Respiratory Physiology & Neurobiology 189 (2013) 614–623

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Impact of Bacillus Calmette–Guérin Moreau vaccine on lung remodeling in experimental asthma

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ARTICLE INFO

Article history:
Accepted 22 July 2013

Keywords:
Asthma
BCG vaccine
Airway remodeling
Eosinophilia
Airway resistance

ABSTRACT

We analyzed the effects of different administration routes and application times of the BCG-Moreau strain on airway and lung inflammation and remodeling in a murine model of allergic asthma. BALB/c mice ($n = 168$) were divided into two groups. The first group received BCG-Moreau strain while the second group received saline using the same protocol. BCG or saline were intradermally or intranasally injected one or two months before the induction of asthma. Mice were further sensitized and challenged with ovalbumin or received saline. Twenty-four hours after the last challenge, BCG prevented the triggering of pro-inflammatory cytokines, probably by increasing Foxp3 and interleukin (IL)-10, modulating eosinophil infiltration and collagen fiber deposition, thus reducing airway hyperresponsiveness. In conclusion, BCG-Moreau prevented lung remodeling in the present model of allergic asthma, regardless of administration route and time of vaccination. These beneficial effects may be related to the increase in regulatory T cells and to IL-10 production in tandem with decreased Th2 cytokines (IL-4, IL-5, and IL-13).

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1. Introduction

Asthma is a chronic inflammatory disease affecting the airways and lung parenchyma (Bateman et al., 2008), associated with remodeling characterized by the following ultrastructural changes: subepithelial fibrosis, mucous metaplasia, airway wall thickening, smooth muscle cell hypertrophy and hyperplasia, myofibroblast hyperplasia, vascular proliferation, and extracellular matrix abnormalities (Al-Muhsen et al., 2011). These changes accelerate decline in lung function (Holgate, 2008) despite treatment with corticosteroids. Since lung remodeling is usually related to established inflammation, it may be hypothesized that early treatment with immunoregulatory agents could prevent damage.

Recent studies have demonstrated the Bacillus Calmette–Guérin (BCG) vaccine to be effective at reducing inflammation and hyperresponsiveness in animal models (Lagranderie et al., 2008) and in humans with asthma (Choi and Koh, 2002, 2003; Cohon et al., 2007). However, the effectiveness of this treatment seems to be affected by aspects of vaccine delivery: experimental studies report better control of the inflammatory process of asthma with intranasal administration compared to the intradermal route (Choi et al., 2007; Erb et al., 1998), even though the latter is more commonly used in humans (Sarinho et al., 2010; Shirtcliffe et al., 2004). Furthermore, there is controversy regarding the best time of BCG administration before induction of allergy (Erb et al., 1998; Nahori et al., 2001; Ozeki et al., 2011). Additionally, a strain-dependent effect of BCG cannot be ruled out. In this line, the Moreau strain, which is widely used for tuberculosis control in Brazil (Benevolo-de-Andrade et al., 2005), has been observed to induce an adaptive immunity while increasing cytokines from T helper 1 (Th1) and regulatory T cells (Treg) (Wu et al., 2007), suggesting that this vaccine could be a potential tool for prevention of allergic asthma.

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http://dx.doi.org/10.1016/j.resp.2013.07.025

Based on the aforementioned, we used a murine model of allergic asthma to analyze the effects of different routes of administration and application times of the BCG-Moreau strain on pulmonary inflammation, remodeling process, and lung function. Moreover, possible mechanisms of action were investigated.

2. Materials and methods

This study was approved by the Ethics Committee of the Carlos Chagas Filho Institute of Biophysics, Health Sciences Center, Federal University of Rio de Janeiro, Brazil (CEUA-CCS, IBCCF 019).

2.1. Animal preparation

A total of 168 newly weaned male BALB/c mice (10–15 g) were randomly divided into two groups. The first group ($n = 84$) received 25 μL of a solution of 10^6 UFC lyophilized BCG Moreau strain resuspended in saline while the second group ($n = 84$) received saline. BCG or saline were intradermally ($n = 42$) or intranasally ($n = 42$) injected one or two months before the induction of allergic asthma.

These groups were further randomized into two other groups. In the ovalbumin group (OVA), mice were immunized using an adjuvant-free protocol with intraperitoneal injection of ovalbumin (10 μg in 0.1 mL sterile saline) on each of seven alternate days. Forty days after the beginning of sensitization, 20 μg of OVA in 20 μL sterile saline were intratracheally instilled. This procedure was performed three times at 3-day intervals. The control group (C) received saline using the same protocol.

Eighty-four animals were used for analysis of lung mechanics and histology, and a second group of 84 animals was used for analysis of airway responsiveness and bronchoalveolar lavage fluid (BALF). The BCG Moreau vaccine was donated by the Ataulpho de Paiva Foundation, Brazil.

2.2. Assessment of lung mechanics

Twenty-four hours after the last challenge, mice were sedated (diazepam 1 mg i.p.), anesthetized (thiopental sodium 20 mg/kg i.p.), tracheotomized, paralyzed (vecuronium bromide, 0.005 mg/kg i.v.), and mechanically ventilated with the following settings: respiratory frequency 100 breaths/min, tidal volume (V_T) 0.2 mL, and fraction of inspired oxygen (FiO_2) 0.21. The anterior chest wall was surgically removed and a positive end-expiratory pressure (PEEP) of 2 cmH_2O was applied, and the lung mechanics were computed. At the end of the experiment, the lungs were prepared for histology and molecular biology. Airflow, volume and tracheal pressure (Ptr) were measured (Hsia et al., 2010). In an open chest preparation, Ptr reflects transpulmonary pressure (PL). Lung static elastance and airway resistance were computed by the end-inflation occlusion method (Bates et al., 1985) using the ANADAT data analysis software (RHT-InfoData, Inc., Montreal, Quebec, Canada).

2.3. Airway responsiveness

Twenty-four hours after the last challenge, airway responsiveness was measured. Increasing doses of methacholine (Sigma Chemical Co., Saint Louis, MI, USA) (100, 300, 1000, 3000, and 10,000 μg/kg) were administered via a silastic catheter placed in the jugular vein. Data were stored at 30 s, 1, 3, and 5 min after agonist injection. Shortly after each intravenous infusion of methacholine, the maximal increase in Ptr was reached, and the respective airflow was measured at this moment (Antunes et al., 2009). Respiratory system resistance (R) was obtained using the equation of motion of the respiratory system: $Ptr(t) = E \cdot V(t) + R \cdot V'(t)$, where ($t$) is time.

2.4. Lung histology

The right lung was removed, fixed in 4% buffered formaldehyde, paraffin-embedded, and cut into 4 μm-thick slices, which were stained with hematoxylin and eosin (Vetec Química Fina, Rio de Janeiro, Brazil). Fraction area of collapsed and normal lung areas were determined by the point-counting technique at a magnification of 200× across 10 random, non-coincident microscopic fields (Hsia et al., 2010). Points falling on collapsed or normal pulmonary areas were counted and divided by the total number of points in each microscopic field. Polymorphonuclear (PMN) and mononuclear (MN) cells and lung tissue were evaluated at 1000× magnification. Points falling on PMN and MN cells were counted and divided by the total number of points falling on lung tissue in each microscopic field.

Airway bronchoconstriction index was determined by counting the points falling on the airway lumen and those falling on airway smooth muscle and on the epithelium, at a magnification of 400×. The number of intercepts (NI) of the lines with the epithelial basal membrane is proportional to the airway perimeter, and the number of points (NP) falling on the airway lumen is proportional to airway area; thus, the magnitude of bronchoconstriction was computed as $CI = NI/NP^{1/2}$. Measurements were performed in five airways from each animal at 400× magnification.

Collagen fibers (Picrosirius-polarization method) (Montes, 1996) were quantified in alveolar septa and airways with the aid of a digital analysis system and specific software (Image-Pro®Plus 5.1 for Windows® Media Cybernetics – Silver Spring, MD, USA) under 200× magnification. The area occupied by fibers was determined by digital densitometric recognition. To avoid any bias due to alveolar collapse, the areas occupied by collagen fibers in each alveolar septum were divided by the area. The results were expressed as the percentage of collagen fiber content per tissue area (%). Collagen fiber content was quantified in the whole circumference of the two largest, transversally cut airways present in the sections. Results were expressed as the area of collagen fibers divided by the perimeter of the basement membrane ($\mu m^2/\mu m$).

