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Research paper

Therapeutic efficacy of pulmonary live tuberculosis vaccines against established asthma by subverting local immune environment

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ABSTRACT

Background: Substantial recent advances in the comprehension of the molecular and cellular mechanisms behind asthma have evidenced the importance of the lung immune environment for disease outcome, making modulation of local immune responses an attractive therapeutic target against this pathology. Live attenuated mycobacteria, such as the tuberculosis vaccine BCG, have been classically linked with a type 1 response, and proposed as possible modulators of the type 2 response usually associated with asthma.
Methods: In this study we used different acute and chronic murine models of asthma to investigate the therapeutic efficacy of intranasal delivery of the live tuberculosis vaccines BCG and MTBVAC by regulating the lung immune environment associated with airway hyperresponsiveness (AHR).
Findings: Intranasal administration of BCG, or the novel tuberculosis vaccine candidate MTBVAC, abrogated AHR-associated hallmarks, including eosinophilia and lung remodeling. This correlated with the re-polarization of allergen-induced M2 macrophages towards an M1 phenotype, as well as with the induction of a strong allergen-specific Th1 response. Importantly, vaccine treatment was effective in a scenario of established chronic asthma where a strong eosinophil infiltration was already present prior to immunization. We finally compared the nebulization efficiency of clinical formulations of MTBVAC and BCG using a standard commercial nebulizer for potential aerosol application.
Interpretation: Our results demonstrate that pulmonary live tuberculosis vaccines efficiently revert established asthma in mice. These data support the further exploration of this approach as potential therapy against asthma.
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1. Introduction

Asthma has reached pandemic levels, with more than 300 million individuals affected all around the world. Asthma is especially prevalent in developed countries compared to low- and middle-income scenarios. One of the most accepted explanations for these differences comes from the named "Hygiene hypothesis", which suggests that asthma development is favoured by the lower exposure of children to determined environmental factors [1]. In this regard, exposure to certain microorganisms and mites (as those present in farms) during early life stages might contribute to educating the immune system, leading to the acquisition of higher tolerance to allergens [2,3].

Asthma is a heterogeneous disease characterized by chronic airway inflammation and remodeling. Even though asthma can be associated with different types of inflammatory response, type 2 inflammation is present in more than 80% of asthma cases in children. T helper (Th) lymphocytes with a Th2 profile are present in most of the patients, producing cytokines as IL-4, IL-5 or IL-13, which are responsible for some of the characteristic clinical symptomatology. IL-5 plays a central role in the survival and recruitment of eosinophils, one of the main players in asthma, and whose presence in sputum represents one of the most accepted biomarkers for the

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Research in context

Evidence before this study

The current tuberculosis (TB) vaccine, BCG, is the most administered vaccine in history. Since BCG, a live-attenuated vaccine, is classically considered a Th1 response-promoter, the benefits of intradermal BCG vaccination for asthma have been widely assessed, although epidemiological evidences are controversial, with no clear benefits demonstrated. Conversely, different studies suggest a relationship between higher tuberculosis infection rates and lower prevalence of asthma. Indeed, we recently demonstrated this correlation in TB-infected mice subjected to an experimental model of allergen-induced asthma. Since tuberculosis infection is mostly acquired by the respiratory route, whereas BCG is given intradermally, evidences from human and animal studies suggest that the beneficial effects of live mycobacteria against asthma might rest on that bacteria physically reach the lungs to induce an asthma antagonistic response at a local level.

Added value of this study

In the present study, in an attempt to mimic the natural route of tuberculosis infection, we investigated the therapeutic efficacy of intranasal live-attenuated mycobacterial tuberculosis vaccines in different acute and chronic murine models of airway hyperresponsiveness (AHR). Our results showed that intranasal vaccine administration reverted AHR-associated response in all scenarios assayed, both in short-term and long-term acute models, and also in an established asthma scenario induced by chronic allergen exposure, where a strong airway eosinophilia was already present prior to vaccine administration. Our results indicate that vaccine treatment subverted asthma-associated type 2 response, including repolarization of allergen-specific Th2 lymphocytes into Th1. In addition, we described for the first time the use of the live-attenuated mycobacterial TB vaccine MTBVAC against asthma. MTBVAC is a vaccine candidate currently under clinical evaluation, and therefore alternative applications of this vaccine are relevant from a translational point of view.

Implications of all the available evidences

From regulatory point of view, aerosol (but not intranasal) delivery might be the only accepted route of administration for live tuberculosis vaccines into the lung compartment. In this study, we have evaluated the efficacy of nebulization in vitro of two clinical formulations of MTBVAC and BCG in a standard commercial nebulizer, demonstrating the feasibility of reaching therapeutic bacterial doses using standard nebulization devices. Altogether, our data support the further exploration of pulmonary application of live-attenuated mycobacteria as potential therapy against asthma.

diagnosis of the disease. In addition, IL-4 and IL-13 trigger airway remodeling by inducing proliferation of airway epithelial cells as well as exacerbated mucus production [4].

In addition to adaptive response, over the last years different studies have evidenced the crucial importance of lung innate populations for asthma triggering. Indeed, allergen presentation through MHC-II molecules from antigen-presenting cells (APC) results essential for the induction of allergen-specific T cells [5]. Asthma has been linked with a pathological macrophage polarization towards an M2 phenotype, as exacerbated levels of M2 macrophages have been found in asthma animal models [6,7] as well as in samples from patients [8]. M2 macrophages adopt regulatory skills and trigger an immune modulatory environment that impairs Th1 response and favours expansion of Th2 cells [9]. M2 is a simplified terminology that encloses different subsets of macrophages with regulatory skills. Thus, three different types of M2 macrophages have been defined, M2a, M2b and M2c, each with its own peculiarities. In the particular case of M2a macrophages, their presence has been linked with an induction of Th2 adaptive response [10]. With regard to allergic asthma, M2a macrophages can contribute to triggering the allergen-specific T cell response by at least two different ways, including presentation of allergen-derived peptides to T lymphocytes through MHC-II molecules, and by secretion of cytokines such as IL-4, which drive T cell response towards a Th2 profile [11]. Therapies targeting M2 macrophages have been shown to alleviate allergic responsiveness [12]. Thus, exacerbated M2 macrophage activation represents a highly attractive opportunity to design novel immunomodulatory treatments targeting this misbalance in lung macrophage populations [13].

The current tuberculosis vaccine BCG is the most administered vaccine in history. As live-attenuated *Mycobacterium bovis*, BCG vaccine is classically considered a Th1 response-promoting stimulus and as such, the benefits of intradermal BCG vaccination for asthma have been widely discussed [14]. Different observational studies suggest that vaccination with BCG could provide protection in the development of certain allergies, including ashtma [15]. Remarkably, an interventional study conducted in South Korea evidenced that the group of asthma patients treated with BCG presented an improvement of lung function in the next days following vaccination, in comparison with the placebo group [16].

At a preclinical level, whole-cell BCG, either live or inactivated, as well as different mycobacterial components, have been extensively proven to be efficient against asthma in different animal models [17-19]. However, most of these results have been obtained with BCG delivered prior to or concurrently with allergen sensitization. As a result, the therapeutic capacity of BCG to revert established asthma has not been elucidated. In addition, previous studies have focused mainly on the Th1/Th2 response balance without considering other components of the immune system, such as pulmonary macrophages, which seem to play major role during asthma development.

In the present study, we mimicked the natural route of tuberculosis infection by intranasal delivery of live tuberculosis vaccines, to evaluate the therapeutic efficacy of this approach in different models of airway hyperresponsiveness (AHR). We hypothesized that direct interplay between vaccines and the lung compartment might modulate the immune environment associated with asthma. Our results revealed that BCG was able to re-educate M2 macrophages induced by allergen administration towards an M1 phenotype, as well as to convert allergen-specific Th2 lymphocytes to Th1. In addition, we assessed for the first time the efficacy of a live-attenuated *Mycobacterium tuberculosis* vaccine, called MTBVAC, against asthma. MTBVAC is currently under clinical evaluation, and to date it remains the first and only live vaccine based on attenuated *M. tuberculosis* that has reached the clinic [20-22]. Importantly, our data showed strong therapeutic efficacy of both BCG and MTBVAC in allergen-challenged mice in a scenario of established disease, demonstrating the potential of live attenuated tuberculosis vaccines as therapy for asthma.

2. Materials and methods

2.1. Ethics

Experimental work was conducted in agreement with the Spanish Policy for Animal Protection RD53/2013 and the European Union Directive 2010/63 for the protection of animals used for experimental and other scientific purposes. Experimental procedures were

approved by the Ethics Committee for Animal Experiments of University of Zaragoza. (protocol PI22/15).

