# Correlation between systemic allergen desensitisation and long-term asthma protection in mice following intravenous administration of the live tuberculosis vaccine MTBVAC

Silvia Calvo,[a,b] Jose Manuel Rodrigo-Muñoz,[b,c] Raquel Tarancón,[a,b,e] Santiago Uranga,[a,b] Carlos Martín,[a,b] Victoria del Pozo,[b,c,d] and Nacho Aguiló[a,b,*]

[a]Grupo de Genética de Micobacterias, Departamento de Microbiología y Medicina Preventiva, Facultad de Medicina, Universidad de Zaragoza, IIS-Aragon, Zaragoza, Spain
[b]CIBER de Enfermedades Respiratorias (CIBERES), Instituto de Salud Carlos III (ISCIII), Madrid, Spain
[c]Immunoallergy Laboratory, Immunology Department, Instituto de Investigación Sanitaria Fundación Jiménez Díaz (IIS-FJD, UAM), Madrid, Spain
[d]Universidad Autónoma de Madrid, Madrid, Spain

eBioMedicine
2024;107: 105272
Published Online xxx
https://doi.org/10.1016/j.ebiom.2024.105272

## Summary

**Background** MTBVAC is a live attenuated tuberculosis vaccine, currently undergoing phase III evaluation for tuberculosis prevention. In previous preclinical studies, we found that local pulmonary administration of MTBVAC via the intranasal route had a strong therapeutic effect against asthma. This effect correlated with the abrogation of allergen-specific Th2 response in the lungs.

**Methods** Using different mouse models of asthma, we investigated the effect of MTBVAC administered by intravenous (IV) route and its potential as immunotherapeutic agent to induce desensitisation of allergen-specific responses at a systemic level. We explored the effects of this process in the efficacy against airway hyperresponsiveness (AHR) induced by exposure to different allergens.

**Findings** IV MTBVAC was highly efficient at reducing AHR induced by different allergens. Additionally, IV MTBVAC was found to be well-tolerated, being progressively eliminated from the different organs analysed. From a mechanistic standpoint, we observed that MTBVAC intravenous, but not intranasal, impaired allergen-specific Th2 response in both lungs and spleen. This reduction at a systemic level correlated with long-term therapeutic protection against allergen exposure. Our results also revealed differential immunological mechanisms governing systemic and local pulmonary allergen desensitisation processes. Notably, in a cohort of patients with asthma sensitive to house dust mite (HDM), *in vitro* incubation of peripheral blood mononuclear cells (PBMCs) with MTBVAC prevented allergen-specific production of Th2 cytokines IL-4 and IL-5.

**Interpretation** Altogether, our results suggest that intravenous MTBVAC could be a plausible allergen desensitising approach for treatment of asthma, and could provide long-term protection against allergen exposure.

**Funding** MCIN/AEI/10.13039/501100011033 [grants number RTI2018-097625-B-I00 and PID2022-138624OB-I00]; Consorcio Centro de Investigación Biomédica en Red- (Groups CB06/06/0020 and CB06/06/0013), Instituto de Salud Carlos III.



**Keywords:** Eosinophilia; Asthma; Live tuberculosis vaccines; MTBVAC; Th2/Th1 lymphocytes; Intravenous inoculation

## Introduction

Asthma is a common chronic respiratory condition characterised by the inflammation of the respiratory airways, leading to symptoms such as wheezing, coughing, and shortness of breath. The Global Asthma Report estimates that over 300 million people worldwide

*Corresponding author. Grupo de Genética de Micobacterias, Departamento de Microbiología y Medicina Preventiva, Facultad de Medicina, Universidad de Zaragoza, IIS-Aragon, Zaragoza, Spain.
*E-mail address:* naguilo@unizar.es (N. Aguiló).
[e]Present address: Exopol S.L., Spain.

## Research in context

### Evidence before this study

Live tuberculosis (TB) vaccines, particularly BCG, have been previously associated with protection against asthma due to their capacity to trigger Th1 responses, which are antagonists to the Th2 pathological responses responsible of type 2 allergies. MTBVAC is a live tuberculosis vaccine candidate currently under clinical evaluation to replace BCG against TB. MTBVAC has demonstrated to be effective in murine asthma models when administered by pulmonary route. This protection correlated with the impairment of allergen-specific Th2 responses specifically in the lungs. However, capacity of MTBVAC to modulate Th2 cells in central lymphoid organs has not been assessed. Systemic Th2 cells with memory markers have been found in patients with asthma, and it is assumed that these pathogenic cells are responsible of perpetuating asthma symptoms upon exposure to the reactive allergen. Therefore, therapies targeting central memory immune responses would be of particular interest to trigger allergen desensitisation.

### Added value of this study

In the present study, we aimed to evaluate whether MTBVAC intravenous (IV) administration could limit airway hyperresponsiveness (AHR) induced by exposure to different types of allergens, as well as to assess how systemic treatment delivery might modulate systemic allergen-induced responses. Our results revealed that a single IV inoculation of MTBVAC abrogated lung and bronchoalveolar lavage (BAL) eosinophilia induced by allergen exposure, to levels comparable to pulmonary MTBVAC. Nevertheless, only IV delivery impaired allergen-specific responses in the spleen, suggesting differential immunological mechanisms leading to systemic and local pulmonary allergen desensitisation processes. Importantly, we found that systemic desensitisation induced by IV MTBVAC correlated with long-term protection against asthma.

### Implications of all the available evidence

Current immunotherapies against allergy pursue induction of tolerance of the immune system against a specific allergen. However, these therapies present some issues, including the duration of the treatment, and the high number of allergen inoculations needed to reach an efficient desensitisation, with the corresponding potential safety events associated with the exposure to the allergen, which can be severe in a proportion of patients. Our results suggest that IV MTBVAC is well-tolerated and it could be a plausible desensitising approach in patients with asthma, overcoming some of the issues currently associated with allergen immunotherapy. Importantly, MTBVAC triggered heterologous protection against different allergens, suggesting that it could be a desensitising therapy for a wide range of patients.

suffer from asthma. While high-income countries generally exhibit higher asthma rates, the prevalence in low-income and middle-income countries is augmenting, due to factors such as urbanisation and changes in lifestyle and environmental factors.[1]

Although the immunological drivers of asthma are diverse, type 2 inflammation is present in more than 80% of asthma cases. Allergen-specific T helper (Th) lymphocytes with a Th2 profile, as well as innate lymphoid cells type 2 (ILC2), produce type 2 cytokines as IL-4, IL-5 or IL-13, which are responsible for most of the characteristic clinical symptomatology. IL-5 plays a central role in the survival and recruitment of eosinophils, a key component in asthma pathology and a widely accepted biomarker for disease diagnosis.[2]

*Mycobacteria* belonging to *Mycobacterium tuberculosis* complex (MTBC), including the current tuberculosis vaccine BCG (Bacillus of Calmette-Guerin), have been classically considered as strong Th1 response-promoting stimuli, and their potential limiting some asthma features has been widely discussed.[3] 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.[4–6] Mechanistically, different studies have proposed the induction of Th1- and T regulatory (Treg)- mediated responses as the mediators that balance the Th2 allergic responses in preclinical models of asthma.[5,7]

MTBVAC is a live attenuated mycobacterium based on the rational attenuation of a *Mycobacterium tuberculosis* clinical isolate, by deletion of the virulence genes *phoP* and *fadD26*.[8] MTBVAC has been widely tested in different animal models by intradermal route, demonstrating to be well-tolerated and efficient at preventing tuberculosis in mice, guinea pigs and non-human primates (NHP).[9] Since 2012, MTBVAC is under clinical development as tuberculosis vaccine in newborns and adults.[10,11] In 2022, MTBVAC started efficacy assessment in a phase III trial against tuberculosis in newborns. During the last years, intravenous (IV) route has been reported as a promising way to deliver the tuberculosis vaccine BCG in different animal models, including non-human primates (NHPs).[12,13] Data in mice indicate that following IV administration, BCG spreads to different organs, including the bone marrow (BM), spleen, liver or lungs, where it can persist for several weeks.[14,15]

We previously demonstrated that intranasal administration of MTBVAC in mice reduced asthma-associated symptoms such as airway eosinophilia and lung remodelling.[16] Mechanistically, MTBVAC pulmonary delivery impaired allergen-specific Th2 response at a local lung level, decreasing production of cytokines as

IL-5 or IL-13. These cytokines play a direct role in mediating allergic asthma symptoms, and are primary targets of recent-generation biological treatments for this disease.[2]

In this study, we aimed to assess the application of MTBVAC delivered by IV route in different relevant mouse models of asthma that reflect some of the main features of this disease, as airway eosinophilia and lung epithelium remodelling. We evaluated whether systemic MTBVAC induced allergen desensitisation not only in the lungs, but also in a central lymphoid organ as the spleen. Our results demonstrated the efficacy of IV MTBVAC in reducing airway hyperresponsiveness (AHR) following exposure to different allergens. Importantly, IV MTBVAC proved to be well-tolerated with no sign of acute toxicity. Our results highlighted the different immunological pathways driving local and systemic allergen desensitisation, and revealed that systemic suppression of allergen-specific Th2 response correlated with long-term protection against allergen-induced AHR.

## Methods

### 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 (protocols PI41/17, PI46/18, PI24/20, PI51/20 and PI67/20). This study adheres to ARRIVE guidelines for the transparent reporting of research involving animals.

Human peripheral blood mononuclear cell (PBMC) samples used in the study were collected in the context of the MEGA project,[17] a study conducted in different Spanish hospitals to create a cohort of patients with asthma with varying grades of severity, in order to gain greater insight into the mechanisms underlying the genesis and course of this disease. This study was designed according to the principles of the Declaration of Helsinki (18th World Medical Assembly, 1964) and Hong Kong (1989). The purpose of the study was explained to each patient before obtaining their written consent to participate. The research project was approved by the Clinical Research Ethics Committee of all hospitals participating in the study in accordance with Personal Data Protection Act 15/1999, Biomedical Research Act 14/2007, and Biomedical Research Royal Decree 1716/2011. In the present study, the samples analysed came from the Fundación Jiménez Díaz University Hospital. The ethics committee from this institution (CEIM-FJD) approved the acquisition and storage of the patient samples in a biobank, and the subsequent analyses conducted with them (Approval Number PIC002-19_FJD).

