

Enhanced expression of PD-L1 and IFN- γ on dendritic cells is associated with BCG-induced Th2 inhibition



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ABSTRACT

Accumulating evidence indicates that the exposure to *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) prevents the development of allergy and the airway dendritic cells (DCs) may be involved in this protective effect. However, studies to better characterize the specific interactions between BCG and DCs and their role in this mycobacteria-mediated Th2 cell suppression are still ongoing. This study aimed to evaluate the effect of the neonatal BCG vaccination in the innate immune response in a mouse model of ovalbumin (OVA)-induced airway inflammation. BCG treated neonatal BALB/c mice were sensitized and challenged with aerosolized OVA. Twenty-four hours after the last challenge, samples were collected for analysis. The intranasal BCG treatment inhibited the allergic Th2-response by decreasing the allergen-induced eosinophilic inflammation, EPO activity, CCL11, IL-25, TSLP, IL-4 and IL-5 lung levels, and serum levels of IgE. Mycobacteria treatment increased lung levels of IL-10 and TGF- β , and the TLR2 and TLR4 expressions by pulmonary CD11c⁺CD103⁺CD8 α ⁺ DCs. Additionally an enhanced expression of PD-L1 was observed besides an increased production of IFN- γ by these cells. These results indicated that neonatal BCG vaccination inhibits key features of allergic airway inflammation, probably by promoting T regulatory immune response via an enhanced expression of TLR2, TLR4 and PD-L1 on DCs.

1. Introduction

Atopic asthma is a potentially life-threatening chronic respiratory disease characterized by episodes of reversible airway narrowing, bronchial hyperresponsiveness, mucus over-production and airway inflammation associated with an increase of T helper 2 (Th2)-type cytokines and IgE synthesis [1,2]. The asthma incidence is increasing leading to major health and economic consequences [3]. Although conventional pharmacological therapies are highly effective for controlling disease in most individuals, many patients have concerns about side-effects and treatment may not be effective in cases of difficult-to-control asthma [4]. In addition, such interventions provide short-term relief from disease symptoms, not altering the underlying immune response to an allergen. Therefore, there remains a clear need for new strategies that have specific and long-lasting effects for the treatment of asthma [5].

The hygiene hypothesis postulates that reduced exposure to microorganisms should increase immune reactivity, favoring the

development of allergic diseases [6]. Even though the original concept has been modified [7], the hypothesis that microbial experience modulates our immune systems has been strengthened by solid epidemiological [8,9] and experimental data [10,11]. In keeping with these findings there have been extensive efforts to subvert the Th2 immune response by treatment with microbial products, paving the way for future therapies [12]. In this context, a number of studies have shown that *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) treatment is capable of ameliorating an established allergen-driven inflammatory response [13], probably resulting from an immune deviation from a pathogenic Th2 towards protective T helper 1 (Th1) and/or T regulatory (Treg) responses [13,14]. Although several models have been used to evaluate this mycobacteria effect, the immunological mechanisms by which BCG could reprogram effector T cell lineage commitment in allergic inflammation has not been clearly defined.

It is now well established that the development of a Th2-response is fundamentally influenced by a fine balance between the functions of innate immune and epithelial cells [15,16]. In this way, as a critical link

Abbreviations: BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; DC, dendritic cells; HE, hematoxylin and eosin; OVA, ovalbumin; PD, programmed death; PD-L, programmed death ligand; SR, sirius red; TLRs, toll-like receptors; TSLP, thymic stromal lymphopoietin; Treg, regulatory T cells

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in innate and adaptive immune responses, dendritic cells (DCs) have a key role in determining how allergic immune responses are initiated and perpetuated. Thus, it may be possible that these cells are mediating the immunomodulatory effects of BCG on allergy, since DCs interact with microbes using pattern recognition receptors [17], including toll-like receptors (TLRs), which play an important role in the pathogenesis of asthma [18–20]. Experimental studies have demonstrated that the recognition of mycobacterial components by TLRs on DCs, specifically TLR2, TLR4 and TLR9, has been implicated in a mycobacteria-induced protective effect [13,21], suggesting that the manipulation of these receptors by mycobacteria-derived molecules may be an approach to treat patients with asthma. Additionally, other pathways regulated by DCs, involved in the fine tuning of the T-cell activation and tolerance, would be influenced by mycobacteria. These pathways are provided by interactions between co-signaling molecules in DCs and surface molecules expressed on T lymphocytes [22]. Depending on the type of molecules involved, this interaction can promote the development of an inflammatory response or may favor immune regulation. The receptor programmed death-1 (PD-1, CD279) and its ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) deliver inhibitory signals that regulate the balance between T cell activation and tolerance [23,24]. A clear understanding of the PD-1/PD-L pathway could open new opportunities for treatment of asthma. Previous findings evidence that the function of PD-1 family members in asthma is complex, with some results suggesting a protective role [25–28] and others indicating a pro-asthmatic role for either PD-L1 [27] or PD-L2 [29]. In regards to mycobacteria it was previously reported that *Mycobacterium tuberculosis* infection induces development of CD4⁺CD25⁺FoxP3⁺ Treg cells through a process that depends on PD-1 axis [30,31], suggesting that PD-1/PD-L pathway might underlie the observed anti-allergic BCG effect. Thus, DCs may play a central role in mycobacteria-mediated inhibition of allergic asthma-like reactions. The present study aimed to evaluate the influence of BCG vaccination on TLR2, TLR4, TLR9, PD-L1 and PD-L2 expressions in pulmonary DCs, by using a neonatal BALB/c mouse model of ovalbumin (OVA)-induced airway inflammation to achieve insights that might improve current asthma therapies.

2. Materials and methods

2.1. Mice

BALB/c mice approximately 10 days old were obtained from Universidade Federal de Juiz de Fora (UFJF) animal care facility and were housed in microisolator cages with a 12 h light/dark cycle, receiving chow and water *ad libitum*. Animals were grouped according to treatment as follows (n = 5–8): (i) PBS (sensitizations and airway challenges with phosphate-buffered saline - PBS); (ii) BCG (sensitizations and airway challenges with PBS plus BCG treatment); (iii) OVA (sensitizations and airway challenges with OVA); (iv) OVA/BCG (sensitizations and airway challenges with OVA plus BCG treatment). All animal experimentation was in accordance with internationally accepted principles for laboratory animal use and care and was approved by the UFJF Ethics Committee on the use of laboratory animals (CEEA - UFJF No. 027/2008).

