

BCG Priming of Dendritic Cells Enhances T Regulatory and Th1 Function and Suppresses Allergen-Induced Th2 Function in vitro and in vivo

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Key Words

Cytokine • Allergy • Inflammation • Lung

Abstract

Background: The inverse correlation of mycobacterial infection with asthma prevalence and the inhibitory effects of vaccination with Bacille Calmette-Guérin (BCG) on airway hyperreactivity in asthma models suggest modulation of dendritic cell (DC) and T cell functions by mycobacterial compounds. **Methods:** To delineate these immunological effects, the immunogenicity of BCG Copenhagen, BCG Chicago and BCG Pasteur was compared in a mouse model. Bone marrow-derived dendritic cells (BMDCs) from BALB/c mice were stimulated with ovalbumin (OVA) with or without BCG. BMDCs were phenotypically characterized by flow cytometry, and we used ELISA to measure the cytokine production of BMDCs as well as of co-cultivated allergen-specific T cells in response to OVA-pulsed. Immunomodulatory effects of BCG were studied in a model of allergic airway inflammation by adoptive transfer of allergen-pulsed BMDCs. **Results:** Immunomodulation with BCG induced production of IL-10 and IL-12 by BMDCs. Co-cultured allergen-specific T cells produced less IL-5, IL-13 and IFN- γ but more IL-10. Also the number of FoxP3⁺ regulatory T cells was enhanced. Strongest effects were seen with BCG Chicago and BCG Pas-

teur. In vivo, administration of BCG modulated OVA-pulsed BMDCs then reduced eosinophilic airway inflammation but enhanced infiltration with granulocytes. Airway hyperreactivity and mucus production were reduced and more FoxP3⁺ T cells were observed. **Conclusion:** BCG-induced suppression of Th2-type allergic airway inflammation was associated with enhancement of regulatory T cell function but also of Th1-associated neutrophilic airway inflammation. These findings raise concerns regarding the safety profile of BCG as a potential tool for prevention and therapy of allergic airway disease.

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Introduction

Epidemiological studies have described an inverse association between mycobacterial infection and atopy and asthma [1, 2]. The first report to create a lot of excitement about this topic illustrated that in *Bacillus Calmette-Guérin* (BCG)-vaccinated school children from an endemic tuberculosis region in Japan, skin test reactivity to tuberculin and asthma prevalence were inversely associated [3]. Focusing on the history of infection rather than vaccination, it was shown that *Mycobacterium tuberculosis* infection in childhood significantly reduced the oc-

currence of subsequent asthma in women [4]. In the same line, international tuberculosis notification rates were inversely associated with the prevalence of asthma symptoms in children [5]. A probable explanation for this negative association was derived from animal models of allergic airway disease showing that, after systemic infection with BCG, sensitized animals produced significantly less Th2-type cytokines and specific serum IgE antibodies while developing little sign of airway hyperreactivity and inflammation [6, 7]. All these observations are in line with the conception of the 'hygiene hypothesis', which postulates that reduced microbial stimulation in early life leads to weakened immune regulation and thus enhanced Th2-type responses to allergens [8, 9].

For the initial polarization of a T cell response against a given antigen/allergen, the activation status of dendritic cells (DCs), which are the main antigen-presenting cells, is crucial [10]. In this context it is remarkable that mycobacteria express a strong capacity to promote IL-12 and IFN- γ production by antigen-presenting cells via Toll-like receptor-dependent stimulation [11–14]. Since those cytokines are the main driving forces for Th1 cell differentiation-antagonizing Th2-polarized allergic immune responses [15], this expression might very well explain the anti-allergic capacities of mycobacteria and/or BCG vaccines.

However, it has to be noted critically that as well as the above cited studies, other epidemiological data exist indicating that an association between an asthmatic phenotype and BCG vaccination in early life was not found [16, 17]. The epidemiological evidence for an inverse relationship between asthma and BCG vaccination seems much more complex [16, 17]. Direct comparison of the different retrospective studies is difficult, because of variable vaccination schedules and the different BCG strains used. Since BCG was first used for vaccinating humans in 1921, the original BCG-Pasteur type has been distributed to various countries, resulting in the development of various daughter strains until the 1960s/1970s when it became possible to prepare seed lots. Recent molecular analysis of these lots identified multiple deletions in the genome of the offspring [18]. The various strains differ not only in growth, colony morphology, residual virulence and protection against tuberculosis in animal models [19], but also in their capacity to induce Th1 responses [20, 21]. Genetic variations of immunogenic proteins such as mpb64, mpb70 or mpb80 might be responsible [18].

Here we compared different BCG strains in their capacity to modulate DC and T cell function regarding

Th1/Th2 polarization. We selected the strains BCG Copenhagen, BCG Chicago and BCG Pasteur because they are among the most commonly used in tuberculosis vaccination. We then applied the most potent BCG strain in terms of suppression of Th2 immunity in a mouse model of allergic airway disease and tested its capacity to serve as a potential tool for allergy prevention.

Materials and Methods

Animals

Specific pathogen-free female BALB/c mice (6–8 weeks old) and OVA T cell receptor transgenic DO11.10 mice were obtained from the Federal Institute of Risk Assessment, Berlin, Germany. All experiments were approved by the local Office for Occupational Safety, Protection of Health and Technical Safety (LaGetSi).

Bacterial Strains

Mycobacterium bovis BCG substrains: BCG Copenhagen (Danish strain 1331), BCG Chicago (Tice) and BCG Pasteur were obtained from Ulrich Schaible, Department of Immunology, Max Planck Institute for Infection Biology, Berlin, Germany (strain, standard preparation containing $2-8 \times 10^5$ CFU/0.1 ml).

In vitro Stimulation of BMDCs

Bone marrow-derived dendritic cells (BMDCs) were generated from bone marrow of BALB/c mice in the presence of high doses of GM-CSF as previously described [22]. Briefly, freshly prepared bone marrow cells were cultured in Click's RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS (PAA Laboratories, Linz, Austria), 2 mM L-glutamine, 10 mM HEPES, 60 μ g/ml penicillin and 20 μ g/ml gentamicin in the presence of 10 ng/ml GM-CSF (PeproTech, Rocky Hill, N.J., USA). Cultures were fed with GM-CSF on days 3, 6 and 8. After 10 days, non-adherent cells were counted, resuspended at 1×10^6 cells/ml and stimulated for 18 h with OVA (100 μ g/ml; Sigma-Aldrich, St. Louis, Mo., USA), BCG Copenhagen, BCG Chicago or BCG Pasteur (10×10^6 CFU/ml). BMDCs resuspended only with supplemented RPMI medium were used as controls. The culture supernatants were harvested and tested for the presence of cytokines by ELISA 24 h later. In parallel, the expression levels of CD80, CD86 and MHC class II on the surface of the BMDCs were determined by flow cytometry after exclusion of dead cells by PI staining (data not shown).