2.2. Bacteria

BCG Danish SSI (Pfizer), BCG Pasteur (strain 1173P2, Institut Pasteur Paris, France), MTBVAC(20) (University of Zaragoza) and MTBVACΔ*erp* [23] (University of Zaragoza) strains were grown at 37 °C in Middlebrook 7H9 broth (Difco) supplemented with ADC 10% (Difco) and 0.05% (v/v) Tween-80 (Sigma), Sauton's medium or on solid Middlebrook 7H10 (Difco) agar medium supplemented with ADC 10%. BCG Pasteur and MTBVAC were transformed with the replicative pJKD6 plasmid encoding green fluorescent protein (GFP) (a kind gift from Luciana Leite, Butantan Institute, Brazil). MTBVAC heat-killed was obtained by boiling an MTBVAC culture at 100 °C for 15 min. MTBVAC produced under GMP conditions was provided by Biofabri (Spain). OncoTICE® was purchased from MSD. Bacterial suspensions for vaccination were prepared in PBS from glycerol stocks previously quantified by plating serial dilutions.

2.3. Animal studies

All mice were kept under controlled conditions and observed for any sign of disease.

For induction of OVA-specific AHR, 8 to 10 weeks old C57BL/6 (Janvier Biolabs) female mice were sensitized by two intraperitoneal injections of 50 μg chicken egg ovalbumin (lyophilized powder, $\geq$98% (Sigma)) with 2 mg aluminum hydroxide (Sigma, St. Louis, MO) one week apart. One week after second sensitization, mice were given a single intranasal administration of BCG, MTBVAC or MTBVAC-derived vaccine at the dose indicated in the figure legends, resuspended in 40μl of PBS. In the acute model, four weeks after vaccine administration, animals were intranasally challenged with 100 μg OVA in sterile PBS for 3 consecutive days and one day after the last challenge, mice were humanely sacrificed. In some of the experiments, OVA challenge was delayed until 4 months after BCG vaccination. In the chronic OVA model, three weeks after immunization, mice were intranasally challenged wth 10 μg OVA twice per week during eight weeks. In this case, vaccines were administered at week 9 of the procedure, in the middle of the challenge phase. For HDM-induced chronic AHR, mice were intranasally challenged twice a week for three consecutive weeks with 10μg HDM. Vaccines were delivered at week 4 of the experiment (i.e., one week after primary HDM challenge), and one month later, mice were intranasally challenged with 10 μg HDM for three consecutive days. One day after last HDM administration animals were humanely sacrificed. Dexamethasone treatment was given according to [24]. Briefly, OVA-sensitized mice were treated intraperitoneally with 5 mg/kg dexamethasone (DEX) (Dexamethasone-Water Soluble, Sigma-Aldrich) the day prior to initiating the challenge phase, and then three additional DEX inoculations were given 1 hour before each OVA intranasal administration.

For bronchoalveolar lavage (BAL) collection, trachea was cannulated and BAL performed with 0.8 ml of ice-cold PBS. Supernatant was separated from cells by centrifugation for 5 min at 4500 xg.

Lungs were removed aseptically. For obtaining cellular suspensions, they were added to HEPES buffer (HEPES 10 mM; NaCl 0,15 M; KCl 5 mM; MgCl2 1 mM; CaCl2 1,8 mM pH 7,4) containing collagenase D 100 mg/ml (Roche) and DNAseI 400 IU (AppliChem), incubated at 37 °C for 30 min, and homogenised using GentleMACS (Miltenyi Biotech) dissociator with the lung specific program according to manufacturer instructions. Afterwards, residual red blood cells were lysed using Red Blood Cells Lysing Buffer (Sigma) and the homogenized filtered to eliminate tissue debris. For bacterial burden determination, lungs were homogenized with the GentleMACS, using the RNA protocol, and then plated onto agar medium 7H10 supplemented with ADC. In the case of histological analysis, lungs were fixed with 4% formaldehyde solution for 24 h prior to the staining procedure.

2.4. Flow cytometry analysis

10^6 lung or BAL cells were incubated for 15 min at 4 °C with Fc receptor blocking reagent (Miltenyi Biotech). Then, eosinophil, neutrophil and macrophage presence was determined by extracellular staining with the following antibodies: CD45-FITC (RRID: AB_2658216), siglecF-APC (RRID:AB_2653441), Ly-6G-Vioblue (RRID:AB_2751964), CD11c-PE (RRID:AB_2654707), CD11b-PerCP/Cy5.5 (RRID:AB_2751174) from Miltenyi Biotech. Eosinophils were defined as $SSC^{high}CD45^{+}CD11b^{+}SiglecF^{+}CD11c^{-}$; neutrophils as $CD45^{+}Ly6G^{+}CD11b^{+}CD11c^{-}$; and Alveolar Macrophages as $CD45^{+}SiglecF^{+}CD11c^{+}CD11b^{dim}$ cells.

For intracellular staining (ICS), after labeling membrane proteins with the above-mentioned antibodies, in addition to MHCII-Vioblue (RRID:AB_2652908) and CD86-PE (RRID:AB_2660746) (Miltenyi), and CD206-APC (RRID:AB_2739133) (BD Biosciences), cells were fixed and permeabilized with the FoxP3 staining set (Miltenyi Biotech), according to manufacturer instructions. As intracellular antibodies, we used iNOS-APC (RRID:AB_2727527) and iNOS-PE (RRID: AB_2727486) (Miltenyi), and Arg1-APC (RRID:AB_2734835)(eBiosciences). Cells were acquired using a Gallios flow cytometer (Beckman Coulter) and analyzed with Weasel software.

2.5. Cytokine analysis

Quantification of IL-5, IL-4, IL-13 and IFN-γ was performed using specific commercial ELISA kits following manufacturer instructions (Mabtech Biotech). Cytokine determination in the lungs was done from organ explants. These were prepared by cutting the lung into small pieces and incubating them overnight at 37 °C in 0.5 ml of culture medium.

To analyze OVA specific response, mediastinal lymph nodes were removed aseptically and mechanically disrupted for cell collection. 2×10^6 cells were incubated with or without OVA at 1 mg/ml for 96 h. Then, supernatant was collected to determine cytokine concentration. OVA-specific response for each cytokine was calculated as the difference between cytokine concentration obtained following OVA stimulation minus the unstimulated control. For ICS, cells were incubated with 1 mg/ml OVA or 1 μg/ml of anti CD3/CD28 (RRID: AB_394590; RRID:AB_394763, respectively) (BD Biosciences) for 24 h, and 10 μg/ml Brefeldin A (Sigma) was added during the last six hours. For surface staining, cells were labelled with anti-CD4-FITC (RRID:AB_394582) (BD Biosciences) and anti-CD3-PerCPVio700 (RRID:AB_2752207) (Miltenyi Biotec) in culture medium with 10% FCS. Then, cells were fixed and permeabilized with the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) following manufacturer instructions, and stained with anti-IFNγ-APC (RRID: AB_2784369) and anti-IL5-PE (RRID:AB_2660015) (Miltenyi Biotech).

2.6. qRT-PCR

For RNA extraction, lungs were immersed into TRIzol reagent (Invitrogen) just upon harvesting, and frozen immediately on dry ice. Once thawed, lungs were homogenised with the GentleMACS, using the RNA 0.2 protocol. 200 μl of chloroform were added per ml of TRIzol and after vigorous vortexing, tubes were centrifuged at 18,000 x g during one hour at 4 °C. Aqueous upper phase containing eukaryotic RNA was recovered, added to 700 μl of isopropanol and centrifuged at 18,000 x g during 10 min at 4 °C. The resulting pellet was washed with 70% EtOH and stored at −20 °C. Residual DNA was eliminated by DNAse treatment, RNA was purified with an extraction based on phenol-acid-chloroform and precipitated ON at −20 °C with isopropanol

and sodium acetate. cDNA libraries were constructed for gene expression analysis by RT-qPCR. Primer pairs used in the present study were the following:

Gene	5′ sequence	3′ sequence
Actin	5′-ACCAGTTCGCCATGGATGAC	5′-TGCCGGAGCCGTTGTC
18S	5′-TTCGTATTGCGCCGCTAGA	5′-CTTTCGCTCTGGTCCGTCTT
Gata3	5′-GACCCGAAACCGGAAGATGT	5′-GCGCGTCATGCACCTTTT
Il12a	5′-ACGCAGCACTTCAGAATCACA	5′-CACCAGCATGCCCTTGTCTA
Il12b	5′-TGGAGCACTCCCCATTCCT	5′-TGCGCTGGATTCGAACAA
Ifng	5′-TTGGCTTTGCAGCTCTTCCT	5′-TGACTGTGCCGTGGCAGTA
Il5	5′TTGACAAGCAATGAGACGATGAG	5′-TCCAATGCATAGCTGGTGATTT
Il4	5′-GGAGATGGATGTGCCAAACG	5′-CGAGCTCACTCTCTGTGGTGTT
Il13	5′-TTGAGGAGCTGAGCAACATCAC	5′-CCATGCTGCCGTTGCA
Stat1	5′-CTCTGGAATGATGGGTGCATT	5′-TTGAGCAGAGCGCGTTCTC
Stat4	5′-CATTTGCAACCCAAGGAGATG	5′-TGGCAGCCCTCGTTTCC
Stat6	5′-AACTGCAACGGCTCTATGTTGA	5′-AGCCAGTCAGCCAGGAGATG
Tnf	5′-CAGCCGATGGGTTGTACCTT	5′-GGCAGCCTTGTCCCTTGA
T Bet	5′-ACCTGTTGTGGTCCAAGTTCAA	5′-GCCGTCCTTGCTTAGTGATGA
Ifnb1	5′-CCCTATGGAGATGACGGAGAAG	5′-GAGCATCTCTTGGATGGCAAA
Ccl11	5′-GACCAGGTTGGGCAAAGAGA	5′-GGCATCCTGGACCCACTTCT
Ym1	5′-GTCTGGCCCCTGGACATG	5′AGAGGGAAATGTCTCTGGTGACA
Il1b	5′-AGTTGACGGACCCCAAAAGA	5′-GGACAGCCCAGGTCAAAGG
Retnla	5′-CAGCTGATGGTCCCAGTGAA	5′TTCCTTGACCTTATTCTCCACGAT
Nos2	5′-GGATCTTCCCAGGCAACCA	5′-TCCACAACTCGCTCCAAGATT
Arg1	5′-GCTCCAAGCCAAAGTCCTTAGA	5′-CCTCGAGGCTGTCCTTTTGA