2.2. BCG vaccination and ovalbumin-induced airway inflammation

Mice were intranasally treated on day 0 with 25 µL of 2×10^5 CFU/mL (1×10^3 CFU/mice) of *Mycobacterium bovis* BCG (Moreau substrain, Ataulpho de Paiva Foundation, Rio de Janeiro, Brazil) or PBS. At 3 days after vaccination, mice were once sensitized intraperitoneally (i.p.) with 1.5 µg of OVA (Grade V, Sigma-Aldrich, USA) adsorbed to 250 µg of alum (INVIVOGEN, USA). Animals were challenged for 20 min with aerosolized OVA 1% or PBS (Inaltec Plus, Nevoni, Brazil) on days 16, 17, 23 and 24 (Fig. 1A). Twenty-four hours after the last OVA challenge mice were anesthetized i.p. with a solution of ketamine (90 mg/kg,

Syntec, Brazil) and xylazine (10 mg/kg, Calmion, Agener União, Brazil) and exsanguinated via the brachial plexus. Then the chest cavity was opened to remove the lung lobes.

2.3. Histological analysis

The right upper lung lobe of each animal was fixed in 10% buffered formalin and submitted to routine histologic processing. Tissue sections were stained with hematoxylin and eosin (HE), and sirius red (SR) (Sigma-Aldrich Corp., USA) and examined in blinded fashion on an optical microscope (Zeiss, Germany) at 100×, 400× and 1000× magnification. The peribronchiolar area was scored on a scale from 0 to 5: 0, no inflammation; 1, a few inflammatory cells; 2, a layer of inflammatory cells around the structure evaluated; 3, ring of inflammatory cells containing 2 to 4 layers; 4, focal inflammatory cells clustered around the structure; 5, intense inflammatory infiltrate [32]. Eosinophils were quantified per 100 µm² of the SR stained lungs, at 1000× magnification.

2.4. Eosinophil peroxidase activity

For determination of eosinophil peroxidase (EPO) activity 100 mg of lung tissue was homogenized in 1.9 mL PBS, and centrifuged at $12,000 \times g$ (Jouan, Thermo Electron Corporation, USA) for 10 min at 4 °C. The pellet was suspended in 1.9 mL of 0.5% HTAB in PBS. The samples were frozen three times in liquid nitrogen and centrifuged at $10,000 \times g$ for 10 min at 4 °C (Thermo Electron CR312, USA). Using a 96-well plate 100 µL of substrate solution (1.5 mL of o-phenyldiamine added to 8.5 mL of tris buffer plus 7.5 µL of H₂O₂) was added to 50 µL of each sample. After 30 min, the reaction was stopped with 50 µL of H₂SO₄ 1 M and the absorbance measured at 492 nm in a microplate reader (SPECTRAMAX 190, Molecular Devices, USA).

2.5. Measurement of cytokines and chemokine levels in lungs

In order to evaluate the levels of cytokines and CCL11 in the lung, 100 mg of tissue was homogenized in 1 mL of PBS containing protease inhibitors (0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 2 µL of aprotinin A) and 0.05% Tween 20. Samples were then centrifuged for 15 min at $500 \times g$ (Thermo Electron CR312, USA) and the levels of cytokines IL-4, IL-5, IL-10, TGF-β, IFN-γ and IL-12 (OptEIA, BD Bioscience – Pharmingen, USA), IL-25, TSLP and the chemokine CCL11 (R & D Systems, USA) were measured by ELISA following the manufacturer's instructions.

2.6. Measurement of anti-OVA-specific IgE, IgG1 and IgG2a antibodies

Quantification of anti-OVA-specific IgE, IgG1 and IgG2a antibodies was determined by ELISA. Briefly, a 96-well plate was sensitized with OVA (10 µg/mL), incubated for 18 h at 4 °C and then washed with 0.05% Tween 20-PBS (PBST) and blocked with 10% fetal bovine serum-PBST for 2 h at room temperature. The plate was washed with PBST and 50 µL of each serum (1:20 for IgE, and 1:100 dilutions for IgG1 and IgG2a) was added per well and incubated for 18 h at 4 °C and then washed. The biotin-conjugated rat anti-mouse IgE, IgG1 or IgG2a (1:1000; BD Bioscience – Pharmingen, USA) was added, and after 1 h of incubation at room temperature a substrate solution (0.1 M citric acid, 0.2 M sodium phosphate, distilled water, OPD, and 0.03% hydrogen peroxide) was added. The reaction was stopped with H₂SO₄ 1 M and the optical density measured at 492 nm in a microplate reader (Spectramax 190; Molecular Devices).

2.7. Cell staining and flow cytometry

The lungs were mashed through a 70 µm cell strainer and the red blood cells were lysed with ACK buffer and the cell suspension was