Analysis of OVA by *Limulus* amoebocyte lysate test revealed quantities of LPS ranging between 6 and 60 ng/mg protein.

In vitro Stimulation of OVA-Specific T Cells

OVA-specific CD4⁺ T cells were isolated from spleens of DO11.10 mice by magnetic CD11c depletion followed by CD4⁺ selection (Miltenyi Biotec, Bergisch Gladbach, Germany). T cells were co-cultured with OVA/BCG-stimulated BMDCs (proportion DC:CD4 1:10) at 1×10^6 /ml in complete RPMI. Before magnetic cell sorting, dead cells were removed by Ficoll-gradient centrifugation (Ficoll Histopaque®-1083; Sigma-Aldrich). After 6 days of co-culture, cells were harvested, washed and restimulated

with PMA (10 ng/ml, Sigma-Aldrich) and ionomycin (1 µg/ml, Sigma-Aldrich) for 24 h. At this time point no BMDCs were left. IL-4, IL-5, IL-10, IL-13, and IFN-γ levels in supernatants were quantified by ELISA. To assess proliferation, antigen-specific CD4⁺ T cells were labeled with CFSE (Molecular Probes; Invitrogen, Carlsbad, Calif., USA), as previously described [23], and analyzed by flow cytometry.

In vivo Sensitization and Treatment

BALB/c mice were passively sensitized by intravenous injection of 2.5×10^6 OVA-specific CD4⁺ T cells from OVA TCR transgenic DO11.10 mice on day -1. Recipients were subsequently challenged on days 0 and 10 by intranasal application of BMDCs stimulated with 1×10^6 OVA (OVA) or OVA and BCG (OVA/BCG). Control animals received unprimed BMDCs for intranasal challenge (Med).

Determination of Airway Reactivity

Airway reactivity was determined on day 12 by barometric plethysmography (Buxco), as previously described [24, 25]. Mice were challenged with aerosolized PBS or increasing doses of methacholine (6–50 mg/ml) for 3 min. Data are expressed as fold increase of baseline values with PBS, using the dimensionless parameter 'enhanced pause' (P_{enh}).

Bronchoalveolar Fluid

On day 13, animals were sacrificed and tracheas cannulated. Airways were lavaged twice with 0.8 ml ice-cold PBS, and cell numbers were determined as described previously [25]. Bronchoalveolar (BAL) cells were counted, spun onto glass slides using a Shandon Cytospin (Thermo Fisher Scientific, Astmoor, UK) and stained with Diff-Quick (Dade Behring, Marburg, Germany), according to the manufacturer's instructions. Numbers of eosinophils, neutrophils, lymphocytes and macrophages present in the BAL were determined microscopically using standard histological criteria.

Immunoglobulin Production

Total IgE and allergen-specific IgE, IgG1 and IgG2a antibody titers were measured by enzyme-linked immunosorbent assay (ELISA), as described previously [25].

Histochemistry

Staining with haematoxylin and eosin (HE) was carried out using standard protocols on 8-µm transversal lung cryosections.

Lung Cell Suspensions

Lungs were perfused via the right ventricular cavity of the heart with 10 ml of PBS to remove the peripheral blood cells. Lungs were carefully removed and cut into small pieces for enzymatic digestion in PBS containing 175 U/ml collagenase, 50 U/ml DNase (both from Sigma-Aldrich), 1% BSA, and 5 mM EDTA. Lung pieces were digested for 25 min at 37°C and an additional 5 min after adding 80 µl 0.5 M EDTA (pH 7.2.) Thereafter, lungs were homogenized by pipetting with a Pasteur pipette and lung cell suspensions filtered through cell strainers (BD Biosciences, San Jose, Calif., USA). Following a density gradient centrifugation (Lympholyte-M; Cedarline Laboratories, Hornby, Canada), cells were finally washed 3 times in PBS/EDTA/BSA. Pooled peri-

bronchial lymph node cells were obtained by passing them through a nylon mesh, washed 3 times in PBS/EDTA/BSA.

Flow Cytometry

Cells were stained on ice with FITC, PE or Alexa Fluor 647 conjugated antibodies in PBS, 2.5% BSA, 0.1% NaN₃. To block unspecific binding, cells were pre-incubated with 100 µg/ml anti-CD16/32 (2.4G2) and 50 µg/ml rat immunoglobulin (Nordic). T cells were stained with antibodies to CD25 (clone 2E4), CD45RB (M4B4) and ICOS (MIC-280). DCs were stained with antibodies to CD11c (N418), MHCII (M5/114), CD40 (FGK), CD80 (16-10A1), CD86 (GL-1), ICOS-L (MIL-5733), CD200 (3B6), CXCR5 (2G8), CXCR4 (2BII), and CCR5 (C34-3448). B cells were stained with CD80 (16-10A1), CD86 (GL1), IgD (11-26c), CD19 (1D3) and B220 (RA3-6B2). Antibodies were prepared in-house from hybridoma supernatants or bought from BD Biosciences or eBioscience (San Diego, Calif., USA).

For staining of FoxP3 (eBioscience) cells were fixed, permeabilized and stained, as per instructions. Cells were analyzed on a FACSCalibur (BD Biosciences). Cells were gated by scatter characteristics and dead cells were excluded using propidium iodide (0.3 µg/ml; Sigma). Data were processed with CellQuest Pro (BD Biosciences) or FlowJo (TreeStar, Ashland, Oreg., USA) software.

Statistical Analysis

Results are presented as means $\pm$ SD. Means were compared using Mann-Whitney U test, SPSS, version 14.0 (SPSS Inc., Chicago, Ill., USA). $p < 0.05$ was regarded as significant.