2.7. Nebulization studies

MTBVAC® and OncoTICE® GMP vials were resuspended with 1 ml/vial of eluent indicated by manufacturers, concentrations normalized at 10^7 CFU/ml, and 2 ml of each vaccine preparation placed in the reservoir of the clinical nebulizer U100 (OMRON). Nebulizer was connected with a plastic tube to a gas washing flask with 5 ml of sterile water, coupled with a vacuum pump to recover the nebulized fraction. Bacteria were nebulized for 5 min and both nebulized and reservoir fractions were plated on solid agar 7H10 medium supplemented with ADC. Nebulization efficacy index for each vaccine strain was calculated as the percentage of bacteria in the nebulized fraction compared to that in the reservoir.

2.8. Statistics

Mice were randomly distributed in groups of 6 animals per cage prior to start experimental procedures. Results were not blinded for analysis. No statistical method was used to calculate sample size in animal experiments. GraphPrism software was used for statistical analysis. Statistical tests used for each experiment are indicated in the figure legends. All statistical tests used were two-tailed. Outlier values were determined applying the Grubb's test to all data sets, and discarded from the final statistical analysis. Differences were considered significant at $p<0.05$.

2.9. Role of funding source

The funders had no role in study design, data collection, interpretation and analysis, decision to publish or preparation of the manuscript.

3. Results

3.1. Intranasal BCG and MTBVAC prevent AHR in allergen-sensitized mice

Initially, we tested vaccine efficacy in an acute model of AHR driven by ovalbumin (OVA) administration. Appearance of asthma symptoms in humans comes preceded by an asymptomatic phase of allergen sensitization, which is usual to occur at an early life stage during childhood [25]. Thus, to make our conclusions more representative of the real situation, we delivered vaccines over previously sensitized animals (Fig. 1a). With the objective to reach the primary organ (lungs) affected during asthma episodes, we inoculated the vaccines by the intranasal route. Our long experience with this route of administration in mouse models indicate that a substantial proportion of bacteria reaches the lungs under the experimental settings used. The dose of BCG initially administered was 10^6 CFUs. As primary marker of asthmatic responsiveness we measured eosinophil infiltration in lungs and respiratory airways the day after last challenge (Fig. 1b, 1c, S1), relevant from a clinical point of view. Our data revealed a strong ability of intranasal BCG to abrogate eosinophilia both in BAL and lungs (Fig. 1c-e). In addition, we found no changes in other myeloid populations, as neutrophils and alveolar macrophages, suggesting that BCG did not trigger an uncontrolled cellular infiltration into the respiratory airways and indeed, the number of total leukocytes (CD45+ cells) were significantly lower in the BCG OVA group compared to OVA (Fig. 1e). Another typical asthma hallmark is the remodeling of the airway epithelium, which is characterized by a thickening of the alveolar walls and the proliferation of goblet cells, responsible for mucus production and secretion. These features were observed in the lungs from OVA group after staining with Periodic Acid Schiff (PAS) technique, where different layers of epithelial cells could be distinguished in the alveolar walls, with an important proportion of PAS-positive cells (goblet cells). In addition, presence of mucosubstances was found in the airways. Remarkably, BCG treatment prevented this phenotype. Goblet cells were hardly visible and no mucus secretion was observed in alveolar spaces, whereas airway epithelium was restricted to one layer (Fig. 1f).

We also assessed intranasal BCG in a long-term AHR model, in which OVA challenge was performed four months after BCG immunization. BCG also reduced eosinophilia in this model (Fig. 1g). Interestingly, bacterial counting in lungs at the time of sacrifice indicated that BCG persistence was dramatically reduced compared to the one-month model (Fig. S2a), indicating that intranasal BCG could modulate asthma-associated immune environment even after almost complete bacterial clearance, which might suggest a contribution of memory response to the vaccine-protective effect.

MTBVAC is a novel live tuberculosis vaccine, attenuated from a clinical isolate of *Mycobacterium tuberculosis*, the pathogen causative of TB in humans, whereas BCG is a derivative vaccine from *Mycobacterium bovis* [20]. MTBVAC has demonstrated higher efficacy and immunogenicity than BCG against tuberculosis in different preclinical models [26], and is currently under clinical evaluation in newborn [22] (ClinicalTrials.gov Identifier NCT03536117) and adult populations [21] (NCT02933281) in TB-endemic countries in Africa, showing acceptable safety and immunogenicity profile to date. In the present study, we evaluated intranasal MTBVAC for the first time in a model of asthma. Using the OVA-driven acute AHR model described above, we observed a strong capacity of intranasal MTBVAC to abrogate eosinophil infiltration into airways, comparable to that observed with BCG (Fig. 1h).

To address whether vaccine persistence could affect protection against asthma, we compared MTBVAC with two other MTBVAC-derived vaccines: MTBVACΔ*erp* strain, which contains an additional deletion of the gene *erp* resulting in a hyperattenuated phenotype with lower persistence as previously described [23] and demonstrated in this study (Fig. S2b); and MTBVAC killed by heat (MTBVAC HK). MTBVAC HK did not reduce eosinophils following OVA challenge, whereas MTBVACΔ*erp* showed significant reduction as compared to the OVA group but lower than that observed with MTBVAC (Fig. 1i). These results suggested an influence of bacterial persistence on protection. To study this in more detail, we conducted a dose-response experiment with intranasal BCG and MTBVAC. We observed robust correlation of the dose with a stronger eosinophilia reduction, both in BAL (Fig. 1J) and lungs (Fig. 1k). In the case of the lungs, the highest dose groups (10^7 MTBVAC and 10^6 BCG) showed a level of