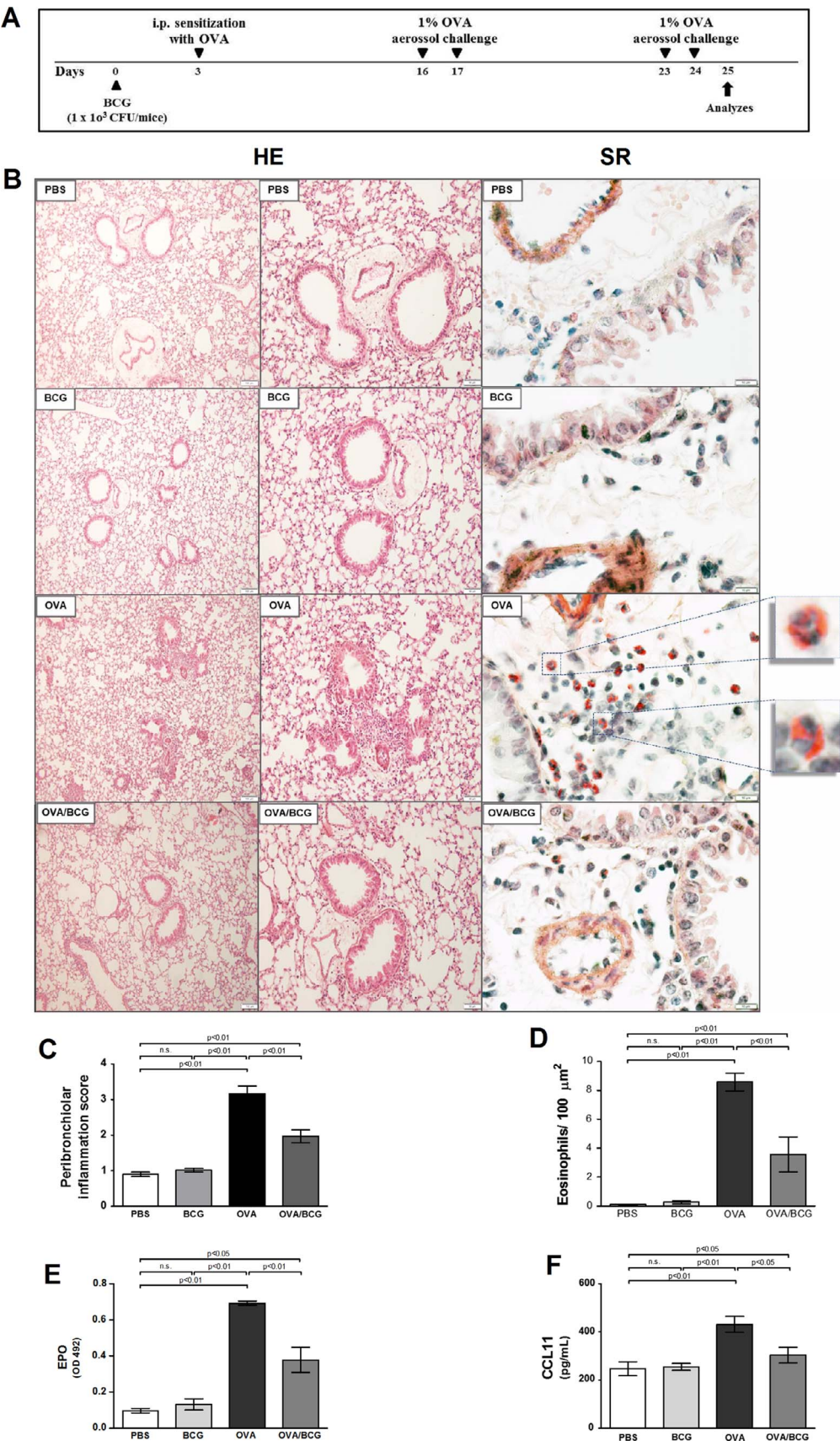


Fig. 1. BCG intranasal vaccination ameliorates parameters of allergic immune response. (A) Mouse model of OVA-induced airway inflammation and BCG vaccination. (B) Samples of lung parenchyma of BALB/c mice treated with phosphate-buffered saline (PBS), *M. bovis* BCG (BCG), ovalbumin (OVA) or ovalbumin plus BCG (OVA/BCG). Lung tissue analysis revealed that BCG vaccination reduced OVA-induced airway inflammation (hematoxylin-eosin – HE) and eosinophil infiltration (Sirius Red – SR). (C) Peribronchiolar inflammation score and (D) number of eosinophils in HE- and SR-stained lung sections, respectively. (E) Eosinophil peroxidase (EPO), and (F) CCL11 measurement in lungs. Five mice per group were analyzed and each image is representative of one group. Magnification of images: HE 100 $\times$ (left) and 400 $\times$ (right) and SR 1000 $\times$. Bars represent the mean $\pm$ SEM. OD indicates optical density.

washed twice in RPMI 1640 and adjusted to 1×10^6 cells per well. Cells were then stained with fluorescent-conjugated antibodies for cell surface markers, including Pacific Blue-anti-CD103 (BioLegend Inc,

USA), FITC-anti-CD11c, PERCP-anti-CD8 α , PE-anti-PD-L1 (CD274), -PD-L2 (CD273) and -TLR4 (BD Biosciences – Pharmingen, USA), PECy7-anti-MHCII and PE-anti-TLR2 (eBioscience, USA). For

intracellular cytokines detection cells were permeabilized and stained with PE-anti-IL-10, -IFN- γ , -IL-12 and -TLR9, (BD Biosciences – Pharmingen, USA). Data acquisition was performed using BD FACSanto™ and was analyzed using CellQuest® software (both Becton Dickinson, USA).

2.8. Statistical analysis

All statistical analyses were performed using Graph Pad prism 5.0 software (GraphPad Software, USA). Numerical data were analyzed by the normality test of Kolmogorov-Smirnov. Subsequently, the unpaired *t* test was used for parametric data and the Mann-Whitney test for nonparametric data. The significance level accepted for the tests was $p \leq 0.05$. Data are expressed as mean and standard error (mean $\pm$ SEM).

3. Results

3.1. BCG vaccination suppress the key features of the OVA-induced airway inflammation

In order to address whether neonatal BCG vaccination was able to reduce the OVA-induced airway inflammation, lung sections were inspected for an overall semiquantitative score of inflammation and eosinophil infiltration. Ovalbumin sensitization and subsequent airway challenge resulted in intense peribronchial and perivascular inflammatory infiltrates (mean score of 3.2, Fig. 1B and C) while the BCG vaccination had no effect on the lung of non-allergic mice (Fig. 1C). When compared with the OVA group, OVA/BCG group showed a reduced inflammatory cell infiltrate (mean score of 2.0, Fig. 1B and C) and significantly decreased eosinophilic infiltration (Fig. 1B), which was confirmed by morphometry analysis with eosinophil counts (Fig. 1D). Also, BCG vaccination significantly reduced the EPO activity (Fig. 1E) and CCL11 levels (Fig. 1F) in the lungs of the allergic mice, compared with the OVA group. Additionally ELISA analysis showed that the OVA group had significantly higher OVA-specific IgE and IgG1 serum levels compared with the PBS or BCG groups (Fig. 2A and B), indicating allergic immunization of these animals, whereas the improvement of airway inflammation by BCG vaccination was correlated with a strong suppression of the OVA-specific IgE levels in serum (Fig. 2A). In contrast, the OVA-specific IgG1 and IgG2a levels did not alter between the OVA and the OVA/BCG groups (Fig. 2B and C), probably due to the analysis were done only 25 days after the BCG vaccination, which might have been not sufficient enough to detect differences between these groups.