Results

BCG Strains Differentially Modulate Activation Status and Cytokine Production by BMDCs

Immature BMDCs were exposed to BCG Copenhagen, BCG Chicago and BCG Pasteur and phenotypical characterized by flow cytometry. All strains induced up-regulation of CD80, CD86 and CD40. The Copenhagen strain enhanced expression of CD80, CD86 and CD40 approximately 2-fold, as compared to unstimulated BMDCs. The Chicago and the Pasteur strains enhanced the mean fluorescence intensity even more than 3-fold (data not shown). No effect was seen on ICOS-L, CD200, CXCR4, CXCR5 and CCR5 (data not shown).

In order to define the pattern of cytokines released by BMDCs that was induced by different BCG strains, we measured IL-10, IL-12 and TNF-α in supernatants of cultured BMDCs after BCG-stimulation. While non-stimulated BMDCs failed to produce significant amounts of these cytokines, an enhanced IL-10 and TNF-α production was detected after stimulation with all BCG strains. While BCG Chicago and BCG Pasteur induced strong secretion of IL-10, the strongest production of IL-12 was detected after stimulation with BCG Pasteur. TNF-α se-

cretion was most enhanced after stimulation with BCG Chicago (fig. 1). The addition of OVA to BCG-stimulated BMDCs did not change the cytokine profile or pattern (data not shown). Pulsing of DCs with OVA was required in view of the in vitro co-culture experiments with allergen-specific T cells and the in vivo experiments with allergen challenges.

BCG-Stimulated BMDCs Modulate Cytokine Production and FoxP3 Expression of Co-Cultured CD4⁺ T cells in vitro

To determine whether BCG-stimulated BMDCs modulate T cell differentiation, we co-cultured OVA-pulsed BMDC with OVA-specific CD4⁺ T cells from DO11.10 mice. CD4⁺ T cells co-cultured with BCG/BMDC produced significantly lower levels of IL-5 and IL-13, in comparison to BMDCs stimulated with OVA only. Notably, the production of IFN- γ also was significantly reduced by stimulation with BCG. In contrast, BCG modulation led to a significant elevation of IL-10, which was most pronounced with the Chicago strain (fig. 2). In our experimental setup these T cells were the only source for cytokine production, since they were washed and transferred to another plate 6 days after co-culture, and then restimulated with PMA and ionomycin.

To analyze the number of regulatory T cells, we stained for the transcription factor FoxP3. With OVA-BMDCs only about 0.9% of the antigen-specific T cells were positive for FoxP3 and CD25. However, this frequency was higher in co-cultures with BCG-stimulated BMDCs, up to 3.3% when the Chicago strain was used (fig. 3).

Taken together, BCG stimulation of BMDCs induced IL-10 secretion by T cells, in particular after stimulation with BCG Chicago. Compared to T cells co-cultured with only OVA-pulsed BMDCs, production of IL-5 and IFN- γ was reduced in co-cultures with BCG-modulated BMDCs.

BCG Modulates BMDC-Induced Airway Inflammation and Hyperreactivity in vivo

To check whether BCG-modulated BMDC exert a regulatory function in vivo, we tested them in an in vivo model of allergic airway inflammation. We transferred allergen-specific CD4⁺ T cells intravenously into naïve BALB/c mice and subsequently administered BCG-modulated OVA-pulsed BMDCs via the airways. As BCG Chicago and BCG Pasteur had been identified as exerting the strongest immunomodulatory effect, we chose BCG Pasteur for our in vivo analysis. As a parameter for airway inflammation we analyzed cells from BAL.

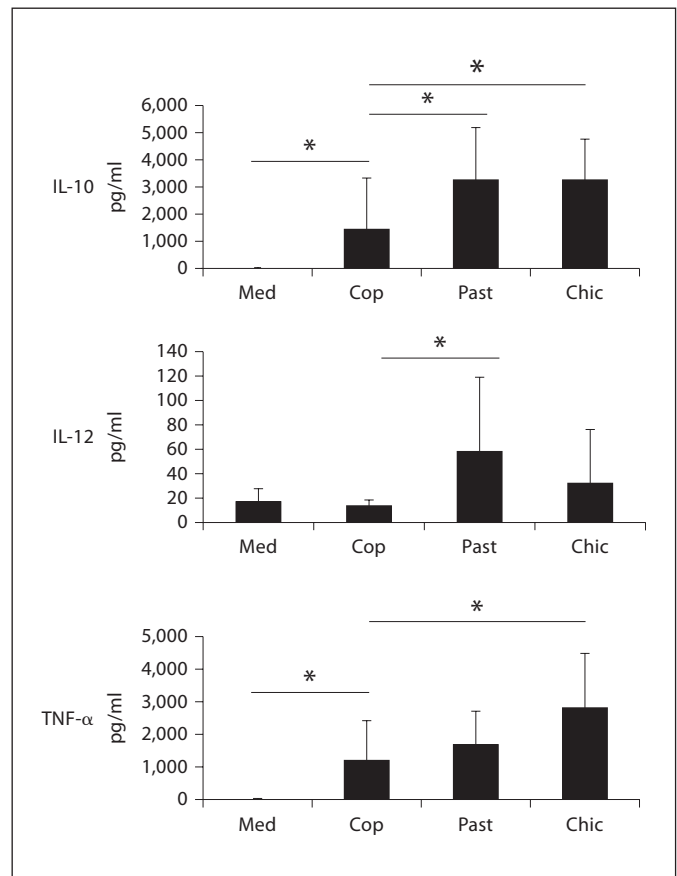


Fig. 1. Cytokine profile of stimulated BMDCs. After 6 days of culture, BMDCs were stimulated with OVA/BCG strains Copenhagen (Cop), Pasteur (Past) or Chicago (Chic). 18 h later supernatants were collected and taken for cytokine analysis by ELISA. Secretion levels of IL-10, IL-12p70, and TNF- α are shown as mean $\pm$ SD of triplicate samples. Med = Controls. * $p < 0.05$.

The total cell number was strongly increased in the positive control group (OVA) compared to the negative control, whereas the total cell number in the BALF of OVA/BCG-treated animals was even triple the negative control (fig. 4b). Following OVA transfer, the numbers of total granulocytes, lymphocytes and eosinophils were significantly increased, compared to the negative control group (fig. 4a). The transfer of OVA/BCG, however, led to an even stronger elevation of neutrophilic granulocytes and monocytes, while eosinophilic infiltration was abrogated.

