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Use of live attenuated Mycobacteria as treatment against asthma and their ability to induce trained immunity

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Tesis Doctoral

USE OF LIVE ATTENUATED MYCOBACTERIA AS TREATMENT AGAINST ASTHMA AND THEIR ABILITY TO INDUCE TRAINED IMMUNITY

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Use of live attenuated Mycobacteria as treatment against asthma and their ability to induce trained immunity

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Directores de la Tesis Doctoral presentada por Raquel Tarancón Iñiguez bajo el título:

Use of live attenuated Mycobacteria as treatment against asthma and their ability to induce trained immunity

EXPONEN:

Que dicha Tesis ha sido realizada bajo su dirección y cumple con las condiciones para optar al grado de Doctor por la Universidad de Zaragoza y a la mención de Doctorado Internacional.

Por lo tanto, emiten el presente **INFORME FAVORABLE**

Zaragoza, 30 de Septiembre de 2019

Fdo. Juan Ignacio Aguiló Anento

Fdo. Carlos Martín Montañés

Esta tesis doctoral ha sido elaborado en el Departamento de Microbiología, Medicina Preventiva y Salud Pública, adscrito al programa de Doctorado del Departamento de Bioquímica y Biología Molecular y Celular, habiendo sido Raquel Tarancón Iñiguez beneficiaria de un contrato predoctoral de Formación de Personal Investigador (FPI) concedido por el Ministerio de Economía y Competitividad del Gobierno de España y Fondo Social Europeo (Referencia: BES-2015-071888)

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A partir de los datos de la presente Tesis Doctoral se han redactado 3 artículos científicos. Uno de los cuales, ha sido publicado y el resto enviados para su publicación.

Tarancon, R., et al., ***Mycobacterium tuberculosis*** **infection prevents asthma and abrogates eosinophilopoiesis in an experimental mode**l. Allergy, 2019

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

Raquel Tarancón, Elena Mata, Santiago Uranga, Ana Belén Gómez, Dessislava Marinova, Isabel Otal, Carlos Martín, Nacho Aguiló

New live attenuated tuberculosis vaccine MTBVAC induces trained immunity and confers protection against experimental lethal pneumonia

Raquel Tarancón, Jorge Domínguez-Andrés, Santiago Uranga, Anaísa V. Ferreira, Laszlo A. Groh, Mirian Domenech, Fernando González-Camacho, Niels P. Riksen, Nacho Aguilo, José Yuste, Carlos Martín, Mihai G. Netea

Además, con el trabajo de esta Tesis se ha realizado una solicitud de patente europea, titulada: "**THERAPEUTIC EFFICACY BY PULMONARY DELIVERY OF LIVE ATTENUATED MYCOBACTERIA**".

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ADC	Albumin-dextrose-catalase
AHR	Airway hyperresponsiveness
AM	Alveolar macrophages
BAL	Bronchoalveolar lavage
Breg	Regulatory types of B cells
casp-1	Caspase-1
CFP10	Culture filtrate protein 10
CFU	Colony-forming units
ChIP	Chromatin immunoprecipitation
DCs	Dendritic cells
EAR	Early asthmatic response
ECP	Eosinophil cationic protein
ECR	Extracellular acidification rate
EDN	Eosinophil-derived neurotoxin
EoE	Eosinophilic esophagitis
EPO	Eosinophil peroxidase
ESAT 6	6-kDa early secretory antigen
FBS	Fetal bovine serum
GDP	Gross domestic product
Gen	Gentamicin
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good Manufacturing Practices
GzmA	Granzyme A
GzmB	Granzyme B
H	Hours
HE	Hematoxilin-Eosin

HRP	Horseradish peroxidase
ICS	Intracellular staining
ICSs	Inhaled corticosteroid
Ig	Immunoglobulin
IL	Interleukin
ILC2 cells	Type 2 innate lymphoid cells
IM	Interstitial macrophages
IN	Intranasal
IP	Intraperitoneal
LABAs	Long acting β2-agonists
LAR	Late asthmatic response
LPS	Lypopolysaccharide
LTBI	Latent tuberculosis infection
LTRA	Leukotriene receptor antagonist
MBP	Major basic protein
MDR	Multidrug resistant
MHC II	Major histocompatibility complex class II
Mo-AMs	Monocyte-derived alveolar macrophages
Mtb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NHANES	National Health and Nutrition Examination Survey
NK	Natural killer
OCR	Oxygen consumption rate
ON	Over night
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PAS	Periodic Acid Schiff
PBMCs	Peripheral blood mononuclear cells

PDIM	Phthiocerol dimycocerosates
PI	Project identification
PPD	Purified protein derivative
PRRs	Pattern recognition receptors
qRT-PCR	quantitative real-time PCR
RD	Region of Difference
ROS	Reactive oxygen species
RR	Rifampicin resistant
SABAs	Short acting β2-agonists
SAR	Systemic acquired resistance
TB	Tuberculosis
TCA	Tricarboxylic acid cycle
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TR-AMs	Tissue-resident alveolar macrophages
Treg	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
TST	Tuberculosis skin test
WHO	World Health Organization
WT	Wilt type
β2ARs	β2 adrenoceptors
β-glucan	β-1, 3-(D)-glucan

ABSTRACT

Asthma is the most common inflammatory disease of the lungs. Its incidence in developed countries reaches 12 %, whereas in developing regions it is 1%. Thus, there is a huge economic burden due to asthma and in addition, current treatments are only symptomatic and do not last in time. In this regard, it has been hypothesised that the Th1 response triggered by attenuated Mycobacteria could impair the Th2 response exacerbated in allergic asthma, and so these bacteria could be used as a more efficient treatment.

On the one hand, tuberculosis infection in humans has been related with a decrease in asthma incidence. In this line, we have demonstrated for the first time that the infection with *Mycobacterium tuberculosis* prevents allergic asthma in a mice model. This protective effect is linked to the reduction of eosinophils production in the bone marrow and to the decrease in the eosinophils infiltration to the airways. Moreover, the infection impairs the Th2 response, by inducing a Th1 immune profile.

On the other hand, there exist already data in the literature concerning the use of BCG against asthma, but the results are controversial and the vaccine was only tested as a preventive treatment. In the present work, we have evaluated the use of BCG and MTBVAC against allergic asthma in mice. MTBVAC is the first live attenuated *Mycobacterium tuberculosis* vaccine that enters in clinical trials, and it has be demonstrated to be safe and immunogenic in humans, so it is called to replace BCG. We have demonstrated that MTBVAC and BCG are effective as prophylactic treatments against allergic asthma. More importantly, for the first time we have proved that they exert therapeutic and comparable effects against established asthma, when administered by the intranasal route. Beneficial effects of both vaccines include the suppression of airways eosinophilia, the impairment of the Th2 cytokines and Th2 cells, and the decrease in the airways remodelling. The therapeutic effect has relevant clinical impact for humans, as patients for asthma could be treated with attenuated Mycobacteria through a nebuliser. Moreover, we demonstrated that the action against asthma mediated by both vaccines is based on the impairment of the Th2 response, through the induction of the Th1 response, and on the re polarization of the M2 macrophages to the M1 profile. Further, the anti-asthma response is dose dependent and it seems to require contact between the bacterium and the lung immune cells.

Interestingly, intranasal MTBVAC and BCG reduced eosinophils in the oesophagus, leading us to hypothesise that both vaccines could be also used as treatments against eosinophilic esophagitis or other food allergies.

Recently, it has been demonstrated the capacity of the human innate immune system to react in an adaptive manner to secondary unspecific stimulus, referred as trained immunity. In addition, it is known that BCG has the capacity to induce this innate immune memory in human cells, both *in vitro* and *in vivo.* It allows BCG to mediate unspecific protective effects against diseases such as bladder cancer or some respiratory diseases.

In this regard, here we have demonstrated that MTBVAC is able to induce trained immunity *in vitro* in human monocytes, and *in vivo* in mice. Importantly, the strength of this response reaches at least similar values to those obtained with BCG, if not higher. In addition, we have determined that MTBVAC-trained monocytes exhibit changes in the metabolism, including the increase in the glycolysis and the requirement of the glutaminolysis. The vaccine also induces epigenetic reprogramming, by the enrichment in the H3K4me3 mark (associated with the activation of the transcription) in the promotor region of the genes *TNFA* and *IL6*, which correlates with the enhanced immune response upon re infection.