2.5. Immunohistochemistry

Right lungs were fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry using monoclonal antibody against α-smooth muscle actin (Dako, Carpenteria, CA, USA) at a 1:500 dilution. The analysis was performed on the slides stained for α-smooth muscle actin applying the point-counting technique. Using a 121-point grid, we calculated the volume proportion of smooth-muscle-specific actin in terminal bronchioles and alveolar ducts as the relation between the number of points falling on actin-stained and non-stained tissue. Measurements were done at 400× magnification in each slide (Hsia et al., 2010).

2.6. Transmission electron microscopy

Three 2 mm × 2 mm × 2 mm slices were cut from three different segments of the left lung and fixed [2.5% glutaraldehyde and phosphate buffer 0.1 M (pH 7.4)] for electron microscopy (JEOL 1010 Transmission Electron Microscope, Tokyo, Japan) analysis. For each electron microscopy image (20/animal), the following structural changes were analyzed: (a) shedding of surface epithelium, (b) airway edema, (c) eosinophil infiltration, (d) neutrophil infiltration, (e) disorganization of ciliated epithelial cells, (f) subepithelial fibrosis, (g) elastic fiber fragmentation, (h) smooth muscle hypertrophy, (i) myofibroblast hyperplasia, and (j) mucous cell hyperplasia. Pathologic findings were graded according to a 5-point semi-quantitative severity-based scoring system as: 0 = normal lung parenchyma,

1 = changes in 1–25%, 2 = changes in 26–50%, 3 = changes in 51–75%, and 4 = changes in 76–100% of examined tissue (Jeffery et al., 1992).

Histological analysis was performed by a blinded pathologist.

2.7. Bronchoalveolar lavage fluid (BALF)

Total leukocyte count in BALF was performed in a Neubauer chamber with optical microscopy after diluting the samples in Türk solution. Differential leukocyte counts were performed in cytospin smears stained by the May–Grünwald–Giemsa method. The amount of interleukin (IL)-4, IL-5, IL-10, IL-12, IL-13, IL-17, interferon (IFN)-γ and transforming growth factor (TGF)-β in the cell-free BALF was evaluated by ELISA in accordance with the manufacturer's instructions (Duo Set, R&D Systems, Minneapolis, USA).

2.8. Foxp3 expression

Quantitative real-time reverse transcription (RT) polymerase chain reaction (PCR) was performed to measure the relative levels of expression of Foxp3 genes in lung tissue (Yang et al., 2009). Total RNA was extracted from the frozen tissues using the SV Total RNA Isolation System (Promega, Rio de Janeiro, Brazil) according to manufacturer instructions. RNA concentrations were measured in a Nanodrop® ND-1000 spectrophotometer. First-strand cDNA was synthesized from total RNA using the GoTaq® 2-Step RT-qPCR System (Promega, Rio de Janeiro, Brazil), according to manufacturer recommendations. Relative mRNA levels were measured with a SYBR green detection system using a Mastercycler ep realplex^2 S (Eppendorf, São Paulo, Brazil). All samples were measured in triplicate. The relative amount of expression of each gene was calculated as the ratio of studied gene to a control gene (acidic ribosomal phosphoprotein P0 [36B4]) and expressed as fold changes relative to C or OVA groups. The following PCR primer was used: 5′-GAGCCAGAAGAGTTTCTCAAGC-3′ and 5′-GCTACGATGCAGCAAGAGC-3′.

2.9. Statistical analysis

Two-way ANOVA followed by Tukey's test was used to compare all data considering route of administration and moment of injection as the study factors. A correlation between mechanical and histological data was analyzed using Spearman's correlation test. A p value less than 0.05 was considered significant. All tests were performed in GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

3. Results

The BCG-Moreau vaccine effectively reduced remodeling and lung inflammation, with positive effects on lung mechanics and morphometry, with no difference between administration route or time.

3.1. Remodeling

Collagen fiber content in the airway and lung parenchyma (Fig. 1A), as well as the amount of α-smooth muscle actin in the terminal bronchiole and alveolar ducts (Fig. 1B) were higher in the SAL-OVA group compared to its respective control (SAL-C). BCG-Moreau therapy, regardless of route and moment of administration, prevented these alterations (Fig. 1A–C). Since no significant difference on lung mechanics and histology were observed in mice treated with saline (data not shown), intradermally and intranasally treated animals were pooled in a single group.

In the SAL-OVA group, electron microscopy evidenced several ultra-structural changes in terminal bronchioles that are characteristic of asthma: shedding of surface epithelium, elastic fiber fragmentation, subepithelial fibrosis, disorganization of ciliated cells, myofibroblast hyperplasia, and smooth muscle hypertrophy. These ultrastructural changes were minimized by administration of BCG-Moreau before the asthma protocol (Table 1 and Fig. 2).

3.2. Lung inflammation

The inflammatory process was evaluated by counting total and differential cells in lung tissue and BALF (Fig. 3). The number of polymorphonuclear cells in lung tissue and of eosinophils in BALF was significantly higher in the SAL-OVA group compared to the other groups (Fig. 3). The administration of BCG-Moreau intradermally or intranasally, one or two months before asthma induction, attenuated the allergen-induced inflammatory process (Fig. 3), with no statistical differences among BCG-treated groups.

3.3. Lung mechanics

Airway hyperresponsiveness, airway resistance (Raw), and lung static elastance (Est, L) were higher in SAL-OVA when compared to SAL-C (Fig. 4). BCG minimized these mechanical changes, with no statistical differences among BCG-treated groups (Fig. 4).

3.4. Lung histology and morphometry

The fraction area of alveolar collapse and the bronchoconstriction index were significantly higher in SAL-OVA than in SAL-C, and the administration of BCG-Moreau prevented these alterations (Fig. 5). Considering all groups together, lung static elastance was well correlated with the fraction area of alveolar collapse, while airway resistance was correlated with the bronchoconstriction index ($p < 0.05$).

3.5. Mechanisms of action of BCG-Moreau vaccine on lung inflammation and remodeling

In order to investigate the possible mechanisms of action of the BCG-Moreau vaccine in the proposed allergic asthma model, cytokines with Th1 (IFN-γ, IL-12), Th2 (IL-4, IL-5 IL-13), Th17 (Th17) and Treg (IL-10), TGF-β profile and the mRNA expression of Foxp3 (Fig. 7) were measured. BCG led to IL-10 and Foxp3 increase, while reducing IL-4, IL-5, and IL-13 in OVA group (Figs. 6 and 7). No significant changes were observed in the other mediators (data not shown).

4. Discussion

In the present study, intranasal and intradermal administration of BCG-Moreau vaccine, one or two months before asthma induction, minimized the inflammatory process. More importantly, BCG-Moreau vaccine prevented airway and lung parenchyma remodeling – as evidenced by the reduction of both collagen fiber content and percentage of smooth muscle-specific actin in terminal bronchioles and alveolar ducts, maintenance of airway epithelium integrity and by the decrease in subepithelial fibrosis, fragmentation of elastic fibers, and hyperplasia of myofibroblasts. Prevention of ultrastructural changes by BCG-Moreau treatment resulted in improved pulmonary function when compared to saline-treated OVA-challenged animals, as assessed by lung mechanics and airway hyperresponsiveness. Furthermore, these beneficial effects were associated with an increase in IL-10 and Foxp3, as well as with a reduction in Th2 cytokines.

