotics and among the Toll-like receptors (TLRs) has an important role [24]. It is well known that TLR4 is an important immune pattern recognition receptor that controls innate and adaptive immune responses and plays an important role in initiating and regulating airway inflammation [25]. Some authors have evidenced that TLR4 plays an important role in exacerbation of allergic airway inflammation and production of serum IgE [26–29]. Therefore, it is relevant to investigate the effect of probiotics on TLR4 signaling in allergic mice.

Some authors have proposed an action mechanism to explain the beneficial effect of probiotic in distant organs from gut [21–23]. In this context, TLRs on dendritic cells located in gut-associated lymphoid tissue (GALT) are important due to fact that there is a rise of the expression as well as the activation of TLRs in response to probiotic recognition [22]. These activated dendritic cells from interaction of probiotic with TLRs secrete cytokines that exerts an important influence on inducing Th0 cell differentiation to Th type immune response. Finally, these differentiated Th cells migrate to the distant organ controlling the inflammation exacerbation as well as immune dysfunction. Thus, the probiotic can be recognized by TLRs present on the surface of dendritic cells and TLRs activated dendritic cells can activate Th1 cells and release anti-inflammatory cytokine (such as IL-10) [22] in order to relief the allergic lung inflammation. However, authors have reported that dendritic cells activated by probiotic in GALT have also ability to migrate to the lung milieu [30]. In this way, the intestinal microbiota may induce Treg cells in the GALT that then spread to the airways could stimulate the bronchial-associated lymphoid tissue (BALT) in response to allergen exposure [22]. Due to these evidences it becomes pertinent to investigate the probiotic effect on TLR4 expression in dendritic cells from lung of asthmatic mice.

Therefore, to guide new therapies, we aim to investigate whether the use of the probiotic *Lactobacillus bulgaricus N45.10* can beneficially modulate the immune response and attenuate lung inflammation during allergic asthma.

2. Methods

2.1. Animals

BALB/c mice, 6 week-old male (6-7-mice/group), under specific pathogen-free conditions were obtained from Development Center of Experimental Models for Medicine and Biology from the Federal University of São Paulo and maintained under standard conditions of temperature (22–25 °C), relative humidity (40–60%), and light/dark cycle (12 h-12 h) with access to food and water ad libitum. The experiment was approved by the Ethics Committee on Animal Research from the Federal University of São Paulo (protocol number 9938270115).

2.2. Asthma model

Sensitizations were performed on days 0 and 7. Mice were sensitized to ovalbumin (OVA) (grade V, Sigma, St. Louis, MO, USA) through intra-peritoneal injections of 0.1 mL alum-precipitated antigen, comprising 10 μg OVA absorbed into 2.25 mg alum (Imject Alum; Pierce, Rockford, IL, USA). Control animals received 0.1 mL of only saline. From day 14 to day 28, the animals were challenged three times a week through exposure to aerosolized OVA (1% in PBS) for 15 min. The control group consisted of mice not sensitization and not challenge with OVA. All determinations were performed 24 h (29 day) after the last OVA challenge.

2.3. Probiotic administration

The probiotic *L. bulgaricus* N45.10 (*Lb*) used in this study was obtained from Liane Pharmacy (Ribeirão Preto, SP, Brazil). Mice received an oral dose of *Lb* (1×10^9 CFU/0.2 mL PBS/mouse/three-times/week) starting one week before primary sensitization until day 28 of the challenge. As a negative control treatment, the mice received only PBS (0.2 mL PBS/three-times/week).

2.4. Experimental groups

All mice were placed in a common box and divided randomly into 4 groups containing seven animals each: (1) control group, which consisted of non-manipulated mice; (2) *Lb* group, where animals were treated with *Lb*; (3) the allergic group, which consisted of mice exposed to the OVA; (4) *Lb* + allergic group: mice pre-treated with Lb and exposed to OVA. *The experimental design is represented in the* Fig. 1.

2.5. Bronchoalveolar lavage fluid (BALF)

After the mice were euthanized with excess anesthetics, the trachea was cannulated, and lungs were rinsed with 0.6 mL of cold PBS (saline). This was followed by 2 additional washings with the same saline volume. Total and differential cell counts of BALF fluid were determined by hemocytometer and cytospin preparation stained with Instant-Prov (Newprov, Brazil). Numbers of eosinophils, macrophages, neutrophils, and lymphocytes were scored by light microscopy.

2.6. Histology and image analysis

Five micrometer slides were stained with hematoxylin and eosin, picrossirius, and periodic acid Shiff and Alcian blue to identify and quantify the density of the eosinophils in the airway walls, the density of collagen fibers in the airway walls, and the neutral and acid mucus in the airway epithelium, respectively. Five airways of all animals were imaged at 400× magnifications using a Nikon Eclipse E-200 microscope camera and the software Image Pro-Plus 4.0. The color threshold for collagen, periodic acid Shiff (PAS), and Alcian blue (AB) were determined and the analyses were performed as detailed in Sections 2.6.1 to 2.6.5.

2.6.1. Eosinophil density in airway wall

The area between the airway basal membrane and the adventitia was quantified using Image Pro-Plus software, and the number of eosinophils was quantified in this area according to the morphological criteria. These results were expressed as the number of eosinophils per square millimeter.

2.6.2. Percentage of mucus

After the airway epithelium area was determined, with the previously determined color threshold for acid mucus (AB positive), the

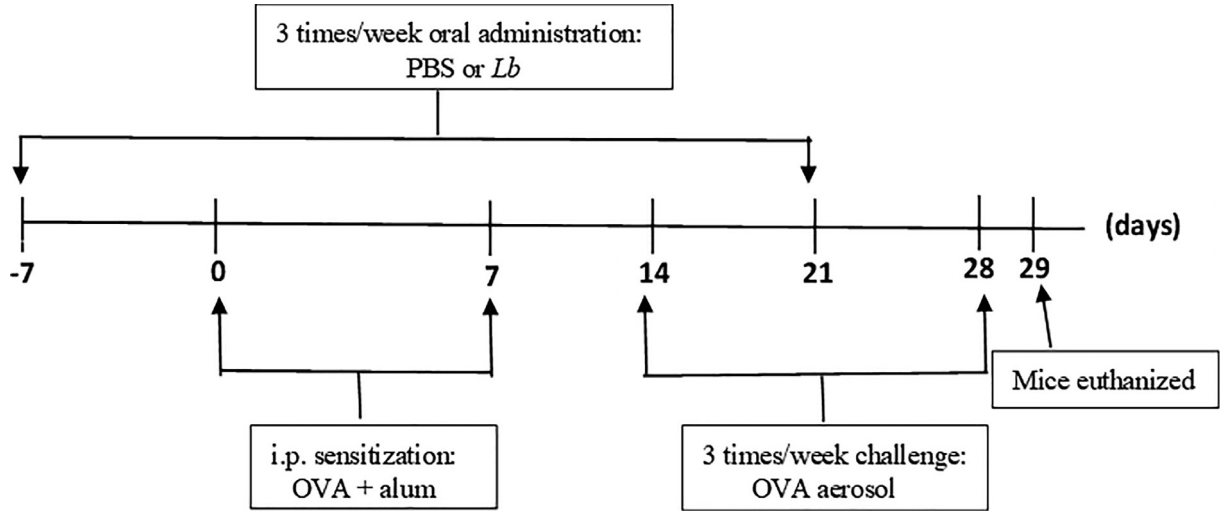

Fig. 1. Time schedule of asthma model. Male BALB/c mice were sensitized intraperitoneally to OVA-Alum on days 0 and 7 and were challenged from day 14 to day 28 with aerosolized OVA. One week before primary sensitization until day 28, mice were treated 3 times a week with either PBS or Lb (1×10^9 CFU/0.2 mL PBS) by oral gavage. One hour after treatment, from day 14 to day 28, mice were challenged 3 times/week with aerosolized OVA. Mice were euthanized on day 29.

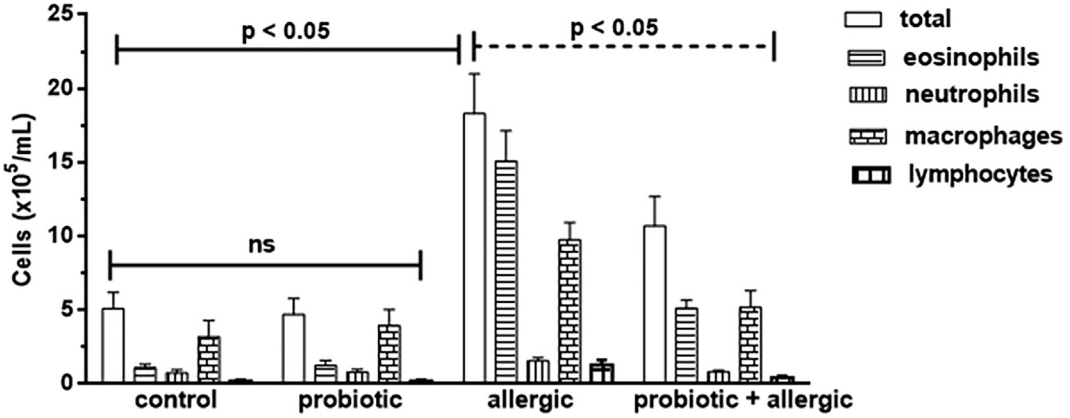

Fig. 2. *L. bulgaricus* N45.10 on inflammatory cell migration. Twenty-four hours after the last OVA challenge, the total cells and inflammatory cells were counted ($\times 10^5$) in BALF in millimeters by the morphometric evaluations of cytospin preparations. Pulmonary inflammation was represented by the influx of specific leukocytes; macrophages, eosinophils, neutrophils, and lymphocytes in BALF fluid. All cell counts were obtained from the normal control group, allergic group, and *Lb* + allergic group. Each bar represents mean ± SEM from 7 different animals. Results were considered significant when $p < 0.05$.

amount of AB area was measured. These results were expressed as the % of AB area compared to the total epithelium.