Taking together, these results provide evidences for the clinical study of MTBVAC as therapy against asthma, and give strong support for its progression throughout the clinical trials as it potentially could exert the same unspecific beneficial effects than BCG.

RESUMEN

El asma es la enfermedad inflamatoria más común de los pulmones. Su incidencia alcanza el 12% en los países desarrollados, mientras que en los países en vías de desarrollo es del 1%. Por lo tanto, el gasto económico que el asma acarrea es muy alto, además los actuales tratamientos están dirigidos a paliar los síntomas y no perduran en el tiempo. En este sentido, se ha postulado que la respuesta Th1 que desencadenan las micobacterias atenuadas podría suprimir la exacerbación de la respuesta Th2 que prima en el asma alérgico, y por tanto, estas bacterias podrían usarse como un tratamiento más eficaz.

Por un lado, la tuberculosis en humanos se ha relacionado con un descenso en la tasa de asma. En relación a esto, hemos demostrado por primera vez que la infección con *Mycobacterium tuberculosis* previene el asma alérgico en modelo de ratón. Este efecto protector implica una reducción en la producción de eosinófilos en la médula ósea y en su infiltración a las vías respiratorias. Además, la infección también produce una disminución de la respuesta Th2, a través de la inducción de la respuesta Th1.

Por otro lado, ya existen datos en la literatura sobre el uso de BCG frente al asma, pero los resultados no son concluyentes y la vacuna sólo se ha probado como tratamiento preventivo. En este trabajo, hemos evaluado el uso de BCG y MTBVAC frente al asma alérgica en ratones. MTBVAC es la primera vacuna viva atenuada a partir de *Mycobacterium tuberculosis* que entra en ensayos clínicos, donde ha demostrado ser segura e inmunogénica, por lo tanto está llamada a remplazar a BCG. Hemos demostrado que tanto BCG como MTBVAC son efectivas como tratamiento preventivo frente al asma alérgica. Aún más importante, hemos probado por primera vez que ejercen un papel terapéutico frente al asma ya establecida, al administrarse por la vía intranasal, y que este efecto es similar entre ambas vacunas. Los efectos beneficiosos de MTBVAC y BCG incluyen la supresión de la eosinofilia en las vías aéreas, la disminución de la producción de citoquinas tipo Th2 y de las células Th2, y una menor remodelación de las vías aéreas. Este efecto terapéutico tiene gran relevancia clínica, ya que los pacientes asmáticos podrían tratarse con micobacterias atenuadas usando un nebulizador. Además, demostramos que la acción frente al asma mediada por ambas vacunas se basa en la disfunción de la respuesta Th2, a través de la inducción de la respuesta Th1, y en la re polarización de los macrófagos M2 a macrófagos M1. La respuesta anti-asma es dosis dependiente, y parece requerir del contacto entre la bacteria y las células inmunes del pulmón.

También es importante el hecho de que MTBVAC y BCG reducen los eosinófilos en los esófagos de ratones, por lo que planteamos la hipótesis de que estas bacterias

puedan usarse como tratamiento frente a la esofagitis eosinofílica o frente a otras alergias alimentarias.

Recientemente, se ha descubierto que el sistema inmune innato en humanos es capaz de responder más fuerte y eficientemente frente a un segundo estímulo, además inespecífico. Esta capacidad se ha descrito con el término "trained immunity" o inmunidad entrenada. Además, se ha demostrado que BCG es capaz de inducir estos rasgos de memoria en las células innatas, tanto *in vitro* como *in vivo*. Gracias a esto, BCG es capaz de mediar efectos inespecíficos beneficiosos frente a enfermedades como el cáncer de vejiga o ciertas enfermedades respiratorias.

En este trabajo hemos demostrado que MTBVAC es capaz de inducir "trained immunity" *in vitro* en monocitos humanos, y también *in vivo* en ratones. Además, la potencia de esta respuesta alcanza al menos valores similares a los obtenidos con BCG, sino superiores. Demostramos que los monocitos entrenados por MTBVAC exhiben cambios en el metabolismo, como son el aumento en la tasa glicolítica y la dependencia de la ruta de la glutaminólisis. La vacuna también induce cambios epigenéticos a través del enriquecimiento de la marca H3K4me3 (asociada a la activación de la transcripción) en la región del promotor de los genes *TNFA* e *IL6*, lo que correlaciona con la respuesta inmune más potente que ocurre tras la reinfección.

Como conclusión, los resultados obtenidos abalan el estudio clínico de MTBVAC como terapia frente al asma, y apoyan su progresión en los ensayos clínicos, ya que la vacuna podría ejercer los mismos beneficios inespecíficos que presenta BCG.

Introduction

Lock up your libraries if you like; but there is no gate, no lock, no bolt that you can set upon the freedom of my mind

Virginia Woolf

1. INTRODUCTION

1.1 ASTHMA

1.1.1 General features

Asthma is one of the most common lung diseases in the world, being the most frequent inflammatory disease of these organs. It is a heterogeneous group of conditions characterised by recurrent but reversible bronchial obstruction caused by mucus accumulation, inflamed airways walls and tightened muscles, which lead to respiratory symptoms (Figure 1). Therefore, the main traits include wheeze, chest tightness, cough, shortness of breath, expiratory airflow limitation and airway hyper-responsiveness (AHR) to some stimuli, such as allergens or exercise. Symptoms vary in intensity over life, and patients may suffer from exacerbations episodes or benefit from periods of remission [1].

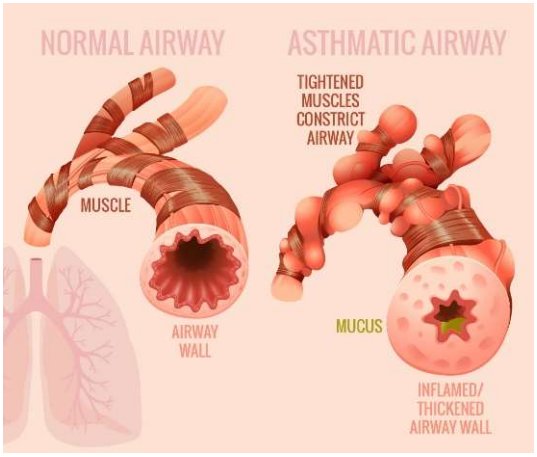


Figure 1. Normal airways versus airways during asthma symptoms [2]. During asthma attacks, there occur swelling, increase of mucus and thickening of muscle around airways.

The word “asthma” comes from a Greek word “aazein” meaning panting [3], but ancient Egyptian, Hebrew, Indian and other medical writings also refer to asthma. In fact, the first written evidence of an asthma attack was described by a Greek physician, Aretaeus of Cappadocia, in the second century of the Common Era[4].

The origin of asthma is not always clear but it is driven by strong genetic and environmental factors and its development is also influenced by immunological factors, age and gender[5].

1.1.2 Epidemiology of asthma

During the last few decades, the prevalence of asthma has increased in many parts of the world, not only in western countries but also in developing countries. In fact, in

countries with high gross domestic product (GDP), it was even rising on a year-by-year basis, until having reached a plateau (Figure 2) [1].

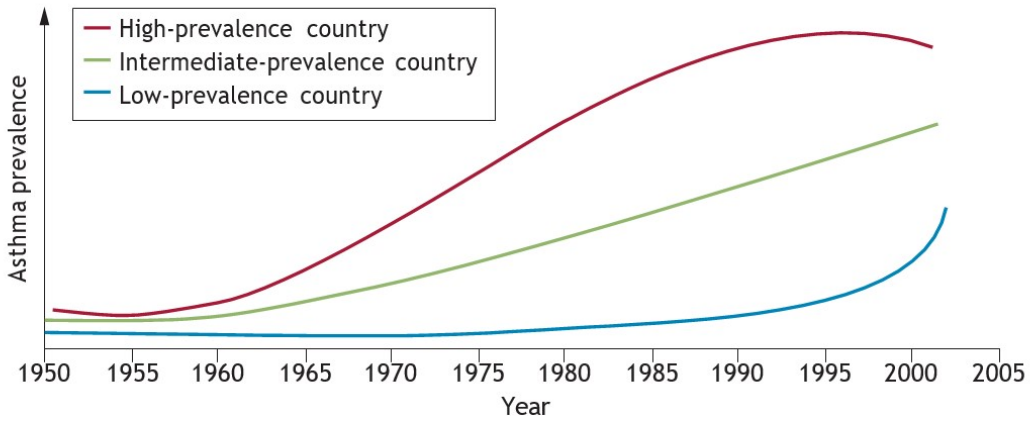


Figure 2. Trends in the prevalence of asthma according to GDP. The prevalence of asthma has plateaued in recent decades in high-prevalence countries, which have a high GDP [1].