3.2. BCG vaccination reduces the levels of epithelial cytokines and drives a Th2 inflammatory response towards a T regulatory cytokine production

To assess the effect of neonatal BCG vaccination on the cytokine

secretion in the inflammatory site, cytokine analysis was performed by ELISA on lung homogenate samples. The levels of IL-25, TSLP, IL-4 and IL-5 were significantly higher in the OVA group than those in the PBS group, while there was a significant overall reduction of these cytokine levels following the BCG vaccination (Fig. 3A–D respectively), when compared with the OVA group. Also, the BCG-treated allergic mice exhibited greatly increased levels of IL-10 (Fig. 3E), TGF- β (Fig. 3F) and IL-12 (Fig. 3H). On the other hand, levels of IFN- γ remained similar in all four groups (Fig. 3G).

3.3. BCG vaccination upregulates TLR2 and TLR4 expression in CD8 α^+ pulmonary dendritic cells

To further characterize the mechanisms by which BCG affects airway allergic response, the expression of TLRs in pulmonary DCs was evaluated by flow cytometry. For this, in order to select mature DCs able to induce T lymphocyte proliferation, cells from processed lung were gated according to size and granularity (Fig. 4A – gate R1). Next, gated cells were selected in accordance with their expression of CD103 and CD11c (Fig. 4A – gate R2), and MHCII and CD8 α^+ markers (Fig. 4A – gate R3). Then, the expression of TLR2, TLR4 and TLR9 by CD103 $^+$ CD11c ^{md} MHCII $^+$ CD8 α^+ pulmonary cells, designated DCs CD8 α^+ , was assessed. Results indicated a decreasing in the expression of TLR2 by DCs CD8 α^+ from the OVA group, when compared with the PBS or BCG groups (Fig. 4B and C). Interestingly, this reduced expression of TLR2 was reverted in BCG-treated allergic mice (Fig. 4C), which also had a significantly large frequency of DCs CD8 α^+ expressing TLR4, when compared with the OVA group (Fig. 4B and D). In regard to the expression of TLR9, no statistically significant differences were found between BCG-treated and untreated allergic mice although it was slightly higher in the OVA/BCG group than those in the OVA group (Fig. 4B e E).

3.4. BCG vaccination induces increases in PD-L1 expression by CD8 α^+ pulmonary dendritic cells

Aimed to investigate whether the BCG-induced anti-inflammatory effect was associated with phenotypic modulation of pulmonary DCs, the frequency of DCs expressing PD-1 ligands was analyzed. Results demonstrated that OVA sensitization and subsequent airway challenge has exerted opposite effects on the expression of these ligands, since DCs CD8 α^+ from the OVA group showed a lower expression of PD-L1 (Fig. 5A and B), while PD-L2 expression was up-regulated in this group (Fig. 5A and C), when compared with the PBS or BCG groups. Intriguingly, neonatal BCG vaccination of allergic mice induced a significantly greater frequency of DC CD8 α^+ PD-L1 $^+$ than untreated allergic mice (Fig. 5A e B) and the PD-L2 expression had a slight reduction compared with the OVA group (Fig. 5A e C) although this difference was not statistically significant.

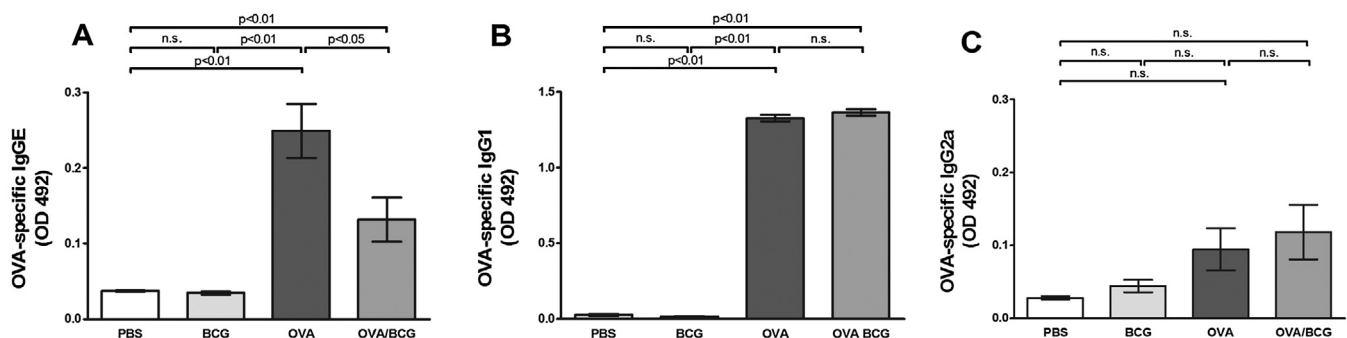


Fig. 2. Effect of BCG vaccination on (OVA)-specific IgE (A), IgG1 (B) and IgG2a (C) serum levels. Values represent means of five mice per group. Bars represent the mean $\pm$ SE. OD indicates optical density.