BALB/c mice were chosen because of their greater ability to develop airway hyperresponsiveness and eosinophilia after chronic

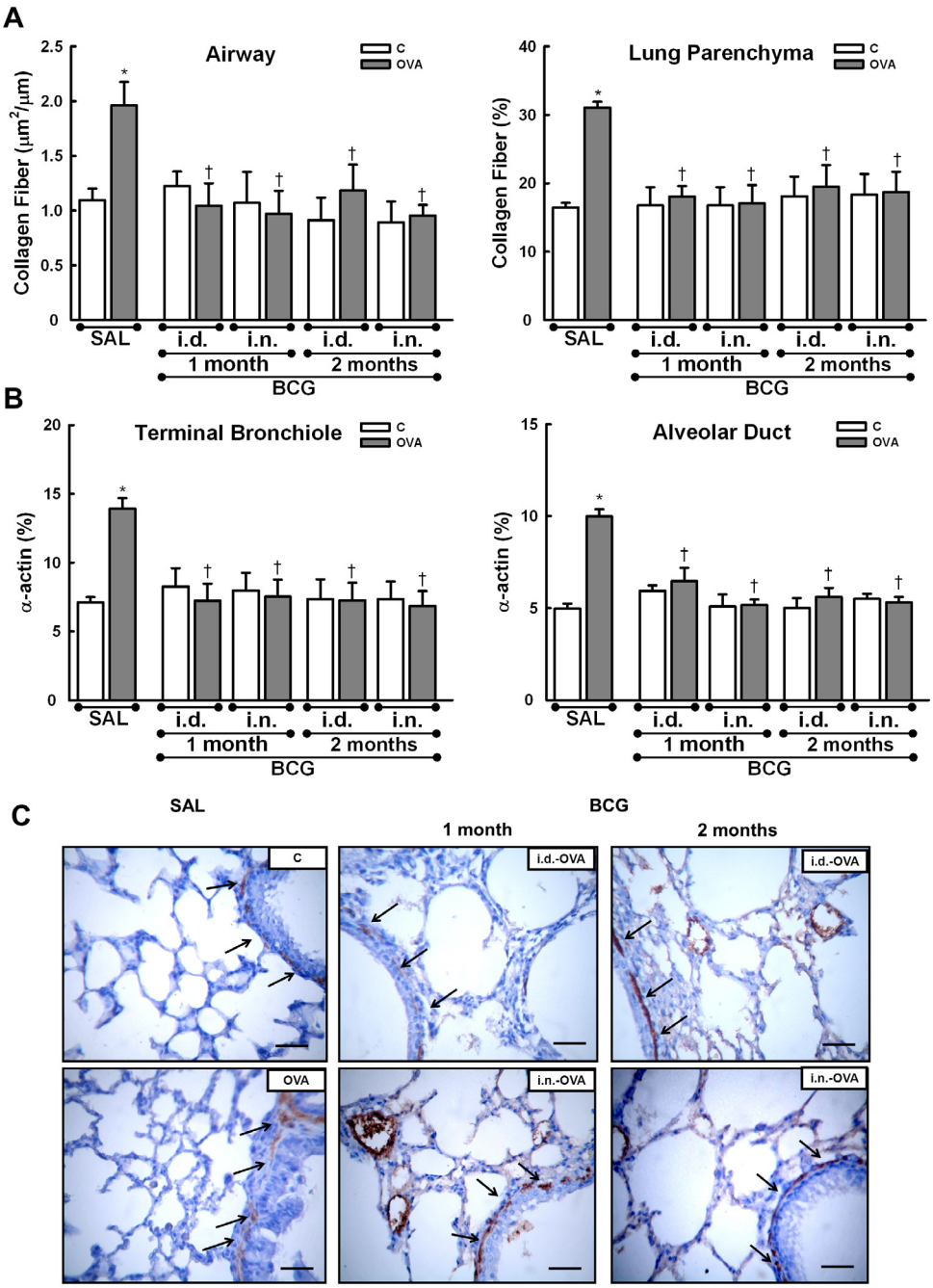

Fig. 1. Collagen fiber content in airway and lung parenchyma (panel A) and fraction area of smooth muscle specific actin in the terminal bronchioles and alveolar ducts (panel B) in SAL and BCG groups treated intradermally (i.d.) or intranasally (i.n.), one or two months before sensitization and challenge with ovalbumin (OVA). Control (C) group received saline using the same protocol. Values are means ± SEM of 6 mice in each group. Photomicrographs of the airways were taken at an original magnification of 400× (panel C). *Significantly different from SAL-C ($p < 0.05$). †Significantly different from SAL-OVA group ($p < 0.05$).

sensitizations and challenges with ovalbumin (Antunes et al., 2009). The present protocol was able to reproduce some aspects of human chronic asthma, such as airway hyperresponsiveness, eosinophilia, smooth muscle hypertrophy, and increased basement membrane thickness (Mestas and Hughes, 2004; Xisto et al., 2005). In this study, the BCG protocol was begun as soon as the mice were weaned, since BCG is usually administered at a very young age (World Health Organization, 2004). Experimental (Erb et al., 1998; Hopfenspirger and Agrawal, 2002; Major et al., 2002; Shen et al., 2008; Tukenmez et al., 1999) and clinical studies (Aaby et al., 2000; Alm et al., 1997; Bager et al., 2003; Choi and Koh, 2002) are controversial concerning the best time for BCG administration. Erb et al. found that the action of this vaccine decreased over time, and that the best results were achieved between two and four weeks before induction of the allergic process (Erb et al., 1998). Conversely, Nahori et al. reported BCG effects lasting more than 8 weeks (Nahori et al., 2001), while Ozeki et al. observed a high amount of BCG mainly in the spleen up to 20 weeks after administration (Ozeki et al., 2011). Based on the aforementioned, we administered BCG-Moreau one or two months before asthma induction. Moreover, previous studies have also suggested an influence of BCG administration route on the vaccine's effectiveness (Choi et al., 2007; Erb et al., 1998; Hopfenspirger and Agrawal, 2002). In this context, Erb et al. argue that BCG should be administered directly into the lung

Table 1
Ultrastructural changes of airways.

Ultrastructural parameters	SAL		BCG			
	C	OVA*	1 month		2 months	
			i.d.-OVA†	i.n.-OVA†	i.d.-OVA†	i.n.-OVA†
Shedding of surface epithelium	0 (0–0)	3 (2.5–3.0)	1 (0.5–1.5)	1 (0.5–1.0)	1 (1.0–2.0)	1 (0.5–1.0)
Airway edema	0 (0–0)	3 (2.5–3.5)	1 (1.0–1.5)	1 (0–1.0)	0 (0–1.0)	0 (0–1.0)
Eosinophils	0 (0–0)	3 (2.5–4.0)	1 (0.5–1.5)	1 (1–2)	1 (0.5–2.0)	1 (0.5–1.0)
Neutrophils	0 (0–0)	2 (1–2)	0 (0–1.0)	1 (0–1.0)	1 (0–1)	0 (0–1.0)
Disorganization of ciliated epithelial cells	0 (0–0)	3 (2.5–3.5)	0 (0–1.0)	0 (0–1.0)	0 (0–1.0)	0 (0–1.0)
Subepithelial fibrosis	0 (0–0)	3 (3.0–4.0)	1 (0–1.0)	1 (0–1.0)	1 (0–1.0)	1 (0.5–1.0)
Smooth muscle hypertrophy	0 (0–0)	3 (2.5–4.0)	1 (1.0–1.5)	1 (0.5–1.0)	1 (1.0–1.5)	1 (0.5–1.5)
Myofibroblast hyperplasia	0 (0–0)	4 (3.0–4.0)	1 (0.5–1.5)	1 (1.0–1.5)	1 (0–1.0)	1 (0.5–1.5)
Elastic fiber fragmentation	0 (0–0)	2 (1.5–2.5)	0 (0–0.5)	0 (0–1.0)	0 (0–1.0)	0 (0–0.5)
Mucous cell hyperplasia	0 (0–0)	3 (2.0–3.5)	1 (0–1.0)	1 (0.5–1.0)	1 (0.5–1.5)	1 (0.5–1.5)

Semiquantitative analysis of the ultrastructural changes in the airway. Mice received saline (SAL) or Bacillus Calmette–Guérin (BCG), intradermally (i.d.) or intranasally (i.n.), one or two months before sensitization and challenge with ovalbumin (OVA). The control (C) group received saline using the same protocol. Values are median (25th percentile–75th percentile) of 5 animals per group. Pathologic findings were graded according to a 5-point semi-quantitative severity-based scoring system: 0 = normal lung parenchyma, 1 = changes in 1–25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100% of the examined tissue.

* Significantly different from SAL-C ($p < 0.05$).

† Significantly different from SAL-OVA group ($p < 0.05$).

to promote better effects (Erb et al., 1998). However, clinical trials have employed the intradermal route for BCG administration (Sarinho et al., 2010; Shirtcliffe et al., 2004). We therefore compared the intradermal and intranasal routes. Erb et al. observed that the route of BCG administration influenced airway eosinophilia, with intranasal infection being superior to intraperitoneal or subcutaneous infection in its ability to reduce airway eosinophilia (Erb et al., 1998). Conversely, our study demonstrated that the administration of BCG-Moreau intradermally or intranasally, one or two months before asthma induction, attenuated the allergen-induced inflammatory process, with no statistical differences between BCG-treated groups. Regarding the BCG vaccine dose, 10^6 CFU was used because it has been associated with a better immune response (Nahori et al., 2001; Yang et al., 2002).