### Bacteria

Mycobacterial strains used were MTBVAC,[8] MTBVACΔerp,[18] MTBVACΔlysA,[19] which are attenuated strains derivative from *Mycobacterium tuberculosis*, by genetic deletion of *phoP* and *fadD26* genes in the case of MTBVAC, and additional deletions of the *erp* and *lysA* genes in the MTBVACΔerp and MTBVACΔlysA substrains, respectively. These bacterial strains have been produced and characterised in our laboratory. Vaccines were grown at 37 °C in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween 80 (Sigma) and 10% (v/v) and ADC (albumin/dextrose/catalase) (Difco), or in Middlebrook 7H10 agar supplemented with 10% ADC. Bacterial suspensions for vaccination were prepared in PBS from glycerol stocks previously quantified by plating serial dilutions. When indicated, MTBVAC was inactivated at 105 °C for 30 min.

### Animal studies

Mice were housed in cages (5–6 mice/cage) in a 12-h light/dark cycle with ad libitum access to food and water. All mice were routinely observed for any sign of disease (at least with a weekly frequency). Mouse strains used in the study were C57BL/6JRj (Janvier Biolabs, RRID:MGI:3028467), B6.129S7-Ifngtm1Ts/J (Ifng−/−) (The Jackson Laboratory, RRID:IMSR_JAX:002286), B6.129S7-Rag1tm1Mom/J (Rag1−/−) (The Jackson Laboratory, RRID:IMSR_JAX:002096) and B6.129S(C)-Batf3tm1Kmm/J (BatF3−/−) (A gift from David Sancho, CNIC (Madrid), RRID:IMSR_JAX:013755). All strains were bred at our animal facilities. We used in all cases mice derived from our breeding colonies, except for the wild-type C57BL/6JRj strain, in which case, for some experiments we complemented the number of animals required with mice directly purchased to Janvier Biolabs. Eight-to-ten weeks-old male and female mice were used.

For induction of ovalbumin (OVA)-specific AHR, mice were sensitised twice with 50 μg chicken egg OVA (lyophilised powder, 98% (Sigma)) and 2 mg $AlOH_3$ (Sigma), administered intraperitoneally one week apart. One week later, mice received a single intranasal or IV administration of MTBVAC vaccine at the dose $5.10^6$ colony-forming units (CFUs). Four weeks later, animals were intranasally challenged with 100 μg OVA for three consecutive days and one day after mice were culled for subsequent analyses. For house dust mite (HDM)- and *Alternaria alternata*-induced AHR, mice were intranasally challenged twice a week for three consecutive weeks with 10 μg of allergen (HDM from Citeq and *Alternaria* extract from DIATER). MTBVAC was delivered at week 4, and one month later, mice were intranasally challenged with 10 μg of allergen for three consecutive days, and culled the day after. In the HDM chronic asthma model, additional HDM challenges were added at 3 and 6 months of experiment. For Treg depletion studies, 200 μg/dose of anti-CD25

neutralising antibodies (clone PC61, AssayGenie, Cat number IVMB0148) were administered intraperitoneally five times during the last 15 days of experiment, three days apart.

Splenocyte transfer protocol was adapted from.[20] Briefly, donor mice were sensitised with OVA as described above. Splenocytes were harvested and cultured for 48 h at 37 °C with 1 mg/ml OVA. A suspension of $10^7$ splenocytes was administered intravenously to each recipient mouse, and three days later they were intranasally challenged with OVA.

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

Lungs or spleen were removed aseptically. Cellular suspensions were generated using the GentleMACS dissociator (Miltenyi), according to manufacturer instructions. For organ explant preparation, lungs or spleen were cut into small pieces and incubated in 1 ml of Gibco AIM V serum-free medium (ThermoFisher) overnight. The resulting supernatant was collected and stored at –80 °C until further analysis.

For CD4+ T cells isolation from spleen cellular suspension, a commercial kit based on magnetic beads (Miltenyi Biotec) was used according to manufacturer instructions.

For bacterial burden determination, organs were homogenised with the GentleMACS, using the RNA protocol, and then plated onto agar medium 7H10 supplemented with ADC.

### Human PBMCs studies

Frozen human PBMCs were obtained from the sample biobank generated inside MEGA project at the Fundación Jiménez Díaz Hospital.[17] A total of 12 PBMC samples were analysed, each of them corresponding to a different patient sensitive to HDM (positivity determined by prick test). Four of the samples came from men and eight from women. The median age of the patients was 46.5 years old (Min = 27; Max = 70). BrdU (bromodeoxyuridine) incorporation assay was analysed in 9 samples and cytokine evaluation was assessed in the 12 samples.

For the BrdU test, we used a well with PBMCs incubated with phytohemagglutinin (PHA) 1 μg/mL as positive control of activation. This control was used to discard samples that did not respond to PHA, as we considered that the integrity of the cells contained within these samples could be compromised. Four samples were negative for PHA stimulation and discarded from the study, and therefore, BrdU incorporation assay was analysed in 5 samples.

At day 0, PBMCs were thawed and cultured in a U-bottom 96-well plate at a concentration of $10^6$ cells/ml and incubated with or without 10 μg/mL HDM. After 24 h, MTBVAC was added at MOI 1 5 or 10 bacteria per cell. After 96 h of infection, supernatants were collected and stored at –80 °C for further analysis by ELISA. For proliferation assays, BrdU was added at day 5 to the wells at a concentration of 1:1000 to perform the BrdU Cell Proliferation Assay, following the manufacturer's instructions (Sigma).

### Flow cytometry analysis

All antibodies were considered as validated by the manufacturer. No additional validation was conducted. BAL or lung cells were incubated for 15 min at 4 °C with Fc receptor blocking reagent (Miltenyi Biotech). Eosinophil populations were 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 $SSC^{high}CD45^{+}SiglecF^{+}CD11c^{-}$ (Supplementary Fig. S1).

For determination of Treg lymphocytes, lung cells were stained with the following antibodies from Miltenyi Biotech: CD3-PerCP-Vio700 (RRID:AB_2657088), CD4-PE (RRID:AB_2811459) and FoxP3-APC (RRID:AB_2651768). CD3 and CD4 markers were stained extracellularly, and FoxP3 intracellularly using the FoxP3 staining set (Miltenyi Biotec) according to manufacturer instructions. Data were acquired with a Beckman Gallios analyzer.

### Cytokine analysis

Quantification of mouse IL-5, IFNγ, IL-6, TNF-α and IL-10 (Mabtech Biotech), and human IL-5 and IL-4 (R&D Systems) was determined using specific commercial ELISA kits in accordance with the manufacturer's instructions.

To analyse OVA-specific response in spleen or lungs, $2 \times 10^6$ cells were incubated with or without 1 mg/ml OVA for 96 h, and supernatant was collected cytokine determination. Allergen-specific response for each cytokine was calculated by subtracting the cytokine concentrations obtained from the unstimulated control.

### Measurement of eosinophil peroxidase (EPO) activity

The protocol was adapted from.[21] Lung cells were resuspended at $2.10^7$ cells/ml in 0.5% cetyltrimethylammonium chloride (CTAC; Sigma) and centrifuged at 4000 rpm for 20 min. Then, the supernatant was aliquoted and stored at –80 °C for further evaluation of EPO activity by an enzymatic reaction. Supernatants were diluted 1:10 in a 50 mM HEPES buffer (pH 6.5) containing 6 mM KBr, and 75 μl of each sample was added to wells of a 96-well flat-bottomed tissue culture plate. EPO substrate solution was freshly prepared by combining 3 mM OPD in 50 mM HEPES

buffer (pH 6.5) with 6 mM KBr and 8.8 mM $H_2O_2$. 75 µl of this EPO substrate solution was added to each well, and the reaction was stopped after 30 s by adding 150 µl of 4N $H_2SO_4$. The absorbance was read at 490 nm.

### Histological analyses

Left lung was fixed in formaldehyde 4%, and then stained using Periodic acid–Schiff (PAS) technique. Images were obtained with a microscope Leica DM5000B. To quantify the degree of lung damage we established affectation grades based on the level of epithelium disorganisation and multi layering, smooth muscle engrossment, goblet cells neoplasia and presence of mucosubstances in the bronchioles. We analysed 167 bronchioles from the *Alternaria*-positive group, 223 from the vaccinated group and 80 from the negative control animals. The percentage of bronchioles corresponding to each grade was calculated, scoring the degree of from 0 (no affected) to 4 (most severe).

### Statistics

Mice were randomly distributed in groups of six animals per cage prior to start experimental procedures. Results were not blinded for analysis. Sample sise in animal experiments was defined according to our previous experience. To calculate sample size, we used the G*power software (version 3.1.9.6), considering an alpha value of 0.05 and a power of 0.9. In addition, based on mean and standard deviation (SD) values from previous experiments, we used a hypothetical effect size value of 1 for sample size calculations. The number of biological replicates and repetitions of each experiment is indicated in figure legends. GraphPrism software (Version 8) was used for graphical representation and statistical analysis. Flow cytometry data was analysed using Weasel software (version 3.0.2). Statistical tests used for each experiment are indicated in the figure legends. All tests applied were two-sided. We used t-test to compare two experimental groups, whereas ANOVA (parametric) or Krustal–Wallis (non-parametric) tests were applied to compare more than two groups. Data normality distribution was analysed using the Shapiro–Wilk test. No outlier value was discarded from the final statistical analysis. Differences were considered significant at $p < 0.05$.

### Role of funders

Funders did not have any role in study design, data collection, data analyses, interpretation, or writing of report.

## Results

### IV MTBVAC is effective and well-tolerated against allergen-induced AHR

We initially assessed the efficacy of IV MTBVAC in asthma preclinical models induced by exposure to different allergens, previously optimised in our laboratory.[16] Using a standard model driven by OVA sensitisation (Fig. 1a), we found that a single MTBVAC intravenous administration ($5 \times 10^6$ CFUs), inoculated over sensitised mice, drove to a highly significant reduction of eosinophils compared to untreated group, both in lungs and BAL (Fig. 1b). In addition, we measured functionality of lung eosinophils by analysing the EPO activity, an enzyme mostly expressed by activated eosinophils and found elevated in sputum from patients with asthma.[22] Our results indicated a significantly reduced EPO activity in the MTBVAC-treated group (Fig. 1c). Finally, we demonstrated that BAL levels of IL-5, a major eosinophil recruitment cytokine, were also decreased by MTBVAC (Fig. 1d).