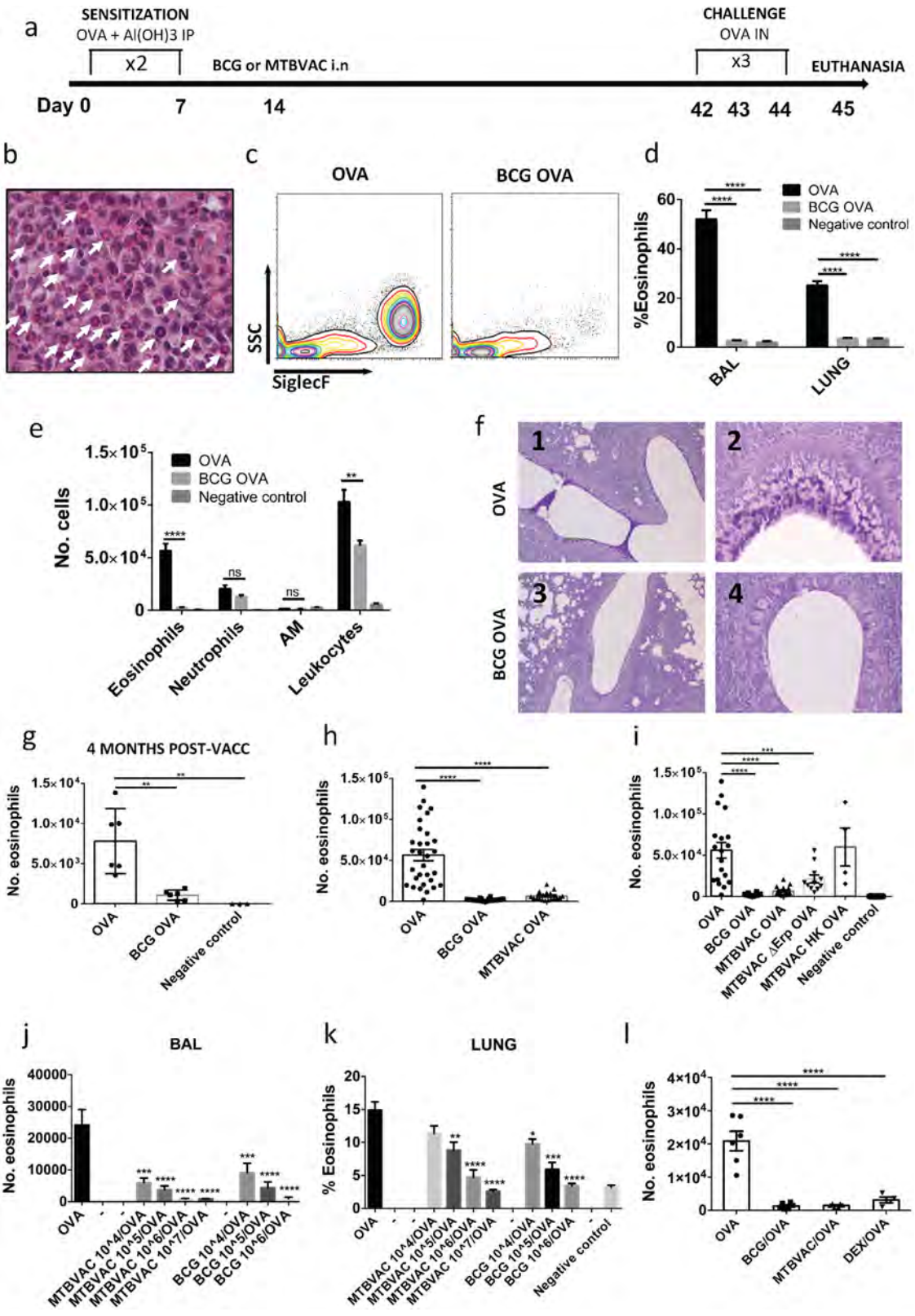

Fig. 1. Intranasal BCG and MTBVAC prevent allergic airway responsiveness in allergen-sensitized mice. (a) Eosinophils were determined by flow cytometry in an OVA-driven acute AHR model, with mice treated intranasally with 10^6 BCG CFUs one week after second sensitization. (b) A representative hematoxilin-eosin lung image of OVA-challenged mice. Presence of eosinophils is highlighted with arrows. (c) Eosinophils were quantified by flow cytometry, corresponding to a population SSC^{high}SiglecF+$CD11b^{+}CD11c^{-}$. A representative diagram is shown in the figure. (d) Percentage of eosinophils in BAL and lungs with respect to CD45+ cells. (e) Total number of leukocytes, eosinophils, neutrophils and alveolar macrophages (AMs) in BAL determined by flow cytometry. (f) Representative images of PAS-stained fixed lungs from OVA-challenged mice untreated (1,2) or BCG-treated (3,4). (g) Eosinophils in BAL using long-term AHR model, in which OVA challenge was performed 4 months after BCG immunization. (h) Eosinophils in BAL following intranasal treatment with 10^6 MTBVAC or BCG CFU. (i) Total number of eosinophils in BAL following intranasal treatment with 10^6 MTBVAC, or the derivative strains MTBVACΔ*erp* and MTBVAC Heat-Killed (HK). (j, k) Eosinophils in BAL (j) and lungs (k) following intranasal treatment with increasing doses of MTBVAC or BCG. (l) BAL eosinophils comparison following BCG, MTBVAC and

eosinophils similar to the negative control, and therefore we used these doses for the subsequent experiments. Finally, we assessed eosinophilia following intranasal MTBVAC or BCG in comparison with systemic administration of dexamethasone (DEX), which is a standard drug used in asthmatic patients. Or results showed comparable eosinophil reduction induced by intranasal MTBVAC and BCG and systemic DEX administration (Fig. 1l).

3.2. Intranasal vaccination subverts asthma-associated lung immune environment

We hypothesized that the beneficial effects against AHR observed upon intranasal vaccination could be related with the ability of the vaccine to locally interact with resident phagocytic cells in the lungs. To assess *in vivo* infected cell populations following intranasal immunization, we delivered a BCG strain that expressed the green fluorescent protein (GFP). Our data demonstrated that level of BCG infection was efficient enough to discern infected cells by flow cytometry (Fig. 2a). Using a panel of antibodies to detect myeloid cell markers, we identified that the majority of BCG-infected cells corresponded to alveolar macrophages (AMs). Noteworthy, an important proportion of infected AMs expressed high levels of iNOS and CD86, two well-known markers of classical macrophage activation (or M1 phenotype) (Fig. 2a). We next compared macrophage polarization of total lung macrophages in OVA-challenged mice treated or not with BCG. Percentage of iNOS- and CD86-positive macrophages was substantially higher in the vaccinated group, whereas no difference was found in the case of MHC-II, with most of the macrophages positive for this marker in both groups (Fig. 2b). Importantly, expression of CD206, a classical marker associated with M2 polarization, was greater in macrophages from OVA group compared to those of the vaccinated group, which expressed similar levels of CD206 as naïve cells (Fig. 2c). To complement these data, we performed another experiment in which we isolated lung RNA from untreated or BCG-treated OVA-challenged mice. Expression analysis of genes linked with macrophage polarization clearly demonstrated that BCG immunization led to an upregulation of the M1 profile-associated genes *Nos2* and *Il1b*, whereas it inhibited expression of the M2 activation markers *Ym1*, *Arg1* and *Retlna* (Fig. 2d). Interestingly, in a dose-response experiment with MTBVAC and BCG, iNOS expression was found highest in the groups treated with the most protective vaccine doses (10^7 MTBVAC and 10^6 BCG) (Fig. 2e), suggesting a correlation between M1 phenotype and protection against asthma. Altogether, our data demonstrated the enrichment of M2-like macrophages during asthmatic responsiveness, and suggested that the therapeutic efficacy of live attenuated mycobacteria could rest on the reversion of this response by inducing classical activation of lung macrophages.

We next studied pulmonary Th1 and Th2 responses, whose misbalance towards the latter one has been classically connected with the immune pathogenesis of asthma. Gene expression analysis of lung RNA revealed that BCG vaccination led to induction of Th1-associated genes as *Ifng*, *Il12a*, or the transcription factor *Tbet*. Conversely, genes codifying for typical Th2 cytokines and chemokines, as IL-5, IL-4, IL-13 or CCL-1, were down modulated by BCG (Fig. 3a) compared to untreated mice. In consonance with these results, IL-5 and IL-4 cytokines were found elevated in BAL and lungs respectively in the OVA control group, whereas IFNγ was increased when treated with BCG (Fig. 3b, c). In addition to total cytokine levels, we also studied allergen-specific T cells after *ex vivo* OVA stimulation of harvested lymphocytes from mediastinal lymph nodes. Data showed a higher IL-4, IL-5 and IL-13, and lower IFNγ OVA-specific production in the OVA group compared to BCG-vaccinated mice (Fig. 3d-g). Intranasal MTBVAC showed a similar ability as BCG to shift CD4+ cells towards Th1 profile (Fig. S3). We also found a correlation between vaccine-induced Th1 profile and bacterial dose, indicating the importance of bacterial persistence for the shift of Th2 response toward Th1 (Fig S4).

Importantly, using intracellular staining and flow cytometry we directly visualized cytokine-producing CD4+ *T* cells expressing the memory marker CD44. Both following stimulation with CD3/CD28 or with OVA, we observed an opposite T cell response polarization between OVA controls and BCG-treated mice. BCG treatment abrogated OVA-specific IL-5-producing cells, whereas it triggered allergen-specific Th1 IFNγ-producing CD4+ lymphocytes (Fig. 3h, i). This suggests that T cell-driven IFNγ production in BCG-treated mice is not only generated by expanded lymphocytes that recognize mycobacterial antigens, but also by allergen-specific T cells, that might be re-shaped from a Th2 towards a Th1 profile due to vaccine-associated inflammatory environment.

3.3. BCG and MTBVAC inhibit eosinophilia and lung remodeling in a scenario of established asthma

With the aim to elucidate the therapeutic potential of live attenuated vaccines against established asthma, we conducted experiments using a chronic AHR model with multiple OVA challenges, where vaccines were delivered halfway through the challenge phase (Fig. 4a). BAL eosinophils were determined in an additional group sacrificed the day prior to vaccination, at week 8, confirming eosinophil infiltration at the time of vaccine administration (Fig. 4b). Data at the end of the procedure, at week 13, revealed that eosinophils were reduced in the treated group, indicating the capacity of BCG to overcome established allergen-induced eosinophilia (Fig. 4c).

In another experiment, we also observed that MTBVAC was able to revert eosinophilia under similar experimental settings as BCG (Fig. 4d). In this experiment we additionally assessed lung remodeling by PAS staining and histological evaluation. Our data revealed an important disorganization of the alveolar epithelium surface in the OVA control group, as well as a substantial presence of goblet cells. This phenotype was not observed following MTBVAC treatment (Fig. 4e). We also analyzed by flow cytometry expression of the M2 and M1 polarization markers Arg-1 and iNOS, respectively. A significant proportion of Arg-1-positive macrophages was found in the OVA group but not in the MTBVAC-treated mice. Conversely, iNOS-positive macrophages were detected in the MTBVAC group, suggesting the ability of the vaccine to re-polarize asthma-associated M2 macrophages towards M1 profile (Fig. 4f, g).