Previous genomic analyses of BCG vaccines demonstrate that there is genetic variability among the strains, leading to controversies regarding BCG efficacy (Davids et al., 2006; Wu et al., 2007). However, the present results suggest that the protective efficacy of BCG-Moreau remains unaltered. According to recent molecular studies (Brosch et al., 2007), BCG-Moreau, which has been used in Brazil for vaccine production since the 1920s (Berredo-Pinho et al., 2011), belongs to the group of "older" strains that are closer to the attenuated strain originally derived by Calmette and Guérin. The BCG-Moreau strain is used in 5% of the BCG vaccines produced in the world (Benevolo-de-Andrade et al., 2005). Allied to its availability (Hayashi et al., 2009) and meaningful role in vaccine preparations, the BCG-Moreau strain has immunogenic effects, as shown herein. In this line, this is the first study to date describing the ability of

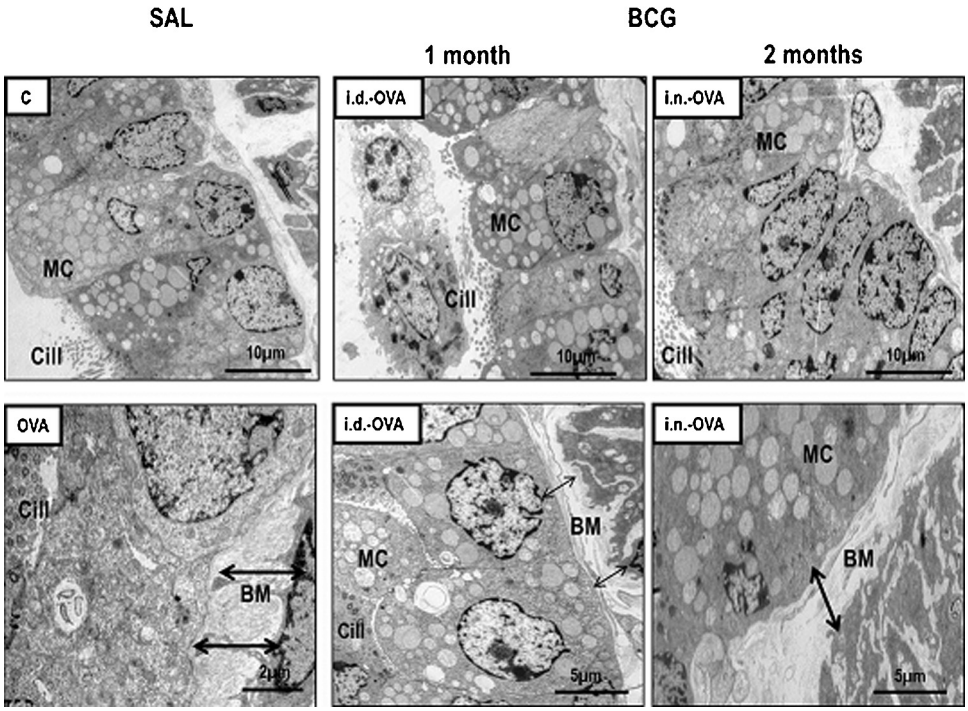

Fig. 2. Electron microscopy of the ultrastructural changes in the airway. Mice received saline (SAL) or Bacillus Calmette–Guérin (BCG), intradermally (i.d.) or intranasally (i.n.), one or two months before animals were sensitized and challenged with ovalbumin (OVA). Control (C) group received saline using the same protocol. MC, Mucous cells; Cill, ciliated cells; BM, basement membrane.

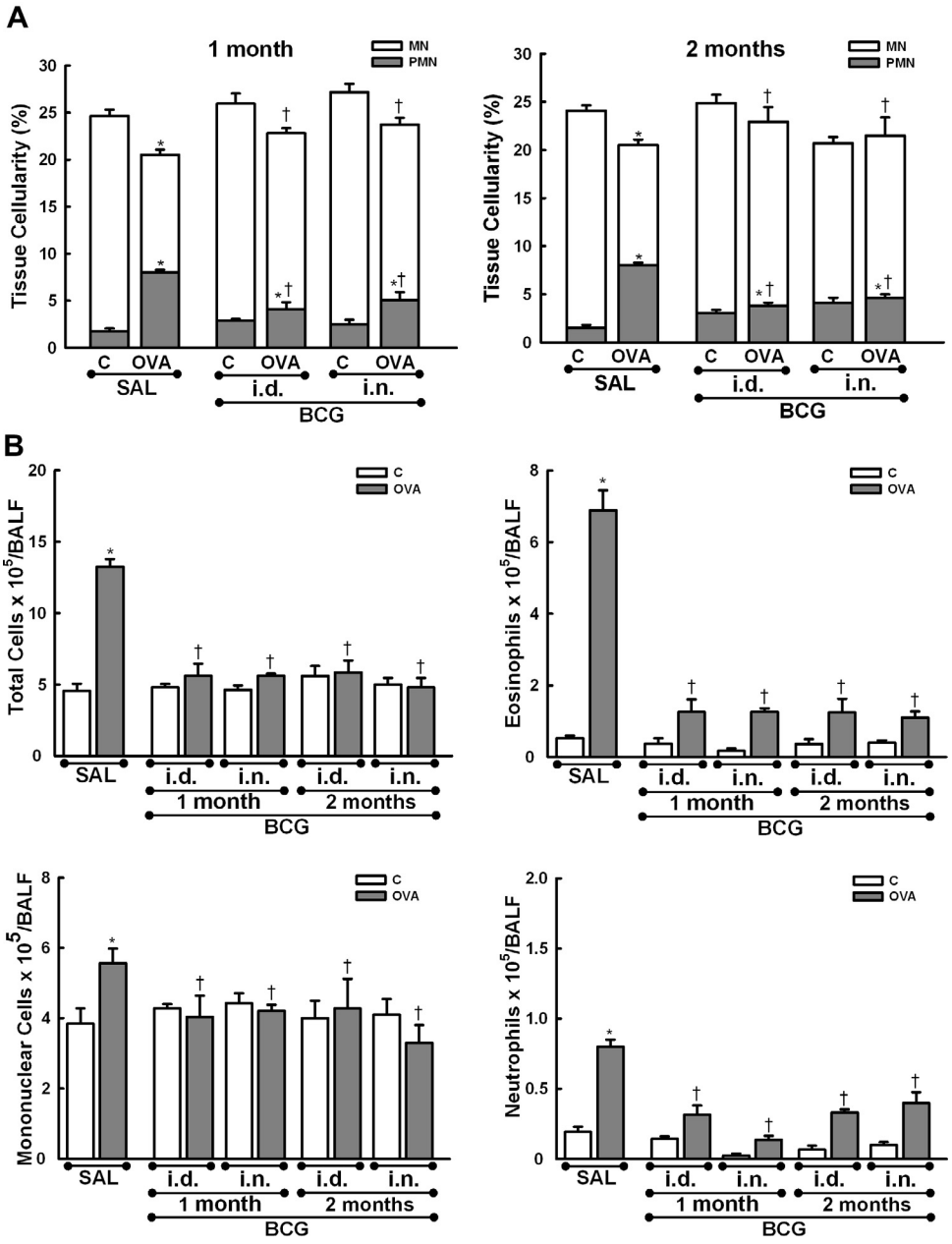

Fig. 3. Tissue cellularity – MN, mononuclear cells; PMN, polymorphonuclear cells (panel A); total cells, mononuclear cells, eosinophils and neutrophils in bronchoalveolar lavage fluid (BALF) (panel B). Mice were treated with saline (SAL) or Bacillus Calmette–Guérin (BCG), intradermally (i.d.) or intranasally (i.n.), one or two months before sensitization and challenge with ovalbumin (OVA). Control (C) group received saline using the same protocol. Values are mean ± SEM of 6 mice in each group. Values are means ± SEM of 6 mice in each group. *Significantly different from SAL-C ($p < 0.05$). †Significantly different from SAL-OVA group ($p < 0.05$).

the BCG-Moreau strain to reduce inflammation and remodeling in experimental asthma.