The efficacy of IV MTBVAC was further investigated in models induced by exposure to two clinically relevant allergens: *Alternaria alternata*, a fungus allergen associated with severe asthma cases,[23] and HDM, one of the most prevalent allergens. In contrast to the OVA experiments, in these cases MTBVAC was administered after several allergen pulmonary challenges, in a scenario of established asthma where eosinophilia was already elevated at the time of treatment delivery (Fig. 2a and b). In the *Alternaria* model, IV MTBVAC reduced eosinophils significantly in BAL (Fig. 2b), and marginally in lungs ($p = 0.0564$ by unpaired t-test) (Fig. 2c). In addition, we also analysed the impact of the treatment over epithelial remodeling. Mice treated with IV MTBVAC showed a reduced level of remodeling compared to untreated controls (Fig. 2d and e). Using a pre-established scoring system to quantify the degree of epithelial alteration, we found that in MTBVAC-treated mice, about 50% of the bronchioles analysed were unaffected, and those affected showed lower severity compared to the untreated group, with no bronchioles presenting the highest grade of severity (Fig. 2d).

In the case of HDM exposure (Fig. 2f), IV MTBVAC significantly abrogated BAL eosinophilia (Fig. 2g). To assess the importance of MTBVAC persistence for the therapeutic effect of the bacteria, we included groups treated with two hyper attenuated MTBVAC substrains: MTBVACΔErp,[18] with an additional virulence gene (*erp*) deleted; and MTBVACΔLys,[19] which is an auxotroph MTBVAC strain uncapable to generate the essential amino acid lysine. Both strains have previously shown a strong hyper attenuated profile in immunodeficient mouse models, in comparison to MTBVAC.[18,19] We also included an additional group of mice treated with heat-killed MTBVAC (MTBVAC HK). Globally, our results demonstrated no statistically significant efficacy of hyper attenuated and inactivated MTBVAC compared to untreated control (Fig. 2g).

Considering potential safety concerns associated with the systemic inoculation of live-attenuated bacteria, we evaluated the tolerability and toxicity of IV MTBVAC.

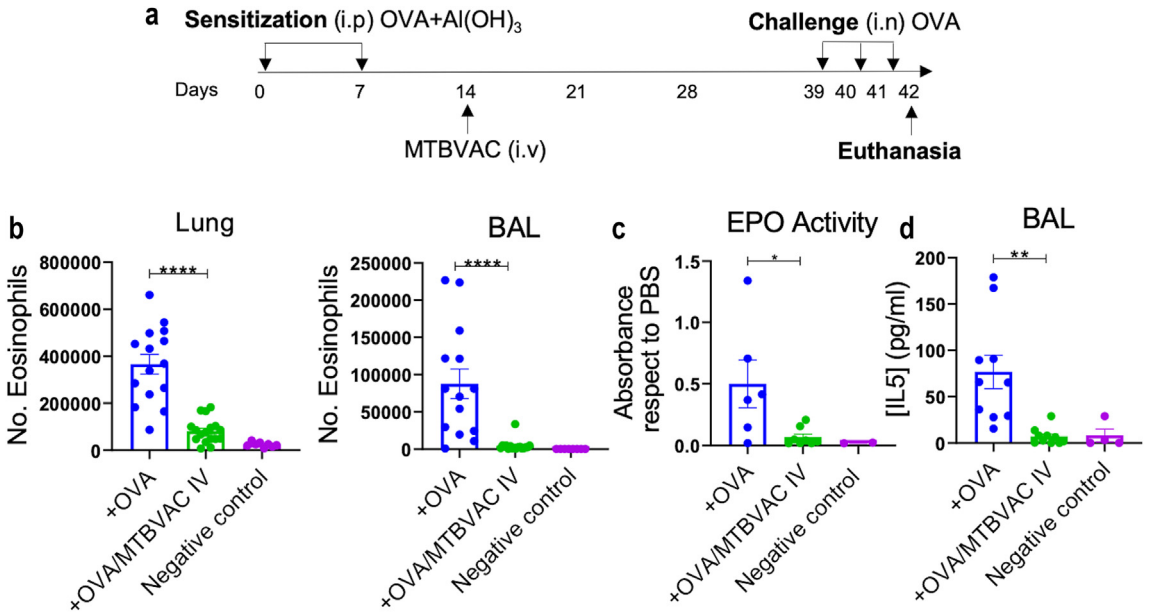


***Fig. 1:*** **IV MTBVAC efficacy in an OVA-driven asthma model**. (a) Model of OVA-induced acute asthma. Mice received IV administration of $5 \times 10^6$ CFUs of MTBVAC one week after second sensitisation. (b) Eosinophils in lungs and BAL were quantified by flow cytometry as$CD45^+SSC^{high}SiglecF^+CD11b^+CD11c^-$ cells. Data pooled from 3 independent experiments (n = 14–18 for OVA-challenged groups and n = 8 for negative controls). (c) EPO activity in lung homogenates. Data from one experiment (n = 6–8 for OVA-challenged groups and n = 2 for negative controls). (d) IL-5 levels in BAL fluid. Data pooled from 2 independent experiments (n = 10 for OVA-challenged groups and n = 4 for negative controls). Data depicted as mean ± SEM. *p < 0.05; **p < 0.01; ****p < 0.0001, by Tukey's multiple comparisons test for one-way ANOVA (b) and Unpaired t-test (c and d).

Over a six-month post-treatment period, we did not find any signs, including weight loss or changes in animal behaviour or food consumption, that could evidence acute or chronic toxicity associated with the treatment. No fever episodes were detected at early timepoints post-injection (Supplementary Fig. S2a), and serum levels of pro-inflammatory cytokines TNFα and IL-6 were normal at four weeks after treatment (Supplementary Fig. S2b), suggesting that MTBVAC was not triggering acute major inflammatory processes. We found transient splenomegaly at one-month post-treatment that resolved at three months (Supplementary Fig. S2c). Finally, we evaluated persistence and bacterial biodistribution up to six months after treatment inoculation. MTBVAC colonised lymphoid (lymphoid nodes and spleen) and non-lymphoid (liver and lungs) at one-month post injection, comparably to the described previously with BCG,[15] and then it was progressively cleared. At the last timepoint analysed, six months, MTBVAC bacteria were still found in spleen, lungs and lymph nodes although at much lower levels compared to one-month. Altogether, these results suggest that IV MTBVAC is well tolerated, it does not cause acute toxicity or inflammation, and it is progressively eliminated during the subsequent months after treatment administration (Supplementary Fig. S2d).

### MTBVAC intravenous, but not intranasal, abrogates systemic allergen-specific Th2 responses

We previously found that intranasal MTBVAC impaired allergen-specific Th2 cells specifically in the lungs.[16] Given the systemic nature of the IV route, we hypothesised that IV MTBVAC could induce allergen desensitisation not only in the lungs, but also in primary lymphoid compartments such as the spleen. Our data revealed that IL-5 levels significantly increased in the spleen following allergen sensitisation, and intravenous but not intranasal treatment administration impaired this process (Fig. 3a). Consistent with this result, only IV delivery impaired the induction of OVA-specific IL-5-producing splenocytes (Fig. 3b). Unlike the observed in spleen, pulmonary production of IL-5 was abrogated both by intranasal and intravenous routes (Fig. 3c), which correlated with the reduction of eosinophils induced by both routes following OVA intranasal challenges (Fig. 3d).

These differing results between both routes of administration suggested distinct immunological mechanisms underlying local and systemic desensitisation induced by MTBVAC. To study them separately, we compartmentalised these two events by conducting experiments of splenocyte transfer from donor sensitised mice to recipient mice that received the allergen