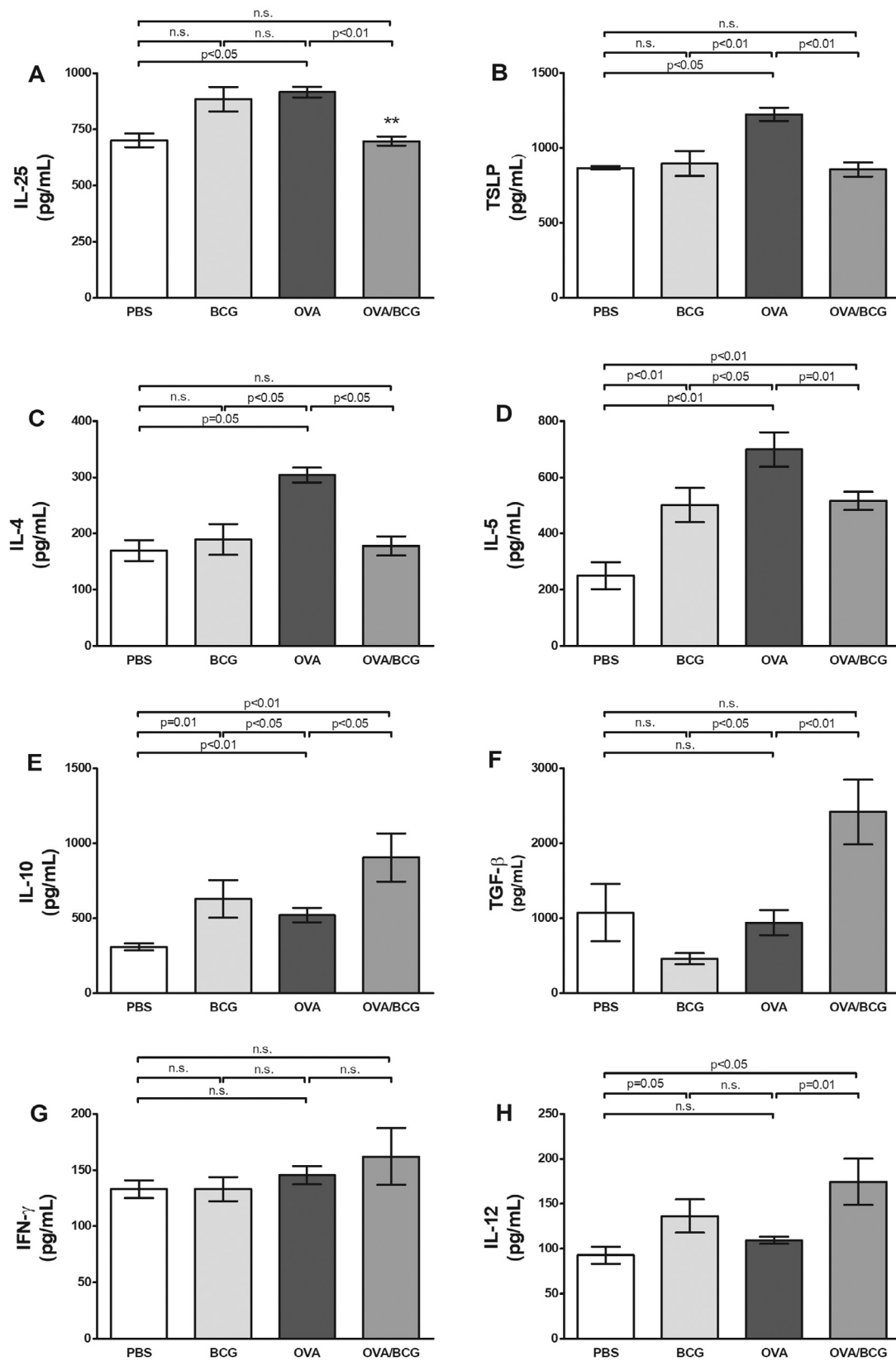


Fig. 3. Effect of BCG on the modulation of cytokine responses in the lungs of mice. (A) IL-25, (B) TSLP, (C) IL-4, (D) IL-5, (E) IL-10, (F) TGF- β , (G) IFN- γ and (H) IL-12 were detected by ELISA. The data represent the mean of results from five mice per group and bars represent the mean $\pm$ SEM.