To analyze if changes in cellular infiltrations led to histological alterations, we determined the intensity of cell influx and mucus production by HE staining in centrally located lung tissue. Following transfer of OVA-

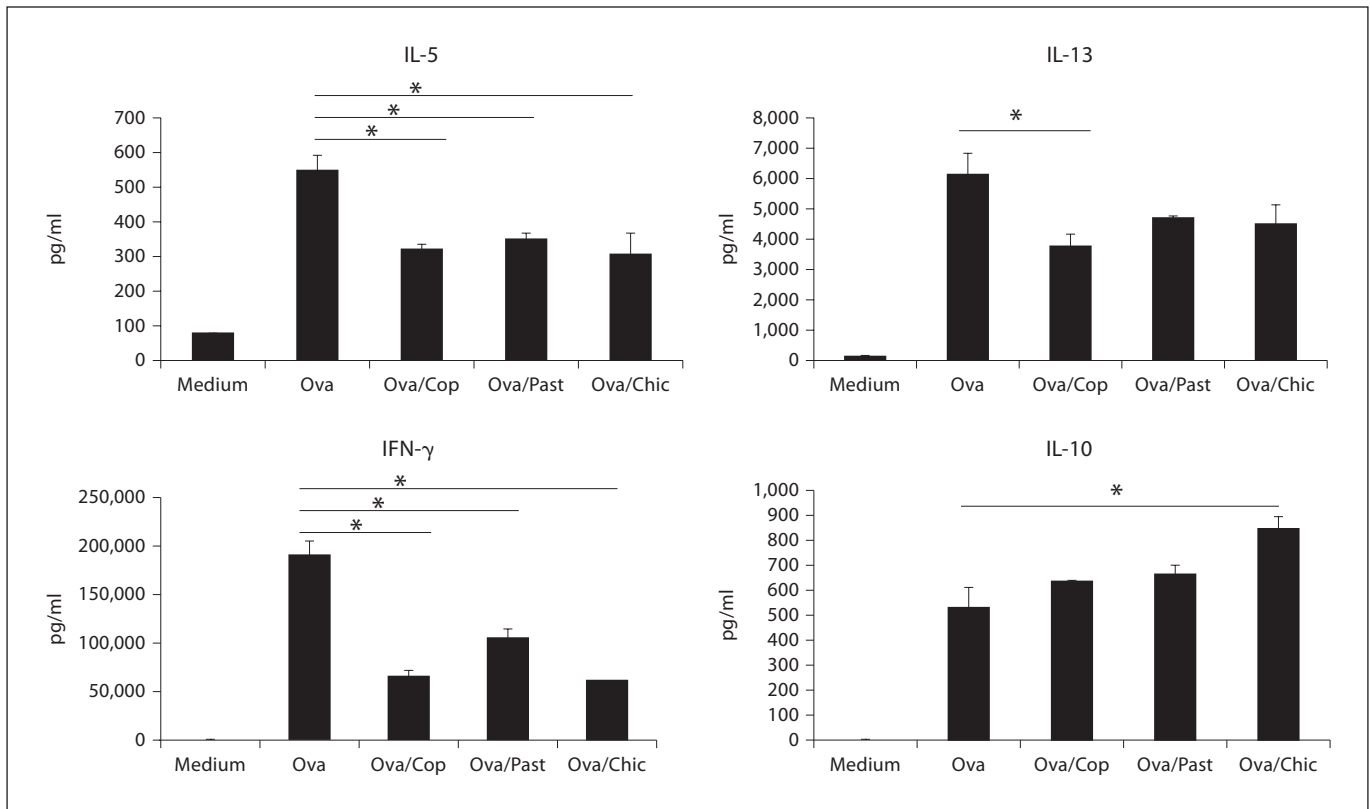


Fig. 2. Cytokine profile of CD4⁺ T cells after co-culture with stimulated BMDCs. BMDCs were pulsed with OVA/BCG strains Copenhagen (Cop), Pasteur (Past) or Chicago (Chic) and co-cultured with CD4⁺ T cells from OVA-TCR transgenic DO11.10 mice. Unstimulated BMDC were co-cultured with CD4⁺ cells as negative

control (medium). After 6 days, cells were restimulated with PMA and ionomycin. 24 h later, supernatants were collected to determine cytokine levels by ELISA. Data are mean $\pm$ SD of triplicate samples. * $p < 0.05$.

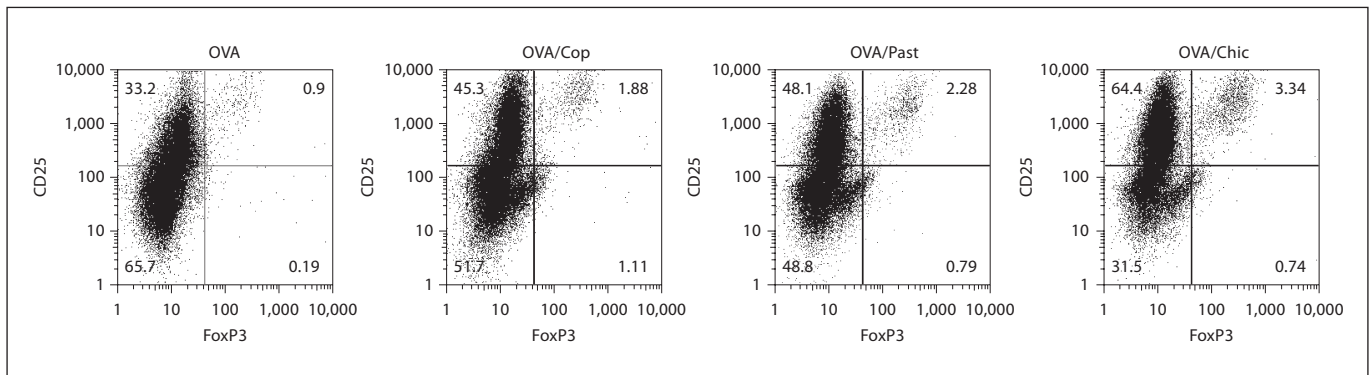


Fig. 3. FoxP3 staining of CD4⁺ T cells after co-culture with stimulated BMDCs. BMDCs were pulsed with OVA/BCG strains Copenhagen (Cop), Pasteur (Past) or Chicago (Chic) before co-culture with isolated CD4⁺ T cells from OVA-TCR transgenic

DO11.10 mice. Unstimulated BMDC were co-cultured with isolated CD4⁺ T cells as negative control (medium). After a 6-day co-culture, cells were stained for intracellular FoxP3 and analysed by flow cytometry.