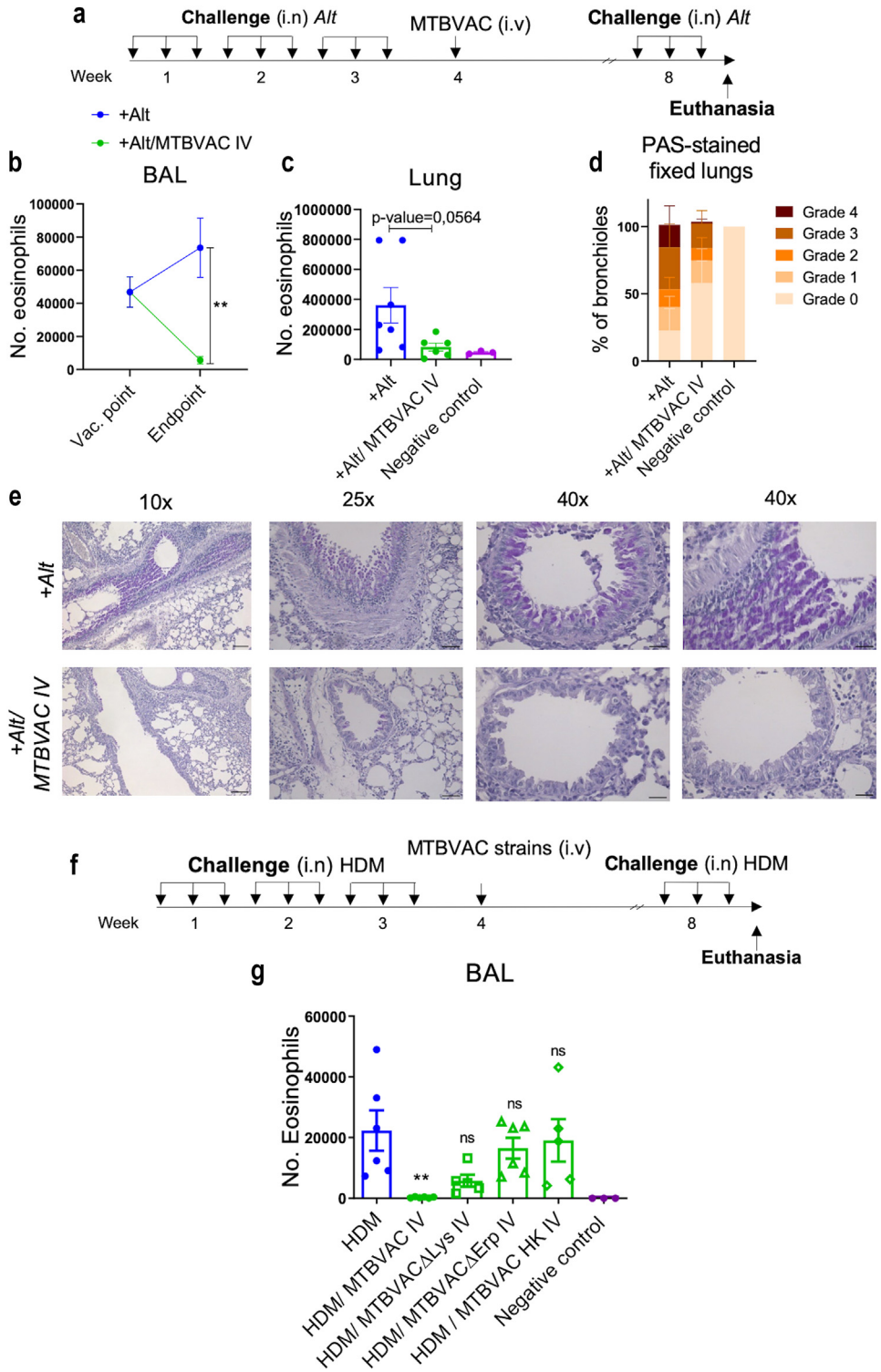


***Fig. 2:* IV MTBVAC efficacy in two models of established asthma**. (a) Model of *Alternaria*-driven asthma. Mice were challenged three times a week during three consecutive weeks with 10 μg of *Alternaria* extract (*Alt*) by intranasal route. $5 \times 10^6$ CFUs of IV MTBVAC was administered the week after. (b) Comparison of BAL eosinophilia at 4 (vaccination point) and 8 weeks (endpoint) of experiment. (c) Eosinophils in lung at endpoint. (d) Pathological score of bronchioles analysed from PAS-stained lungs at endpoint. e) Representative images of PAS-stained fixed lungs from *Alternaria*-challenged mice. Image magnification is displayed in the figure. Scale bars displayed in the images correspond to a length

challenges, and in which we evaluated AHR induction (Fig. 3e). As recipient mice we used Rag1−/− strain, which lacks of lymphocytes. Control experiments indicated that Rag1−/− mice did not develop eosinophilia following sensitisation and challenge with OVA, revealing that adaptive lymphocytes are needed to trigger AHR in this model (Supplementary Fig. S3a and b). Prior to transfer, splenocytes from donor mice were expanded *ex vivo* for 48 h in the presence of allergen (Supplementary Fig. S4a). Transfer of these splenocytes to Rag1−/− mice and the subsequent OVA challenge led to an increase of lung eosinophilia (Supplementary Fig. S4b). Of note, AHR was not induced following a transfer of a CD4+ T cells-depleted splenocyte fraction, whereas it was triggered after transferring isolated CD4+ lymphocytes, emphasising the critical role of CD4+ T cells inducing AHR in this model (Supplementary Fig. S4b). As expected, incubation of OVA-sensitised mouse splenocytes with the allergen promoted production of IL-5, and this process was abrogated when donor mice were inoculated with MTBVAC by intravenous route, but not by intranasal (Fig. 3f). This pattern of IL-5 production in donor splenocytes correlated with the increment of lung and BAL eosinophilia in recipient mice, which it was observed in the untreated and MTBVAC intranasally-treated groups but not in the IV MTBVAC group (Fig. 3g). Moreover, eosinophilia induction was accompanied by significantly higher levels of lung EPO activity (Fig. 3h).

Given previous studies describing the role of Treg cells in mediating AHR reduction by certain formulations of BCG,[5] we assessed the contribution of these lymphocytes to MTBVAC therapeutic effect against OVA-driven asthma. To this end, Treg cells were depleted using anti CD25 neutralising antibodies (Supplementary Fig. S5a). Our results indicated that, despite the efficient depletion of Treg induced by antibody treatment (Supplementary Fig. S5b), MTBVAC treatment led to reduction of BAL eosinophilia regardless of Treg absence (Supplementary Fig. S5c). These data suggested that Treg-mediated responses were dispensable in the context of the treatment with MTBVAC by IV route.

Altogether, our results suggest that allergen desensitisation at lung level, achieved by both MTBVAC intranasal and IV delivery, is particularly determinant for preventing AHR. Additionally, our results indicate that IV administration mediates the desensitisation of allergen-specific response at a systemic level, which may also contribute to the therapeutic effect of MTBVAC induced by this route.

### MTBVAC induces allergen-specific desensitisation of PBMCs from patients with allergy

To evaluate the translational potential of IV MTBVAC as a desensitising agent, we conducted experiments using PBMCs from patients known to be sensitive to HDM.[17] These cells were incubated with HDM to activate allergen-specific cells, and we added subsequently MTBVAC to the culture (Fig. 4a), to assess its capacity to modulate allergen-specific cellular activation. We initially measured HDM-induced expansion of PBMCs using BrdU proliferation assay. Comparing PBMCs from healthy donors with those from patients, we observed that HDM-induced BrdU incorporation only occurred in the patient samples and not in healthy controls, confirming the specificity of the assay (Supplementary Fig. S6). We then incubated PBMCs from each patient with HDM in the presence of increasing multiplicities of infection (MOIs) of MTBVAC. We observed a significant reduction of BrdU incorporation in the presence of the bacteria, with a dose-dependent tendency (Fig. 4b). These results suggested that MTBVAC could modulate allergen-induced expansion of immune cells.

In addition, we measured production of Th2 cytokines IL-4 and IL-5 in cell supernatants following allergen incubation. Our results revealed that MTBVAC reduced Th2 cytokine production in all the specimens that produced IL-4 and IL-5 following HDM exposure. A reduction of both cytokines was found at all the MOIs tested, although only in the case of IL-5 this tendency was statistically significant (Fig. 4c and d). MTBVAC-driven Th2 cytokine reduction was particularly evident when both cytokines were analysed together (Fig. 4e). These results suggest the potential of MTBVAC to induce desensitisation of allergen-specific Th2 cells in a human context.

### Role of IFNγ and cDC1s in systemic and local MTBVAC-induced desensitisation

Next, we explored the immunological mechanisms behind MTBVAC protection. Given that MTBVAC is an intracellular microorganism with capacity to trigger Th1 cellular responses,[24] including IFNγ production, we wondered whether this ability could contribute to impair Th2 responses. Firstly, we confirmed that IFNγ was produced in the spleen from IV MTBVAC-treated mice (Fig. 5a). We then studied MTBVAC capacity to prevent AHR in the absence of IFNγ. Unexpectedly, our data revealed that IV MTBVAC abrogated AHR induction in IFNγ−/− mice, both in terms of eosinophilia reduction

of 8 mm (10x), 3.2 mm (25x) and 2 mm (40x). Data from one experiment (n = 6–7 for *Alternaria*-challenged groups and n = 3 for negative controls). (f) Model of HDM-specific chronic asthma. (g) Eosinophils in BAL at endpoint. Data from one experiment (n = 5–6 for HDM-challenged groups and n = 3 for negative controls). Data depicted as mean ± SEM. *p < 0.05; **p < 0.01, by Unpaired t-test (c), Krustal–Wallis test for one-way ANOVA (g) and Tukey's multiple comparisons test for two-way ANOVA (b).