Nowadays, there are 339 million people affected by asthma, according to the global asthma report from 2018[6]. Importantly, in 2015, 0.40 million people died from asthma[7].

In developed western countries, the prevalence tends to be 10%-12%, far higher than the ≤1% observed in the lowest-income and most rural countries [1, 8]. Moreover, asthma is more common in urban areas and there is a greater disease prevalence with greater distance from the equator and with each successive lower poverty level group[9].

More specifically, data from the World Health Organization (WHO) estimate that the prevalence of reported asthma in younger adults is around 4.5% of the global population, with Australia, Northern and Western Europe, and Brazil as the countries with the highest percentage. The average prevalence between adolescents is 14.1%, where the highest one (≥20%) was generally observed in English speaking countries of Australasia, Europe and North America, and in parts of Latin America[6]. On the other hand, the lowest incidence in adolescents (lower than 5%) occurs in the Indian subcontinent, Asia-Pacific, Eastern Mediterranean, and Northern and Eastern Europe (Figure 3)[10]. Finally, in children, the global prevalence was 11.5%, ranging from 6.8% in the Indian subcontinent to 21.7% in Oceania[10].

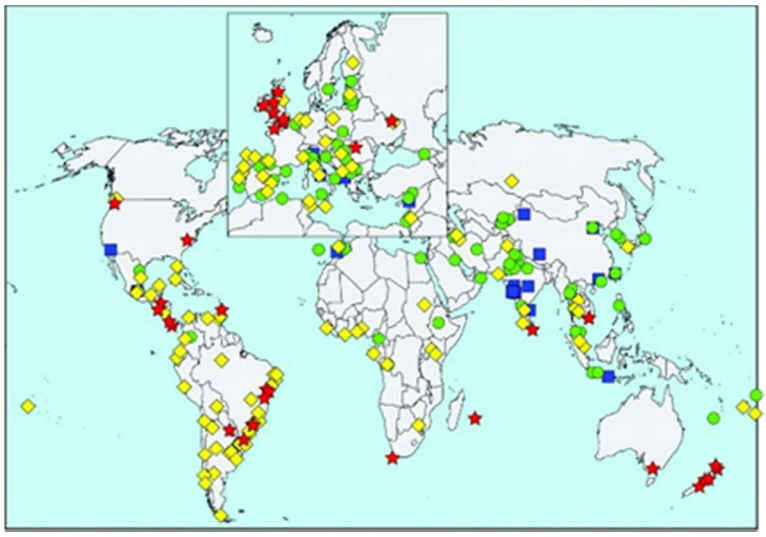


Figure 3. Asthma prevalence in 13–14 year age group. The symbols indicate prevalence values of <5% (blue square), 5 to <10% (green circle), 10 to <20% (yellow diamond) and >20% (red star) [10].

Since the last 1970s, asthma is considered an epidemic and there exist multiple hypothesis trying to explain the cause of such a high increase. A decline of microbial diversity, as the base of the hygiene or most recently named microbiome theory, is the most widespread hypothesis, since the microbial exposure is needed to regulate the immune response [11]. However, other factors could be influencing the increase epidemic observed in less affluent regions, such as the microbiome composition, or the fact that different microbial exposures may induce different effects in geographically-distinct human populations [5, 12].

1.1.3 Economic burden of asthma

Concerning the economic burden, unfortunately reliable national estimates of costs are not available for the vast majority of developing countries. The most current report [6] about the global economic burden estimates that annual direct costs varied from less than US$150 per patient (Abu Dhabi, United Arab Emirates) to more than US$3000 per patient (United States of America), a 20-fold difference. For example, the total annual asthma costs in the USA increased from US$12 billion in 1994 to US$56 billion in 2011. Total costs of asthma for people aged 15 to 64 living in Europe were €19.3 billion (US$24.7 billion) during 1999-2002, while in the United Kingdom the costs were £5 billion (US$9.8 billion). Moreover, in both North America and Europe, asthma medicines were the largest component of direct medical costs [13, 14]. Finally, according to the WHO, in 2004, the world total asthma costs probably exceeded those of tuberculosis and HIV/AIDS combined [15].

1.1.4 Asthma phenotypes

Asthma is a disease with many phenotypes that share a chronic inflammation of the lungs, several respiratory trends, reversible obstruction, and airways hyper-responsiveness (AHR) and remodelling. Until recently, the disease was classified into allergic (extrinsic) or non-allergic asthma [16], depending on the origin. Nowadays, due to the discovery of the vast heterogeneity in the disease, the classification has become more complex. There is a phenotype of T2-type asthma, whose origin is the exacerbation of the Th2 response, and a non-T2 type asthma, where the Th2 response seems to be normal and which includes very late–onset of asthmatics, obesity associated asthma, smoking-related and neutrophilic asthma. Finally there exists asthma in which affected individuals show little inflammation (Figure 4).[17]

T2-type inflammation occurs in >80% of children and in the majority of adults with asthma. It includes, most often, eosinophilic allergic asthma and less frequently, eosinophilic non-allergic asthma [1]. Allergic asthma is defined by the need of allergic sensitization and by a correlation between allergen exposure and asthma symptoms. Further, it is more common in male. Approximately 90% of asthma cases in childhood and more than 50% of asthma cases in adults are allergic [18], so it is the most common type of asthma, encompassing two thirds of asthmatics[19]. At the end, the burden due to allergic asthma is much higher than the healthcare costs necessary for non-allergic patients[19]. Non-allergic asthma is more frequently associated with nonsteroidal anti-inflammatory hypersensitivity. Interestingly, these patients are older than the ones with the allergic profile, and they are mainly female, however, the etiology and mechanisms involved have not been totally elucidated[20]. It is still an eosinophilic asthma, which is controlled by type 2 innate lymphoid cells (ILC2 cells) acting together with basophils and which occurs in some cases of eosinophilic severe asthma[21].

IgE levels are normally lower in non-allergic asthmatics than in allergic patients, but they both have a predisposition toward Th2 inflammation in the airway due to innate factors such as IL-33 or IL-25[22] and they both present eosinophilic airway inflammation[20, 23].

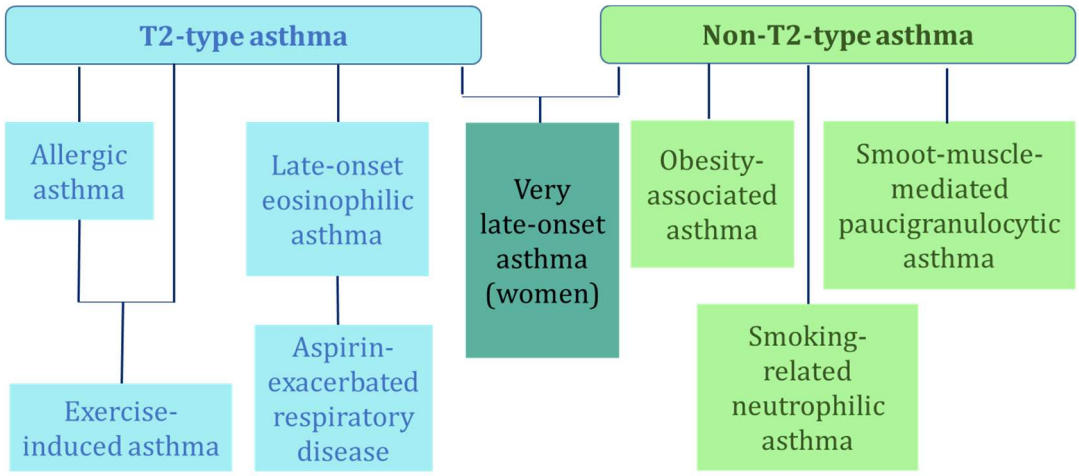


Figure 4. Asthma phenotypes. There exist T2-type asthma (including allergic and non-allergic profiles) and non-T2-type asthma (including smoking, obesity or neutrophilic associated asthma). Adapted from [1].