2.6.3. Percentage of collagen fibers deposition in the airway wall

First, the area between the airway basal membrane and the airway adventitia was determined. Next, using the previously determined color threshold for picrossirius, the picrossirius-positive area was quantified. These results were expressed as the % of collagen (picrossirius positive) area compared to the total measured area.

2.6.4. Peribronchial edema

To measure the amount of peribronchial edema, transversely sectioned non-cartilaginous airways were selected and magnified 1000×. The numbers of points of the interacting eyepiece falling on areas of edema were quantified in three randomly selected areas of each airway wall.

2.6.5. Index of bronchoconstriction

The airway bronchoconstriction index was assessed as the number of points hitting the airway lumen divided by the root square of the number of intercepts between the lines of the grid and the airway basal membrane. Measurements were performed in five airways from each animal at 400× magnification.

2.7. Quantification of IgE level in serum

Serum was obtained from the blood sample by centrifugation of 3000 × g for 10 min at 4 °C. The total IgE in serum was measured using enzyme-linked immunosorbent assay (ELISA) with an anti-mouse IgE ELISA kit (Invitrogen, SP, Brazil) according to the manufacturer's instructions. OVA-specific IgE determination serum samples were added followed by the addition of biotin-labeled OVA (Sigma, SP, Brazil) according to the manufacturer's instructions.

2.8. Leukotriene and prostaglandin in BALF

BALF levels of LTC_4 and PGD_2 were measured using ELISA according to the manufacturer's instructions. All assays were performed in duplicate. Briefly, the serum samples were added in duplicate to 96-well plates with 100 µl per well. The appropriate biotin conjugated antibodies were added to each well. The samples were incubated at room temperature for 2 h. The wells were then aspirated, and each well was washed 5 times. The substrate solutions were added to each well and then incubated for 30 min at room temperature in the dark. The

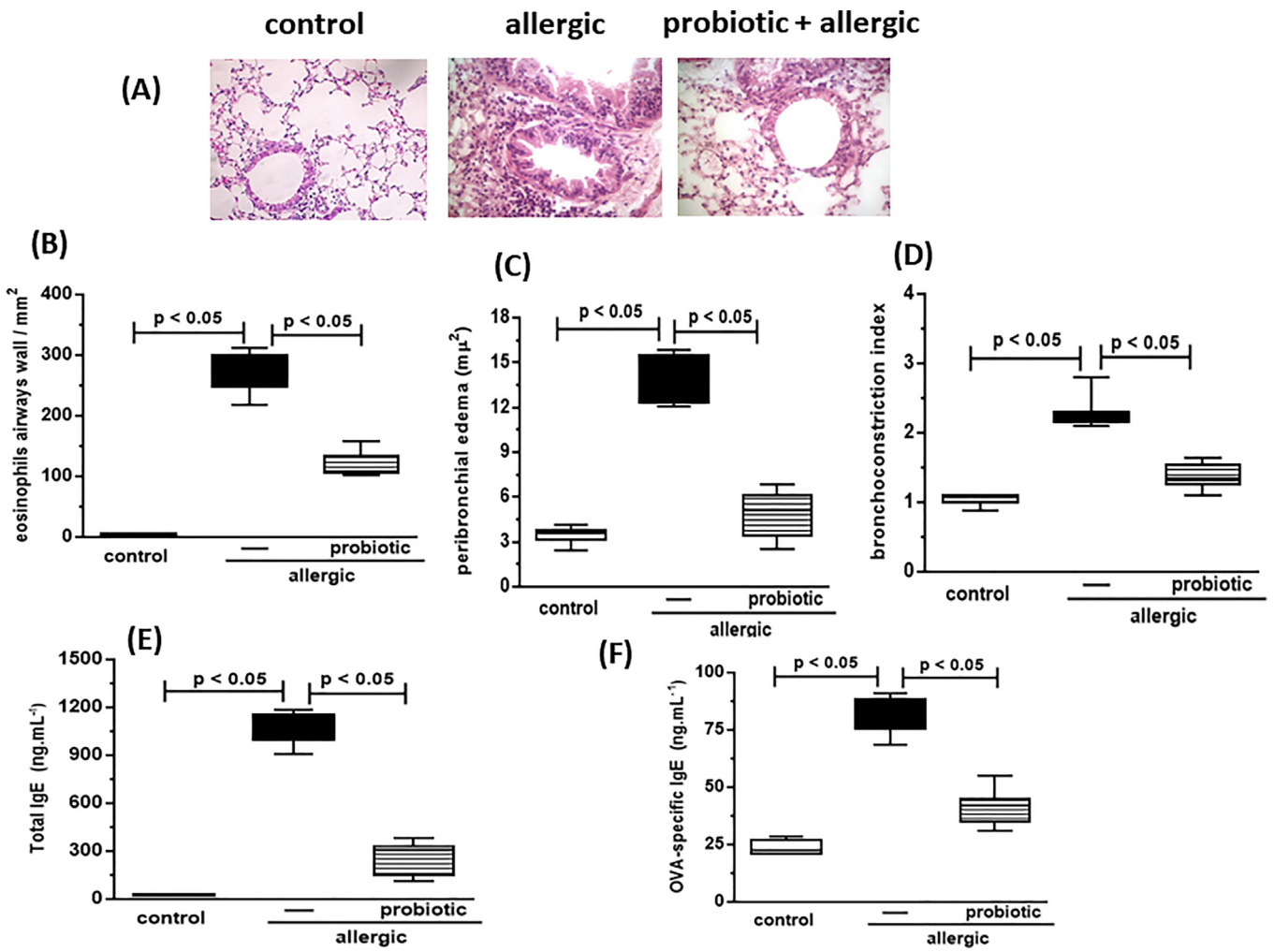

Fig. 3. *L. bulgaricus* N45.10 on airway morphometry and IgE levels in serum. Sections (5 μm) of formalin-fixed lungs were stained with hematoxylin and eosin for histological examination in control, allergic, and *Lb* + allergic groups. Mice received an oral dose of *Lb* (1×10^9 CFU/0.2 mL PBS/mouse/three-times/week) starting one week before primary sensitization until day 28 during the challenge. Control and allergic groups received PBS as treatment. (A) To identify and quantify the migration cell (line shows the peribronchial region with or without edema, asterisk indicates the absence or presence of bronchoconstriction, arrow represents the cellular migration; original magnification, ×200). (B) Quantification of eosinophils in airway wall sites per area (mm^2), (C) peribronchiolar edema sites per area (μm^2), (D) bronchoconstriction index measured as described in Material and Methods section. Blood serum obtained from all mice was assayed for (E) total IgE and (F) OVA-specific IgE by using enzyme-linked immunosorbent assay (ELISA). The IgE levels in serum were measured 24 h after the antigen challenge. Each bar represents mean ± SEM from 7 different animals. Results were considered significant when $p < 0.05$.

optical density (OD) of each well was determined using a microplate reader (BioRad Model 680, USA), which was set to 450 nm. A standard curve was created of the average of the OD triplicate readings.

2.9. Cytokines measurement in BALF and serum

The levels of cytokines from Th1 (IL- 2, IL-10, IL-12, IFN-γ) and Th2 (eotaxin, IL-4, IL-5, IL-13), Th17 (IL-17A) axis, and IL-33 in BALF or serum were assessed using ELISA Kits in accordance with the manufacturer's instructions. Values are expressed as pg/mL deduced from standard runs in parallel with recombinant cytokines.