Studies have shown that the correlation between number of eosinophils and pulmonary airway reactivity has a critical impact on disease severity and number of exacerbations (Bousquet et al., 1990). An asthma model using eosinophil-deficient genetically modified PHIL mice demonstrated the essential role of eosinophils in airway hyperresponsiveness and pulmonary mucus accumulation (Lee et al., 2004). Eosinophils may also contribute to airway remodeling, In this line, total ablation of eosinophil lineage reduced asthma-induced airway remodeling, as demonstrated by a decrease in peribronchiolar collagen deposition and fewer smooth muscle-specific actin positive cells (Humbles et al., 2004). Moreover, eosinophils produce a multitude of fibrogenic factors, such as TGF-β, IL-11, IL-17, and IL-25 (Hamid and Tulic, 2009). In fact, eosinophils are the major source of TGF-β in asthmatic airways (Minshall et al., 1997; Ohno et al., 1996), and they are an important source of numerous cytokines (e.g. IL-4, IL-5, IL-10, IL-13) that may influence the innate and adaptive immune responses associated with asthma (Hamid and Tulic, 2009). Eosinophils also produce lipid mediators such as cysteinyl leukotrienes (Weller et al., 1983) and PGD_2 (Luna-Gomes et al., 2011), which contribute to the recruitment of innate and adaptive immunity cells, edema formation, and bronchoconstriction (Barnes, 2011). Thus, an immunomodulatory therapy that reduces eosinophil recruitment may prevent inflammation and remodeling in allergic airways. BCG treatment reduced both mononuclear and PMN accumulation in the airways, but its most prominent effect seems to be on

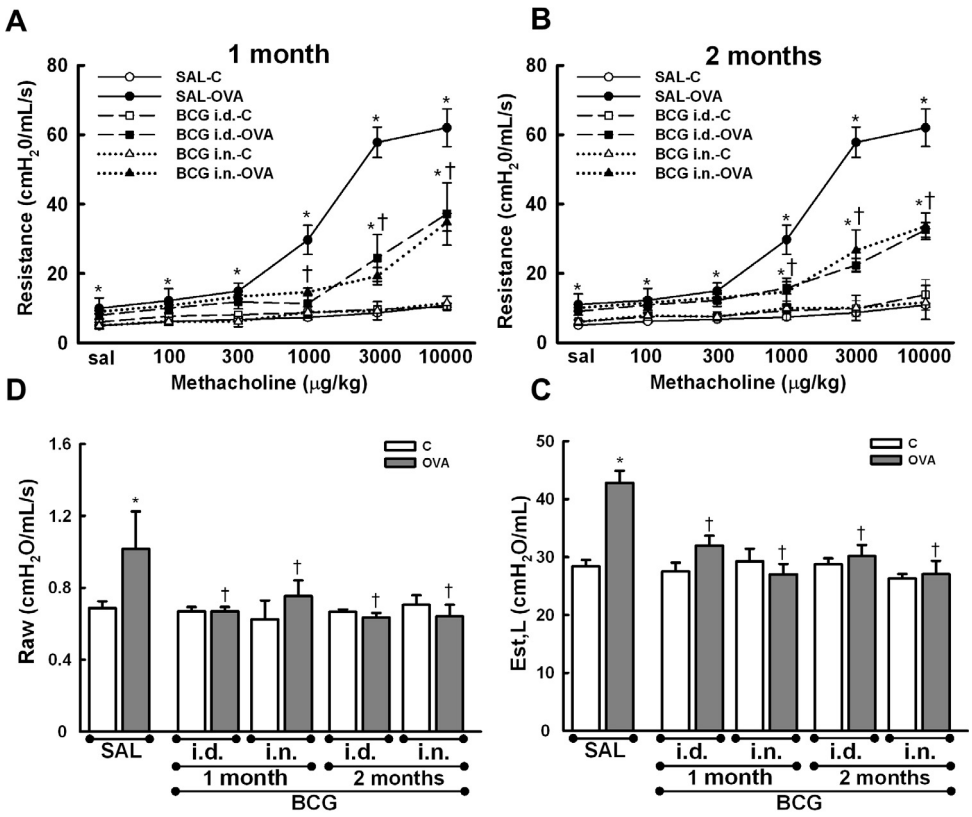

Fig. 4. Airway responsiveness induced by increasing methacholine doses (100, 300, 1000, 3000, and 10,000 μg/kg) (panel A); airway resistance (Raw) (panel B); lung static elastance (Est, L) (panel C). Mice received saline (SAL) or Bacillus Calmette–Guérin (BCG), intradermally (i.d.) or intranasally (i.n.), one or two months before sensitization and challenge with ovalbumin (OVA). Control (C) group received saline using the same protocol. Values are means ± SEM of 6 mice in each group. *Significantly different from SAL-C ($p < 0.05$). †Significantly different from SAL-OVA group ($p < 0.05$).

eosinophils, which were markedly reduced in BALF. This reduction may be associated with the decrease in IL-4, IL-5, and IL-13 (Hamid and Tulic, 2009).

IL-13 produced by T cells, eosinophils, or innate helper cells (Hamid and Tulic, 2009; Holgate, 2012) has a central role in airway hyperresponsiveness development (Grunig et al., 1998; Wills-Karp et al., 1998). BCG treatment completely abrogated IL-13 production in lungs of antigen-challenged mice, suggesting a causative relation with inhibition of lung parenchyma remodeling. Meanwhile, BCG treatment had no clear effect on TGF-β production in OVA-challenged mice. This lack of a clear-cut effect of BCG may reflect the somewhat controversial role of TGF-β in asthma. While TGF-β promotes goblet-cell and smooth muscle hyperplasia and subepithelial fibrosis, aggravating airway hyperresponsiveness (Halwani et al., 2011), it may also limit airway remodeling by inhibiting tissue damage through inhibition of T and inflammatory cells (Holgate, 2012).

The asthma model used in this study promoted a stereotypical Th2 cytokine profile with increase in cytokines related to airway and lung parenchyma inflammation and remodeling processes. BCG prevented asthma-associated alterations through modification of the adaptive immune response, which led to reduced levels of IL-4, IL-5, and IL-13 after antigen challenge. Bilenki et al. showed that BCG may reduce allergic inflammation of the airways through induction of a Th1-skewed response by mycobacterium activated dendritic cells. Transfer of dendritic cells from BCG-infected mice to mice sensitized with ragweed extract induced higher IFN-γ and IL-12 while inhibiting IL-4, 5, -9, and -13 allergen-induced production by spleen and draining lymph node cell cultures, indicating a Th1-dominated immune response (Bilenki et al., 2010). Several experimental studies in Th2-mediated diseases, including asthma, have shown an inhibition of Th2 compared to Th1 stimulus (Erb et al., 1998; Koh et al., 2001; Lagranderie et al., 2010; Tukenmez et al., 1999). However, we did not find an increase in Th1 response-associated cytokines (IFN-γ and IL-12), thus indicating that a Th1-dependent inhibition of the allergic response is unlikely in our model. Such differences may arise from variations in study design, administration route of BCG, the specific BCG strain used, or the time elapsed between BCG administration and allergic challenge. We strived to reproduce as closely as possible the effects of BCG vaccination as done in public health campaigns around the world and particularly in Brazil.

Regulatory T cells (Tregs) also seem to counteract Th2 response in allergic subjects (Holgate, 2012); thus, induction of Tregs may represent an additional potential mechanism of BCG protection in asthma (Ahrens et al., 2009). Regardless of route or time of administration, BCG promoted an increase in Foxp3 gene expression in lung, suggesting an increase in Tregs. Furthermore, this increase in Foxp3 expression was independent of OVA sensitizations and challenges, as observed in the control groups. Increase in Foxp3 was paralleled by an increase in IL-10 production after antigen challenge; this suggests that BCG may reduce asthma inflammation by favoring accumulation of IL-10-producing Tregs in lungs. IL-10 (Bilenki et al., 2010; Gao et al., 2012) and Tregs (Gao et al., 2012) have also been shown to play a central role in BCG-induced decrease in allergic inflammation.