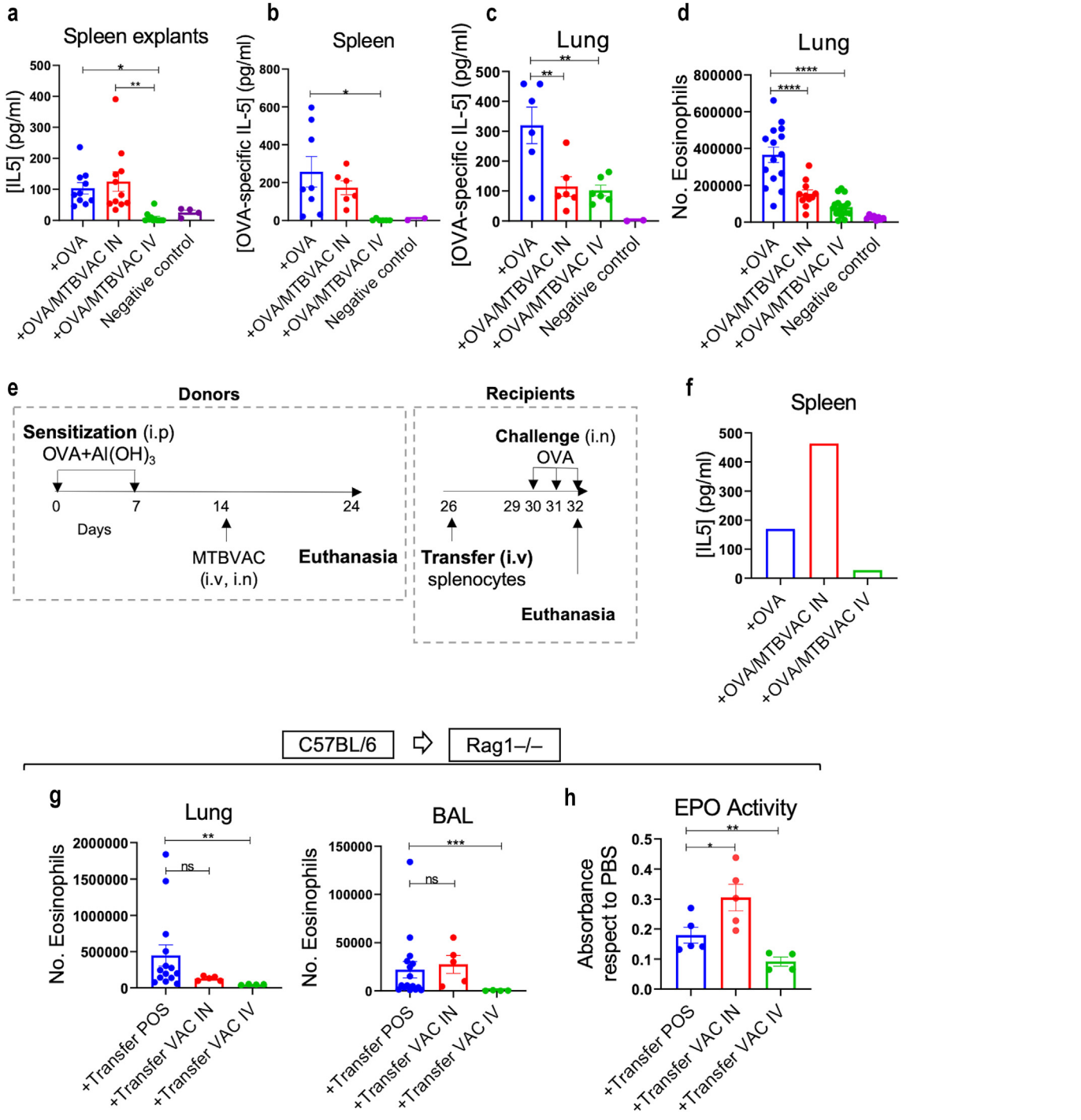


***Fig. 3:* MTBVAC intravenous, but not intranasal, abrogates systemic allergen-specific Th2 responses**. (a) IL-5 determination in spleen homogenates in the OVA-driven asthma model. Data pooled from 2 independent experiments (n = 10–11 for OVA-challenged groups and n = 4 for negative controls). (b, c) OVA-specific IL-5 production by spleen (b) and lung (c) cells, following *ex vivo* stimulation with OVA. Data are displayed after subtraction of the baseline value obtained by incubation without OVA. Data from one experiment (n = 6–8 for OVA-challenged groups and n = 2 for negative controls). (d) Lung eosinophilia. Data pooled from 3 independent experiments (n = 11–17 for OVA-challenged groups and n = 8 for negative controls). (e) Scheme of transfer experiments. Splenocytes from OVA-sensitised donor mice are expanded *ex vivo* with the allergen, and transferred to Rag1–/– recipient mice, followed by OVA challenge to assess lung eosinophilia. (f) IL-5 production by the pool of donor mouse splenocytes after 48 h of incubation with OVA. (g) Lung and BAL eosinophilia from OVA-challenged recipient mice,

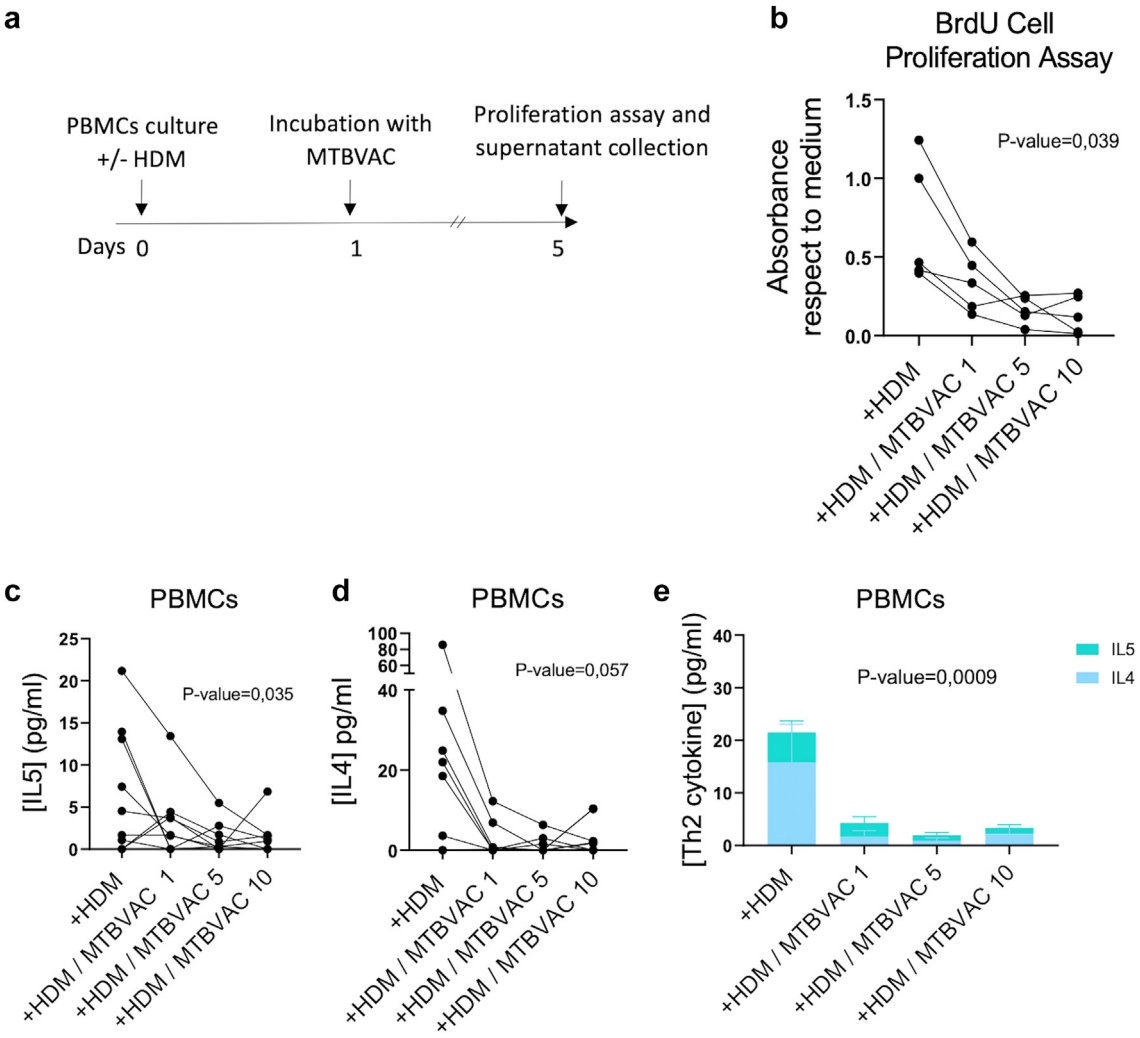


***Fig. 4:*** **MTBVAC induces allergen-specific desensitisation of PBMCs from patients with allergy**. (a) Experimental scheme of the PMBCs study. (b) BrdU proliferation assay of PBMCs from patients in response to HDM, following MTBVAC incubation with increasing MOI (1, 5 and 10 bacteria per cell). Data from one experiment (n = 5 patients). (c) IL-5, (d) IL-4 and (e) both cytokines production in PBMCs from patients in response to HDM. Data shown after subtracting baseline values obtained from unstimulated controls (n = 12). (b–d) Individual values for each patient are displayed. (e) Data shown as mean ± SEM. p values are indicated, obtained by Repeated Measured one-way ANOVA (b–d) and Repeated Measured two-way ANOVA (e) tests.

(Fig. 5b) and decrease of lung EPO activity (Fig. 5c), to similar levels as wild-type controls. Analysis of OVA-specific IL-5 producing cells in spleen and lungs evidenced that IFNγ is particularly critical for systemic desensitisation (in the spleen), but not at a pulmonary level (Fig. 5d). Splenocyte transfer experiments from sensitised IFNγ−/− donors to Rag1−/− recipient mice corroborated this finding. Transfer of splenocytes from MTBVAC-treated IFNγ−/− mice did not provide any protection in the recipient animals against OVA-induced AHR (Fig. 5e, f, g), confirming that IFNγ has a crucial role inducing systemic desensitisation driven by IV MTBVAC. These results confirm that the reduction of pulmonary Th2 responses by MTBVAC appears

transferred with splenocytes from indicated donor groups: POS: OVA-sensitised; VAC: OVA-sensitised and treated with MTBVAC (intranasal (IN) or IV). (h) EPO activity in lung homogenates from recipient mice. (f, h) Data from one experiment (n = 4–5 recipient mice). Data depicted as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, by Tukey's multiple comparisons test for one-way ANOVA (a, b, c and d), and Kruskal–Wallis test for one-way ANOVA (g and h).