2.10. Real-Time polymerase chain reaction (PCR) for STAT6, GATA-3, RORγt, T-bet, and foxp3 in lung tissue

For mRNA analysis, the thoracic cavity of the mice was exposed, and their heart and lung were removed in bloc, 24 h after last OVA challenge. The pulmonary artery was cannulated and then the pulmonary vasculature was perfused with ice cold sterile phosphate buffer solution (PBS) using a peristaltic pump (Thermo Fisher Scientific, Suwannee, GA, USA) to remove the intravascular blood. Lung fragments were cut into 5 mm pieces using a tissue chopper, flash frozen in liquid nitrogen and stored at −80 °C for real-time polymerase chain reaction (RT-PCR) analysis of genes expression. For that assay, total RNA was isolated from lung by TRIzol reagent (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's protocol. RNA was subjected to DNase I digestion, followed by reverse transcription to cDNA. PCR was performed in a 7000-sequence detection system (ABI Prism; Applied Biosystems, Foster City, CA, USA) using the SYBRGreen core reaction kit (Applied Biosystems). The STAT6 mRNA primers used for quantification were forward primer 5′-CCTGGTCGGTTCAGATGC TTT-3′ and reverse primer 5′-GTGCGGCAAGATGCTGTTTC-3′. The primers used for GATA-3 mRNA quantification were forward primer 5′-GAGGTGGACGTACTTTTTAACATCG-3′ and reverse primer 5′-GGCA TACCTGGCTCCCGT-3′. Primers used for RORγt mRNA quantification were forward primer 5′-CCGCTGAGAGGGCTTCAC-3′ and reverse primer 5′-TGCAGGAGTAGGCCACATTACA-3′. For *T*-bet mRNA quantification were used forward primer 5′-GCCAGGGAACCGCTTATATG-3′ and reverse primer 5′-GACGATCATCTGGGTCACATTGT-3′. Primers used to quantify foxp3 mRNA were forward primer 5′-CCCAGGAAAG

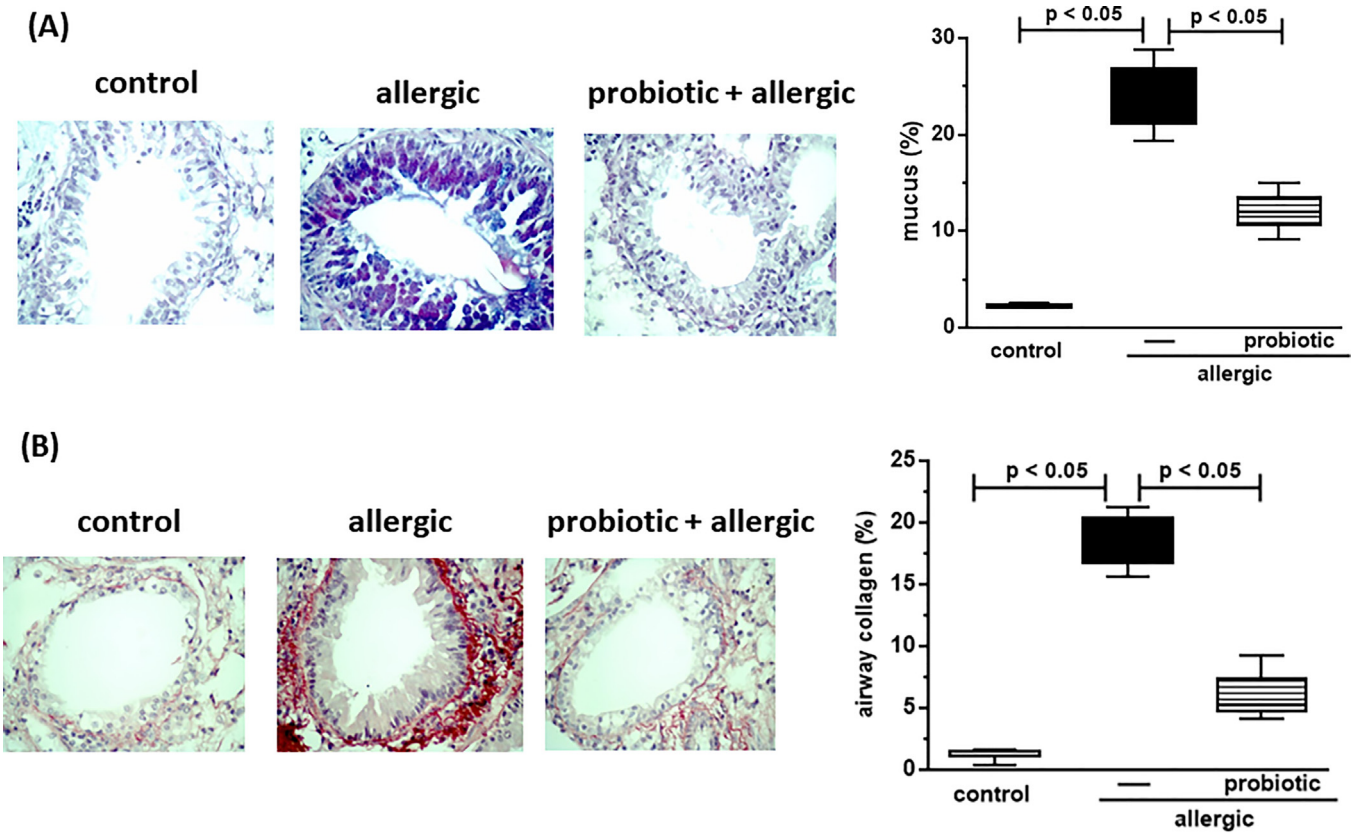

Fig. 4. *L. bulgaricus* N45.10 on mucus and collagen production in lung. The mucus and collagen production were examined in control, allergic, and *Lb* + allergic groups. Mice received an oral dose of *Lb* (1×10^9 CFU/0.2 mL PBS/mouse/three-times/week) initiating one week before primary sensitization until day 28 of the challenge. Control and allergic group received PBS as treatment. (A) Sections of formalin-fixed lungs were stained with Periodic Acid Schiff (PAS) before histological examination using light microscopy (original magnification, ×200). One representative image from 7 different animals, for one independent experiment is presented. Quantification of neutral mucus and acid mucus (%) was analyzed in lung in all mice groups. (B) Sections of formalin-fixed lungs were stained with Picrossirius before examination with light microscopy (original magnification, ×200). One representative image from 7 different animals is shown. Quantification of airways collagen (%) was analyzed in lung in all mice groups. Each bar represents mean ± SEM from 7 different animals. Results were considered significant when $p < 0.05$.

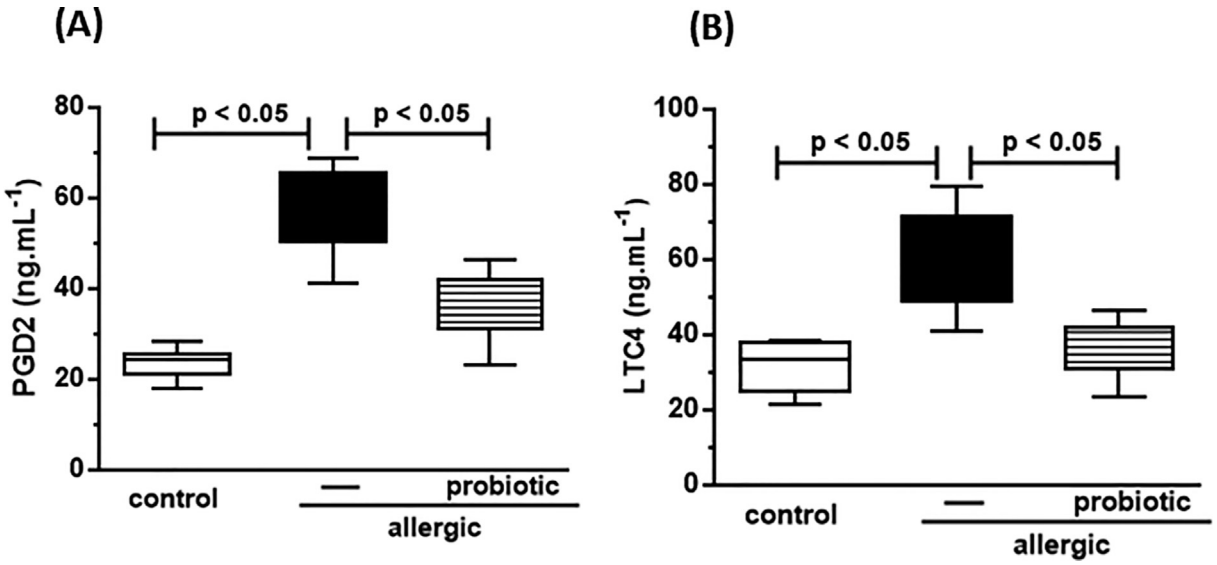

Fig. 5. *L. bulgaricus* N45.10 on eicosanoids in BALF. BALF obtained from control, allergic, and *Lb* + allergic groups was prepared for analysis of PGD_2 and LTC_4. Mice received an oral dose of *Lb* (1×10^9 CFU/0.2 mL PBS/mouse/three-times/week) initiating one week before primary sensitization until day 28 of the challenge. Control and allergic groups received PBS as treatment. PGD_2 (A) and LTC_4 (B) were assayed by enzyme-linked immunosorbent assay (ELISA). Both PGD_2 and LTC_4 levels in BALF were assayed 24 h after the last OVA challenge. Each bar represents mean ± SEM from 7 different animals. Results were considered significant when $p < 0.05$.