Asthma is a chronic inflammatory disease in which an exacerbated Th2 response is a central component that leads to changes in airway responsiveness and structure, as well as function impairment (Hamid and Tulic, 2009). The focus of this study was to investigate if BCG-Moreau vaccination would have a positive impact on asthmatic airway function due to its immunomodulatory

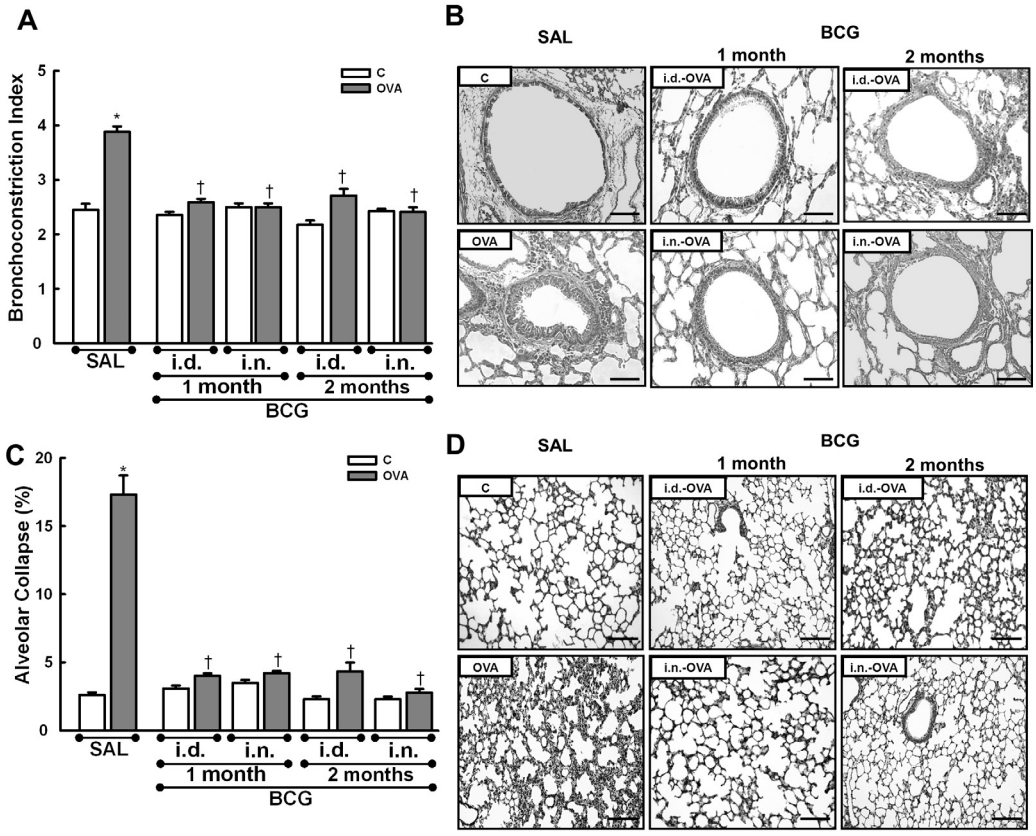

Fig. 5. Bronchoconstriction index (panel A) and fraction area of alveolar collapse (panel C). Mice received saline (SAL) or Bacillus Calmette–Guérin (BCG), intradermally (i.d.) or intranasally (i.n.), one or two months before sensitization and challenge with ovalbumin (OVA). Control (C) group received saline using the same protocol. Values are means ± SEM of 6 mice in each group. Representative photomicrographs of airways and lung parenchyma taken from an original magnification of 400× (panel B) and of 200× (panel D), respectively. *Significantly different from SAL-C ($p < 0.05$). †Significantly different from SAL-OVA group ($p < 0.05$).

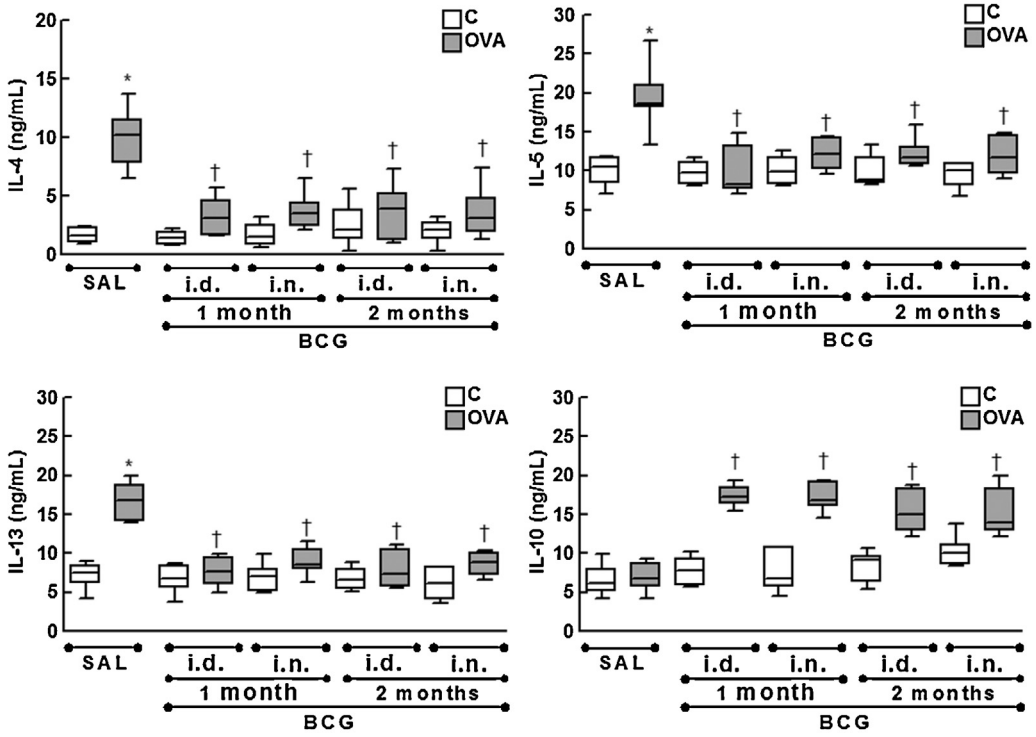

Fig. 6. Interleukin (IL)-4, IL-5, IL-13 and IL-10 in bronchoalveolar lavage fluid (BALF). Mice received saline (SAL) or Bacillus Calmette–Guérin (BCG), intradermally (i.d.) or intranasally (i.n.), one or two months before sensitization and challenge with ovalbumin (OVA). Control (C) group received saline using the same protocol. Values are means ± SEM of 6 mice in each group. *Significantly different from SAL-C ($p < 0.05$). †Significantly different from SAL-OVA group ($p < 0.05$).

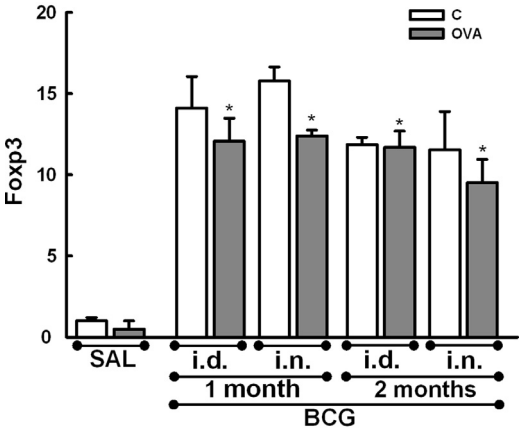

Fig. 7. Foxp3 mRNA expression in mice receiving saline (SAL) or Bacillus Calmette–Guérin (BCG), intradermally (i.d.) or intranasally (i.n.), one or two months before sensitization and challenge with ovalbumin (OVA). Control (C) group received saline using the same protocol. Values are mean ± SEM of 5 mice in each group. †Significantly different from SAL-OVA ($p < 0.05$).

effects. BCG-Moreau vaccination completely abrogated allergen-induced increases in airway resistance and elastance due its effect of reducing bronchoconstriction and alveolar collapse, respectively. Moreover, it significantly inhibited the airway hyperresponsiveness that is a hallmark of asthma. Improvement of airway function was paralleled by inhibition of airway remodeling. The number of α-smooth muscle actin-positive myofibroblasts was reduced in lung tissue in BCG-OVA compared to SAL-OVA group, which may be associated with the observed reduction in collagen deposition and subepithelial fibrosis.

The strengths of this paper are the use of BCG-Moreau, a strain widely used in children vaccination against tuberculosis in Brazil, and the modulation of lung remodeling. We believe that these strengths sufficiently counterbalance limitations such as the use of only one mouse strain (precluding extrapolation of the results to other strains) and the fact that a prophylactic approach was tested (making the results inapplicable to therapeutic management).

The present study has limitations that need to be addressed: (1) it has been described that the presence of viable organisms and granulomas in the lungs needs to be observed in order to characterize BCG immunization. However, this was not observed in our study, probably due analysis at a later time point, more than 60 days (Shaler et al., 2011). Thus, further studies should be performed earlier, following BCG administration, to establish the granulomatous inflammation; (2) we hypothesized that the benefits we observed were associated with increased Treg cells or IL-10. However, for the study to be truly mechanistic, we should have demonstrated that the BCG vaccine could no longer protect against OVA-induced asthma in the absence of Tregs or IL-10. Further studies are therefore warranted to address this point.

5. Conclusion

In conclusion, in the present murine model of allergic asthma, the BCG-Moreau strain prevented airway and lung parenchyma remodeling, regardless of administration route and time of vaccination. These beneficial effects may be related to an increase in the number of Treg cells and in the production of IL-10 in tandem with a decrease in Th2 (IL-4, IL-5, and IL-13) cytokines.