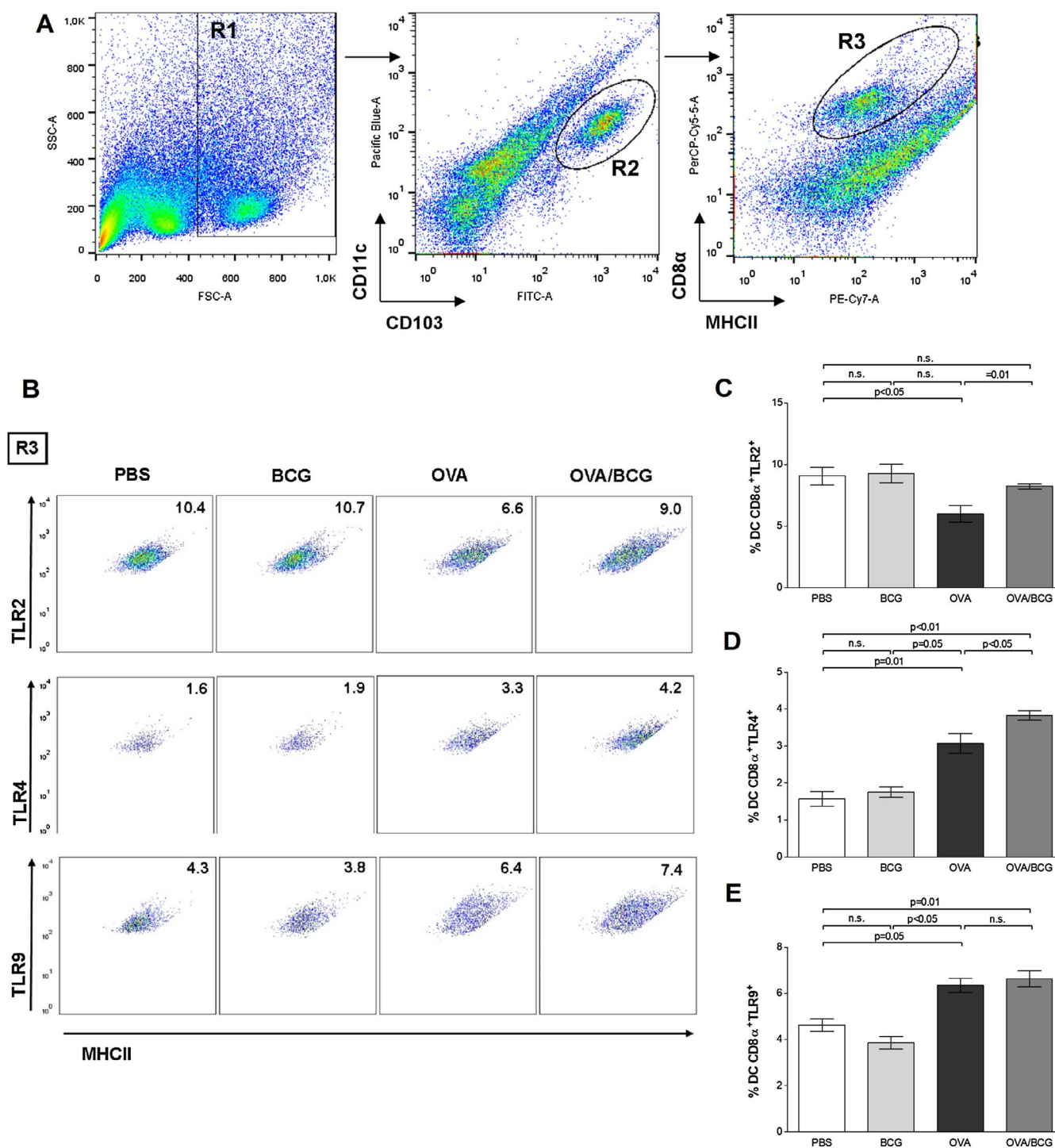


Fig. 4. BCG vaccination increases the expression of TLR2 and TLR4 in pulmonary dendritic cells (DCs). (A) Gating strategy for identify DCs in BALB/c mouse lungs by flow cytometer is illustrated. Live DCs were first gated (R1) according to cell size (FSC) and granularity (SSC) and then identified by multicolour flow cytometry as CD103 $^+$ CD11c ^{md} (R2), MHCII $^+$ CD8 α^+ population (R3). After, pulmonary DCs (CD103 $^+$ CD11c ^{md} MHCII $^+$ CD8 α^+) were analyzed for the expression of TLRs. (B) Representative flow cytometry dot plots of the percentage of pulmonary DCs expressing TLR2, TLR4 and TLR9 for phosphate-buffered saline (PBS), *M. bovis* BCG (BCG), ovalbumin (OVA) and ovalbumin plus BCG (OVA/BCG) groups. Numbers in quadrants represent percentage events in each throughout. Frequency of DCs expressing (C) TLR2, (D) TLR4 and (E) TLR9. Data are representative of at least two independent experiments, from five to eight mice per group, and bars represent the mean $\pm$ SEM.

3.5. BCG vaccination induces IFN- γ -producing CD8 α^+ pulmonary dendritic cells

In order to determine the involvement of CD8 α^+ DCs in the inhibition of asthma-like reactions by BCG, the intracellular IL-10, IFN- γ and IL-12 production was assessed. Data indicated that the OVA-challenge increased the percentage of DC CD8 α^+ producing IL-10, IFN- γ

and IL-12, when compared with the PBS or BCG groups (Fig. 5A,D–F). Interestingly, the neonatal BCG administration to OVA-challenged mice resulted in decreased production of IL-10 in comparison with the OVA group (Fig. 5A and D). This effect was accompanied by a significantly larger frequency of DC CD8 α^+ expressing IFN- γ (Fig. 5A and E), whereas BCG did not influence the IL-12 production between treated and untreated allergic mice (Fig. 5A and F).