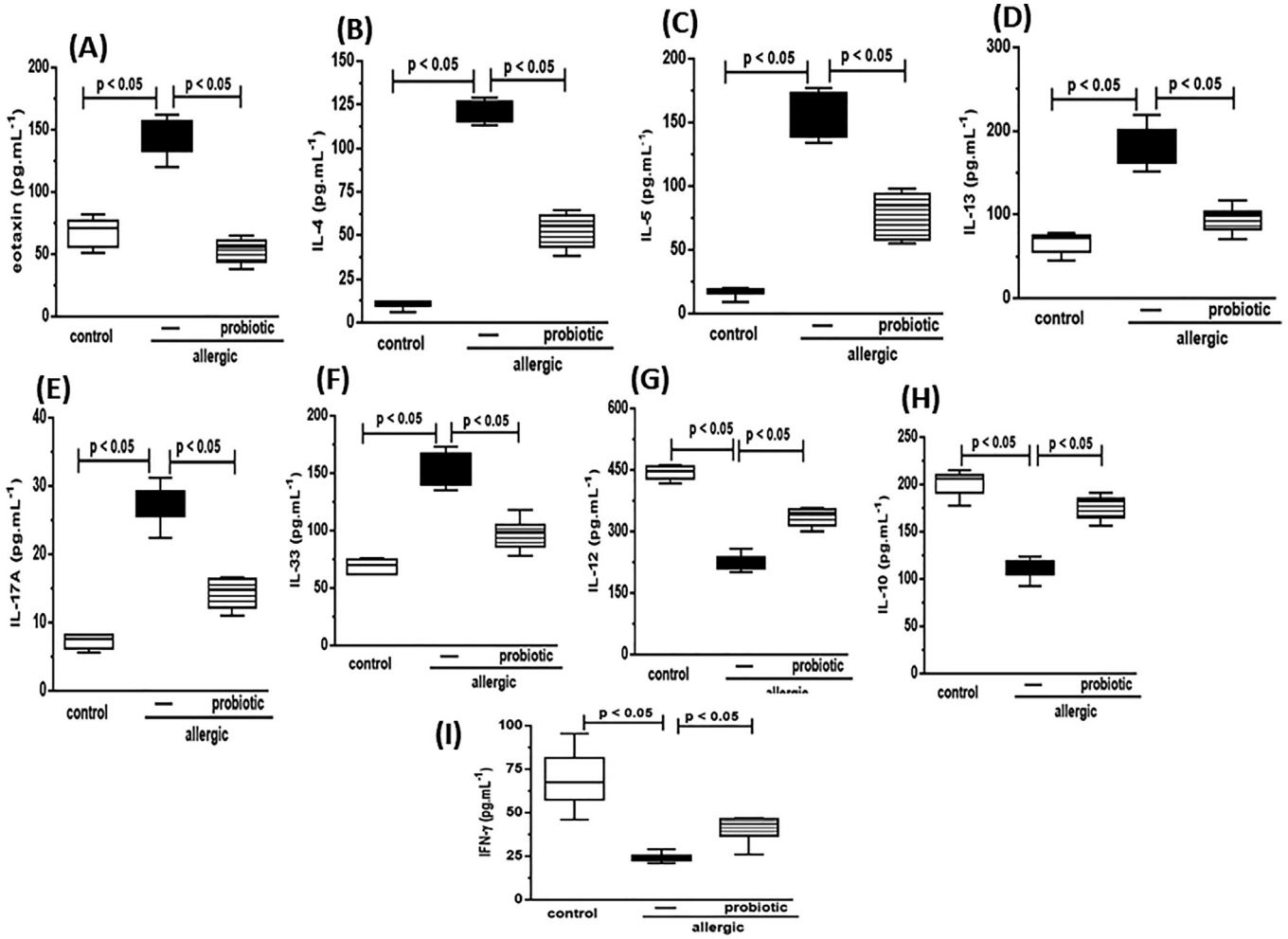

Fig. 6. *L. bulgaricus* N45.10 on cytokines levels in BALF. BALF obtained from control, allergic, and *Lb* + allergic groups were prepared for analysis of pro- and anti-inflammatory cytokines. Mice received an oral dose of *Lb* (1×10^9 CFU/0.2 mL PBS/mouse/three-times/week) initiating one week before primary sensitization until day 28 of the challenge. Control and allergic groups received PBS as treatment. Eotaxin (A), IL-4 (B), IL-5 (C), IL-13 (D), IL-17 (E), IL-33 (F), IL-12 (G), IL-10 (H), and IFN-γ (I) were assayed by enzyme-linked immunosorbent assay (ELISA). The cytokines levels in BALF were assayed 24 h after the last OVA challenge. Each bar represents mean ± SEM from 7 different animals. Results were considered significant when $p < 0.05$.

ACAGCAACCTT-3′ and reverse primer 5′-TTCTCACAACCAGGCCAC TTG-3′. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-CTCTACCCACGGCAAGTTCAA-3′ and reverse 5′-GGGATG ACCTTGCCCACAGC-3′, were used as control. Quantitative values for transcription factors and GAPDH mRNA transcription were obtained from the threshold cycle number, where the increase in the signal associated with an exponential growth of PCR products begins to be detected. Melting curves were generated at the end of every run to ensure product uniformity. The relative target gene expression level was normalized based on GAPDH expression as endogenous RNA control. ΔCt values of the samples were determined by subtracting the average Ct value of STAT6, GATA-3, RORγt, *T*-bet, or foxp3 mRNA from the average Ct value of the internal control GAPDH. As it is uncommon to use ΔC_t as relative data due to this logarithmic characteristic, the $2^{-\Delta^{Ct}}$ parameter was used to express the relative expression data. Results are expressed as a ratio relative to the sum of GAPDH transcript level as internal control.

2.11. Immunohistochemical for STAT6 and T-bet

For immunohistochemistry analysis, the paraffin-embedded sections of lung tissues were deparaffinized with xylene and then rehydrated. Section slides were incubated with 3% hydrogen peroxide for 10 min and then in 5% BSA in PBS blocking solution for 20 min, and then incubated with anti-STAT6 or anti-*T*-bet antibody (Cell signaling Technology) in blocking solution overnight at 4 °C. After washing with PBS, the slides were treated with biotinylated secondary antibody for 20 min, streptavidin-HRP (horseradish peroxidase) for 20 min, and 3,3N-Diaminobenzidine Tertrahydrochloride for 10 min. The slides were then washed, and counter stained with hematoxylin. Slides were evaluated by microscopy, and the positive cells exhibited yellow or brown particles.

2.12. Activation of dendritic cells and expression of Toll-like receptors

Lung cells after being lysed were washed in PBS/0.1% BSA buffer, and staining was done using a permeabilization buffer (PBS/0.1% BSA/0.1% saponin) to detect intracellular expression, followed by incubation with the antibodies: anti-CD11c APC-conjugated; anti-MHCII PeCy7-conjugated; anti-CD80 FITC-conjugated; anti-CD86 PE-conjugated; anti-TLR1, TLR2, and TLR6 PE-conjugated; and anti-TLR4, TLR5, and TLR9 FIT-conjugated. All the antibodies were supplied by BD Biosciences and eBiosciences (San Diego, CA, USA). Fluorescence was measured on a FACSCanto reading II (Becton Dickson®, San Jose, CA, USA) obtained in 100,000 events/sample, and analyses were performed using the Flow Jo software (TreeStar, Ashland, OR, USA). The percentage of each antibody specific stained subpopulation obtained from five different animals per group was analyzed on dendritic cells ($CD11c^+$