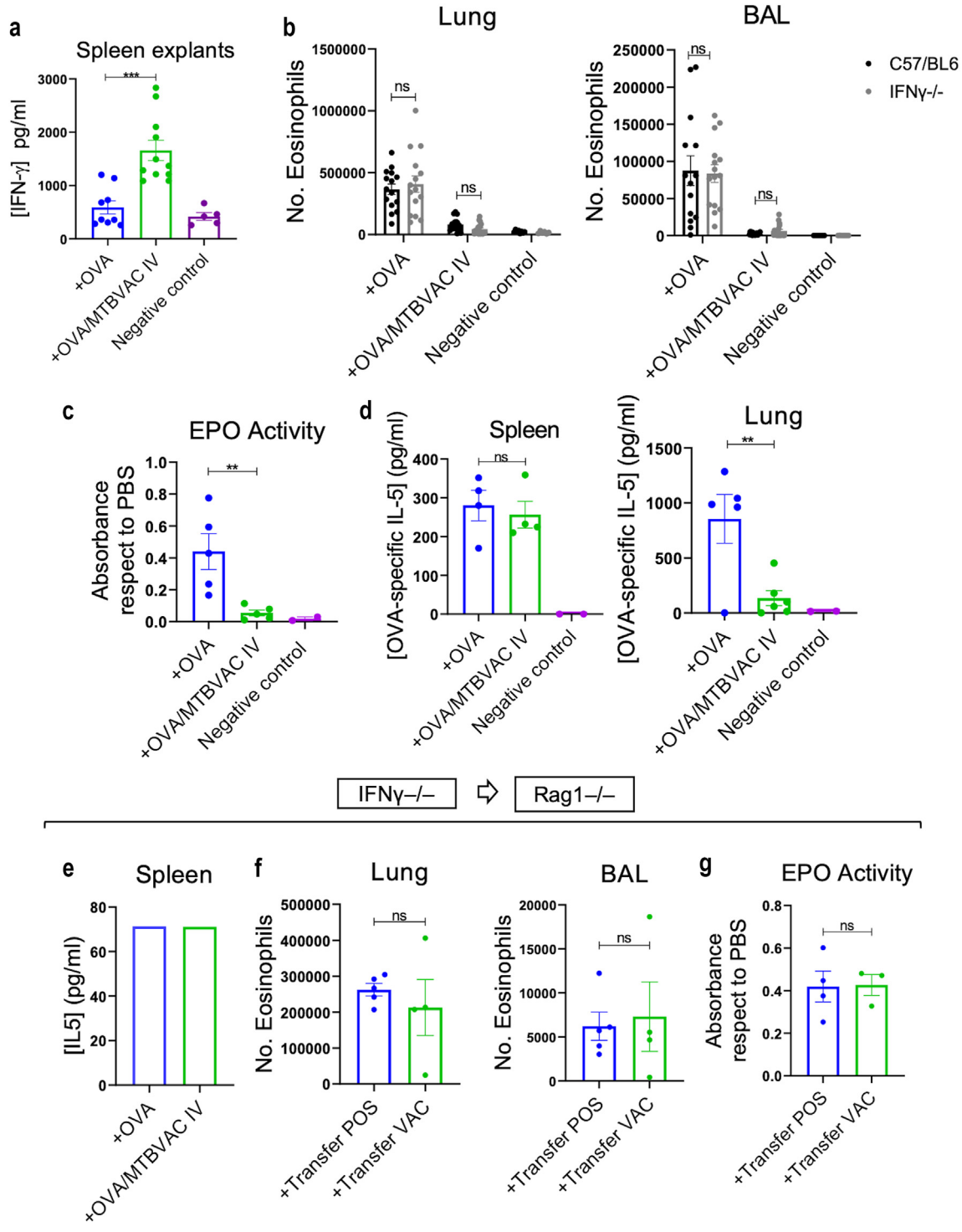


***Fig. 5:* Contribution of IFNγ to MTBVAC-induced allergen desensitisation**. (a) IFNγ production in spleen homogenates in the OVA-driven AHR model. Data pooled from 2 independent experiments (n = 9–11 for OVA-challenged groups and n = 5 for negative controls). (b) Lung and BAL eosinophilia comparison between wild-type (WT) and IFNγ−/−mice. Data pooled from 3 independent experiments (n = 15–17 for OVA-challenged groups and n = 6 for negative controls). (c) EPO activity in lung homogenate from IFNγ−/−mice. Data from one experiment (n = 5 for OVA-challenged groups and n = 2 for negative controls). (d) OVA-specific IL-5 production by spleen and lung cells of IFNγ−/− mice following *ex vivo* stimulation with OVA. Data are displayed after subtraction of the baseline value obtained by incubation without OVA. Data

to be more critical than systemic desensitisation in preventing AHR development, a finding consistent with the comparable efficacy shown by intranasal and IV MTBVAC (Fig. 3d).

Type 1 conventional dendritic cells (cDC1s) contribute to protection against allergen-induced asthma in mice.[25] Since IV attenuated mycobacteria activate cDC1 cells,[26] we evaluated a possible contribution of this cellular subset to MTBVAC protection against asthma. We used mice lacking BatF3, a transcription factor crucial for the differentiation of cDC1s.[27] Our data revealed that the capacity of IV MTBVAC to prevent AHR in BatF3−/− mice was significantly impaired in the lung compartment, in comparison to wild-type controls (Fig. 6a). Concordantly, we observed that in the absence of cDC1s, IV MTBVAC did not reduce OVA-specific IL-5 production in the lungs, suggesting that cDC1 cells mediate MTBVAC-induced allergen desensitisation at a pulmonary level (Fig. 6b). Conversely, MTBVAC-induced reduction of IL-5 in splenocytes was found in the BatF3−/− mice (Fig. 6b), indicating that the contribution of cDC1 may be restricted to pulmonary compartment. In agreement with this observation, transfer of splenocytes from MTBVAC-treated BatF3−/− mice to Rag1−/− recipients resulted in a reduction of OVA-driven AHR (Fig. 6c–e), confirming that cDC1s would not be involved in systemic desensitisation induced by the treatment. However, when splenocytes were transferred from wild-type mice to BatF3−/− recipient mice, which were subsequently challenged with OVA, we found no protection against AHR in the group receiving splenocytes from IV MTBVAC-treated mice (Fig. 6f–h). This result demonstrates that cDC1s critically contribute in the lungs to trigger allergen desensitisation, and this is a crucial event induced by MTBVAC to reduce AHR.

### Systemic desensitisation induced by IV MTBVAC correlates with long-term protection against asthma

We next assessed the duration of the protection provided by IV MTBVAC compared to intranasal route. We hypothesised that systemic reduction of allergen-specific Th2 cells in the spleen, which is specifically induced by the IV route, could impact the duration of the protection mediated by MTBVAC. Thus, we conducted long-term experiments using an adapted HDM-driven chronic asthma model, extending the time frame after MTBVAC administration to six months (Fig. 7a). An early analysis at month one post-treatment inoculation revealed similar level of protection between both routes of administration. However, data at longer timepoints indicated a significant waning effect in the protection mediated by intranasal MTBVAC, which it was not observed following IV administration (Fig. 7b), where long-term protection was maintained. This result suggests that systemic desensitisation differentially induced by IV MTBVAC is crucial for maintaining robust durable treatment responses.

## Discussion

The present study proposes a therapeutic approach based on the IV delivery of the live tuberculosis vaccine, MTBVAC, and its characterisation as an immunotherapeutic agent against asthma. A single MTBVAC systemic administration proved to trigger durable responses against allergen-induced AHR. Importantly, our data with hyper attenuated or inactivated MTBVAC strains suggest that a certain level of bacterial persistence in the host is important for treatment efficacy, and an extra attenuation results detrimental for the treatment to reduce effectively allergen-induced AHR. Noteworthy, unlike our results, a previous study demonstrated AHR reduction following treatment with another heat-killed strain, derived from *Mycobacterium vaccae*.[28] However, comparing both studies, we found important differences. In addition to the inactivated mycobacteria used (MTBVAC derives from *M. tuberculosis*) and the administration route (*M. vaccae* was administered subcutaneously), HK *M. vaccae* was administered twice and at doses higher than the tested by us (up to $10^8$). All these differences in the experimental setting might explain the discordant outcomes obtained in both studies.

The use of the current tuberculosis vaccine, BCG, had been previously proposed as an effective therapy against asthma, based on its capacity to induce cellular responses that could counterbalance the pathological Th2 responses elicited during asthma.[29] Our results indicate that both systemic and respiratory MTBVAC administration effectively abrogated allergen-specific Th2 responses in the lungs, resulting in a strong reduction of different AHR readouts at short-term post-vaccination. These data align with findings associating Th2 cells accumulation in the lungs with asthma features,[30] and the hypothesis that modulation of these cells would be efficient at preventing asthma.[31] Our results

from one experiment (n = 4–7 for OVA-challenged groups and n = 2 for negative controls). (e, g) Transfer of splenocytes from OVA-sensitised IFNγ−/− mice to Rag1−/− mice followed by OVA challenge. (e) IL-5 production by donor mouse splenocytes after 48 h of incubation with OVA. (f) Lung and BAL eosinophilia from OVA-challenged recipient mice, transferred with splenocytes from indicated donor groups: POS: OVA-sensitised; VAC: OVA-sensitised and treated with MTBVAC. (g) EPO activity in lung homogenates from recipient mice. (e, g) Data from one experiment (n = 4–5 recipient mice per group). Data depicted as mean ± SEM. *p < 0.05; ***p < 0.001, by Unpaired t-test (a, c, d, f, and g), by Tukey's multiple comparisons test for two-way ANOVA (b).