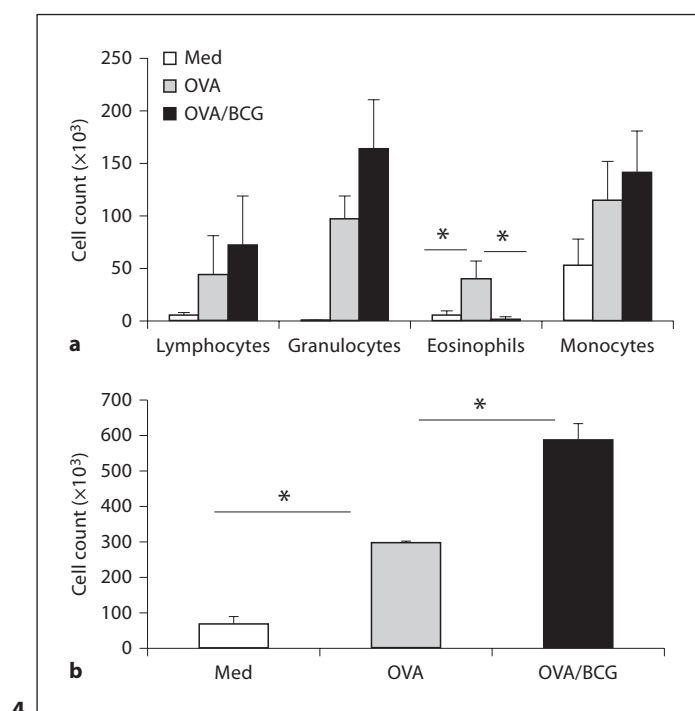
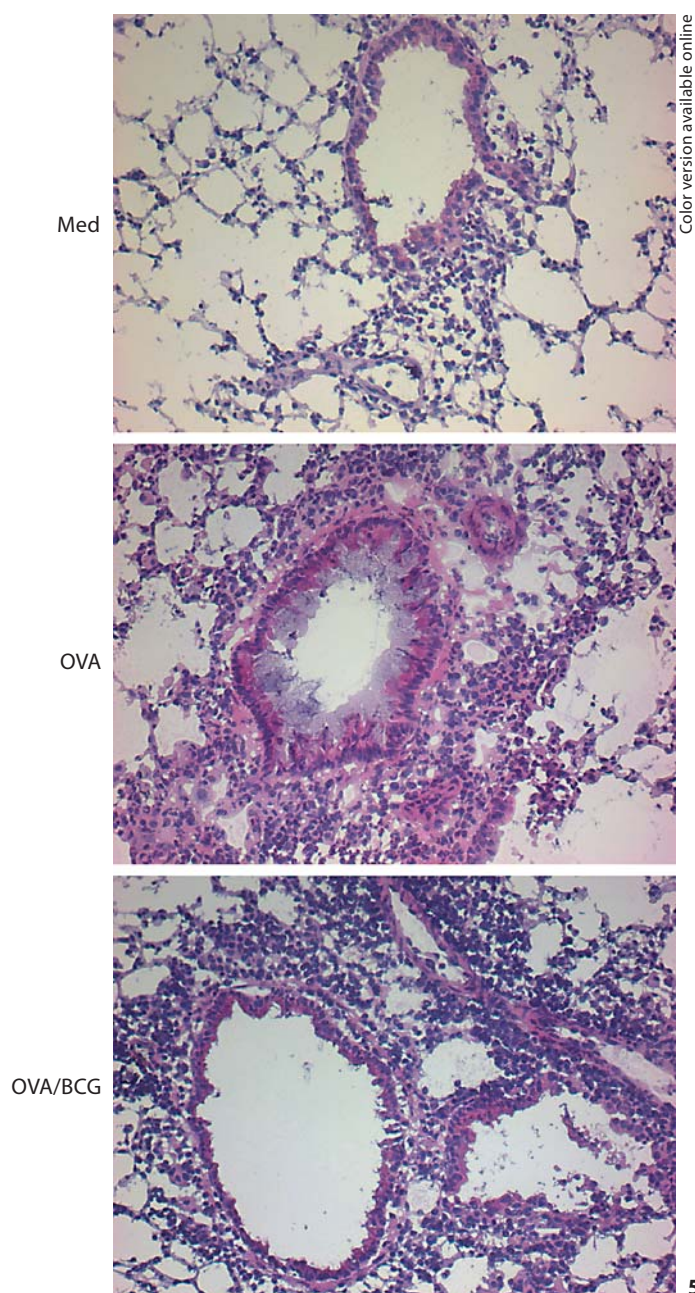


Fig. 4. Cell count and differentiation in bronchoalveolar fluid. BALB/c mice were passively sensitized by intravenous injection of 2.5×10^6 OVA-specific CD4⁺ T cells from OVA TCR transgenic DO11.10 mice on day -1. Recipients were subsequently challenged on days 0 and 10 by intranasal application of 1×10^6 BMDCs stimulated with OVA (OVA) or OVA and BCG (OVA/BCG). Control animals received unprimed BMDCs for intranasal challenge (Med). 48 h after the last challenge animals were sacrificed and tracheas cannulated for BAL. Cells from BALF were counted, spun onto glass slides and stained with DiffQuick® before microscopy. Total cell number and numbers of different cell types are shown as mean $\pm$ SD of 5 animals per group in a representative assay from a series of 3 independent experiments. * $p < 0.05$.

Fig. 5. Light photomicrographs of HE-stained cryosections of lung tissues. BALB/c mice were passively sensitized by intravenous injection of 2.5×10^6 OVA-specific CD4⁺ T cells from OVA TCR transgenic DO11.10 mice on day -1. Recipients were subsequently challenged on days 0 and 10 by intranasal application of 1×10^6 BMDCs stimulated with OVA (OVA) or OVA and BCG (OVA/BCG). Control animals received unprimed BMDCs for intranasal challenge (Med). On day 13, animals were sacrificed and lungs frozen in liquid nitrogen. 10- μ m cryosections from lung tissue were fixed and stained with HE.



pulsed BMDCs, numbers of mucus producing goblet cells were increased and overall mucus production was enhanced in comparison to negative controls (Med; fig. 5). In contrast, lung tissue of OVA/BCG-treated animals displayed no signs of increased mucus production, although interstitial lymphoid infiltration was similar to

the OVA group (fig. 5). Moreover, the airway lumen of OVA/BCG-treated animals was shown to be much less narrow than the lumen of OVA-treated mice.