Funding

This research was supported by Center of Excellence Program (PRONEX-FAPERJ), Brazilian Council for Scientific and Technological Development (CNPq), Carlos Chagas Filho Rio de Janeiro State Research Supporting Foundation (FAPERJ), National Institute of Science and Technology of Drugs and Medicine (INCT-INOFAR), Coordination for the Improvement of Higher Level Personnel (CAPES), Coordination Theme 1 (Health) of the European Community's FP7 (HEALTH-F4-2011-282095).

Conflict of interest

The authors declare no conflict of the interest.

Acknowledgements

The authors would like to express their gratitude to Mr. Andre Benedito da Silva for animal care, Mrs. Ana Lucia Neves da Silva for her help with microscopy, and Ms. Claudia Buchweitz and Mrs. Moira Elizabeth Schöttler for their assistance in editing the manuscript.

References

Aaby, P., Shaheen, S.O., Heyes, C.B., Goudiaby, A., Hall, A.J., Shiell, A.W., Jensen, H., Marchant, A., 2000. Early BCG vaccination and reduction in atopy in Guinea-Bissau. Clin. Exp. Allergy 30, 644–650.

Ahrens, B., Gruber, C., Rha, R.D., Freund, T., Quarcoo, D., Awagyan, A., Hutloff, A., Dittrich, A.M., Wahn, U., Hamelmann, E., 2009. BCG priming of dendritic cells enhances T regulatory and Th1 function and suppresses allergen-induced Th2 function in vitro and in vivo. Int. Arch. Allergy Immunol. 150, 210–220.

Al-Muhsen, S., Johnson, J.R., Hamid, Q., 2011. Remodeling in asthma. J. Allergy Clin. Immunol. 128, 451–462, quiz 463–464.

Alm, J.S., Lilja, G., Pershagen, G., Scheynius, A., 1997. Early BCG vaccination and development of atopy. Lancet 350, 400–403.

Antunes, M.A., Abreu, S.C., Damaceno-Rodrigues, N.R., Parra, E.R., Capelozzi, V.L., Pinart, M., Romero, P.V., Silva, P.M., Martins, M.A., Rocco, P.R., 2009. Different strains of mice present distinct lung tissue mechanics and extracellular matrix composition in a model of chronic allergic asthma. Respir. Physiol. Neurobiol. 165, 202–207.

Bager, P., Rostgaard, K., Nielsen, N.M., Melbye, M., Westergaard, T., 2003. Age at bacille Calmette–Guerin vaccination and risk of allergy and asthma. Clin. Exp. Allergy 33, 1512–1517.

Barnes, P.J., 2011. Pathophysiology of allergic inflammation. Immunol. Rev. 242, 31–50.

Bateman, E.D., Hurd, S.S., Barnes, P.J., Bousquet, J., Drazen, J.M., FitzGerald, M., Gibson, P., Ohta, K., O'Byrne, P., Pedersen, S.E., Pizzichini, E., Sullivan, S.D., Wenzel, S.E., Zar, H.J., 2008. Global strategy for asthma management and prevention: GINA executive summary. Eur. Respir. J. 31, 143–178.

Bates, J.H., Rossi, A., Milic-Emili, J., 1985. Analysis of the behavior of the respiratory system with constant inspiratory flow. J. Appl. Physiol. 58, 1840–1848.

Benevolo-de-Andrade, T.C., Monteiro-Maia, R., Cosgrove, C., Castello-Branco, L.R., 2005. BCG Moreau Rio de Janeiro: an oral vaccine against tuberculosis – review. Mem. Inst. Oswaldo Cruz 100, 459–465.

Berredo-Pinho, M., Kalume, D.E., Correa, P.R., Gomes, L.H., Pereira, M.P., da Silva, R.F., Castello-Branco, L.R., Degrave, W.M., Mendonca-Lima, L., 2011. Proteomic profile of culture filtrate from the Brazilian vaccine strain *Mycobacterium bovis* BCG Moreau compared to *M. bovis* BCG Pasteur. BMC Microbiol. 11, 80.

Bilenki, L., Gao, X., Wang, S., Yang, J., Fan, Y., Han, X., Qiu, H., Yang, X., 2010. Dendritic cells from mycobacteria-infected mice inhibits established allergic airway inflammatory responses to ragweed via IL-10- and IL-12-secreting mechanisms. J. Immunol. 184, 7288–7296.

Bousquet, J., Chanez, P., Lacoste, J.Y., Barneon, G., Ghavanian, N., Enander, I., Venge, P., Ahlstedt, S., Simony-Lafontaine, J., Godard, P., et al., 1990. Eosinophilic inflammation in asthma. N. Engl. J. Med. 323, 1033–1039.

Brosch, R., Gordon, S.V., Garnier, T., Eiglmeier, K., Frigui, W., Valenti, P., Dos Santos, S., Duthoy, S., Lacroix, C., Garcia-Pelayo, C., Inwald, J.K., Golby, P., Garcia, J.N., Hewinson, R.G., Behr, M.A., Quail, M.A., Churcher, C., Barrell, B.G., Parkhill, J., Cole, S.T., 2007. Genome plasticity of BCG and impact on vaccine efficacy. Proc. Natl. Acad. Sci. U.S.A. 104, 5596–5601.

Choi, I.S., Koh, Y.I., 2002. Therapeutic effects of BCG vaccination in adult asthmatic patients: a randomized, controlled trial. Ann. Allergy Asthma Immunol. 88, 584–591.

Choi, I.S., Koh, Y.I., 2003. Effects of BCG revaccination on asthma. Allergy 58, 1114–1116.

Choi, I.S., Lin, X.H., Koh, Y.A., Cui, Y., 2007. Inoculation route-dependent and allergen-specific suppressive effects of bacille Calmette–Guerin vaccination on asthmatic reactions in BALB/c mice. Lung 185, 179–186.

Cohon, A., Arruda, L.K., Martins, M.A., Guilherme, L., Kalil, J., 2007. Evaluation of BCG administration as an adjuvant to specific immunotherapy in asthmatic children with mite allergy. J. Allergy Clin. Immunol. 120, 210–213.

Davids, V., Hanekom, W.A., Mansoor, N., Gamieldien, H., Gelderbloem, S.J., Hawkridge, A., Hussey, G.D., Hughes, E.J., Soler, J., Murray, R.A., Ress, S.R., Kaplan, G., 2006. The effect of bacille Calmette–Guerin vaccine strain and route of administration on induced immune responses in vaccinated infants. J. Infect. Dis. 193, 531–536.

Erb, K.J., Holloway, J.W., Sobeck, A., Moll, H., Le Gros, G., 1998. Infection of mice with *Mycobacterium bovis*-Bacillus Calmette–Guerin (BCG) suppresses allergen-induced airway eosinophilia. J. Exp. Med. 187, 561–569.

Gao, X., Bai, H., Cheng, J., Fan, Y., Wang, S., Jiao, L., Xiu, N., Yang, X., 2012. CD8alpha+ and CD8alpha− DC subsets from BCG-infected mice inhibit allergic Th2-cell responses by enhancing Th1-cell and Treg-cell activity respectively. Eur. J. Immunol. 42, 165–175.

Grunig, G., Warnock, M., Wakil, A.E., Venkayya, R., Brombacher, F., Rennick, D.M., Sheppard, D., Mohrs, M., Donaldson, D.D., Locksley, R.M., Corry, D.B., 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. Science 282, 2261–2263.

Halwani, R., Al-Muhsen, S., Al-Jahdali, H., Hamid, Q., 2011. Role of transforming growth factor-beta in airway remodeling in asthma. Am. J. Respir. Cell Mol. Biol. 44, 127–133.

Hamid, Q., Tulic, M., 2009. Immunobiology of asthma. Annu. Rev. Physiol. 71, 489–507.

Hayashi, D., Takii, T., Fujiwara, N., Fujita, Y., Yano, I., Yamamoto, S., Kondo, M., Yasuda, E., Inagaki, E., Kanai, K., Fujiwara, A., Kawarazaki, A., Chiba, T., Onozaki, K., 2009. Comparable studies of immunostimulating activities in vitro among *Mycobacterium bovis* bacillus Calmette–Guerin (BCG) substrains. FEMS Immunol. Med. Microbiol. 56, 116–128.

Holgate, S.T., 2008. Pathogenesis of asthma. Clin. Exp. Allergy 38, 872–897.