On the other hand, considering predominant inflammatory cells, asthma can be classified as eosinophilic, neutrophilic or paucigranulocytic. Neutrophilic inflammation is rare, it is usually associated with pre-school wheezing and it is controlled by the Th17 subset of helper T cells, so it is a non-T2-type asthma [21]. For its part, eosinophilic phenotype is more severe than the paucigranulocytic phenotype, it occurs in atopic children and it is the most frequent phenotype regarding exacerbations [24].

1.1.5 Allergic asthma

Clemens Peter Freiherr coined the term Allergy for the first time in 1906 in the Münchener Medizinische Wochenschrift as "specifically altered reactivity of the organism"[25].

Nowadays, allergy is known as an immunological hypersensitivity triggered by an exacerbation of the allergen-specific Th2 response. It includes multiple clinical manifestations such as anaphylaxis, urticaria, angioedema, allergic rhinoconjunctivitis, allergic asthma, serum sickness, allergic vasculitis, hypersensitivity pneumonitis, atopic dermatitis (eczema), eosinophilic esophagitis, contact dermatitis and granulomatous reactions, and finally food- or drug – induced hypersensitivity reactions.

Allergic asthma was already recognized in the 19th century, but mechanisms explaining the disease took some years to be discovered. In 1919, Maximillian Ramirez noticed that blood transfusion could transfer allergic asthma and passively sensitize the recipient[26]. However, it was not until 1968 when the WHO published in an official report the existence of the fifth immunoglobulin class, IgE, which was responsible for the asthma transfer previously described[27].

1.1.5.1 Molecular mechanisms in allergic asthma

The most common asthma phenotype, allergic asthma, is triggered by two subsequential phases, the first one is called sensitization and it is followed by further allergen encounters.

Sensitization phase

In susceptible individuals, the allergen sensitivity is initiated when environmental allergens meet the mucosal epithelia [4]. There, epithelial cell secretes Interleukin (IL)-25, IL-33 and thymic stromal lymphopoietin (TSLP). These sensing cytokines induce ILC2s, which start producing the T2-cytokines IL-5, IL-9 and IL-13. In addition, the sensing cytokines signal to the allergen- presenting dendritic cells (DCs) to take up incoming allergens and to bring them to the lymph nodes [28-30].

Then, airway DCs process the allergen into small peptides, which will be presented at the selective major histocompatibility complex class II (MHC II) to the T cell receptors of naïve T cells. Moreover, co-stimulatory signals between DCs and T cells are required to differentiate T cell into TH2-type T cells. Specifically, these signals are mediated by CD28, which interacts with CD80 and CD86, also mediated by ICOS (expressed by naïve T cells after activation), which binds to its own ligand ICOS-L (expressed by APCs and T cells) and finally by OX40, which binds to OX40-ligand (OX40L).[31]

At this point, Th2-type T cells secrete IL-3, IL-4, IL-5, IL-9, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Some of these allergen-specific Th2 cells migrate to the B cell follicle. There, by the stimulation caused by the previous cytokines (overall IL-4 and IL-13) they induce the class switching to the immunoglobulin heavy chain in B cells, and the production of allergen-specific IgE. Finally, these IgE bind to the high-affinity FcεRI on the surface of mast cells and basophils.[28, 29]

Others Th2-cells, recruited by chemoattractants, relocate to the airway mucosa to elicit the T2-type inflammatory response and the secretion of pro-allergic cytokines. ILC2 are also providing Th2 cytokines [1] (Figure 5).

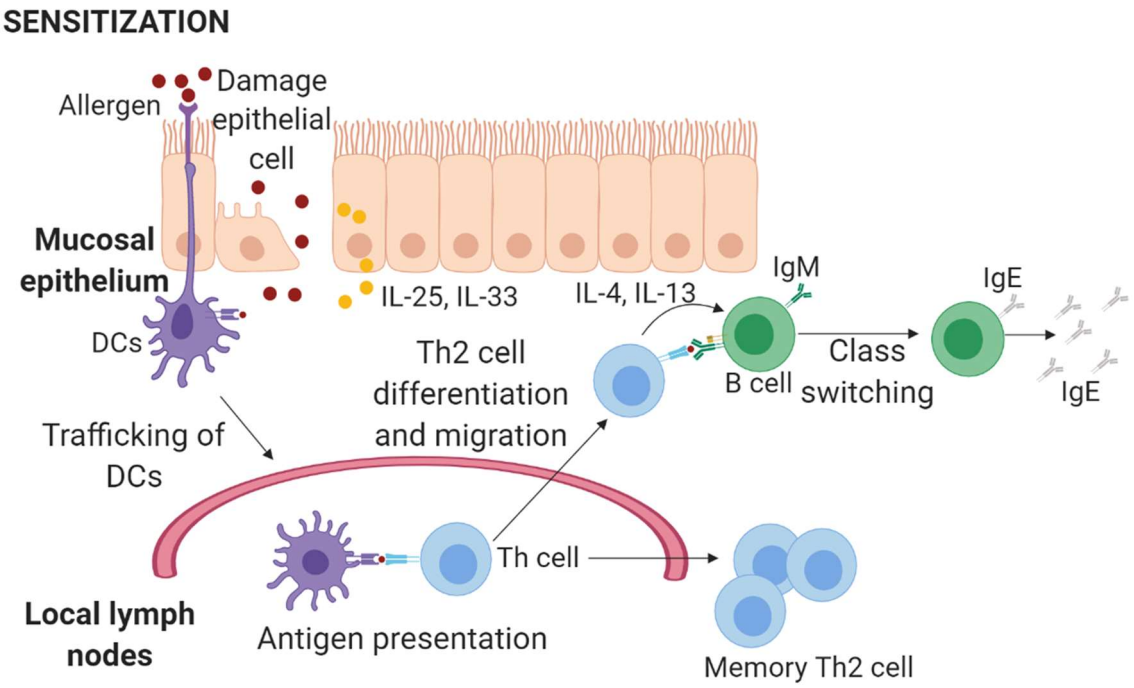


Figure 5. Sensitization phase. Upon allergen exposure, epithelial cells secrete IL-25, IL-33 and TSLP. Moreover, ILC2s, produce T2-cytokines, such as IL-5, IL-9 and IL-13. Then, airway DCs present the allergen to naïve T cells which differentiate to Th2 cells. These cells secrete T2 cytokines and induce the class switching in B cells and the production of allergen-specific IgE.

Effector phase

After the sensitization phase, further exposures to the same allergen lead to the cross-linking of the IgE in the surface of mast cells. It results in mast-cell activation, degranulation and in the release of histamine, prostaglandin D2 and leukotriene C4. This causes a rapid bronchoconstriction response, called early asthmatic response (EAR) and it lasts for 5–90 minutes. Afterwards, there is a late asthmatic response (LAR) during over 3-12 hours. In this phase, attracting by chemoattractants produced by mast cells and other cells, there is an infiltration of leukocytes, especially eosinophils, whose formation at the bone marrow, survival and recruitment, is stimulated by IL-5. There is also a Th2 cytokine release from mast cells and T cells and an increase in AHR and mucus, also mediated by IL-13 [28-30, 32] (Figure 6).