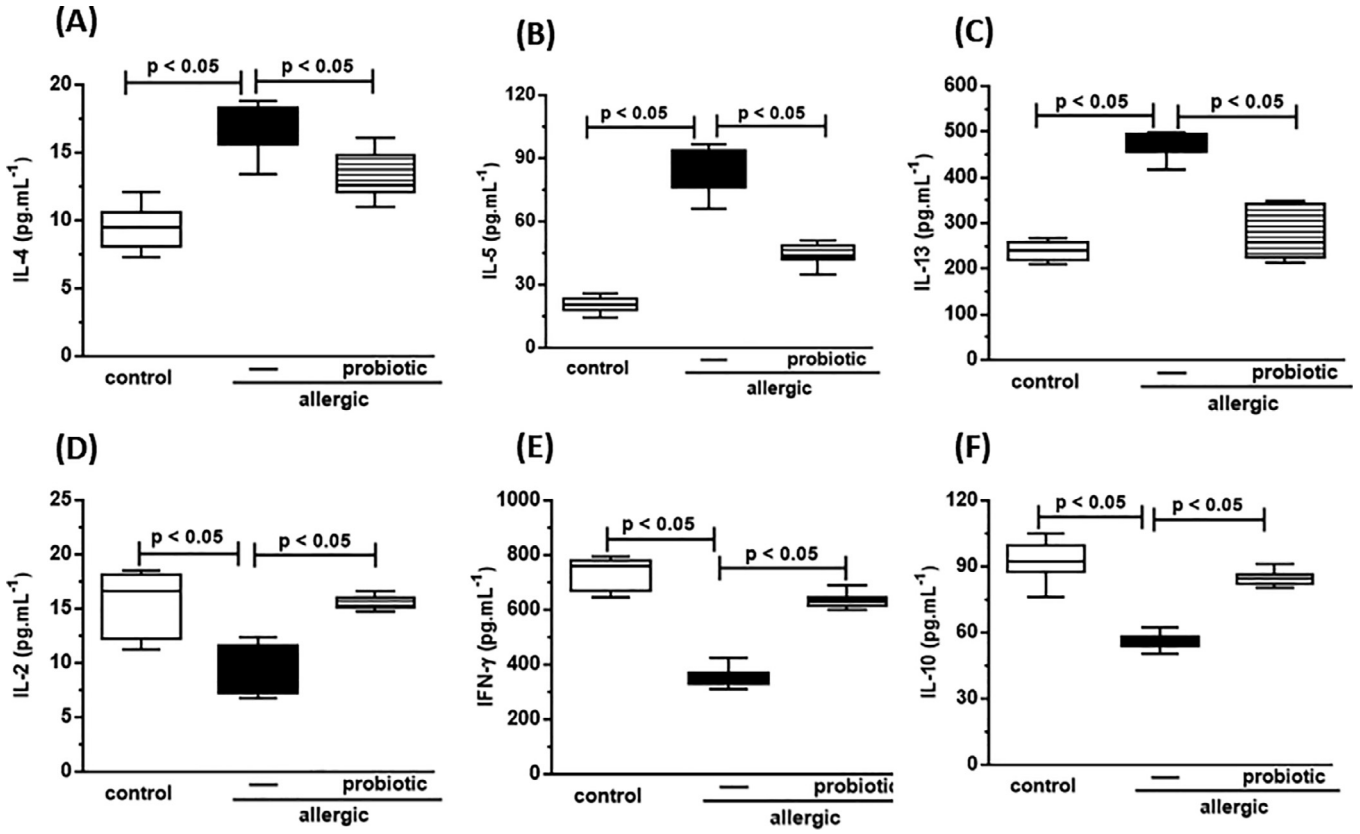

Fig. 7. *L. bulgaricus* N45.10 on cytokines levels in serum. Serum obtained from control, allergic, and *Lb* + allergic groups was prepared for analysis of pro- and anti-inflammatory cytokines. Mice received an oral dose of *Lb* (1×10^9 CFU/0.2 mL PBS/mouse/three-times/week) initiating one week before primary sensitization until day 28 of the challenge. Control and allergic groups received PBS as treatment. IL-4 (A), IL-5 (B), IL-13 (C), IL-2 (D), INF-γ (E), and IL-10 (F) were assayed by enzyme-linked immunosorbent assay (ELISA). The cytokines levels in serum were assayed 24 h after the last OVA challenge. Each bar represents mean ± SEM from 7 different animals. Results were considered significant when $p < 0.05$.

MHCII$^+$) and gated based on their characteristic size (FSC) and granularity (SSC).

2.13. Statistical analysis

Statistical significance was analyzed using PRISM 5 (GraphPad Software, La Jolla, CA, USA). Values for all measurements are expressed as mean ± SEM, and the *p* values for significance were set to 0.05. Differences were tested using the unpaired Student *t* test or the Mann-Whitney test for samples with nonparametric distributions.

3. Results

3.1. Lung inflammation and airway remodeling.

Eosinophils-rich leukocytes infiltration plays a key role in allergic diseases such as asthma. The effect of *Lb* on airway inflammation determined using the differential cell counts revealed that the total cells, macrophages, eosinophils, neutrophils, and lymphocytes (Fig. 2) were significantly increased in the allergic group compared to the control group (non-sensitized and non-challenged with OVA mice). However, the lung infiltrating cells were significantly decreased in the allergic group that received *Lb*. Given that *Lb* inhibits eosinophils-rich leukocytes infiltration into the BALF, we evaluated whether *Lb* can impose the anti-inflammatory effect on lung tissue. Histological alterations in lung parenchyma and peribronchiolar region were observed in the allergic group and were partially reverted after *Lb* administration (Fig. 3A). In fact, *Lb* administration reduced eosinophilic inflammation (3B), appearance of peribronchial edema (3C), and index of bronchoconstriction (3D) induced by OVA challenge. In addition, the oral feeding with *Lb* in the control group had no effect on the infiltration of cells into lung and in all the parameters analyzed. The serum levels of total IgE (3E) and OVA-specific IgE (3F) were significantly augmented in the allergic group compared to the control group. However, in *Lb*-treated mice, the total IgE and OVA-specific IgE in serum was significantly decreased compared to the allergic group. The increase in mucus secretion in lungs of the allergic group was reversed by *Lb* (Fig. 4A). The same result can be observed in the secretion and deposition of collagen in the airway wall (4B). No significant differences were observed among the control and the *Lb* groups.

3.2. LTC_4 and PGD_2 in BALF

Airway inflammation is associated with activation of mast cell which is capable to secrete high levels of lipid inflammatory mediators such as LTC_4 and PGD_2. To investigate the effect of *Lb* on these lipidic mediators, the levels of LTC4 and PGD2 were measured in BALF by ELISA 24 h after the last OVA challenge. Fig. 5 illustrates that the antigenic challenge produces an increase of both the LTC4 (5A) and PGD2 (5B) concentration when compared to control group. In addition, the concentration of theses eicosanoids in BALF from allergic mice reduced after *Lb* treatment in comparison with asthmatic mice. No significant differences were observed among LTC_4 and PGD_2 levels from the control and *Lb* groups.

3.3. Th1/Th2/Th17/Treg in BALF and serum

The imbalance between cytokines from different lymphocytic responses plays a pivotal role in development and perpetuation of allergic asthma symptoms. To investigate if *Lb* can positively interfere to protect

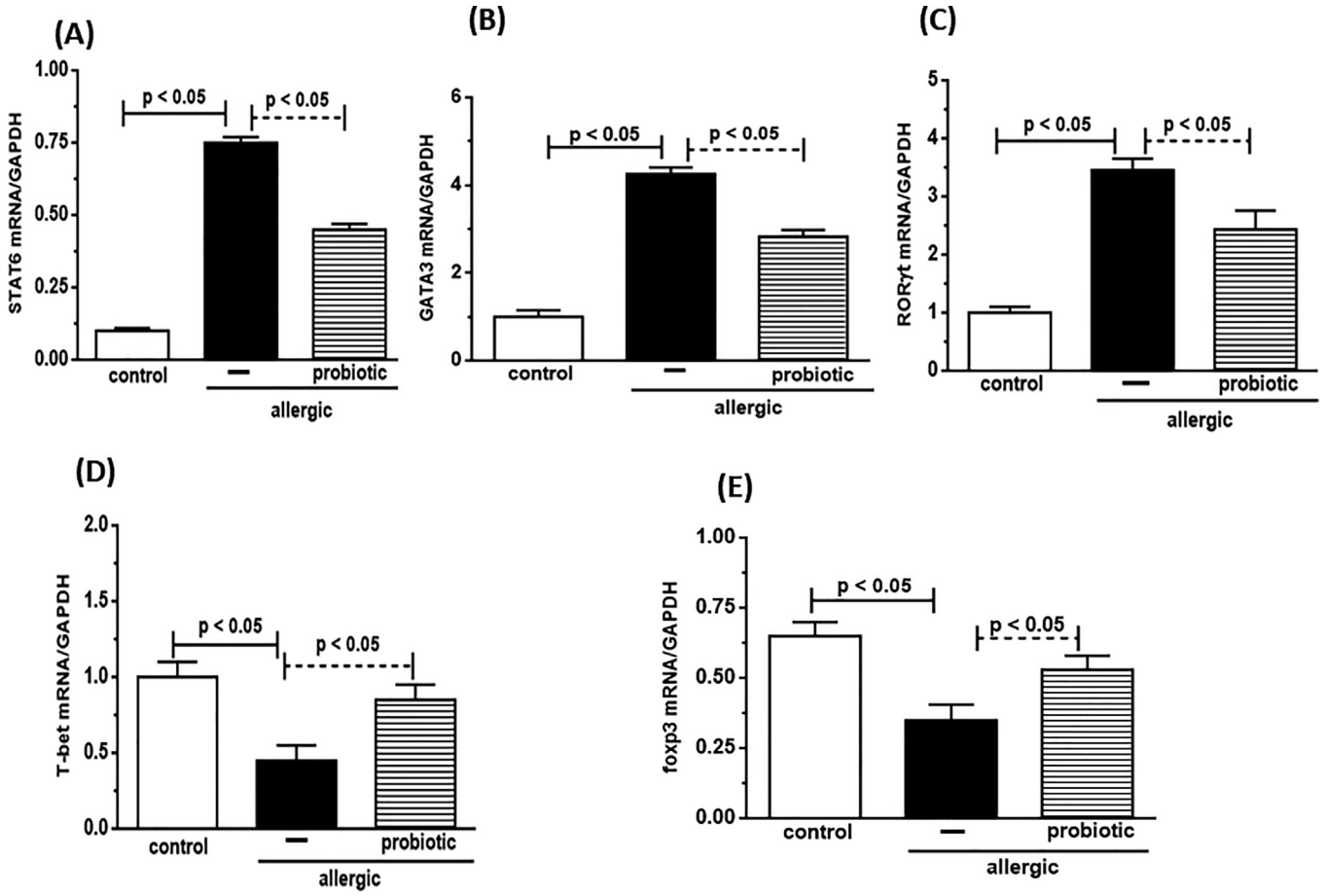

Fig. 8. *L. bulgaricus* N45.10 on transcription factors expression in lung. The mRNA expression of the STAT6, GATA-3, RORγt, foxp3, and *T*-bet in lung from the control, allergic, and *Lb* + allergic groups is illustrated. Mice received an oral dose of *Lb* (1×10^9 CFU/0.2 mL PBS/mouse/three-times/week) initiating one week before primary sensitization until day 28 of the challenge. Control and allergic groups received PBS as treatment. The mRNA expression for STAT6 (A), GATA-3 (B), RORγt (C), *T*-bet (D), and foxp3 (E) in lung tissue were evaluated through Real Time-PCR 24 h after the last OVA challenge. Each bar represents mean ± SEM from 7 different animals. Results were considered significant when $p < 0.05$.