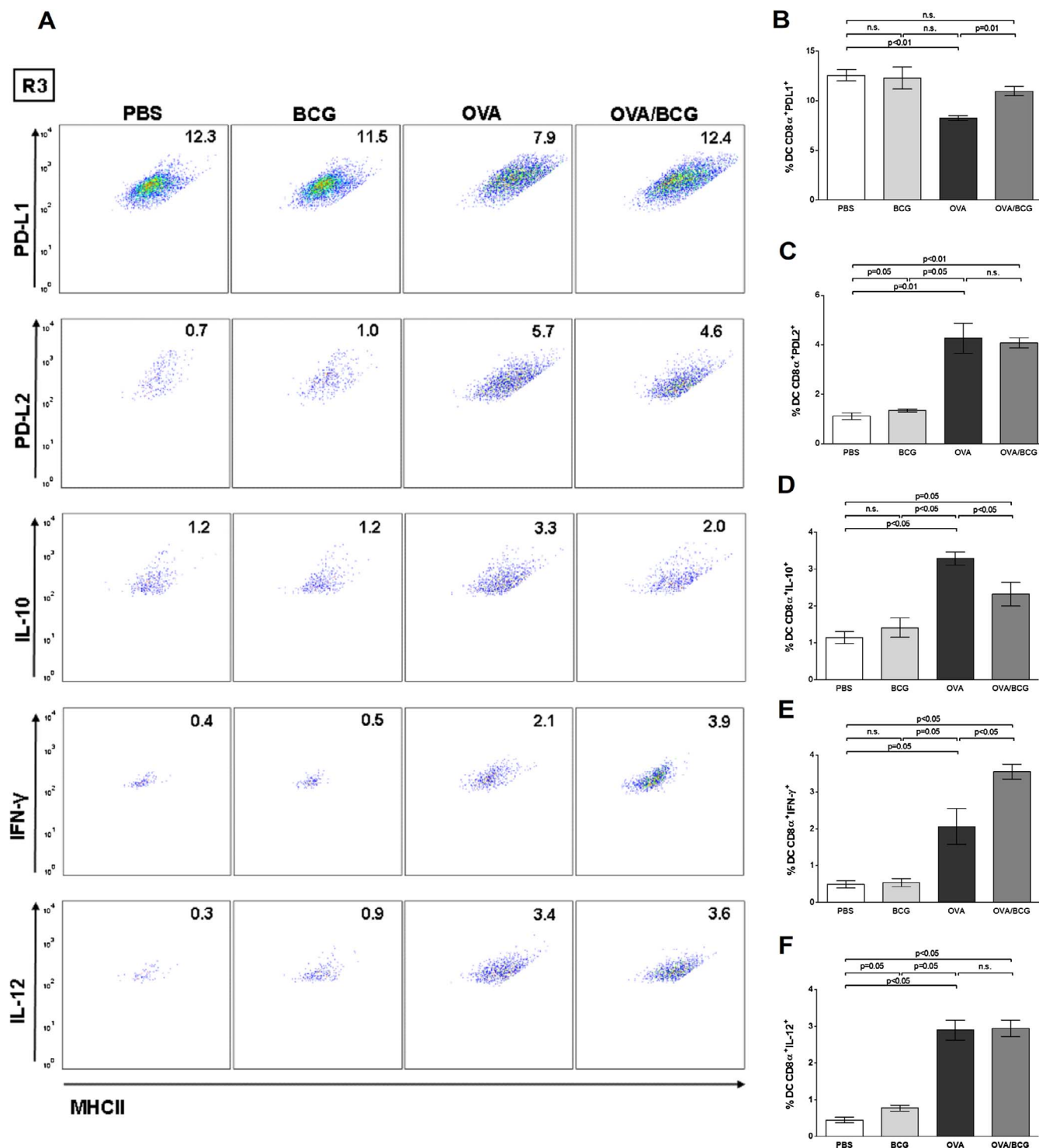


Fig. 5. BCG vaccination increases the expression of PD-L1 and IFN- γ in pulmonary dendritic cells (DCs). Pulmonary DCs gated as described in Fig. 3 (R3: CD103⁺CD11c^{int}MHCII⁺CD8 α ⁺) were analyzed for the expression of PD-L receptors and intracellular cytokines. (A) Representative flow cytometry plots showing percentage of pulmonary DCs expressing PD-L1, PD-L2, IL-10, IFN- γ and IL-12 for phosphate-buffered saline (PBS), *M. bovis* BCG (BCG), ovalbumin (OVA) and ovalbumin plus BCG (OVA/BCG) groups. Numbers in quadrants represent percentage events in each throughout. Frequency of DCs expressing (B) PD-L1, (C) PD-L2, (D) IL-10, (E) IFN- γ and (F) IL-12. Data are representative of at least two independent experiments, from five to eight mice per group, and bars represent the mean $\pm$ SEM.

4. Discussion

In the recent decades, the potential use of BCG for the management of asthma has been the topic of significant interest, since there is extensive evidence in mouse models that therapy with mycobacteria inhibits allergen-induced asthma-like responses [14]. In the current study, the effect of giving BCG to neonatal mouse before the exposure to

the allergen was evaluated. The results obtained extended previous reports showing that the intranasal BCG administration suppresses the OVA-induced airway inflammation [14], reducing the mainly pathogenic allergic features, such as airway eosinophilic inflammation and IgE production by B cells. Additionally, BCG-treated allergic mice showed Treg-like cytokine production instead of Th2-like responses. This BCG protective effect was associated with an increased expression

of TLR2, TLR4 and PD-L1 by pulmonary CD103⁺CD11c^{md}MHCII⁺CD8 α ⁺ DCs, providing some insight into the molecular basis by which DCs from BCG-treated allergic mice may modulate allergen-specific Th2 responses. Interestingly, the BCG administration induces IFN- γ production in pulmonary DCs obtained from allergic mice.

Recently, it has become clear that the interaction of the microbial environment with the innate and adaptive immune response during childhood is crucial for a balanced immune system [8], emphasizing the central role of the airway mucosal DCs and adjacent epithelial cells in the initiation and persistence of atopic asthma, as in the development of lung tolerance. In this context, considerably evidence has been accumulated demonstrating that DCs can also exert regulatory functions, highlighting these cells as one of the most accessible arrays of targets for development of new treatment for allergy. In regards to the mycobacteria, it has been demonstrated that, besides macrophages, DCs are the major target cells of BCG [33] and these cells help to modulate ensuing adaptive immune responses against mycobacteria [34]. In keeping with this thought, in the present work multiple mechanisms would be involved in the BCG-associated reduced Th2 responses, including some resulting from interactions between mycobacteria and specific receptors in DCs. Because of this, the expression of TLR2, TLR4 and TLR9 by CD103⁺CD11c^{md}MHCII⁺CD8 α ⁺ DCs was investigated, since it has been demonstrated that some components of mycobacteria are recognized by these receptors on DCs [35–37]. Analysis demonstrated that both TLR2 and TLR4 expression were upregulated in BCG-treated allergic mice, suggesting that signaling pathways induced by these interactions may be involved in the mycobacteria modulatory mechanism. The role of these receptors in the regulation of the adaptive immune response has been evidenced from studies showing the induction or activation of Treg cells through DC-TLR signaling [38]. In this way, it was reported that TLR4-deficient mice have a defective induction of IL-10-secreting Treg cells [39] and TLR2 signaling promotes the induction of these cells [40]. Specifically, TLR2 [21,41] and TLR4 [21,42,43] agonists were able to suppress an asthmatic allergic response, either by inducing Treg or Th1 immune responses. Also in the current work the signaling of these TLRs on DCs by BCG may have induced a regulatory response which modulates the allergic airway inflammation, since the high expressions of TLR2 and TLR4 were associated with a significant increase of regulatory cytokines in lung tissue [38,39]. These observations are supported by previous studies indicating that DCs have the ability to initiate a tolerogenic response during allergic sensitization through inducing Treg cells [25,44]. Additionally, the activation of TLR4 was linked to an increase in Tregs suppressive capability [45]. It is important to mention that TLR4 can also be activated by LPS, a common contaminant of OVA, which has the potential to contribute to the development of asthma in several different ways [46]. Although every effort was made to maintain sterile procedures to minimize the endotoxin contamination in all OVA preparations, it is well known that the completely remove of LPS remains a major technical hurdle in mouse models of asthma, given the extremely low levels of endotoxin that can elicit significant biological effects [47]. Then, a study using TLR2- and TLR4-deficient mice is crucial to confirm the involvement of these receptors in the BCG anti-allergic effect observed in this asthma model.