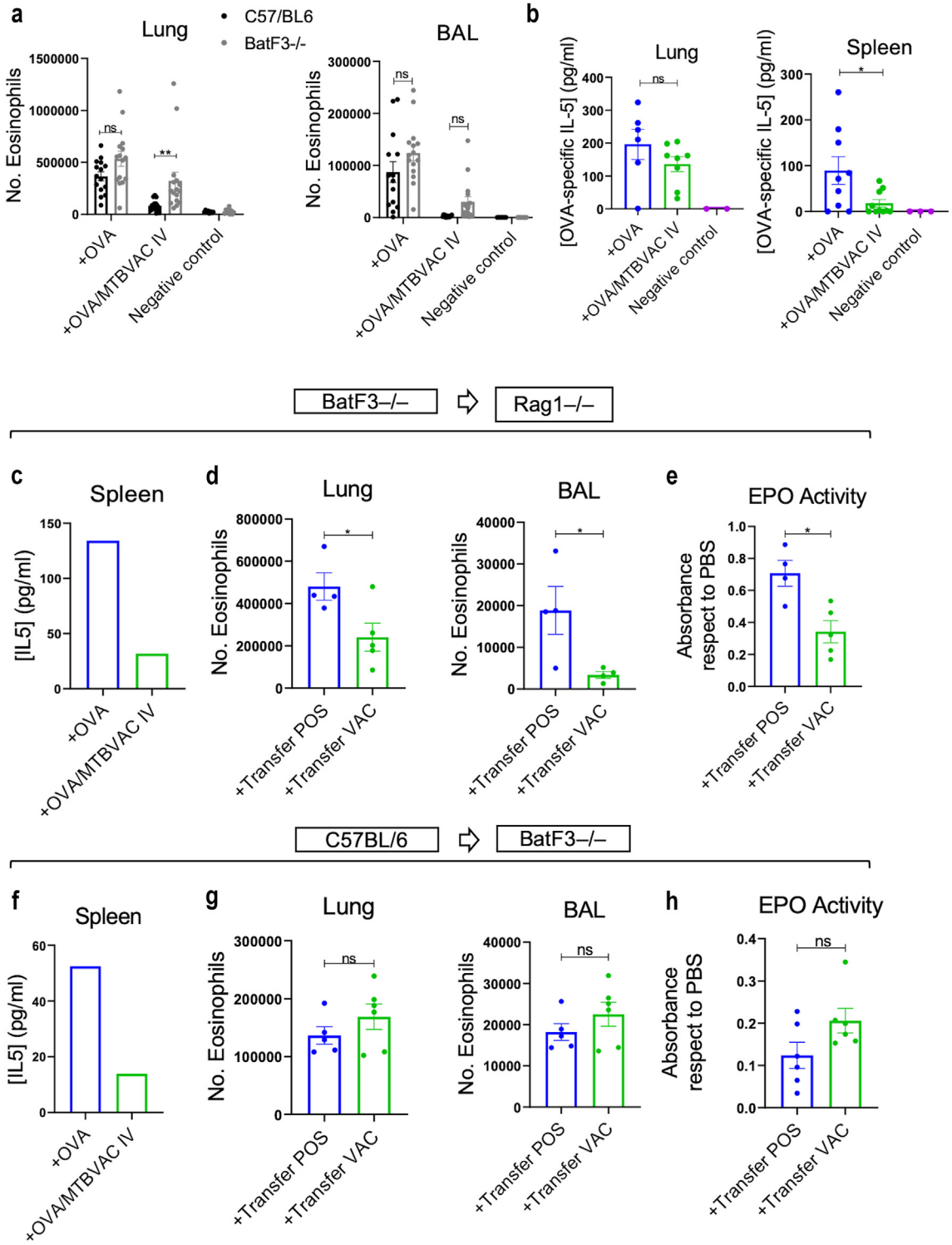


***Fig. 6*: cDC1 are required for MTBVAC-induced lung desensitisation**. (a) Lung and BAL eosinophilia comparison between WT and BatF3−/−mice. Data pooled from 3 independent experiments (n = 15–16 for OVA-challenged groups and n = 9 for negative controls). (b) Allergen-specific IL-5 production lung and spleen cells, following *ex vivo* stimulation with OVA. Data are displayed after subtraction of the baseline value obtained by incubation without OVA. Data from one experiment (n = 6–10 for OVA-challenged groups and n = 3 for negative controls). (c, e) Transfer of splenocytes from OVA-sensitised BatF3−/− mice to Rag1−/− mice followed by OVA challenge. Data from one experiment (n = 4–5

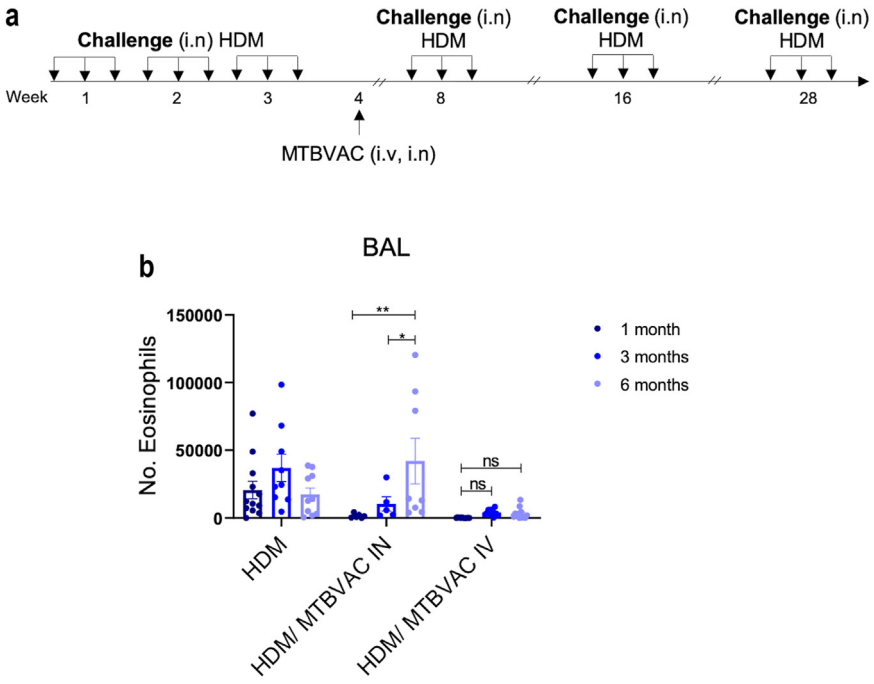


***Fig. 7:*** **Intravenous, but not intranasal, MTBVAC induces durable efficacy against asthma**. (a) Model of HDM-specific chronic asthma comparing efficacy of intravenous and intranasal MTBVAC administered at week 4 of experiment. (b) BAL eosinophilia at 1, 3 and 6 months post-MTBVAC treatment. Data from three experiments (n = 9–12 for HDM-challenged groups, n = 5–8 for MTBVAC IN-treated groups, and n = 11–13 for MTBVAC IV-treated groups). Data depicted as mean ± SEM. *p < 0.05; **p < 0.01, by Tukey's multiple comparisons test for two-way ANOVA.

also suggest that Treg lymphocytes are dispensable for the protective effect given by MTBVAC. In disagreement with this result, a previous study described that Treg cell contribution was crucial for the AHR-reducing capacity of an inactivated BCG formulation based on extended freeze-drying (EFD).[5] However, if we focus on live BCG, more comparable to MTBVAC from a biological perspective,[8] previous studies indicated that the effect of live BCG at modulating AHR is independent of Treg,[7,32] as we have observed with MTBVAC. Indeed, in the study where live and EFD-inactivated BCG were compared, only the EFD formulation induced Treg expansion, as well as IL-10 production.[5] This publication evidences the importance that bacterial formulation might have for the interaction of BCG with different immunological compartments and the consequent differential responses triggered.

A crucial finding in our study was the observation that only IV MTBVAC (and not intranasal) led to a reduction of allergen specific IL-5-production in a primary lymphoid organ as the spleen. These results could definitely have an impact in terms of the duration of the protection induced by the bacteria. Indeed, we demonstrated that at six months post-treatment, the group of mice treated with IV MTBVAC was still protected against a challenge with the allergen, whereas the animals treated by intranasal route, which induced local but not systemic desensitisation, developed AHR upon long-term re-challenge. Our data regarding MTBVAC persistence revealed that at six months after vaccination, bacterial number had dramatically decreased in the analysed organs, suggesting that the protection observed at long-term is not a consequence of the inflammatory response generated by bacterial presence, but likely a

recipient mice per group). (c) IL-5 production by the splenocytes from donor mice after 48 h of incubation with OVA. (d) Lung and BAL eosinophilia from OVA-challenged recipient mice, transferred with splenocytes from indicated donor groups: POS: OVA-sensitised; VAC: OVA-sensitised and treated with MTBVAC. (e) EPO activity in lung homogenates from recipient mice. (f, h) Transfer of splenocytes from WT to BatF3−/− mice. Data from one experiment (n = 5–6 recipient mice per group). (f) IL-5 production by donor mouse splenocytes. (g) Lung and BAL eosinophilia from recipient mice. (h) EPO activity in lung homogenates from recipient mice. Data depicted as mean ± SEM. *p < 0.05; by Tukey's multiple comparisons test for two-way ANOVA (a), by Unpaired t-test (b, d, e, g and h).

consequence of the lack of allergen-specific central memory response that could repopulate the lungs with effector Th2 cells following allergen exposure. In line with this argument, allergen-specific Th2 cells with central memory markers have been found in blood from patients with asthma,[33] and it is assumed that these pathogenic long-lived T cells might be ultimately responsible of the perpetuation of asthma throughout lifetime.[34] Upon allergen exposure, specific memory CD4+ T cells would migrate to lungs where they efficiently recognise allergen-derived peptides presented by APCs, and respond quickly by secreting Th2-associated cytokines.[35] A therapy impairing central memory allergen-specific response might definitely have a potential impact on reducing sensitivity of patients to allergen exposure. Our results with human samples may indicate the potential translatability of our preclinical data to further clinical studies, and suggest the capacity of MTBVAC as a desensitising agent in a human context.

Our study highlights the differential immunological mechanisms underlying systemic and local desensitisation induced by MTBVAC. We found that IFNγ resulted crucial for elimination of allergen-specific IL-5-producing cells in the spleen but not in the lungs, suggesting a high grade of compartmentalisation of the MTBVAC-triggered response between non-mucosal and mucosal tissues. Lung cDC1s could contribute to this compartmentalisation. Our data reveal that cDC1s are important for IV MTBVAC to reduce allergen-specific Th2 cells in the lungs, whereas they are dispensable for desensitisation at a systemic level. cDC1s have been reported as major contributors to antigen presentation in the lungs, including presentation of allergens.[25] Moreover, cDC1s are well known to be major IL-12 producers,[27] crucially contributing to the induction of Th1 responses and limitation of Th2 polarisation.[36] Therefore, our results are compatible with the hypothesis that MTBVAC would be inducing activation of cDC1s in the lungs, which could subsequently present allergen-derived peptides to T cells in the presence of IL-12, conforming an environment that would facilitate abrogation of Th2 cells. In agreement with this hypothesis, a previous study demonstrated that BatF3−/− mice resulted more sensible to chronic AHR induced by HDM exposure, due to lack of IL-12-producing cDC1 cells.[25] In another study, authors demonstrated that AHR protection induced by inflammatory components from *Helicobacter pilory* was strictly dependent on BatF3-expressing cDC1 cells.[37]

Even though we consider that our results are robust and they support the conclusions, the study has some limitations. One of them is that, despite the demonstration of IV MTBVAC efficacy in three different models of allergen-induced AHR, this result has been only tested in one mouse strain (C57BL/6). In this regard, further studies should demonstrate MTBVAC efficacy in other genetic backgrounds, ideally in both inbred and outbred. In addition, though we consider that the data shown with human samples are promising regarding the transference potential of MTBVAC as approach against asthma, the number of samples analysed are low and only specific from one allergen, so they likely do not represent the big heterogeneity of the patients with asthma in the clinic. Therefore, further studies should be focused on testing the experimental procedure optimised in this study with a higher number of samples, and from different study cohorts to increment their heterogeneity.