We next assessed the therapeutic efficacy of intranasal BCG and MTBVAC in a model of established AHR induced by house dust mites (HDM), a relevant allergen in clinical asthma. Allergen-induced airway responsiveness was triggered by successive intranasal HDM challenges (Fig. 5a), which elicited a strong eosinophil infiltration in the BAL (Fig. 5b). BCG and MTBVAC administration resulted in abolishment of BAL eosinophilia, as measured in frequency (Fig. 5b) and absolute number of cells (Fig. 5c). PAS-stained lungs showed that HDM exposure in untreated mice strongly induced proliferation of the alveolar epithelium and appearance of goblet cells, a phenotype that was not observed in the vaccine-treated groups (Fig. 5d).

Finally, we observed that vaccine administration diminished production of Th2 cytokines and substantially increased IFNγ in BAL (Fig. 5e, g) and lymph node cells following stimulation with HDM (Fig. 5f, h).

3.4. MTBVAC and BCG aerosolization feasibility with a clinical nebulizer

There are strong regulatory safety concerns about intranasal delivery of vaccines in humans [27]. However, aerosol administration

dexamethasone (DEX) treatment. Data in the graphs are pooled means±SEM from three independent experiments (d, e, h), two (i), or one (g, j, k, l). A minimum of 6 mice were used per group and experiment. $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$, by two-way ANOVA (a, e,) or by one-way ANOVA (g, h, i, j, k, l), with Bonferroni post-test.

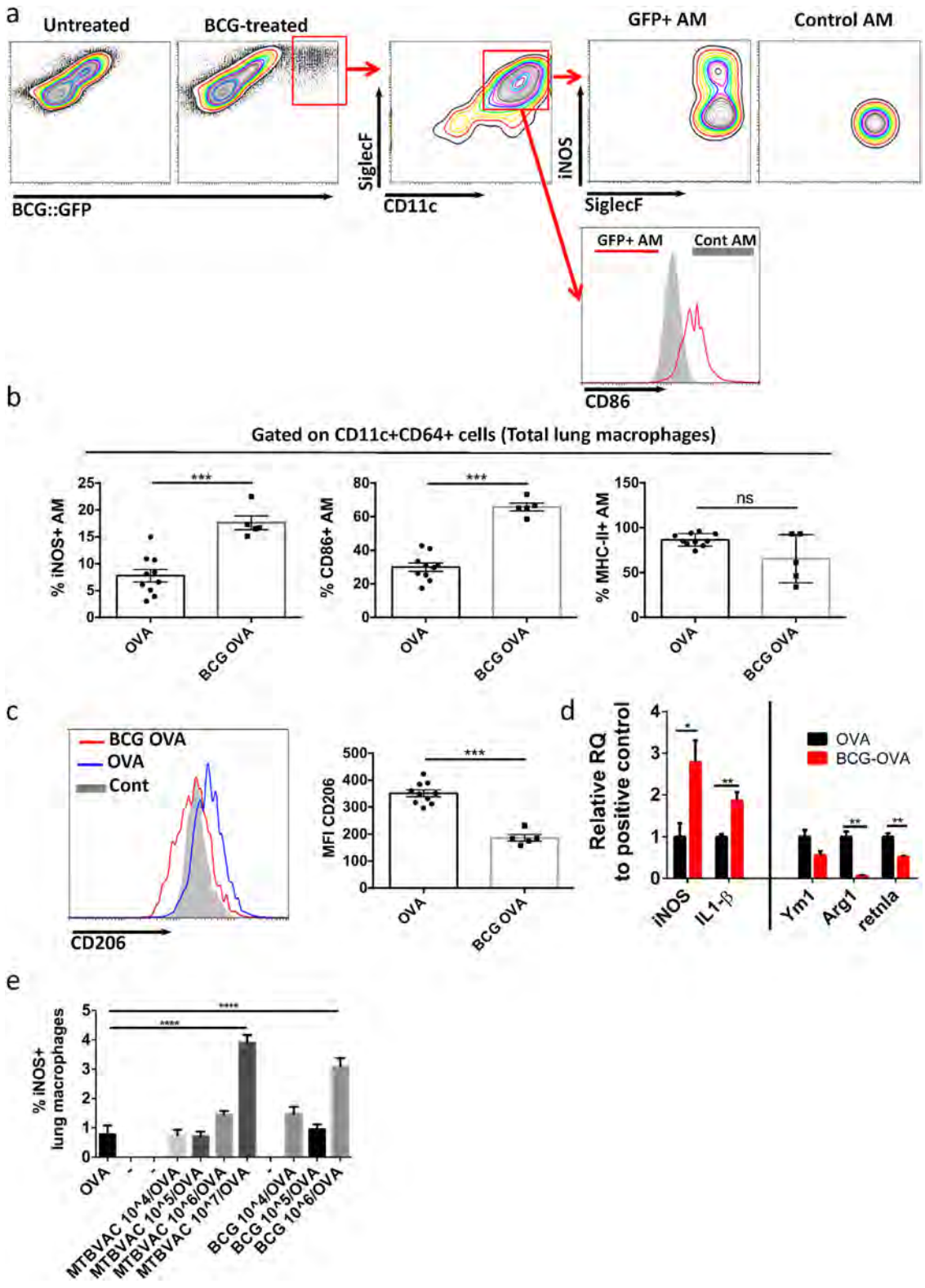

Fig. 2. BCG intranasal infects lung resident macrophages and induces classical activation. (a) Groups of mice were immunized with 10^6 CFU of GFP-expressing BCG. One month later, infected cells were monitored and characterized by flow cytometry. Expression of M1-polarization markers iNOS and CD86 was analyzed. Representative diagrams are shown in the figure. (b) Percentage of iNOS-, CD86- and MHC- positive lung macrophages in OVA-challenged untreated or BCG-treated. (c) Surface expression of the M2-activation marker CD206. Representative overlay histogram showing CD206 surface expression of indicated experimental groups. Graph shows comparison of Mean Fluorescence Intensity (MFI) corresponding to CD206 level of expression. (d) M1 and M2 activation markers measured by qRT-PCR in lungs from OVA-challenged mice, untreated or BCG-treated. (e) Percentage of iNOS-positive cells in lung macrophages following intranasal treatment with increasing doses of MTBVAC or BCG. Data in the graphs are representative mean±SEM from two independent experiments (b, c) or one (d, e). A minimum of 6 mice was used per group and experiment. $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; by unpaired single (b, c) or multiple (d) t-student test; or by one-way ANOVA (e) with Bonferroni post-test.