Airway responsiveness of spontaneously breathing, conscious mice to aerosolized methacholine was assessed by barometric whole-body plethysmography [24, 25].

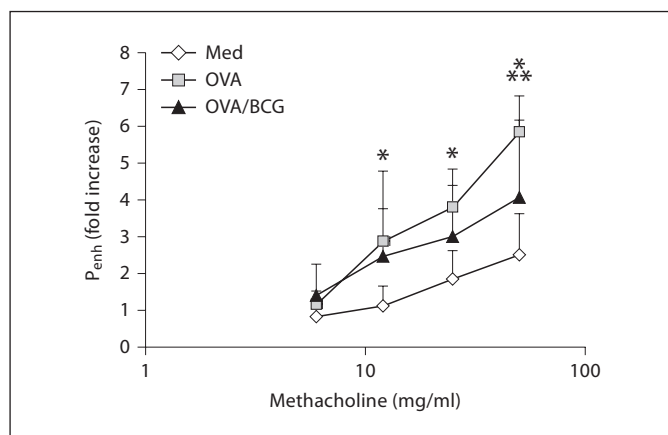


Fig. 6. Airway reactivity after in vivo sensitization and treatment. BALB/c mice were passively sensitized by intravenous injection of 2.5×10^6 OVA-specific CD4⁺ T cells from OVA TCR transgenic DO11.10 mice on day -1. Recipients were subsequently challenged on days 0 and 10 by intranasal application of 1×10^6 BMDCs stimulated with OVA (OVA) or OVA and BCG (OVA/BCG). Control animals received unprimed BMDCs for intranasal challenge (Med). 24 h after the last allergen challenge airway reactivity was measured as previously described. Fold increase of P_{enh} after 3 min of provocation with methacholine (50 mg/ml) is shown as mean $\pm$ SD of 5 animals per group in a representative trial of a series of 3. * $p < 0.05$, Med vs. OVA; ** $p < 0.05$, OVA/BCG vs. OVA.

Following transfer of OVA-BMDCs in mice (OVA), airway reactivity to increasing doses of methacholine was significantly increased compared to negative control (Med). Compared to the OVA group, airway reactivity of OVA/BCG-treated animals was reduced but still higher than in the control group (fig. 6).

The in vivo transfer of BCG-modulated BMDCs in naïve mice did not lead to different serum levels of total IgE (data not shown). However, OVA-specific IgG1 and IgG2a were significantly enhanced in the OVA/BCG-treated group compared to OVA. On the other hand, a relevant production of OVA-specific IgE was not observed after transfer of any kind of BMDCs (fig. 7a).

BCG Modulates BMDC-Induced Immune Responses in vivo

Phenotypic characterization of cells from local lymph nodes and digested lungs was performed by flow cytometry. The total cell number in lymph node (LN) and digested lungs was significantly increased after transfer of OVA-pulsed BMDC, compared to medium control (data not shown). The transfer of OVA/BCG-modulated BMDCs led to a stronger increase of total cell numbers in LN

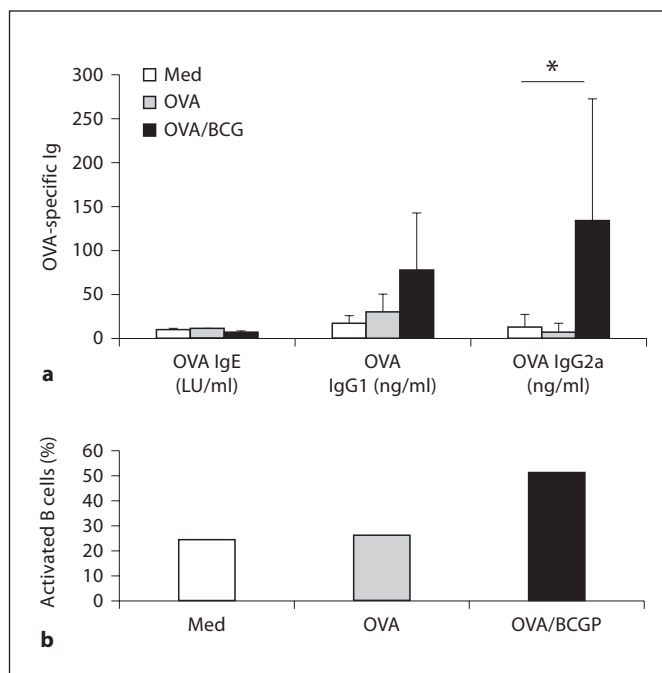
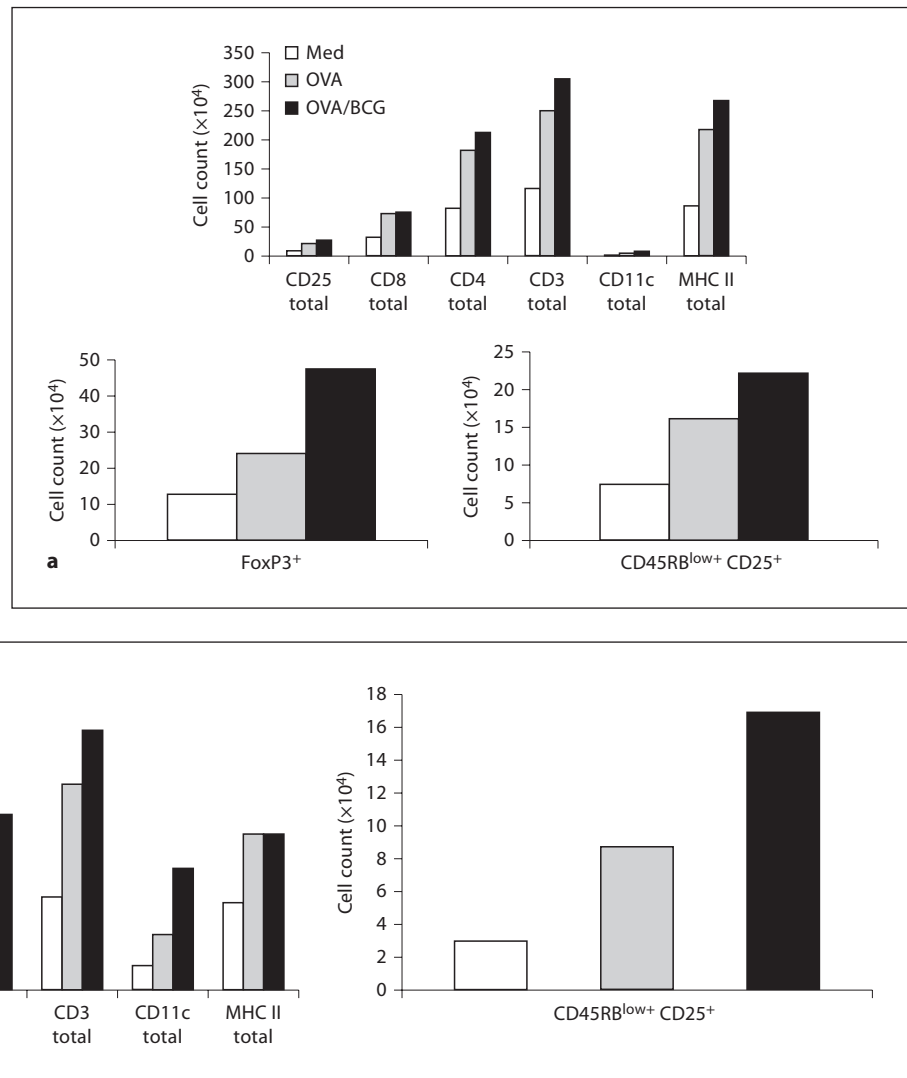