the allergic mice of oscillation of these chemokines, we evaluated the *Lb* effect on secretion of Th1, Th2, and Th17 cytokines in BALF and serum. Fig. 6 represents the increase of cytokines concentration eotaxin (6A), IL-4 (6B), IL-5 (6C), IL-13 (6D), IL-17A (6E), and IL-33 (6F) in BALF from allergic mice in comparison with control group. Inversely, the antigenic challenge decreased the cytokines concentration IL-12 (6G), IL-10 (6H), and IFN-γ (6I) *in* BALF from allergic mice compared to control group. Fig. 6 also illustrates the that *Lb* decreased these cytokines, including IL-4, IL-5, IL-13, IL-17A, IL-33, and eotaxin levels in BALF from allergic mice compared to allergic mice not treated with *Lb*; however, *Lb* reversed the fall of IL-12, IL-10, and IFN-γ concentration in BALF from allergic mice. In serum (Fig. 7), the cytokines concentration IL-4 (7A), IL-5 (7B), and IL-13 (7C) presented higher levels in allergic mice than in control group. Oppositely, the antigenic challenge reduced cytokines level *IL-2* (7D), IFN-γ (7E), and IL-10 (7F) *in serum* compared to control group. No significant differences in the cytokines and chemokines levels were observed among the control and *Lb* groups.

3.4. mRNA of transcription factors in lung

An important cellular correlation involving signaling proteins and transcription factors is responsible for the synthesis of chemical mediators capable of modulating the inflammatory response in allergic asthma. Thus, we investigated if the action mechanism of *Lb* can interfere with asthma-related transcription factors. Fig. 8 represents the antigenic challenge effect with OVA on mRNA expression of STAT6 (8A), GATA-3 (8B), RORγt (8C), *T*-bet (8D), and foxp3 (8E). The mRNA expression for STAT6, GATA-3, as well as RORγt in lung tissue from allergic mice increased compared to control group. On the contrary, the mRNA expression of *T*-bet and foxp3 was markedly decreased after antigenic challenge (allergic group). When the allergic animals were treated with *Lb*, the expression of transcription factors STAT6 (8A), GATA-3 (8B), and RORγt (8C) were significantly reduced in comparison with allergic mice that did not receive *Lb*. Otherwise, the *Lb* restored the mRNA expression for *T*-bet (8D) and foxp3 (8E) to values close to those found in the control group. No significant differences in transcription factor mRNA expression were observed among the control and the *Lb* groups.

3.5. STAT6 and T-bet proteins in lung

Fig. 9 illustrates the lung localization of STAT6 and *T*-bet in mice of the control, allergic, and *Lr*-treated allergic groups. Lung localization of both STAT6 and *T*-bet marked with immunohistochemical staining are represented in (9A) and (9B), respectively. The presence of STAT6 and *T*-bet in allergic mice lungs was upregulated and downregulated, respectively, when compared to the control groups; however, in *Lb*-treated allergic mice the immunohistochemical stain illustrated that STAT6 and *T*-bet were both partially restored to values close to respective control groups. No significant differences in STAT6 and *T*-bet proteins were observed between the control and the *Lb* groups.

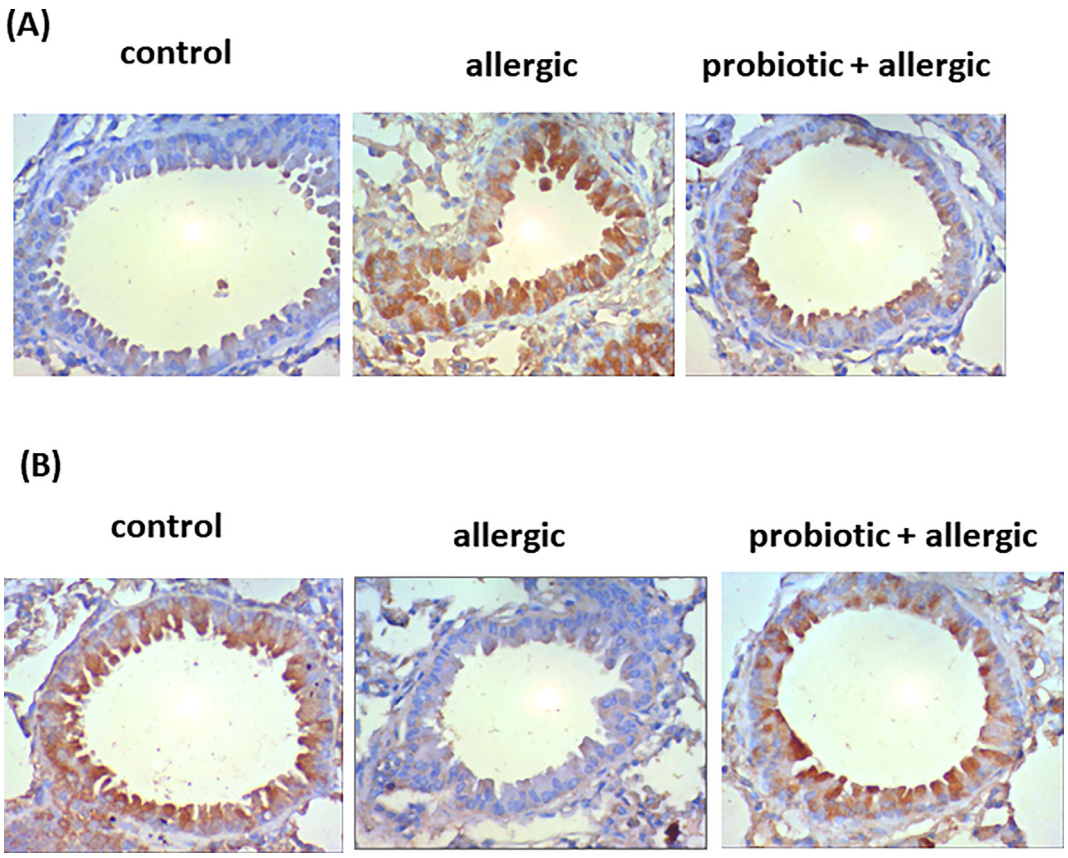

Fig. 9. *L. bulgaricus* N45.10 on STAT6 and *T*-bet protein in lung. The changes on STAT6 (A) and *T*-bet (B) proteins in mice lung among the experimental groups (control, allergic, and *Lb* + allergic) were determined 24 h after the last challenge and are illustrated here. Mice received an oral dose of *Lb* (1×10^9 CFU/0.2 mL PBS/mouse/three-times/week) initiating one week before primary sensitization until day 28 of the challenge. Control and allergic groups received PBS as treatment. For immunohistochemical localization of STAT6 and *T*-bet in lung, the positive reaction was visualized as a yellowish-brown stain. Each bar represents the mean ± SE from 7 different animals. Results were considered significant when $p < 0.05$.

3.6. Activation of dendritic cells in lung.

Dendritic cells activated by probiotic can migrate from GALT to lung tissue to polarize naïve T lymphocytes into lung environment and to shift the immune response to a suitable lymphocytic response to the pathological condition [22]. Fig. 10 illustrates that the antigenic challenge increased the activation of dendritic cells via upregulation of CD80 (10B) and CD86 (10C) in lung tissue. The oral feeding of allergic mice with Lb upregulated the CD86 activation (10C) to values higher than those found in allergic group. No significant differences in activation of dendritic cells were observed among the control and the Lb groups.