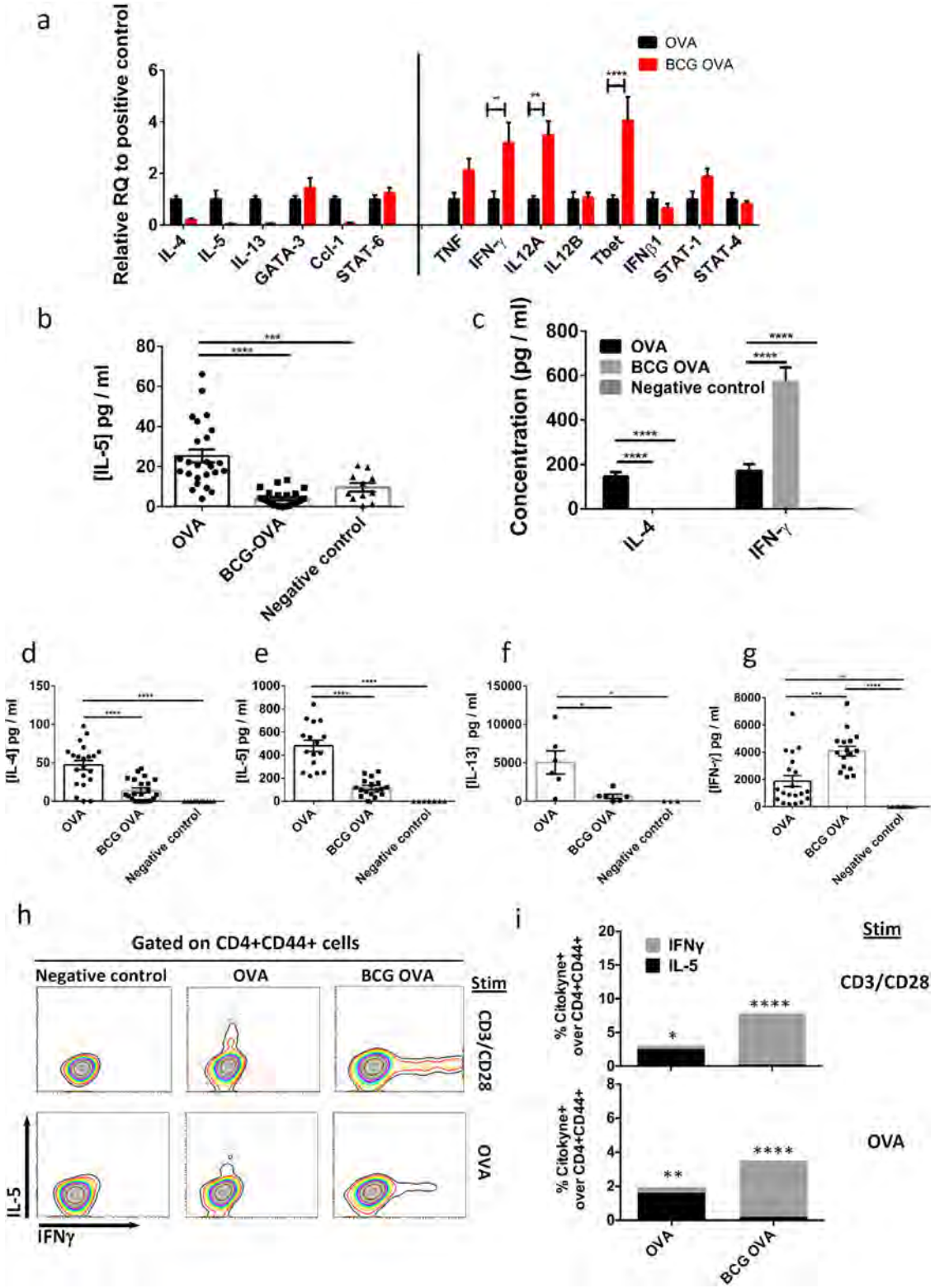

Fig. 3. BCG intranasal reshapes allergen-specific response into a Th1 profile. (a) Th1 and Th2 activation markers measured by qRT-PCR in lungs from OVA-challenged mice, untreated or treated with BCG one week after sensitization. (b) IL-5 determination in BAL. (c) IL-4 and IFNγ determination in lung explants (d-g) Allergen-specific IL-4, IL-5, IL-13 and IFNγ produced by mediastinal lymph node cells, following *ex vivo* stimulation with OVA. Data are represented following subtraction of the value obtained in the absence of allergen. (h, i) IL-5- and IFNγ- producing cells visualized by intracellular staining and flow cytometry following *ex vivo* stimulation with anti CD3/CD28 or with OVA. Representative diagrams are shown. Data in the graphs are pooled means±SEM from three independent experiments (b-e, g) or one (a, i). A minimum of 6 mice was used per group and experiment. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$, by multiple t-student test (a), one-way ANOVA with Bonferroni post-test (b, d-g), and two-way ANOVA with Bonferroni post-test (c, i).

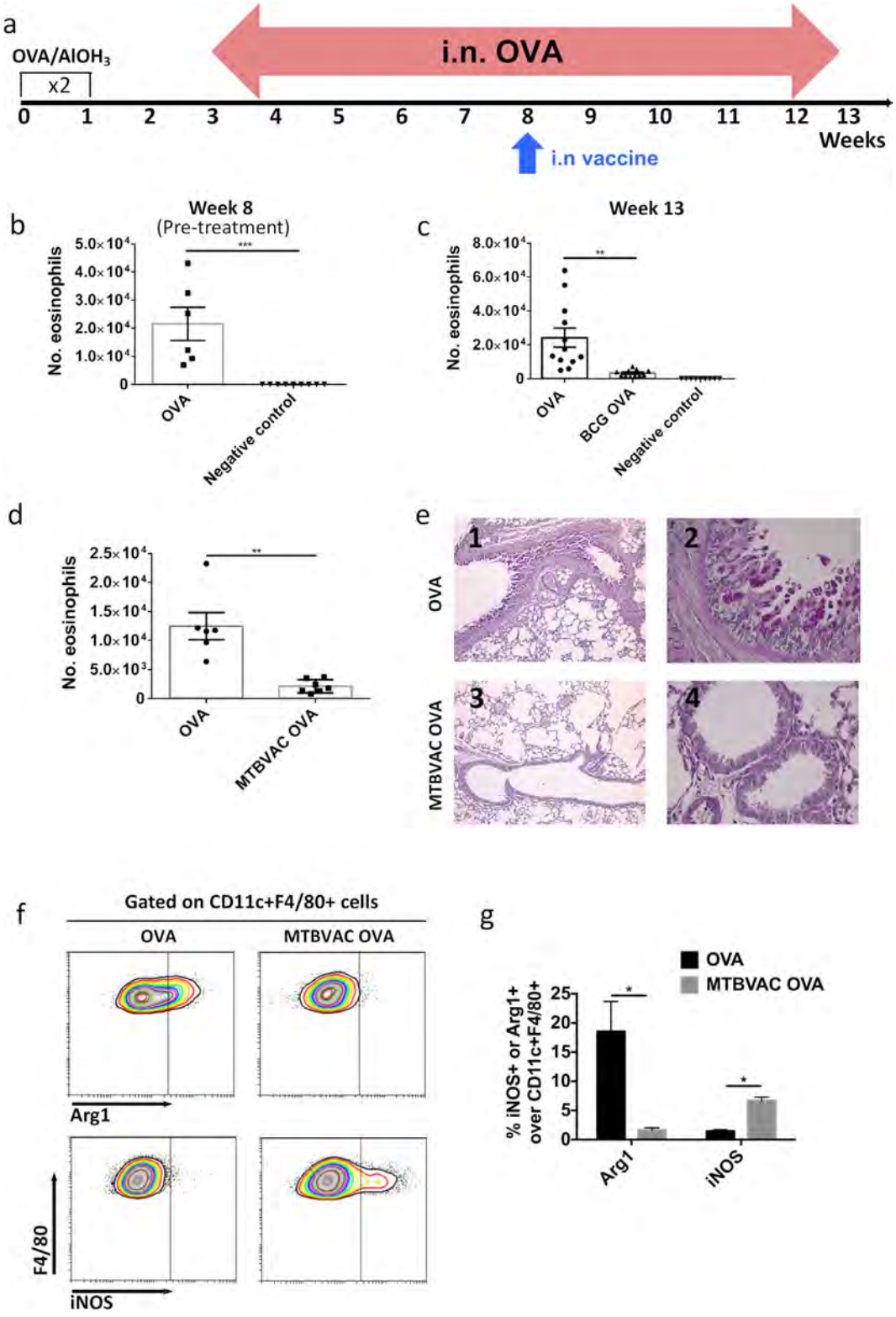

Fig. 4. BCG and MTBVAC intranasal revert established allergic airway responsiveness in an OVA-driven chronic model. (a) Scheme of the experiment based on an OVA-induced chronic model. Sensitized mice were challenged from week 3 to 13 with two intranasal inoculations weekly of OVA 10μg. Vaccines were delivered at week 9, in the half of the challenge phase. (b) Total number of eosinophils in BAL at week 8, the week before vaccine immunization. (c) Total number of eosinophils in BAL at week 13, four weeks after BCG treatment. (d) Total number of eosinophils in BAL at week 13, four weeks after treatment with 10^7 CFU of MTBVAC. (e) Representative images of PAS-stained fixed lungs from OVA-challenged mice untreated or MTBVAC-treated. (f, g) Intracellular expression of iNOS and Arg-1 in lung macrophages at week 13. Representative dot-plot diagrams are shown. Data in the graphs are pooled means±SEM from two independent experiments (b, c), or one (d, g). A minimum of 6 mice was used per group and experiment. $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$, by t-student test (b, e), one-way ANOVA with Bonferroni post-test (b, c), and multiple t-student test (g).