Fig. 7. B cell response after in vivo sensitization and treatment. BALB/c mice were passively sensitized by intravenous injection of 2.5×10^6 OVA-specific CD4⁺ T cells from OVA TCR transgenic DO11.10 mice on day -1. Recipients were subsequently challenged on days 0 and 10 by intranasal application of BMDCs stimulated with 1×10^6 OVA or OVA and BCG. Control animals received unprimed BMDCs for intranasal challenge (Med). On day 13, animals were sacrificed. **a** Blood samples were collected and levels of OVA-specific IgE, IgG1 and IgG2a were determined by ELISA. Mean $\pm$ SD of 5 animals per group is shown of a representative result from a series of 3 independent trials. * $p < 0.05$. **b** Peribronchial lymph node cells were stained with antibodies to CD19, IgD and CD86 and analyzed by flow cytometry. Shown is the percentage of activated (IgD⁺ CD86⁺) B cells.

and digested lungs. However, the total cell number was highest in the spleens from controls, suggesting a recruitment of cells from spleen to the respiratory system (data not shown).

Total numbers of CD3, CD4 and CD8 expressing T cells in the lung significantly increased after OVA-BMDC administration, and further increased after BCG stimulation. Regarding T cell subsets, the strongest influx of cells was detectable in the OVA/BCG group – in local LN as well as in lung digest. The cellular composition (CD3⁺, CD4⁺ and CD8⁺) was comparable between all groups. However, further characterization of the T cells from LN and lung digest revealed a significant increase in numbers of activated CD4⁺CD25⁺, CD4⁺CD45RB^{low} effector/memory

Fig. 8. Lymphocyte subpopulations in bronchial lymph nodes and lung cell suspension. BALB/c mice were passively sensitized by intravenous injection of 2.5×10^6 OVA-specific CD4⁺ T cells from OVA TCR transgenic DO11.10 mice on day -1. Recipients were subsequently challenged on days 0 and 10 by intranasal application of BMDCs stimulated with 1×10^6 OVA or OVA and BCG. Control animals received unprimed BMDCs for intranasal challenge (Med). On day 13, animals were sacrificed. Pooled peribronchial lymph nodes cells (a) or lung cell suspensions (b) were stained with antibodies to T cells: CD3, CD4, CD8, ICOS, CD25 and CD45RB. DCs were stained with antibodies to CD11c and MHCII. For staining of FoxP3, cells were fixed, permeabilized, stained and analyzed by flow cytometry.



phenotype, and FoxP3⁺ regulatory T cells after OVA/BCG transfer, compared to the OVA group (fig. 8a, b).

The proportion of activated B cells (CD19⁺, CD86⁺, IgD⁻) in lymph nodes was doubled in the OVA/BCG group compared to the medium group. This increase was associated with an increased production of OVA-specific IgG1 and IgG2a antibodies, known to be associated with a Th1 cytokine profile in this system (fig. 7b).

In all transfer groups, DCs expressing high levels of CD11c and MHC class II were found to be activated (CD80 and CD86 positive) to similar degrees. However, we observed an almost 3-fold increase in the total numbers of DCs in the LNs and lung digest of OVA/BCG recipients. Interestingly, numbers of myeloid DC (Ly-6G/C⁺ CD11c high) were nearly 5-fold higher in LN from

OVA/BCG recipients compared to LN from the medium group, and twice as high compared to the OVA group. Counts of plasmacytoid DCs (Ly-6G/C⁺ CD11c low) increased significantly after transfer of OVA and OVA/BCG DCs, however less pronounced than myeloid DCs, to a similar degree for both groups (fig. 9).

Discussion

Epidemiological studies in humans and experimental work in animals suggest that mycobacteria can modify allergen-induced airway inflammation [1]. Our present study further delineates this notion, showing that an inhibitory function of BCG might be mediated via modula-

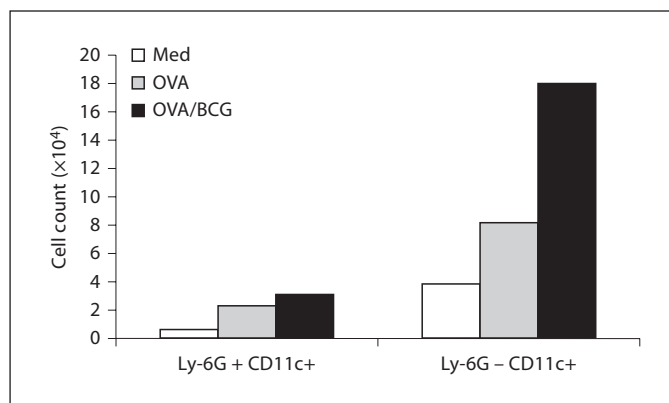


Fig. 9. DC subpopulations in bronchial lymph nodes. BALB/c mice were passively sensitized by intravenous injection of 2.5×10^6 OVA-specific CD4⁺ T cells from OVA TCR transgenic DO11.10 mice on day -1. Recipients were subsequently challenged on days 0 and 10 by intranasal application of 1×10^6 BMDCs stimulated with OVA (OVA) or OVA and BCG (OVA/BCG). Control animals received unprimed BMDCs for intranasal challenge (Med). On day 13, animals were sacrificed. Pooled peribronchial lymph node cells were stained for CD11c and Ly-6G/C and analyzed by flow cytometry.