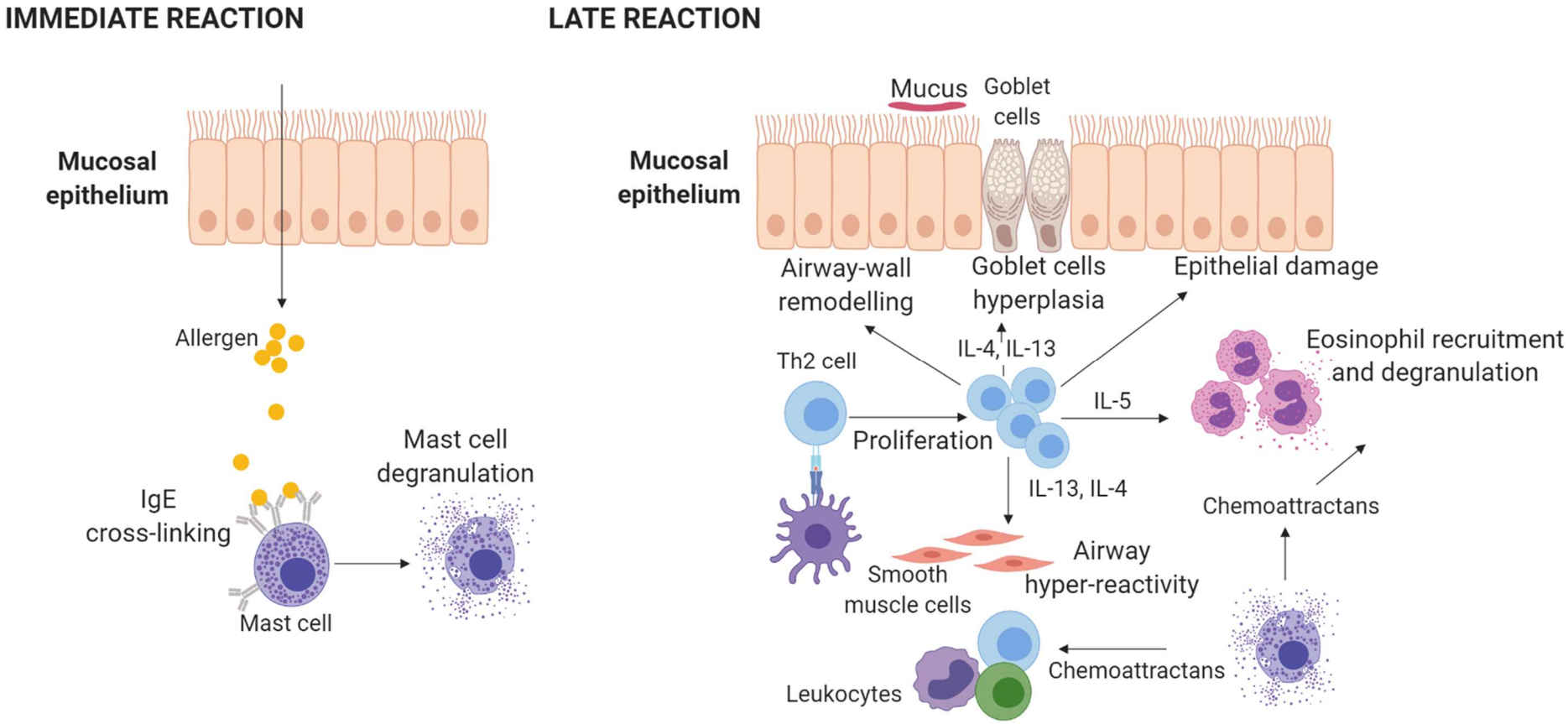


Figure 6. Effector phase. Further exposures to the allergen lead to the cross-linking of the IgE in the surface of mast cells, resulting in mast-cell degranulation, which liberates bronchoconstrictor molecules (immediate reaction). It also induces the recruitment of eosinophils and other inflammatory cells, which mediate the inflammatory allergic response (late reaction)

1.1.6 Eosinophils in asthma

Sixty-nine percent of adult asthma patients have eosinophilic asthma, according to an analysis of National Health and Nutrition Examination Survey (NHANES) [33]. These myeloid cells play a very important role in asthma since they cause airway damage, mucus production, AHR, airway remodelling, fibrosis and exacerbations. Further, they are also direct cause of chronic inflammation in severe asthma [34-37].

Eosinophils have distinct granules containing proteins such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN)). They also contain eicosanoids, including cysteinal leukotrienes, prostaglandins and thromboxane. After being stimulated, they degranulate and produce cytokines such as IL-13 and TGF-β[34].

MBP and EPO are cytotoxic for epithelial cells so they cause tissue damage. In addition, cell damage triggers the activation of repair pathways, which, if excessive, may contribute to structural changes, referred to as airway remodelling. In their turn, IL-13 and leukotrienes cause AHR acting in airway smooth muscle. IL-13 also promotes mucus hypersecretion via enhanced differentiation of goblet cells. Finally, TGF-β is implicated in tissue remodeling via fibroblast proliferation and via increase production of collagen and glycosaminoglycans (Figure 7) [29, 34, 37].

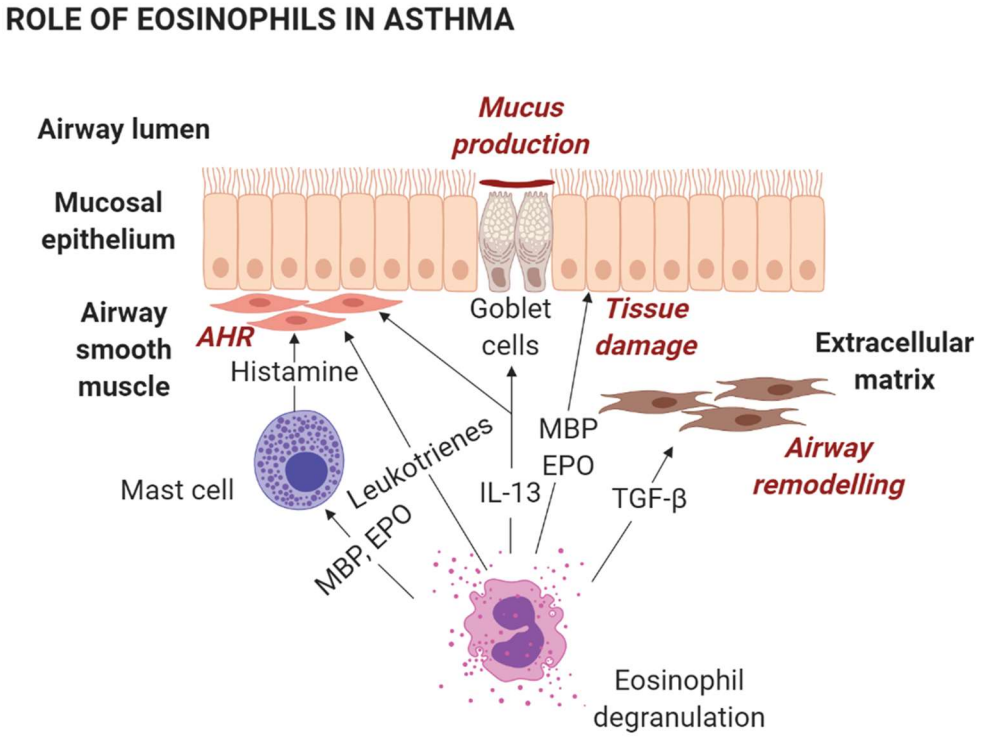


Figure 7. Biology of eosinophils. When eosinophils degranulate, they liberate proteins, eicosanoids and cytokines, producing AHR, airway remodelling, tissue damage and mucus production.

Last but not least, the degree of eosinophilia correlates with disease severity[35] and exacerbation frequency[34, 38-41].

1.1.7 Other important cells population in asthma

Aberrant pulmonary immunity is a central feature of asthma. Lung immunity is highly controlled by events of cell-cell communication and by the function of different cells type. These cells type include the previously mentioned T cells, FoxP3+ regulatory T-cell (Treg), regulatory types of B cells (Bregs), resident alveolar macrophages (AMs), interstitial macrophages (IMs), DCs etc.[42]

Breg and Treg are specifically induced under inflammatory conditions and are capable of suppressing inflammation, enhancing recovery, or inducing tolerance to aeroallergens through the release of the suppressor IL-10 and TGF-β which inhibit over-activated immune response[42]. In allergic diseases, Treg cells are able to suppress both the sensitization and effector phases through different modes of action, which include cell contact dependent mechanisms as well as secreted cytokine-dependent pathways [43, 44]. The role of Treg in asthma has not been totally understood although some studies suggest that defective suppression by Tregs could explain the development of allergic airway inflammation in asthma. For example, it has been demonstrated that Tregs already defective in the umbilical cord blood of newborns are a genetic risk of allergy [45, 46]. On the contrary, in another study, Treg numbers were higher in asthmatic versus healthy children, suggesting that the high number of Tregs in certain patients with allergic asthma might still not be sufficient to control the disease[47].

Recently, it has been discovered the role of lung macrophages in asthma. Lung macrophages consist of AM and IM. The former includes airway macrophages and macrophages that reside in the alveoli, being in direct contact with the environment and isolated by bronchoalveolar lavage (BAL). Their origin is on embryonically derived fetal monocytes that colonize the lung shortly after birth and differentiate into them. Only when AM are damaged or depleted, monocytes recruited from the circulation become the dominant source of new macrophages. In this way, they contribute to replenish the AM niche, where both tissue-resident AM (TR-AMs) and monocyte-derived AM (Mo-AMs) contribute to repopulation (Figure 8). On the other hand, IM are the macrophages that reside in the interstitium, and they are replenished from blood monocytes and from local proliferation [48, 49].