Holgate, S.T., 2012. Innate and adaptive immune responses in asthma. Nat. Med. 18, 673–683.

Hopfenspirger, M.T., Agrawal, D.K., 2002. Airway hyperresponsiveness, late allergic response, and eosinophilia are reversed with mycobacterial antigens in ovalbumin-presensitized mice. J. Immunol. 168, 2516–2522.

Hsia, C.C., Hyde, D.M., Ochs, M., Weibel, E.R., 2010. An official research policy statement of the American Thoracic Society/European Respiratory Society: standards for quantitative assessment of lung structure. Am. J. Respir. Crit. Care Med. 181, 394–418.

Humbles, A.A., Lloyd, C.M., McMillan, S.J., Friend, D.S., Xanthou, G., McKenna, E.E., Ghiran, S., Gerard, N.P., Yu, C., Orkin, S.H., Gerard, C., 2004. A critical role for eosinophils in allergic airways remodeling. Science 305, 1776–1779.

Jeffery, P.K., Godfrey, R.W., Adelroth, E., Nelson, F., Rogers, A., Johansson, S.A., 1992. Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. A quantitative light and electron microscopic study. Am. Rev. Respir. Dis. 145, 890–899.

Koh, Y.I., Choi, I.S., Kim, W.Y., 2001. BCG infection in allergen-presensitized rats suppresses Th2 immune response and prevents the development of allergic asthmatic reaction. J. Clin. Immunol. 21, 51–59.

Lagranderie, M., Abolhassani, M., Vanoirbeek, J., Lefort, J., Nahori, M.A., Lapa, E.S.J.R., Huerre, M., Vargaftig, B., Marchal, G., 2008. *Mycobacterium bovis* BCG killed by extended freeze-drying reduces airway hyperresponsiveness in 2 animal models. J. Allergy Clin. Immunol. 121, 471–478.

Lagranderie, M., Abolhassani, M., Vanoirbeek, J.A., Lima, C., Balazuc, A.M., Vargaftig, B.B., Marchal, G., 2010. *Mycobacterium bovis* bacillus Calmette–Guerin killed by extended freeze-drying targets plasmacytoid dendritic cells to regulate lung inflammation. J. Immunol. 184, 1062–1070.

Lee, J.J., Dimina, D., Macias, M.P., Ochkur, S.I., McGarry, M.P., O'Neill, K.R., Protheroe, C., Pero, R., Nguyen, T., Cormier, S.A., Lenkiewicz, E., Colbert, D., Rinaldi, L., Ackerman, S.J., Irvin, C.G., Lee, N.A., 2004. Defining a link with asthma in mice congenitally deficient in eosinophils. Science 305, 1773–1776.

Luna-Gomes, T., Magalhães, K.G., Mesquita-Santos, F.P., Bakker-Abreu, I., Samico, R.F., Molinaro, R., Calheiros, A.S., Diaz, B.L., Bozza, P.T., Weller, P.F., Bandeira-Melo, C., 2011. Eosinophils as a novel cell source of prostaglandin D2: autocrine role in allergic inflammation. J. Immunol. 15, 6518–6526.

Major, T., Wohlleben, G., Reibetanz, B., Erb, K.J., 2002. Application of heat killed *Mycobacterium bovis*-BCG into the lung inhibits the development of allergen-induced Th2 responses. Vaccine 20, 1532–1540.

Mestas, J., Hughes, C.C., 2004. Of mice and not men: differences between mouse and human immunology. J. Immunol. 172, 2731–2738.

Minshall, E.M., Leung, D.Y., Martin, R.J., Song, Y.L., Cameron, L., Ernst, P., Hamid, Q., 1997. Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma. Am. J. Respir. Cell Mol. Biol. 17, 326–333.

Montes, G.S., 1996. Structural biology of the fibres of the collagenous and elastic systems. Cell Biol. Int. 20, 15–27.

Nahori, M.A., Lagranderie, M., Lefort, J., Thouron, F., Joseph, D., Winter, N., Gicquel, B., Lapa e Silva, J.R., Vargaftig, B.B., 2001. Effects of *Mycobacterium bovis* BCG on the development of allergic inflammation and bronchial hyperresponsiveness in hyper-IgE BP2 mice vaccinated as newborns. Vaccine 19, 1484–1495.

Ohno, I., Nitta, Y., Yamauchi, K., Hoshi, H., Honma, M., Woolley, K., O'Byrne, P., Tamura, G., Jordana, M., Shirato, K., 1996. Transforming growth factor beta 1 (TGF beta 1) gene expression by eosinophils in asthmatic airway inflammation. Am. J. Respir. Cell Mol. Biol. 15, 404–409.

Ozeki, Y., Hirayama, Y., Takii, T., Yamamoto, S., Kobayashi, K., Matsumoto, S., 2011. Loss of anti-mycobacterial efficacy in mice over time following vaccination with *Mycobacterium bovis* bacillus Calmette–Guerin. Vaccine 29, 6881–6887.

Sarinho, E., Kunz, F.C., Bellesi, N., Maia, P.F., M, F.C., Rizzo, J.A., Silva, A.R., 2010. Múltiplas doses de vacina BCG podem proteger contra asma?/Can multiple doses of BCG vaccine protect against asthma? J. Bras. Pneumol. 36, 281–285.

Shaler, C.R., Kugathasan, K., McCormick, S., Damjanovic, D., Horvath, C., Small, C.L., Jeyanathan, M., Chen, X., Yang, P.C., Xing, Z., 2011. Pulmonary mycobacterial granuloma increased IL-10 production contributes to establishing a symbiotic host–microbe microenvironment. Am. J. Pathol. 178, 1622–1634.

Shen, H., Huang, H., Wang, J., Ye, S., Li, W., Wang, K., Zhang, G., Wang, P., 2008. Neonatal vaccination with Bacillus Calmette–Guerin elicits long-term protection in mouse-allergic responses. Allergy 63, 555–563.

Shirtcliffe, P.M., Easthope, S.E., Weatherall, M., Beasley, R., 2004. Effect of repeated intradermal injections of heat-inactivated *Mycobacterium bovis* bacillus Calmette–Guerin in adult asthma. Clin. Exp. Allergy 34, 207–212.

Tukenmez, F., Bahceciler, N.N., Barlan, I.B., Basaran, M.M., 1999. Effect of preimmunization by killed *Mycobacterium bovis* and vaccae on immunoglobulin E response in ovalbumin-sensitized newborn mice. Pediatr. Allergy Immunol. 10, 107–111.

Weller, P.F., Lee, C.W., Foster, D.W., Corey, E.J., Austen, K.F., Lewis, R.A., 1983. Generation and metabolism of 5-lipoxygenase pathway leukotrienes by human eosinophils: predominant production of leukotriene C4. Proc. Natl. Acad. Sci. U.S.A. 80, 7626–7630.

Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L., Donaldson, D.D., 1998. Interleukin-13: central mediator of allergic asthma. Science 282, 2258–2261.

World Health Organization, 2004. BCG vaccine. WHO position paper. Wkly. Epidemiol. Rec. 79, 27–38.

Wu, B., Huang, C., Garcia, L., Ponce de Leon, A., Osornio, J.S., Bobadilla-del-Valle, M., Ferreira, L., Canizales, S., Small, P., Kato-Maeda, M., Krensky, A.M., Clayberger, C., 2007. Unique gene expression profiles in infants vaccinated with different strains of *Mycobacterium bovis* bacille Calmette–Guerin. Infect. Immun. 75, 3658–3664.

Xisto, D.G., Farias, L.L., Ferreira, H.C., Picanco, M.R., Amitrano, D., Lapa, E.S.J.R., Negri, E.M., Mauad, T., Carnielli, D., Silva, L.F., Capelozzi, V.L., Faffe, D.S., Zin, W.A., Rocco, P.R., 2005. Lung parenchyma remodeling in a murine model of chronic allergic inflammation. Am. J. Respir. Crit. Care Med. 171, 829–837.

Yang, M., Yang, C., Nau, F., Pasco, M., Juneja, L.R., Okubo, T., Mine, Y., 2009. Immunomodulatory effects of egg white enzymatic hydrolysates containing immunodominant epitopes in a BALB/c mouse model of egg allergy. J. Agric. Food Chem. 57, 2241–2248.

Yang, X., Fan, Y., Wang, S., Han, X., Yang, J., Bilenki, L., Chen, L., 2002. Mycobacterial infection inhibits established allergic inflammatory responses via alteration of cytokine production and vascular cell adhesion molecule-1 expression. Immunology 105, 336–343.