tion of DC, resulting in up-regulation of regulatory T cell function and down-regulation of Th2-type function in vitro. The level of Treg enhancement was dependent on the specific BCG strain employed, where the secretion of IL-10, IL-12 and TNF- α was higher after stimulation with the Chicago and Pasteur than with the Copenhagen strain. The in vivo inhibition of allergen-induced airway inflammation by BCG-primed DC was associated with a Th1-associated airway inflammation characterized by an influx of high numbers of neutrophilic granulocytes.

In our in vitro analysis we observed that different BCG strains have different effects on BMDC activation and function. The phenotypical analysis revealed a variable pattern of activation markers expressed on the cell surface. BCG Chicago and Pasteur nearly equally led to a strong up-regulation of CD80, CD86 and CD40, whereas stimulation with BCG Copenhagen showed less effect. There was no expression of the co-stimulatory ligands ICOS-L, PD-L1 and PD-L2, nor of chemokine receptors such as CXCR4, CXCR5 and CCR5 (data not shown). The different BCG strains also influenced the cytokine production differently. After stimulation with BCG Copenhagen, the cytokine production was less enhanced compared to BCG Pasteur and Chicago. The latter 2 strains led to a nearly equal amount of IL-10, whereas Pasteur

induced the strongest IL-12, and Chicago the highest TNF- α production. Looking at the 'historical evolution' of BCG strains it is very interesting to find that the daughter strain BCG Chicago (Tice), which has been known since 1934, was mixed with BCG Pasteur (also modified) in the 1950s by Rosenthal because of its diminished state [26]. This succeeding mixture might be an explanation for the similar in vitro effect of Pasteur (lyophilised 1961) and Chicago (Tice) strains compared to the Copenhagen (Danish) strain, in our system. Genetic variations of immunogenic proteins such as mpb64, mpb70 or mpb80 offer a possible explanation for this phenomenon.

Co-cultivation of BCG stimulated BMDC with allergen-specific T cells, which induced a reduced production of Th2 cytokines IL-13 and IL-5, but also a marked reduction of IFN- γ (Th1). This is probably explained by enhanced regulatory DC functions after modulation with BCG, translating into increased production of IL-10 by regulatory T cells. Accordingly, numbers of regulatory T cells expressing FoxP3 [27] were more than doubled after co-culture with BCG Pasteur- or BCG Chicago-stimulated DCs, compared to co-culture of T cells with OVA-primed BMDC. Actually, Hanekom was one of the earliest to describe the ability of BCG to drive Treg in human newborns [28].

It was now an obvious question to ask whether modulation of DC function by BCG translated into immune suppression of airway disease in vivo. For this purpose, we chose the BCG strain Pasteur due to its influence on in vitro cytokine production of BMDCs, especially considering the production of IL-10 and IL-12. Intranasal administration of OVA/BCG Pasteur-stimulated BMDCs into BALB/c mice that had received an intravenous application of allergen-specific naïve T cells resulted in a strong reduction of eosinophilic airway inflammation compared to mice receiving OVA-BMDCs. Moreover, cell hyperplasia and increased mucus formation in the airways was diminished in the group receiving BCG-modulated BMDC. Thus, cardinal features of allergic asthma, closely connected with increased IL-5 [29], IL-4 and IL-13 production [30], were abrogated by immune modulation with BCG.

However, we noticed an enhanced inflammation with neutrophilic granulocytes in the bronchoalveolar lavage from mice receiving BCG-modulated DC, a strong indicator of an increased Th1 response. In the histology of lung specimens, this cell influx was even more impressive, likely affecting the development of airway hyperactivity in mice after administration of BCG/BMDCs. These stainings also showed a reduction in mucus-pro-

ducing goblet cells in the animals receiving BCG-modulated DC compared to those receiving OVA-only-pulsed DC. This difference could be due to either a reduction in Th2 cytokine secretion, possibly due to an increase in regulatory elements or an increase in Th1 secretion. The identification of activated IgD⁺CD86⁺ B cells in FACS analysis after BCG/OVA-BMDC treatment was nicely reflected by an increased OVA-specific IgG2a and IgG1 production also suggesting enhanced Th1 cytokine secretion since IgG2a is associated with such a cytokine pattern (fig. 7a, b).

Induction of airway inflammation via intranasal application of OVA-BMDCs was associated with cell recruitment from spleen to local LN and the lung, which was amplified through BCG/OVA-BMDC treatment. Cellular compositions in local LN and lung-tissue infiltrated cells furthermore revealed an increase of (activated) T cells as well as DCs, with a main emphasis on myeloid DC. Similar to our in vitro results, we detected more CD45RB^{low} CD25⁺ cells expressing FoxP3 in LN and in the lungs from mice treated with BCG-BMDCs (fig. 8a, b), denoting that in addition to changes associated with an increased Th1 cytokine pattern, regulatory elements were also increased in our in vivo system.

Currently, the consequences of treatment strategies aimed at direct antagonization of enhanced Th2 cytokine production in Th2-biased diseases is an ongoing contro-

versial discussion. Some studies have demonstrated that reduction of increased Th2 features by treatment with Th1-promoting tools was associated with a marked symptomatic relief [31–33]. On the other hand, it has been shown that transfer of allergen-specific Th1 cells into airways of naïve mice prior to allergen airway challenges caused severe airway inflammation and did not reduce development of Th2-mediated airway hyperreactivity and inflammation [34, 35]. Our present results support these findings by demonstrating that using BCG to modulate DC induced regulatory functions which were able to suppress some aspects of allergic airway disease, but also stimulated Th1-associated inflammatory responses.

In conclusion, BCG-induced suppression of Th2-type allergic airway inflammation seems to be associated with an up-regulation of both Treg function and Th1-type airway inflammation. These results raise concerns regarding the safety profile of BCG as a potential tool for prevention and therapy of allergic airway disease.

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