In asthma, after allergen exposure, there is a rapid recruitment of monocytes and an increase in Mo-AMs, which promote acute inflammatory responses. However, TR-AMs may proliferate locally and suppress allergic inflammation [48, 49].

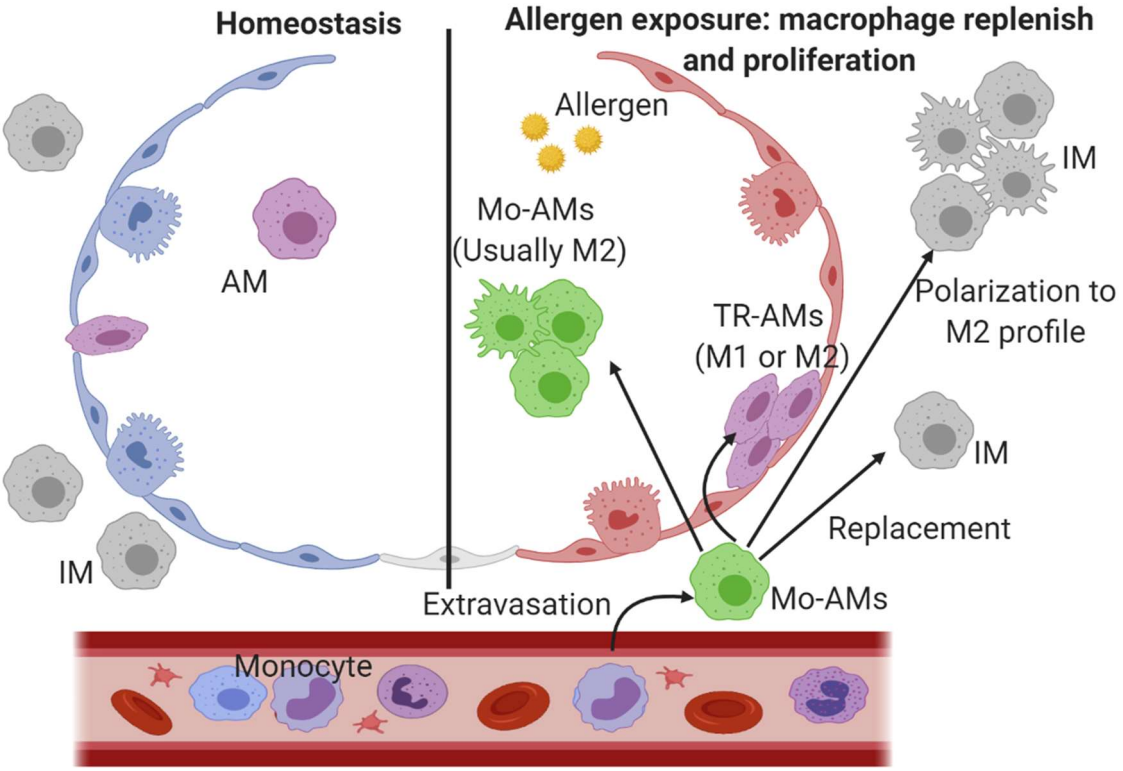


Figure 8. Repopulation strategies of macrophages. Upon allergen exposure, there is a recruitment of monocytes from blood to the lung, which differentiate to both IM and AM.

It has been recently known that macrophages can adopt many different polarisations. From a pro-inflammatory state also known as classically activated or M1 macrophages (activated by IFN-γ), to a pro-repair state, referred as alternatively activated or M2 macrophages (activated by T2 cytokines). Finally, they can reach an anti-inflammatory state with expression of IL-10 (M2-like macrophages). In addition, both AM and IM can polarize to M1 or M2 states.

Alternatively activated (M2 or YM1+) macrophages have been associated with the development of asthma. Further, a high number of these macrophages correlated with having worse symptoms associated with remodelling and development of eosinophilic lung inflammation. Their contribution to disease initiation and progression remains unclear[50] even though it is known that they are induced by IL-4, IL-13 and IL-33 and that they secrete IL-13 and chemokines which induce eosinophils infiltration [51].

1.1.8 Animals model of asthma

It is widely known that animal models of diseases are necessary to understand the pathophysiology of human diseases and identify potential therapeutic targets, allowing preclinical testing of new therapies for humans. Models of allergic asthma in mice are already well established and they use different allergen such as ovalbumin (OVA) [52, 53], the most historically used allergen. This model demonstrated to

induce an immune response that is associated with increased AHR, cellular infiltration into the lungs, increase in eosinophil numbers and elevated levels of inflammatory cytokines in BAL fluid[54].

OVA is often replaced by naturally occurring allergens such as house dust mite (HDM) –a binding lipid protein- which is the most ubiquitous source of indoor allergens inducing allergies and which is highly associated with asthma in humans (Figure 9)[55, 56].

Figure 9. House dust mite [57]. A microscopic electronic image of one of the most common allergen for humans.

On the other hand, there exist acute or chronic allergic asthma models in mice. Acute asthma models are more widely used because they are easy to set up and they reproduce many features of clinical asthma. However being asthma a chronic inflammatory disease resulting from continued or intermittent allergen inhalation, it is also important to develop chronic allergen exposure scenarios. In these models, repeated episodes induce a chronic state of inflammation leading to development of irreversible responses, collectively referred to as airway remodelling and persistent AHR[58]. These changes in the airways persist in the absence of allergens likely because of the progressively irreversible component of airflow obstruction seen in asthma. Murine models of both acute and chronic asthma have been used to investigate prophylactic (therapy administered prior to allergen challenge) and therapeutic (therapy administered after well-established inflammation) drugs[31].

1.1.9 Asthma treatment

As previously described, asthma is a heterogeneous disease with different presentations, outcomes and mechanisms. In general, there are treatments for quick-relief of asthma attacks and for long-term control (Table 1), which prevent symptoms [59, 60], but common drugs are not specific for a particular asthma phenotype.

1.1.9.1 Long-term control

The 2019 GINA recommendation[61] warns that every asthma patients should take inhaled corticosteroid (ICSs) as the cornerstone of the treatment to reduce the risk of serious exacerbations and to control symptoms. These ICSs supress inflammation and reduce asthma symptoms by switching off multiple activated inflammatory genes through reversing histone acetylation via the recruitment of histone deacetylases. Their mainly targets are T2 cytokines such as IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-13, IL-15, TNF-α, GM-CSF, SCF and also chemotactic mediators and adhesion molecules, which reduce the recruitment and survival of inflammatory cells into the airways [62].

In moderate-severe asthma, patients could take together with ICSs long acting β2-agonists (LABAs)[61]. They provide long and sustained relief by airway relaxation through their bronchodilator effects via β2 adrenoceptors (β2ARs) located on airway smooth muscle [63].

As alternative treatment of symptoms and to prevent exacerbations, short acting β2-agonists (SABAs) or leukotriene receptor antagonist (LTRA) can be taken[61]. SABAs act similar to LABAs but have a short half-live[63]. LTRA has a bronchodilator action and it inhibits airway inflammation [64].

Together to these traditional treatments, 2019 GINA[61] recommend to both severe allergic and eosinophilic asthma to take extra treatments based on specific biologic therapies. In this regard, the precision medicine has just arrive for these patients[65]. Anti-IgE (omalizumab) against severe allergic asthma, anti-IL5 (Mepolizumab and Reslizumab) and less often IL-4R antibody (dupilumab) for severe eosinophilic asthma are now available, while not causing any of the unwanted effects of conventional asthma treatments[1, 59]. For example, anti-IL5 antibody is targeted towards IL-5, so it inhibits the terminal differentiation and maturation of eosinophils in the bone marrow and the mobilization of eosinophils and eosinophil precursors into the circulation [59, 66, 67]. The problem with these treatments is that sometimes they are not enough to treat asthma symptoms since a single pathway blockade has only partial efficacy [1, 67].

1.1.9.2 Reliever treatment for asthma attacks

When suffering from an asthma attack, patients can take an extra dose of inhaled SABAs or ICSs-formoterol, another bronchodilator [68].