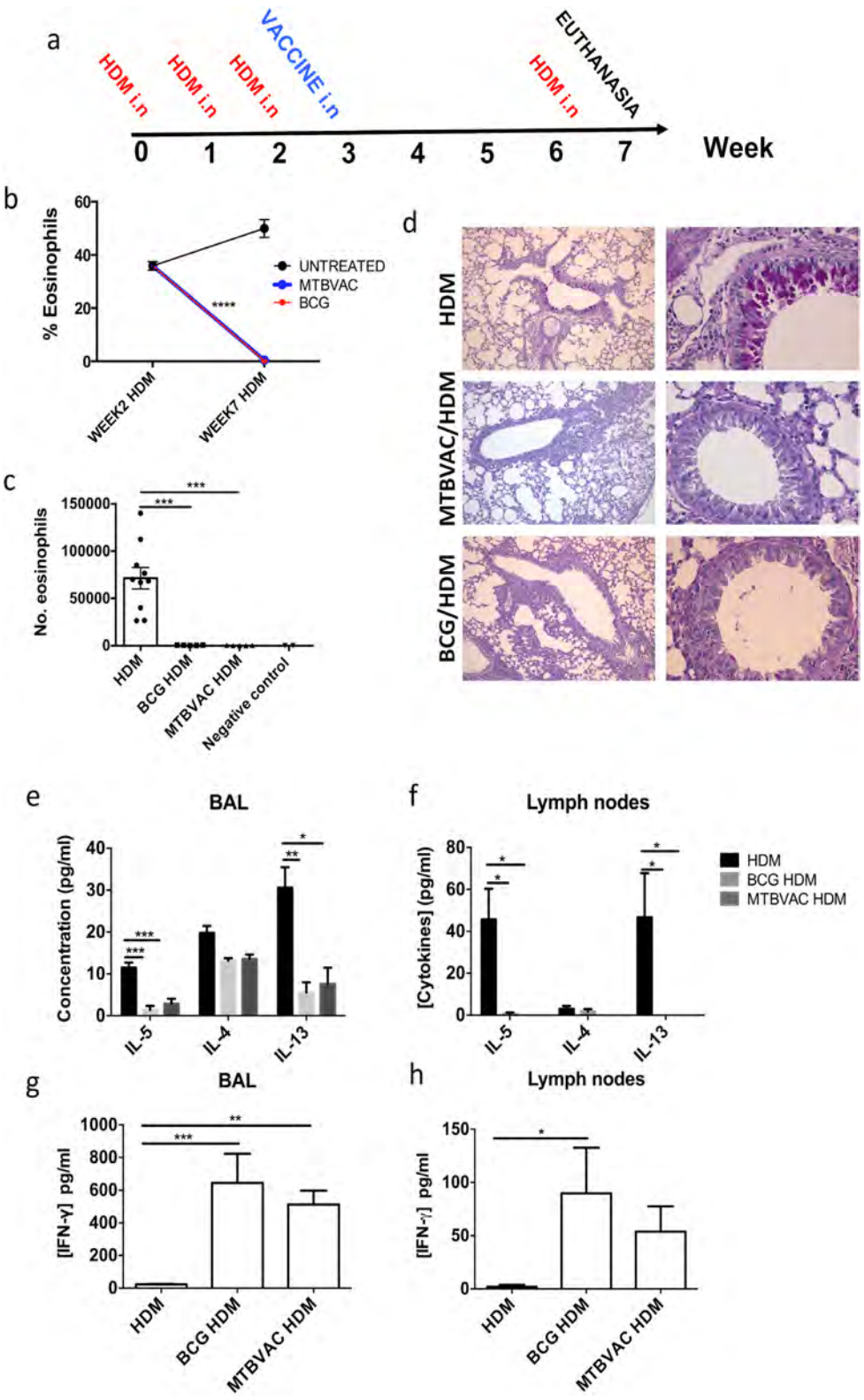

Fig. 5. BCG and MTBVAC intranasal vaccination reverts established allergic airway responsiveness induced by the relevant allergen HDM. (a) Scheme of the experiment. Mice were challenged twice a week during 3 consecutive weeks with intranasal HDM 10μg. Vaccines were delivered the week after. (b) BAL percentage of eosinophils (with respect to CD45+ cells) comparison between week 2 (prior to treatment) and week 7 (end of the procedure), following treatment with MTBVAC or BCG. (c) Total number of eosinophils in BAL at the end of the procedure. (d) Representative images of PAS-stained fixed lungs from HDM-challenged mice untreated or BCG- or MTBVAC-treated. 10x left panels and 40x right panels (e, f) Th2 cytokines IL-5, IL-4 and IL-13 were analyzed in BAL, and in lymph nodes following *ex vivo* stimulation with HDM. (g, h) Th1 cytokine IFNγ- was analyzed in BAL, and in lymph nodes following ex vivo stimulation with HDM. Data in the graphs are mean±SEM from one experiment ($n=6$ mice per group). $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$ by one-way ANOVA with Bonferroni post-test (a, c, g, h), and two-way ANOVA with Bonferroni post-test (e, f).

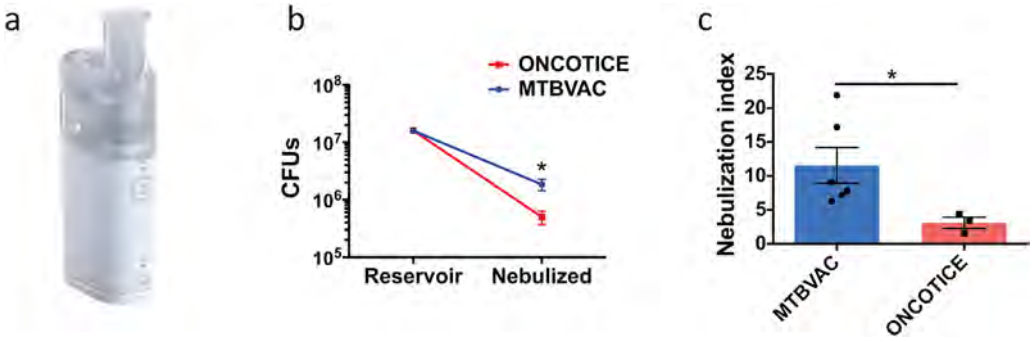

Fig. 6. MTBVAC and BCG are feasibly nebulized with a clinical device. (a) OMRON U100 nebulizer used in the study. (b) MTBVAC and BCG OncoTICE® CFUs determined in nebulized and reservoir fractions. (c) Nebulization efficacy index calculated as the percentage of bacteria recovered in the nebulized fraction compared to the contained in the reservoir. Each dot corresponds to the result obtained with a different filter. Data in the graph represents mean±SEM from six independent nebulizations with MTBVAC, and three with BCG OncoTICE®. *$p<0.05$, by t-student test.

is an accepted way to reach the pulmonary compartment in clinic, and multiple commercial nebulizers authorized for human use are available. From a practical perspective, an important step to bring pulmonary administration of live mycobacteria to clinical development is the feasibility to deliver therapeutic vaccine doses through the aerosol route. To evaluate this, we measured the nebulization efficacy of GMP formulations of MTBVAC and BCG (OncoTICE®) adapted for human use, through the clinical nebulizer OMRON U100 (Fig 6a). Bacterial concentration loaded into the reservoir was similar for both vaccines, around 1.5×10^7 CFUs/ml. After 1 min of nebulization, the mean amount of bacteria recovered in the nebulized fraction was 1.85×10^6 CFUs for MTBVAC and 5×10^5 for BCG (Fig 6b). The mean efficacy of nebulization with respect to the initial bacterial load in the reservoir was 11.7% for MTBVAC and 3.1% for OncoTICE® (Fig. 6c).

Studying different characteristics of BCG and MTBVAC vaccines we noticed a different appearance between MTBVAC and BCG clinical formulations upon reconstitution (Fig S5a), with BCG showing a cloudy suspension, whereas MTBVAC suspension was more clear. It is important to remark that the amount of viable bacteria in both formulations was comparable, and therefore the more turbid aspect of BCG could be a consequence of a higher proportion of dead bacteria produced during the manufacturing process. In addition, this could be related with the higher tendency of BCG to clump during bacterial growth. As we have observed under laboratory conditions, in the absence of detergent, MTBVAC tended to grow in suspension, whereas BCG suspension showed more clumps (Fig. S5b). We also visualized both individual bacilli of MTBVAC and BCG by electronic microscopy. Images revealed lower bacterial length of MTBVAC (<1 μm) in comparison to BCG (>2 μm) (Fig. S5c).

4. Discussion

Data available in the literature evidence a primary role of lung innate cells in the development of asthma. Concretely, alternatively activated or type M2 macrophages are elevated in lungs from asthmatic individuals, both in animal models and humans. Reasons behind this pathological macrophage polarization are not clear. A plausible explanation is that allergens can directly cause damage in the alveolar epithelium, and macrophages might be alternatively activated as reaction to the injury to induce a wound-healing response [28].

In the present study, we evaluated the therapeutic potential of live attenuated tuberculosis vaccines BCG and MTBVAC delivered by the intranasal route in different preclinical models of asthma. Our results are highly robust. Intranasal BCG and MTBVAC revert AHR-associated response in all scenarios assayed, both in short-term and long-term acute models, and also in established asthma induced by OVA or HDM, where a strong airway eosinophilia was already present prior to vaccine administration. Our results showed that airway eosinophilia reduction induced following single intranasal delivery of BCG or MTBVAC was comparable to the observed following dexamethasone (DEX) treatment achieved after four drug systemic inoculations. This reflects a qualitative difference between treatment with vaccines or anti-inflammatory standard drugs. Whereas in the former case, vaccines are administered once and they provided therapeutic efficacy in the long-term by subverting the asthma-associated immune response; in the relative to standard treatments their target are the symptoms instead of the causes of the disease, and therefore they carry out the beneficial effect only in the short-term, with the consequence that the patient is usually subjected to a chronic treatment during the whole life.

BCG and MTBVAC are efficiently internalized by alveolar macrophages upon immunization, leading to the expression of M1 markers like iNOS or CD86. These proinflammatory macrophages might trigger an inflammatory response antagonist to the generated by alternatively activated macrophages usually associated with asthma. Importantly, our analysis with GFP-expressing BCG bacteria indicates that not only infected macrophages adopt an M1 phenotype, but also uninfected cells. This suggests that macrophages initially infected by vaccine bacteria generate a chain of signals that would lead to activation of bystander neighbours, favouring the expansion of a response that globally counterbalances asthma-associated lung environment.