3.7. Expression of Toll-like receptors in dendritic cell

Activated Toll like receptor (TLR) in dendritic cells by probiotic induces secretion of cytokines with ability to attenuate the immune response. Thus, we investigated if *Lb* can interfere in aspects of innate immunity such the TLR expression in dendritic cells from lung tissue of allergic group. Fig. 11 illustrates that among the TLR studies, the expression of TLR1 (11A), TLR4 (11C), and intracellular TLR9 (11D) increased in allergic mice compared to control group, except TLR2 (11B). On the contrary, the treatment with *Lb* reduced the TLR4 (11C) expression in dendritic cells from allergic mice in comparison with allergic group. *Lb* did not alter the expression of all other TLR studied. No significant differences in TLR expression were observed among the control and the *Lb* groups.

4. Discussion

No therapy able is currently available to heal the symptoms of allergic asthma. Current therapies can control the inflammatory process characteristic of asthma, but the onset of immune activation against the antigen and the individuals' predisposition cannot be controlled [31]. The use of corticoids is the main therapy for asthmatic patients. However, although corticosteroids are efficient if used in high doses or for extended periods of time, they have many side effects that increase the treatment cost. In addition, some patients have resistance to the therapeutic effects of corticosteroid, and high doses of corticoids results in an increased risk of systemic side effects [32,33]. For patients with these problems, there is an urgent need to develop new anti-inflammatory therapies with immunomodulatory activity to provide alternative asthma treatments.

We investigated whether *L. bulgaricus N45.10* (*Lb*) can suppress airway inflammation and cytokines secretion during development of mouse asthma model through of allergic asthma-related transcription factor and Th cytokines.

Our results found that after oral feeding with *Lb* the inflammatory cells migration into bronchi, principally eosinophils and lymphocytes in the BALF were reduced. These results seem to be associated with the decrease of both the Th2 immune response and the eotaxin production. In fact, some authors have evidenced that eosinophil recruitment in allergic reactions is regulated through eotaxin production via secretion of Th2 cytokines which guarantee the maintenance of allergic disease [34]. Hence, our results evidenced that the treatment with *Lb* reduced the cytokines secretion from Th2 and Th17 axis while simultaneously increasing the secretion of IL-10, IL-12, and IFN-γ in BALF from allergic

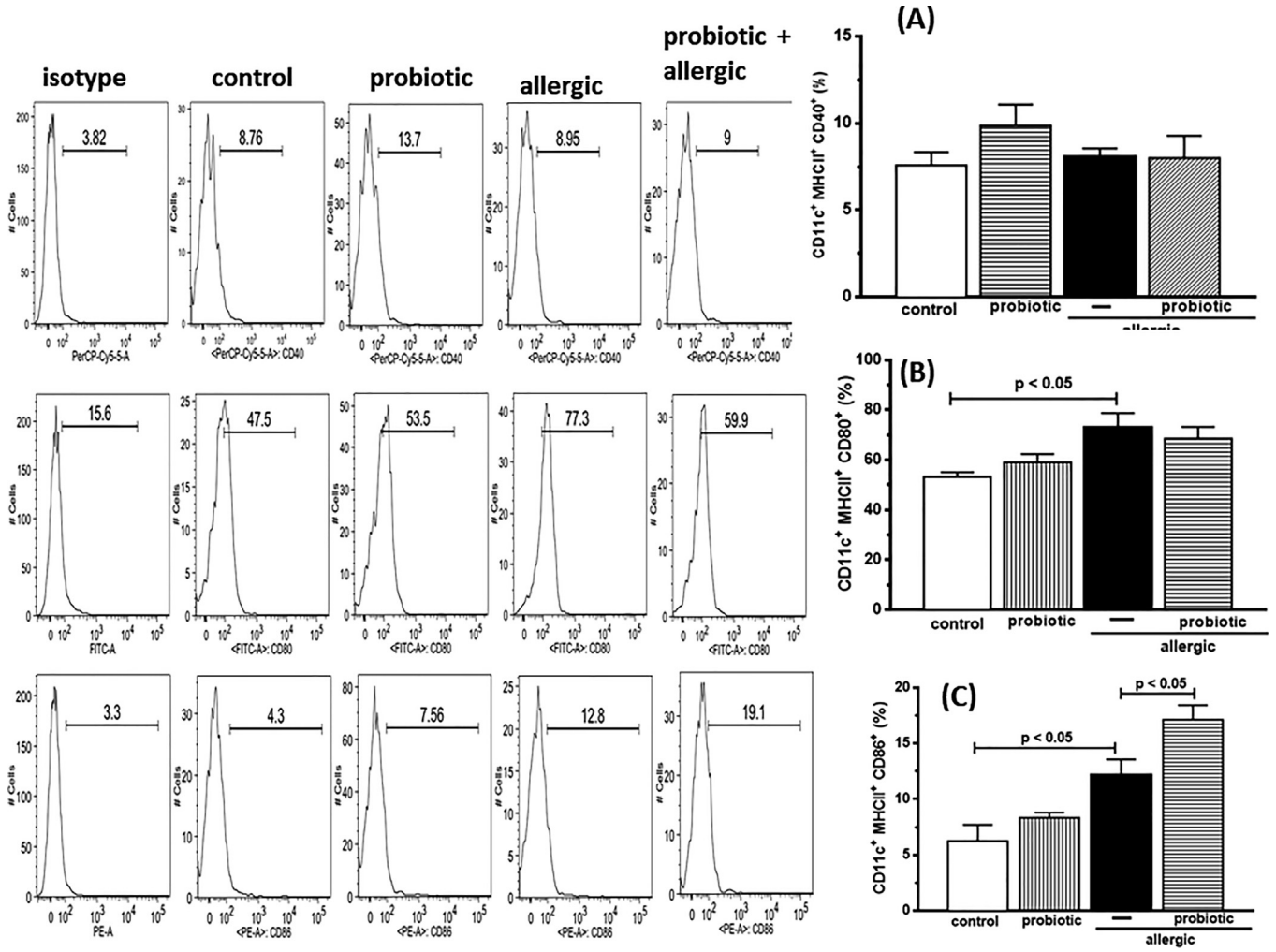

Fig. 10. *L. bulgaricus* N45.10 on dendritic cells activation. Lung cells were collected from control, allergic, and *Lb* + allergic groups. Mice received an oral dose of *Lb* (1×10^9 CFU/0.2 mL PBS/mouse/three-times/week) initiating one week before primary sensitization until day 28 of the challenge. Control and allergic groups received PBS as treatment. Cells were labeled with the indicated antibody and analysed by flow cytometry. Data are represented as the percentage of CD11c + cells expressing (A) CD80, (B) CD40, or (C) MHCII. Each bar represents mean ± SEM from 7 different animals. Results were considered significant when $p < 0.05$.

mice. Our results indicate that the *Lb* anti-inflammatory effect is closely related to modulation of transcription factors related to allergic asthma, which are STAT6, GATA-3, RORγt, *T*-bet, and foxp3.

Another important effect of *Lb* presented herein is the reduction of serum IgE concentration in mice challenged with OVA. This result suggests that the *Lb* can attenuate the allergic immune response because the antigen recognition phase was associated with the increased concentrations of IgE. Therefore, the *Lb* seems to exert a systemic effect on allergic asthma, although it is recognized into gut-associated lymphoid tissue (GALT). Other authors have also found that IgE and Th2 cytokines were reduced in the presence of probiotics in murine model of allergic asthma [35–37]. It is worth highlighting that *Lb* also influenced the secretion of both the cytokines in serum, which reinforces the idea that probiotic effect can spread systemically to control the immune disorder in asthma allergic. Despite the importance of the Th2 immune response in allergic asthma, other inflammatory mediators, such as IL-33 secreted by epithelial cells, can induce airway eosinophilia, production of Th2 cytokines, and airway inflammation in models of allergic disease [38,39]. Our results indicated that *Lb* also interfered in IL-33 secretion from inflammatory cells into BALF from allergic mice.

The reduction of IL-33 secretion into BALF from allergic mice after treatment with *Lb* suggests that the studied probiotic can interfere with the secretion from epithelial cells. In asthma, IL-33 is secreted principally by epithelial cells, which in turn activate lymphoid cells with the ability to secrete IL-4 and IL-13 [40]. This suggests that the immunomodulatory effect of *Lb* in the allergic asthma can be at least in part mediated by downregulating the IL-33 secretion from epithelial cells.

In asthma, overproduction of the lipidic mediators stimulate the release of Th2 cytokines and decrease the Th1 cytokines expressions accompanied by migration of eosinophils and lymphocytes in the lung [41,42]. Our results evidenced that the anti-inflammatory effect of *Lb* is also driven to lipid mediators PGD_2 and LTC_4. The PGD_2 and LTC_4 are eicosanoids derived from lipooxygenase metabolism; thus, this pathway might be involved in anti-asthma effect of *Lb*. These results suggest that after recognition of *Lb* by immune cells, for example dendritic cells, the immunomodulatory effect of *Lb* may at least interfere with different signaling pathways that control the allergic asthma.