	Step 1	Step 2	Step 3	Step 4	Step 5
PREFERRED CONTROLLER To prevent exacerbatins and control symptoms	As-needed low dose ICS-formoterol	Daily dose of ICS or as needed low dose of ICS-formoterol	Low dose ICS-LABA	Medium dose ICS-LABA	High dose ICS-LABA. Refer to asthma phenotype add anti-IgE, anti-IL5/5R, anti-IL4R
Other controller options	Low dose ICS taken whenever SABA is taken	LTRA or low dose ICS taken whenever SABA taken	Medium dose ICS, or low dose ICS-LTRA	High dose ICS, add-on LTRA	Add low dose OCS, but consider side effects
PREFERRED RELIEVER	As needed low dose ICS-formoterol		As needed low dose ICS-formoterol		
Other relievers options	As needed short-acting SABA				

Table 1. Asthma medication options recommended from GINA 2019.

1.1.9.3 Side effects of asthma treatments

Due to the long-term use of ICS, local and systemic side effects may appear. The most prevalent local side effects include dysphonia (being the most common), oropharyngeal candidiasis and cough. On the other hand, systemic side effects are mainly adrenal and growth suppression, osteoporosis, bruising, cataracts, glaucoma, metabolic abnormalities (insulin, glucose etc.) and psychiatric disturbances [69]. Moreover, likely the most important disadvantages of current asthma treatments are the poor control in severe asthma patients and the need to take for life and constantly the medicaments.

1.2 MYCOBACTERIA AS POTENTIAL TREATMENT AGAINST T2-TYPE ASTHMA

Most cases of asthma (more than 80%) are considered T2-type asthma [1], because they are caused by an exacerbated Th2 cell response. Future asthma therapy should address the systemic component of the disease and the aim should be not only to control, but to modify the course of the disease. As biologic new treatments are only blocking one step of the allergic cascade, it could be definitely better to re-educate the immune system through a non-T2 regime and so a non-allergic and a non-eosinophilic profile. One plausible option to modulate this response is to induce a Th1 immune response that impairs the Th2 response responsible for asthma. Therefore, in the present dissertation, as explained below, it is hypothesised that Mycobacteria could be used as a potential treatment against T2-type asthma, since Mycobacteria induce a strong Th1 immune profile[70] that may abrogate the Th2 asthmatic response.

1.2.1 Cross-regulation between Th1 and Th2 response as asthma treatment

The Th1 response is characterized by the production of IFN-γ, GM-CSF, IL-12 and tumor necrosis factor (TNF)-β and it is important for the immune response against intracellular bacteria, fungi, protozoa and viruses [71, 72]. Moreover, Th1 differentiation depends on both T-bet and STAT4 transcription factors [73, 74].

It is widely known that during Th-cell differentiation toward one lineage, the other lineage is normally suppressed. It implies that there is a cross-regulation between the Th2 cell response (humoral) and their opposite Th1 cell response (cellular). This event occurs through the repression of transcription factors important for lineage progression and through cross-inhibition mediated by cytokines. In fact, T-bet suppresses GATA-3 function (the key factor for Th2 response) through tyrosine kinase-mediated interaction between the two transcription factors that interfere with the binding of GATA-3 to its target DNA [75, 76]. On the other hand, there exists a mutual suppression between IFN-γ and IL-4 signalling [77, 78].

Considering this, inducing the Th1 response in a non-pathological way, might be a very remarkable option to prevent or even to treat T2-type asthma.

1.2.2 Mycobacteria induce Th1 response

It is widely accepted that immunity to *Mycobacterium tuberculosis* (Mtb) is associated with Th1-cell activity and the production of IFN-γ and TNF. In response to a mycobacterial infection, CD4+ T lymphocytes recognized mycobacterial antigens presented in MHCII molecules, and after the necessary costimulatory signals, they start the effector phase. Hence, induced by the IL-12 secreted by dendritic cells and

macrophages, they secrete a specific Th1 cytokine profile, including IL-2, TNF-β and mainly IFN-γ, which induces the immune cellular response, typical for intracellular bacterial infections. The latter cytokine is necessary for the control of the infection by Mtb but it cannot control the infection alone [70, 79-81]. Further, the IFN-γ negatively regulates the Th2 response, typical from extracellular pathogens[82] and allergy.

1.2.3 Tuberculosis

Tuberculosis (TB) in humans is normally caused by Mtb and to a lesser extent by other members of the *Mycobacterium tuberculosis* complex (MTBC) such as *Mycobacterium africanum, Mycobacterium canetti, Mycobacterium microti or Mycobacterium bovis*. As TB is an airborne disease, the lung is the main organ affected and responsible for the transmission, but TB may occur in any other part of the body. TB patients suffer from persistent cough, fatigue, weight loss, night sweats, fever and coughing up blood. Even with the improvements in hygienic measures, health care, and quality of life and the antituberculosis therapy or vaccine, TB is still a major public health issue [1].

Nowadays, according to the WHO, TB is the leading cause of death from a single infectious agent, even above HIV/AIDS, with 10 million people developing TB disease in 2017. About 1.7 billion people, 23% of the world's population, are estimated to have a latent TB infection and they are people who potentially can switch to an active TB disease during their lifetime. In addition, TB is also one of the top 10 causes of death (Figure 10)[83], causing, in 2017, an estimated 1.6 million deaths (Figure 11).

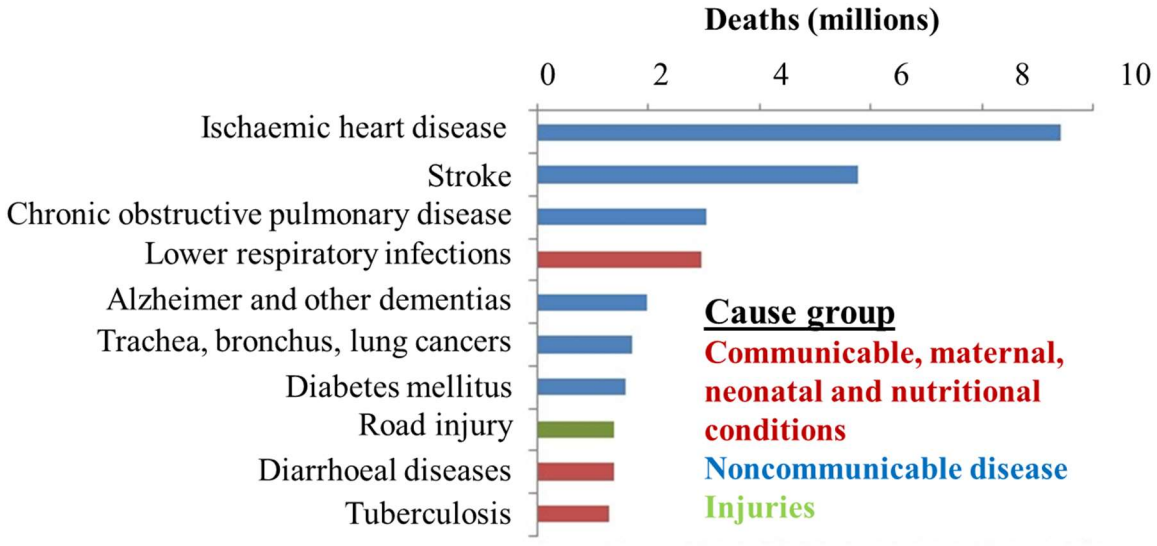


Figure 10. Top 10 global causes of death in 2016. Adapted from [83].