BCG-associated benefits for asthma patients have been extensively studied in the clinic, both in observational studies comparing asthma prevalence among BCG vaccinated populations [15], and in interventional controlled trials with two arms vaccinated either with BCG or placebo [16]. Data are controversial, with studies showing opposite findings. Reasons to explain this controversy can be attributed to different aspects, such as age-group (children or adults), different doses of vaccine administered, previous exposure to mycobacteria, or even use of different BCG substrains. For instance, in an interventional study conducted in 2002 in South Korea, authors found an improvement of lung function in the BCG group with respect to placebo. However, study subjects were mostly BCG-vaccinated, since this vaccine is usually administered during childhood in the country, and importantly, the intradermal BCG dose was extremely high (58.2×10^7 CFUs) (standard intradermal dose is 5×10^5) [16]. These experimental settings are different from other studies performed in countries without a history of BCG vaccination, where a standard dose of BCG would be used. This is the case of another study from 2008 conducted in the Netherlands, where no significant benefit was found in the BCG group [29]. Indeed, at least two meta-analysis reports comparing different published studies suggested poor association between intradermal BCG vaccination and low risk of developing allergies [14,30].

Our data convey that the beneficial effect of BCG might be organ dependent, and therefore BCG needs to be physically present in the lungs to induce an efficient antagonist response against asthma. Indeed, subcutaneous BCG is not protective against asthma in mice [18], a result that we have confirmed with MTBVAC (data not shown). This agrees with our observation that live mycobacteria must interact with resident lung macrophages to activate them in a classical way. Data from the literature regarding asthma incidence and TB infection and disease could endorse this hypothesis. A significant correlation between lower prevalence of asthma and TB notification cases has been reported [31]. A study conducted in individuals with latent TB infection (LTBI) showed a strong association between tuberculosis skin test (TST) positivity and lower prevalence of different types of allergy [32], including asthma. We recently demonstrated this result using an experimental tuberculosis infection mouse model [33]. Interestingly, Obihara et al. found a substantial reduction of asthma incidence in the group with the highest TST value, suggesting a correlation between magnitude of the TB-specific immune response and degree of unspecific protection [32]. TST assay rate of false LTBI positives is high, mainly due to the interference of the test with BCG vaccine or environmental mycobacteria. However, the authors found the lower asthma prevalence in the group with higher TST positivity ($>$20 mm), which makes more likely that this unspecific benefit could be due to latent tuberculosis infection [34,35].

Finally, reports of individual cases have described lower rates of exacerbations in asthmatic patients who suddenly undergo active TB [36]. Since *M. tuberculosis* infection is mostly acquired by the respiratory route (whereas BCG is given intradermally), we speculate that our results might be reflecting a natural unspecific protection against asthma that already occurs in nature.

Allergen-specific CD4+ *T* cells are thought to play a central role in asthma pathogenesis. Use of allergen-MHC-II tetramers has allowed characterization of allergen-specific CD4+ *T* cells in asthmatic individuals, finding tetramer-positive T cell clones that express central memory markers [37]. These long-lived T cells are ultimately responsible of the perpetuation of asthma throughout lifetime. Upon allergen exposure, specific memory CD4+ *T* cells migrate to lungs where they efficiently recognize allergen-derived peptides presented by APCs, and respond quickly by secreting Th2-associated cytokines leading to asthma inflammatory response. Thus, a therapy that pursues asthma abrogation should focus on hampering allergen-specific Th2 memory cells. For instance, immunotherapy based on low-level allergen epitope exposure works by inducing anergy on allergen-specific Th2 T cells [38].

Our results analysing OVA-specific T cells in draining lymph nodes indicate that intranasal vaccines have a strong impact in OVA-specific Th2 cells. Intracellular staining and flow cytometry, which allows direct visualization of cytokine-producing cells, showed a population of IL-5-producing T cells in the OVA group that is diminished upon BCG treatment, as long as a population of OVA-specific IFNγ-producing cells arises. This suggests that live mycobacteria could reshape the phenotype of T cells involved in asthma responsiveness. Interestingly, BCG-induced protection against AHR was also observed in long-term studies (where OVA challenge was delayed until 4 months post immunization), though bacteria were almost totally cleared at the time of analysis, suggesting that vaccines might be triggering allergen-specific memory T cells with a Th1 profile that persisted even after bacterial clearance, and which could provide a long-lasting protection against allergen exposure.

A question that remains unanswered in the present study is whether the T cells that adopt a Th1 profile after immunization are generated *de novo* from naïve T cells, or they are the same Th2 clones induced by allergen exposure, which are re-polarized to Th1 by the effect of the vaccine. Supporting the second hypothesis, T cells present high plasticity and Th2 cells have been shown to become Th1 in the presence of an appropriate environment enriched with interferon and IL-12 [39].

Our results demonstrate in mice the efficacy of intranasal BCG and MTBVAC subverting AHR. Nevertheless, it is unlikely that intranasal route is authorized for live tuberculosis vaccine delivery in clinic. Together with the anatomical limitations associated with this route [40], there are safety concerns with intranasal administration. A flu vaccine intranasally delivered caused transient face palsy episodes in clinic [27]. In addition, proximity of the nose cavity to the brain would be likely a cause of reluctance for regulatory authorities. Conversely, the aerosol route results more plausible [41]. Recent studies in non-human primates (NHP) have demonstrated that BCG reaches the lungs following aerosolization with clinical nebulizers [42,43]. With regard to human studies, BCG has been already administered in the past by the aerosol route without major toxicity issues [44,45], and at present there are at least two on-going clinical trials to assess safety of aerosol BCG (NCT03912207, NCT02709278). Interestingly, our results showed the feasibility of nebulizing GMP formulations of MTBVAC and BCG through a clinical nebulizer, reaching bacterial loads proven to be effective in models of established asthma. Of note, our data indicated that MTBVAC was more efficiently nebulized than BCG. As mentioned above, two distinct phenotypic characteristics between MTBVAC and BCG could account for the different nebulization efficiency of the vaccines. Considering that the mean particle size released by clinical nebulizers is around 5 μm, it is realistic to speculate that clumping and bacterial size could crucially affect the efficacy of vaccine nebulization. In this regard, MTBVAC presents a reduced clumping capacity during growth and smaller bacterial size compared to BCG. This could be due to the loss of most of the extractable surface lipids in MTBVAC as a result of the deletions in the virulence genes *phoP* and *fadD26* [20]. Indeed, *phoP* mutant strains show a reduced cording factor [46], which is highly related to clumping in wild-type strains. Intradermal MTBVAC is in advanced clinical development in TB-endemic countries today with demonstrated safety, at least comparable to BCG, and superior immunogenicity characterized by antigen-specific Th1 responses in both adult and newborn populations [21,22]

Although BCG was initially conceived as a prophylactic vaccine against TB, other different clinical applications have been subsequently proposed. The most remarkable one has been the intravesical application of BCG as bladder cancer therapy, successfully used for the last four decades. However, no previous therapeutic application of pulmonary BCG has been reported up-to-date. From a safety perspective, a key step to move this type of treatment into clinic should be to evaluate the reactogenicity of the vaccine in individuals pre-exposed to mycobacteria, including BCG vaccination and/or *M. tuberculosis* infection. In this regard, a recent study conducted in South Africa described the safety of a pulmonary BCG administered by the intratracheal route [47]. Interestingly, the authors inoculated BCG in adult populations with different mycobacterial sensitization status, including BCG-vaccinated and latent (pulmonary) TB infected, finding no major adverse events associated with intratracheal administration of BCG. In the case of intradermal MTBVAC, a phase IIa dose-escalation study for safety and immunogenicity is currently being performed in *M. tuberculosis* infected and uninfected adults (BCG-vaccinated at birth) in South Africa (NCT02933281).

Over the last years, novel experimental immunotherapies against asthma have emerged, mainly based on the monoclonal antibody-mediated blockade of specific molecules that contribute to asthma-induced inflammation, such as IL-4, IL-5, Il-13 or IgE, showing in many cases positive results. However, these studies also evidence that efficacy achieved by inhibition of a single pathway is in general partial. For instance, IL-5-specific therapy substantially inhibits eosinophilia, whereas outcomes for other measures, such as lung function, are less favourable [48]. Since asthma is a highly complex disease, we speculate that an approach as the proposed in the present study,

with a capacity to interact with both innate and adaptive immune components, could be more efficient dealing with the different aspects of the pathology.

Contributors

C.M. and N.A. designed the experiments and directed the study. R. T., E.M., S.U., A.B.G. and I.O. performed the experiments. R.T., D.M., C. M. and N.A. wrote the manuscript. All authors have read and approved the final version of the manuscript.

Declaration of Competing Interests

Raquel Tarancón, Elena Mata, Santiago Uranga, Dessislava Marinova, Carlos Martín and Nacho Aguiló are co-inventors of the patent "Therapeutic efficacy by pulmonary delivery of live attenuated mycobacteria" held by the University of Zaragoza. Santiago Uranga, Dessislava Marinova, Carlos Martín and Nacho Aguiló are co-inventors of the patent "Compositions for use as a prophylactic agent to those at risk of infection of tuberculosis, or as secondary agents for treating infected tuberculosis patients" held by the University of Zaragoza and Biofabri. Carlos Martín is inventor of the patent "Tuberculosis vaccine" held by the University of Zaragoza. There are no other conflicts of interest.

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Data Sharing Statement

The authors declare that the data supporting the findings of this study are available from the corresponding author upon request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.103186.

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