In conclusion, this study introduces a potential immunotherapy for asthma based on a single IV inoculation of the live tuberculosis vaccine MTBVAC. Results indicate that the bacteria are well-tolerated and progressively eliminated, and induce long-term protection against allergen-induced AHR. The fact that a single inoculation of this immunotherapeutic agent reduces AHR in the different mouse models utilised suggests that this therapy could overcome some of the problems of the current allergen-specific immunotherapies, including the duration of the treatments and the high number of allergen inoculations needed to reach an efficient desensitisation, with the consequent potential side effects associated to allergen exposure. Finally, the observation that this treatment induces heterologous protection against allergens of different nature indicates that IV MTBVAC could be a desensitising therapy for a broad range of patients.

#### Contributors

C.M., V.D.P and N.A. designed the experiments. S.C., J-M-R-M, R.T. and S.U. performed the experiments. S.C., C.M., V.D.P and N.A. wrote the manuscript. N.A. supervised the study. All authors have read and approved the final version of the manuscript. S.C, J-M-R-M and N.A. have accessed and verified the data. V.D.P and N.A. are responsible of the decision to submit the manuscript.

#### Data sharing statement

The authors declare that the data supporting the findings of this study are available after publication, upon request to the corresponding author.

#### Declaration of interests

Raquel Tarancón, Santiago Uranga, 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, 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.

#### Acknowledgements

Authors acknowledge the Scientific and Technical Services from Instituto Aragonés de Ciencias de la Salud (IACS) and Universidad de Zaragoza.

During the preparation of this work the authors used ChatGPT (v3.5) to correct English grammar mistakes. After using this tool/service, the authors have reviewed and edited the content as needed,

confirmed the validity of the text and take full responsibility for the content of the publication.

### Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105272.

### References


1 Cao Y, Chen S, Chen X, et al. Global trends in the incidence and mortality of asthma from 1990 to 2019: an age-period-cohort analysis using the global burden of disease study 2019. *Front Public Health*. 2022;10:1036674.
2 Holgate ST, Wenzel S, Postma DS, Weiss ST, Renz H, Sly PD. Asthma. *Nat Rev Dis Prim*. 2015;1(1):15025.
3 Linehan MF, Nurmatov U, Frank TL, Niven RM, Baxter DN, Sheikh A. Does BCG vaccination protect against childhood asthma? Final results from the Manchester Community Asthma Study retrospective cohort study and updated systematic review and meta-analysis. *J Allergy Clin Immunol*. 2014;133(3):688–695.e14.
4 Erb KJ, Holloway JW, Sobeck A, Moll H, Le Gros G. Infection of mice with Mycobacterium bovis-Bacillus Calmette-Guerin (BCG) suppresses allergen-induced airway eosinophilia. *J Exp Med*. 1998;187(4):561–569.
5 Lagranderie M, Abolhassani M, Vanoirbeek J, et al. Mycobacterium bovis BCG killed by extended freeze-drying reduces airway hyperresponsiveness in 2 animal models. *J Allergy Clin Immunol*. 2008;121(2):471–478.
6 Tsujimura Y, Inada H, Yoneda M, Fujita T, Matsuo K, Yasutomi Y. Effects of mycobacteria major secretion protein, Ag85B, on allergic inflammation in the lung. *PLoS One*. 2014;9(9):e106807.
7 Wang P, Zhang G, Qin X, et al. Inhibition of allergen-induced airway remodeling by neonatal bacillus Calmette-Guerin vaccination is associated with interferon-gamma-producing T cells but not regulatory T cells in mice. *Ann Allergy Asthma Immunol*. 2011;107(2):163–170.
8 Arbues A, Aguilo JI, Gonzalo-Asensio J, et al. Construction, characterisation and preclinical evaluation of MTBVAC, the first live-attenuated M. tuberculosis-based vaccine to enter clinical trials. *Vaccine*. 2013;31(42):4867–4873.
9 Martin C, Marinova D, Aguilo N, Gonzalo-Asensio J. MTBVAC, a live TB vaccine poised to initiate efficacy trials 100 years after BCG. *Vaccine*. 2021;39(50):7277–7285.
10 Spertini F, Audran R, Chakour R, et al. Safety of human immunisation with a live-attenuated Mycobacterium tuberculosis vaccine: a randomised, double-blind, controlled phase I trial. *Lancet Respir Med*. 2015;3(12):953–962.
11 Tameris M, Mearns H, Penn-Nicholson A, et al. Live-attenuated Mycobacterium tuberculosis vaccine MTBVAC versus BCG in adults and neonates: a randomised controlled, double-blind dose-escalation trial. *Lancet Respir Med*. 2019;7(9):757–770.
12 Darrah P, Zeppa J, Maiello P, et al. Prevention of tuberculosis in macaques after intravenous BCG immunisation. *Nature*. 2020;577(7788):95–102.
13 Larson EC, Ellis-Connell AL, Rodgers MA, et al. Intravenous Bacille Calmette-Guerin vaccination protects simian immunodeficiency virus-infected macaques from tuberculosis. *Nat Microbiol*. 2023;8(11):2080–2092.
14 Kaufmann E, Sanz J, Dunn JL, et al. BCG educates hematopoietic stem cells to generate protective innate immunity against tuberculosis. *Cell*. 2018;172(1-2):176–190.e19.
15 Mittrucker HW, Steinhoff U, Kohler A, et al. Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. *Proc Natl Acad Sci U S A*. 2007;104(30):12434–12439.
16 Tarancon R, Mata E, Uranga S, et al. Therapeutic efficacy of pulmonary live tuberculosis vaccines against established asthma by subverting local immune environment. *eBioMedicine*. 2021;64:103186.
17 Muñoz X, Álvarez-Puebla MJ, Arismendi E, et al. The MEGA project: a study of the mechanisms involved in the genesis and disease course of asthma. Asthma cohort creation and long-term follow-up. *Arch Bronconeumol*. 2018;2896:30009.
18 Solans L, Uranga S, Aguilo N, et al. Hyper-attenuated MTBVAC erp mutant protects against tuberculosis in mice. *Vaccine*. 2014;32(40):5192–5197.
19 Broset E, Saubi N, Guitart N, et al. MTBVAC-based TB-HIV vaccine is Safe, elicits HIV-T cell responses, and protects against mycobacterium tuberculosis in mice. *Mol Ther Methods Clin Dev*. 2019;13:253–264.
20 Wise JT, Baginski TJ, Mobley JL. An adoptive transfer model of allergic lung inflammation in mice is mediated by CD4+CD62LlowCD25+ T cells. *J Immunol*. 1999;162(9):5592–5600.
21 Varga SM, Beckman NA, Chu M, Braciale TJ. Sensitive detection and quantitation of mouse eosinophils in tissues using an enzymatic eosinophil peroxidase assay: its use to rapidly measure pulmonary eosinophilia during experimental respiratory syncytial virus infection of mice. *J Immunol Methods*. 2002;262(1-2):111–120.
22 Frigas E, Loegering DA, Solley GO, Farrow GM, Gleich GJ. Elevated levels of the eosinophil granule major basic protein in the sputum of patients with bronchial asthma. *Mayo Clin Proc*. 1981;56(6):345–353.
23 Bush RK, Prochnau JJ. Alternaria-induced asthma. *J Allergy Clin Immunol*. 2004;113(2):227–234.
24 Aguilo N, Gonzalo-Asensio J, Alvarez-Arguedas S, et al. Reactogenicity to major tuberculosis antigens absent in BCG is linked to improved protection against Mycobacterium tuberculosis. *Nat Commun*. 2017;8:16085.
25 Conejero L, Khouili SC, Martinez-Cano S, Izquierdo HM, Brandi P, Sancho D. Lung CD103+ dendritic cells restrain allergic airway inflammation through IL-12 production. *JCI Insight*. 2017;2(10).
26 Moreo E, Jarit-Cabanillas A, Robles-Vera I, et al. Intravenous administration of BCG in mice promotes natural killer and T cell-mediated antitumor immunity in the lung. *Nat Commun*. 2023;14(1):6090.
27 Hildner K, Edelson BT, Purtha WE, et al. Batf 3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science*. 2008;322(5904):1097–1100.
28 Smit JJ, Van Loveren H, Hoekstra MO, Van der Kant PA, Folkerts G, Nijkamp FP. Therapeutic treatment with heat-killed Mycobacterium vaccae (SRL172) in a mild and severe mouse model for allergic asthma. *Eur J Pharmacol*. 2003;470(3):193–199.
29 Kowalewicz-Kulbat M, Locht C. BCG for the prevention and treatment of allergic asthma. *Vaccine*. 2021;39(50):7341–7352.
30 Lai JF, Thompson LJ, Ziegler SF. TSLP drives acute T(H)2-cell differentiation in lungs. *J Allergy Clin Immunol*. 2020;146(6):1406–14018.e7.
31 Bosnjak B, Stelzmueller B, Erb KJ, Epstein MM. Treatment of allergic asthma: modulation of Th2 cells and their responses. *Respir Res*. 2011;12(1):114.
32 Zhang G, Wang P, Qiu Z, et al. Distant lymph nodes serve as pools of Th1 cells induced by neonatal BCG vaccination for the prevention of asthma in mice. *Allergy*. 2013;68(3):330–338.
33 Bateman EA, Ardern-Jones MR, Ogg GS. Persistent central memory phenotype of circulating Fel d 1 peptide/DRB1*0101 tetramer-binding CD4+ T cells. *J Allergy Clin Immunol*. 2006;118(6):1350–1356.
34 Muehling LM, Lawrence MG, Woodfolk JA. Pathogenic CD4(+) T cells in patients with asthma. *J Allergy Clin Immunol*. 2017;140(6):1523–1540.
35 Ling MF, Luster AD. Allergen-specific CD4(+) T cells in human asthma. *Ann Am Thorac Soc*. 2016;13 Suppl 1(Suppl 1):S25–S30.
36 Athie-Morales V, Smits HH, Cantrell DA, Hilkens CM. Sustained IL-12 signaling is required for Th1 development. *J Immunol*. 2004;172(1):61–69.
37 Engler DB, Reuter S, van Wijck Y, et al. Effective treatment of allergic airway inflammation with Helicobacter pylori immunomodulators requires BATF3-dependent dendritic cells and IL-10. *Proc Natl Acad Sci U S A*. 2014;111(32):11810–11815.