Our results found that both the dendritic cells activation and the TLR4 expression in these cells were increased in allergic mice. For *Lb* effect in dendritic cell activation in lung, our results showed that the oral feeding with *Lb* increased the activation dendritic cell to values higher than those from allergic mice. On the contrary, the *Lb* reduced the expression of TLR4 in dendritic cells in lung from allergic mice. In the present manuscript, although *Lb* reduced the TLR4 expression in dendritic cells in lung from allergic mice, the expected immunomodulatory effect would be an increase in TLR4 expression in dendritic cells because the Th2-dependent allergic response observed in

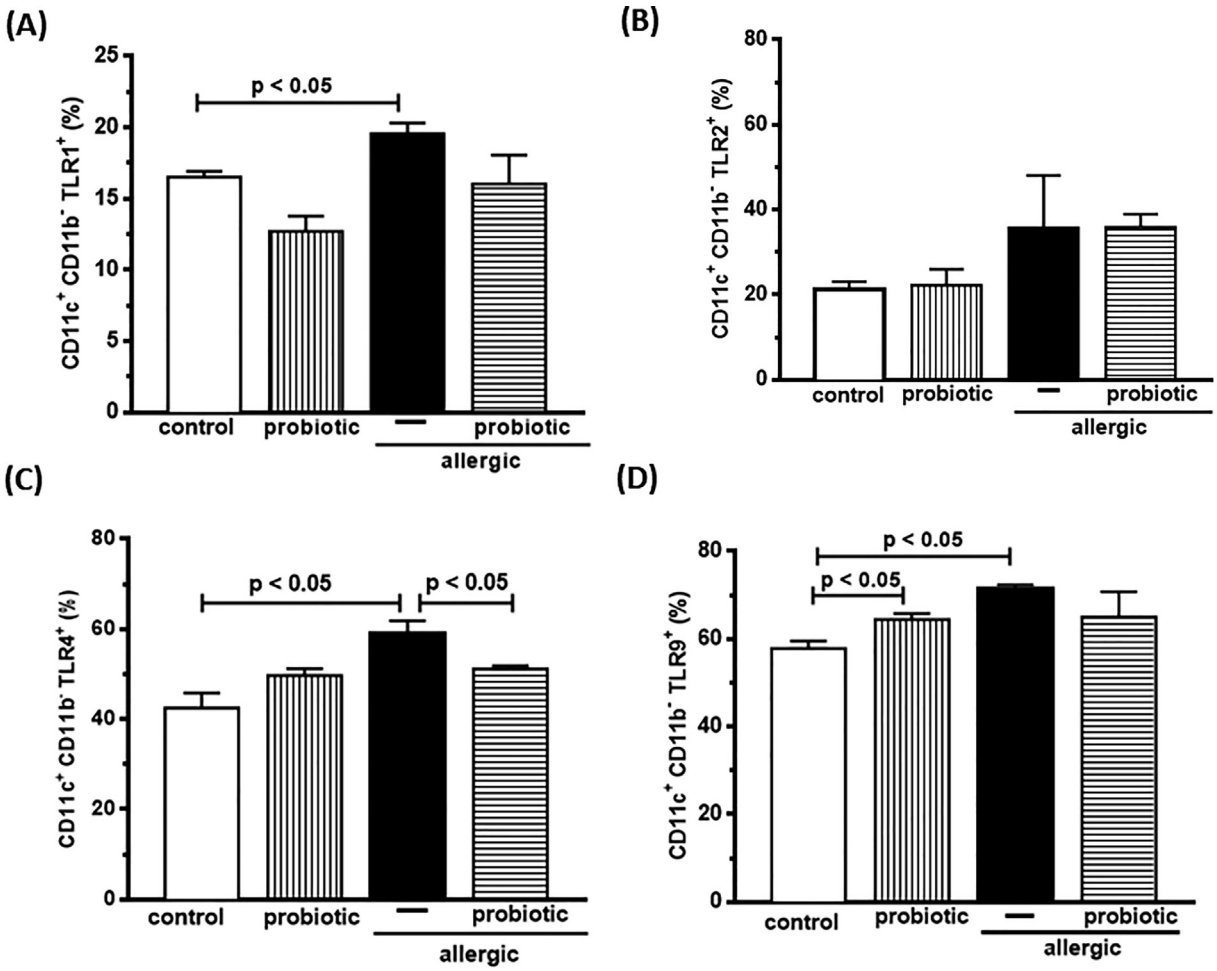

Fig. 11. *L. bulgaricus* N45.10 suppressed the Toll-like receptors expression. Lung cells were collected from control, allergic, and *Lb* + allergic groups. Mice received an oral dose of *Lb* (1×10^9 CFU/0.2 mL PBS/mouse/three-times/week) initiating one week before primary sensitization until day 28 of the challenge. Control and allergic groups received PBS as treatment. Cells were labeled with the indicated antibody and analysed by flow cytometry. Data are represented as the percentage of (A) TLR1, (B) TLR2, (C) TLR4 and (D) TLR9. Each bar represents mean ± SEM from 7 different animals. Results were considered significant when $p < 0.05$.

asthma could be attenuated through expression of TLR4 with consequent secretion of cytokines associated to Th1 response**.** However, some authors have evidenced that TLR4 plays an important role in exacerbation of allergic airway inflammation and production of serum IgE [27–29]. The results described in the present manuscript showed an important increase of TLR4 expression in dendritic cells into lung from allergic mice, which was reduced by *Lb*. Therefore, our results suggest that the oral feeding with *Lb* attenuates the Th2 response in allergic mice by reducing the TLR4 expression in dendritic cells.

Considering that the *Lb* is not translocated to the lung, it is reasonable to suggest that dendritic cells migrated from the gut to the lung already activated with the TLR4 expression increased. However, our results did not determine if the dendritic cells with increased TLR4 expression originate from GALT or were activated by metabolites of *Lb* into lung environment. This question does not compromise the results in the present study that evidences the *Lb* immunomodulatory effect in lung from allergic mice; however, it could contribute to the comprehension of *Lb* action mechanism and the cellular target preferred by probiotic. Other studies should better investigate the mechanism of *Lb* on different populations of immune cells.

The imbalances in Th1/Th2 and Th17/Treg response were found in patients with allergic asthma [5]. Our results also demonstrated that chemokines and cytokines from Th1/Th2 and Th17/Treg were also found imbalanced in allergic mice. On the other hand, we found that *Lb* downregulates the Th2 response (GATA-3, STAT-6, IL-3, IL-4, IL-5, IL-13 and IL-33) and up-regulates the expression of Th1 response (*T*-bet, IFN-γ, IL-10 and IL-12).

It is well known that the STAT6 and the GATA-3 play essential roles in the exacerbation and perpetuation of the allergic inflammatory response [43–45]. We found that *Lb* significantly reduced RNA expression for both transcription factors in lung tissue from allergic mice as well as the concentration of cytokines with Th2 profile. These results suggest that the anti-inflammatory effect of the *Lb* on Th2 cytokines in lung could be due to a reduction of both the STAT6 and the GATA-3 mRNA expression. The IL-4/IL-13/STAT6 pathway is strongly upregulated in detriment to the significant reduction of *T*-bet protein concentration in the lung of allergic individuals [46]. It is worth pointing that *T*-bet cause the tissue-specific expression of Th1 cytokines and suppress the GATA-3 which control expression of Th2 cytokines [47]. In the present study, the pretreatment with *Lb* increased the *T*-bet protein concentration in allergic mice. This indicates that *Lb* interferes in transcription as well as in translation processes to synthetize inflammatory proteins with the goal of ensuring that the immune response can be restored to values closer to the normal level.

The interventions that inhibit the Th2 response by enhancing both the Th1 and Treg response may be useful for allergic asthma treatment. Tregs play a key role in balancing immune responses, and it has been demonstrated that an increased expression of foxp3 is directly associated with augmented suppressive function of these cells [48]. Herein, the pretreatment with *Lb* induced the foxp3 mRNA expression and IL-10 production in lung of allergic animals, suggesting that *Lb* also stimulates Treg response.

It has been reported that Tregs suppress not only the Th2 but also the Th17 responses [49]. IL-17 is essential to the neutrophilic influx into the airways and enhances the Th2 cell-mediated eosinophilic airway inflammation in asthma [50,51]. We also found that *Lb* inhibits the Th17 response as well as the RORγt mRNA expression. These findings indicate that *Lb* administration decreased IL-17 level by downregulation of the RORγt transcription factor.

Although animal studies have provided clear evidence that lactobacillus can have profound immunoregulatory effects and regulate immune responses beyond the GALT, the results of clinical trials have been highly variable [52,53]. Therefore, it is important to consider which strains of bacteria can produce the most efficient immune regulation in accordance with immunologic disorder. Further studies are needed to investigate whether a single strain of bacteria can control disease-specific characteristics or if probiotics are homeostasis regulators capable of inducing a regulatory immune response according to the milieu disease.

Taken together, our studies indicate that the oral feeding with *Lb* modulates the host immune response by preventing the Th2 polarization and Th17 cells while the *Lb* also increases the Th1 response by modulating both the STAT6 and the *T*-bet transcription factors. Finally, these data indicate that the *Lb* could be promising for the development of new strategies to prevent asthma development.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2019.103928.

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