Besides the TLRs signaling, the present study demonstrated that the improvement of airway inflammation was correlated with a suppression of IL-25 and TSLP, suggesting that this would be another mechanism underlying the BCG-protective effect, since it has been shown that these epithelial cytokines are crucial in allergen-induced airway responses [48]. Particularly, TSLP can either directly activate CD11c⁺ DCs to prime naïve CD4⁺ T cells to differentiate into inflammatory Th2 cells or might directly promote the production of Th2 cytokines by T cells [49], which could be enhanced by IL-25 [50]. Interestingly, previous work showed that BCG redirects TSLP-activated DCs away from inducing inflammatory Th2 to Treg response [51]. Taken together, these data suggest that in the present study the airway epithelial cells would be

one important cellular target of BCG and the OVA-induced epithelial cells activation was probably modulated by the mycobacteria treatment, presumably through impairing the function of TSLP at the level of DCs. Nonetheless, further studies are needed to clarify and confirm this supposed mechanism.

It has been previously reported that the molecular basis for the capacity of tolerogenic DCs to modulate T cell responses may be related to their cytokine production and the expression of surface molecules [52,53]. Present results showed that the neonatal BCG vaccination induced the production of IFN- γ in allergic mice DCs, suggesting that these cells coordinated an immune deviation from Th2 pathogenic toward a Th1 protective response. Along with inducing the regulatory response, this Th1 inducer effect would be another mechanism underlying the allergic inflammation-inhibition found in this work. This notion is further strengthened by the high levels of IL-12 found in the lung from the BCG vaccinated mice. Surprisingly, analysis indicated that besides the unaltered frequency of IL-12-producing DCs, the levels of IFN- γ in lung homogenate are also similar between the BCG-treated and untreated allergic mice. Further, the IL-10 expression was down-regulated in DCs obtained from BCG-treated allergic mice. A possible explanation for these apparently discrepant findings could lie in the time of cytokine production and the short-term protocol. Probably, high levels of IL-10 and IL-12 production by DCs occurred at the beginning of the protocol, soon after the BCG administration, as previously reported with an *in vitro* experimental study [34]. Also, increased levels of IFN- γ would be detected in the lungs of the BCG-treated allergic mice in an extended protocol, since it has been shown that the peak response of this Th1 cytokine occurs in the lung at the fourth week after intranasal vaccination [54].

An important finding of the current study is that CD103⁺CD11c^{md}MHCII⁺CD8 α ⁺ DCs from allergic mice had their PD-L1 expression increased after BCG administration, reaching levels similar to mice from the PBS group. This data supports the proposition that the mechanism underlying the BCG-Th2 allergic response-inhibition involves the induction of a regulatory immune response, since previous reports demonstrated that the capacity of DCs to induce Tregs and limit asthma development has been linked to their elevated expression of PD-L1 [25,30,31,55,56]. Also this molecule seems to be directly responsible for the generation of Treg cells when expressed by DCs CD8 α ⁺ and when TGF- β was present [57]. Interestingly, it was demonstrated that the PD-L1 expression induced in DCs upon stimulation with BCG is necessary for mycobacteria-induced Treg expansion [30,31,56] and the TLR2 signaling could play a dominant role in this pathway [56]. On the other hand, in this study the PD-L2 expression increased on the pulmonary DCs after OVA challenge, as demonstrated by previous researches [26]. Otherwise, the expression of this modulatory molecule appears to have a slight tendency towards decreasing after BCG administration. These results suggest that PD-L2 also may play a role in suppressing airway inflammation, since experimental evidence indicates that PD-L2 could act through a receptor other than PD-1, enhancing the T cell proliferation and cytokine production [26,58–60] and exacerbating the Th2 inflammatory response [29]. However, studies are just beginning to elucidate the function of PD-1 ligands in allergy and asthma and some works indicate that PD-L2 expression in the lung protects against the initiation and progression of airway inflammation [27,61]. Some data indicated that the cytokine environment may have an important role to play in differentially regulating PD-L1 and PD-L2 expression and modulating inflammatory responses in the lung microenvironment [26,29]. Therefore, further study will be required to clarify the function of the PD-1/PD-L interactions in the Th2 response development upon BCG therapy.

In the current asthma model, although BCG was administered through the nasal mucosa, making it improbable that mycobacteria reached the airways, BCG therapy actually ameliorates the airway allergic response. Probably, the mycobacteria protective effect started early, during allergen sensitization, when the systemic response could

be modulated, suppressing the OVA-specific IgE production. It could be supposed that after OVA sensitization, allergen-loaded DCs migrated to the draining lymph nodes of allergic mice, where some naïve allergen-specific T cells are activated with Tregs rather than the Th2 pattern due to the microenvironment induced by BCG-activated DCs. Thus, since DCs remain infected with BCG for a much longer time [33], this suppressive effect is extended during allergen challenge, modulating the airway inflammatory response. At the same time the opposite way may also occur with BCG-induced effector T cells, since it has been demonstrated that BCG vaccination-induced Th1 cells migrated from distant lymph nodes into the inflamed lungs upon inhaled OVA challenge, thereby inhibiting the allergen airway inflammation [62].

In conclusion, this study has demonstrated that neonatal BCG vaccination markedly inhibited the development of the Th2-dependent downstream effector phase of allergy. The higher expression of TLR2, TLR4, PD-L1 and IFN- γ by pulmonary DCs associated with the decreased levels of IL-25 and TSLP strengthens the proposition that the innate immune cells are involved in the mechanism underlying the BCG-induced Th2 inhibitory effect. Clearly, additional studies are required to elucidate the functional role of the pulmonary DCs in response to BCG-treatment. Nevertheless, data obtained provide new insights into the mechanism by which BCG exert their protective effect and should help in designing new therapeutic approaches that exploit the DCs for prevention and treatment of allergic diseases, including asthma.

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Disclosures

The authors have no financial conflicts of interest.

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