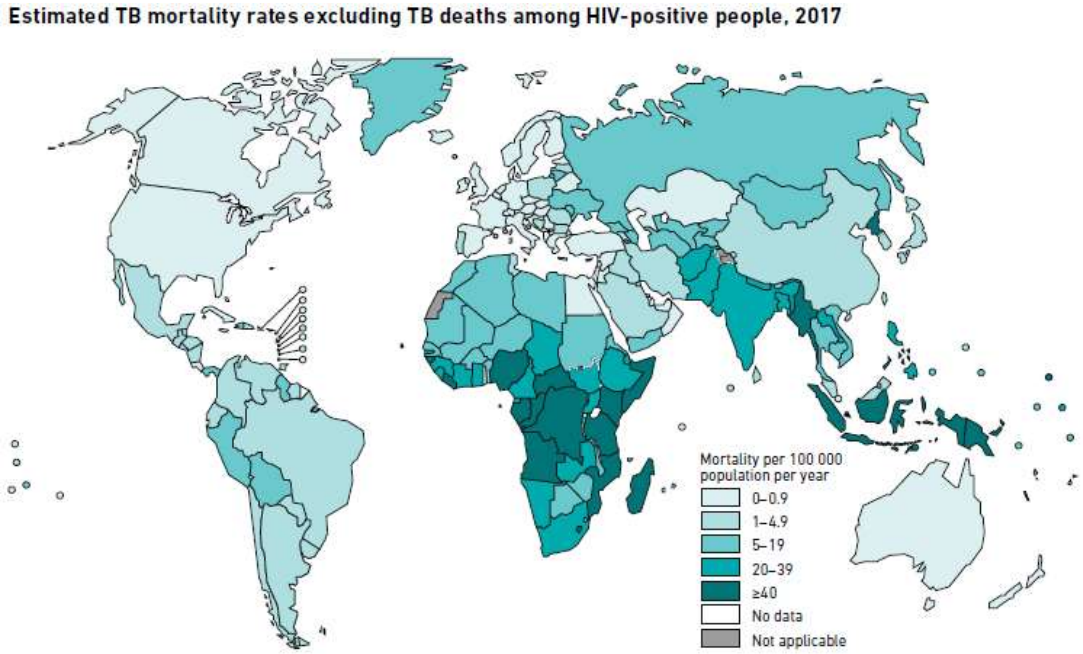


Figure 11. Estimated TB mortality in 2017 (excluding TB deaths among VIH-positive people)[84].

According to the last WHO report[84], the disease is spread worldwide but the distribution is totally heterogeneous: overall 90% were adults (aged ≥15 years), 9% were people living with HIV (72% in Africa) and two thirds were in eight countries: India (27%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%). In fact, only 6% of global cases were in the WHO European Region (3%) and WHO Region of the Americas (3%). In addition, the new cases are also heterogeneously distributed (Figure 12).

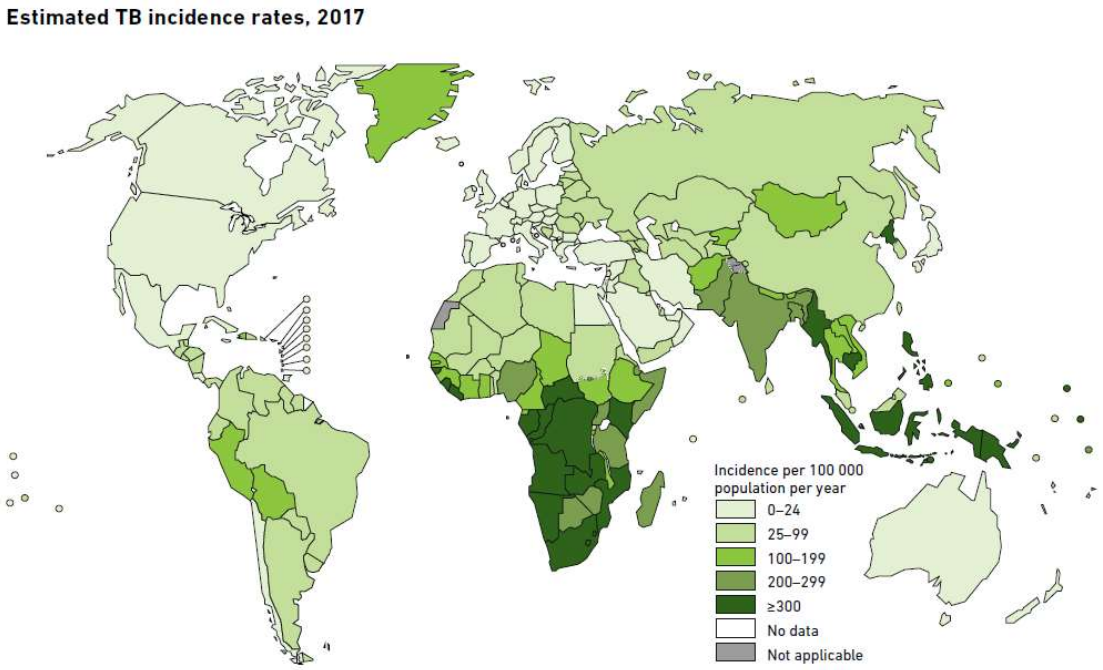


Figure 12. Estimated TB incidence rates in 2017[84].

On the other hand, there is a huge public health crisis concerning the drug-resistant TB, since 3.5% of new TB cases and 18% of previously treated cases had multi-drug/Rifampicin-resistant tuberculosis (MDR/RR-TB)[84].

1.2.4 BCG

BCG is the current vaccine against TB. It is the most administered human vaccine in the world, with more than 3 billion people vaccinated and over 120 million doses administered per year [85] (Figure 13). In fact, 90% of children worldwide are vaccinated. Since 1974, BCG vaccination at birth has been included in the WHO Expanded Programme on Immunization (EPI), always administered by the intradermal route in a concentration of live particles ranges from 50,000 to 3 million per dose depending on the BCG strain [86].

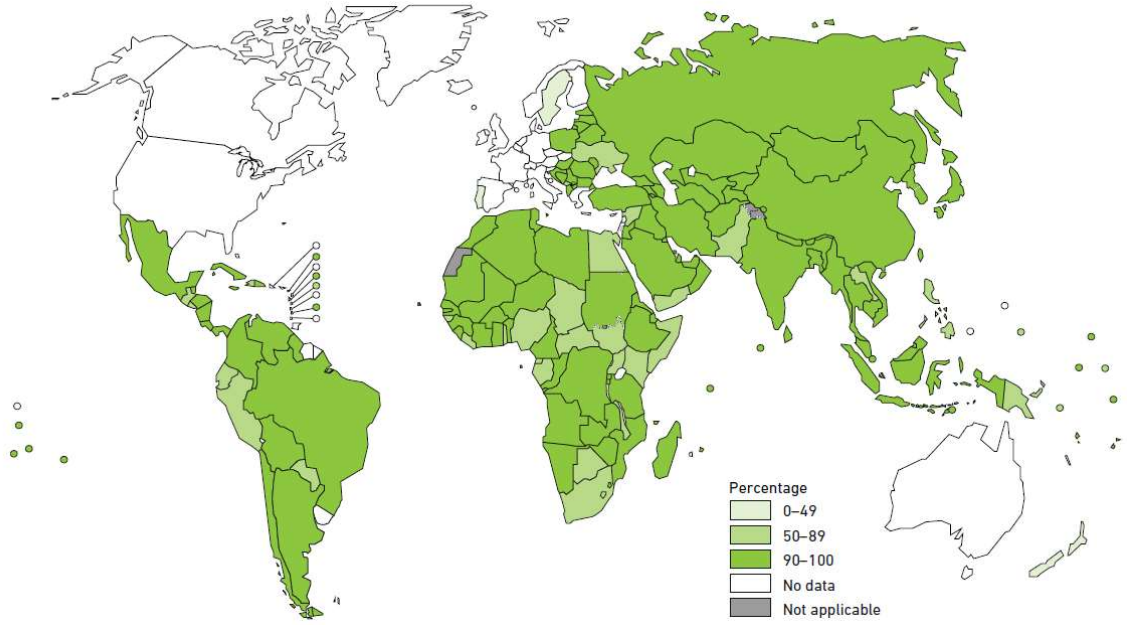


Figure 13. Coverage of BCG vaccination in 2017[84]**.**

BCG comes from a virulent *Mycobacterium bovis* strain which causes a primary cause of TB in cattle and which is member of the MTBC. The virulent strain was supplied in 1902 by Edmond Nocard, originally isolated from milk of a cow suffering from tuberculous mastitis, and attenuated by Albert Calmette and Camille Guérin, so hence the name[87]. In 1908, they both decided to subculture the strain in order to attenuate it and to obtain a TB vaccine. By 1919, having done 230 subcultures, the bacillus was not able to produce disease nor progressive TB when administered into rabbits, cattle, horses or guinea pigs [88, 89]. Therefore, in 1921, the BCG strain was used for the first time as a human vaccine[90] and as early as 1924, the original culture of BCG strain was sub-cultured and distributed from Pasteur Institute of Lille to several laboratories throughout the world [91]. However, the original strain was propagated on non-synthetic culture media and followed distinct passaging protocols. Consequently, it appeared more than 14 genetically and immunogenic distinct BCG sub-strains, referred as daughter strains, until the freeze-drying conservation started by 1960 (Figure 14) [92, 93].

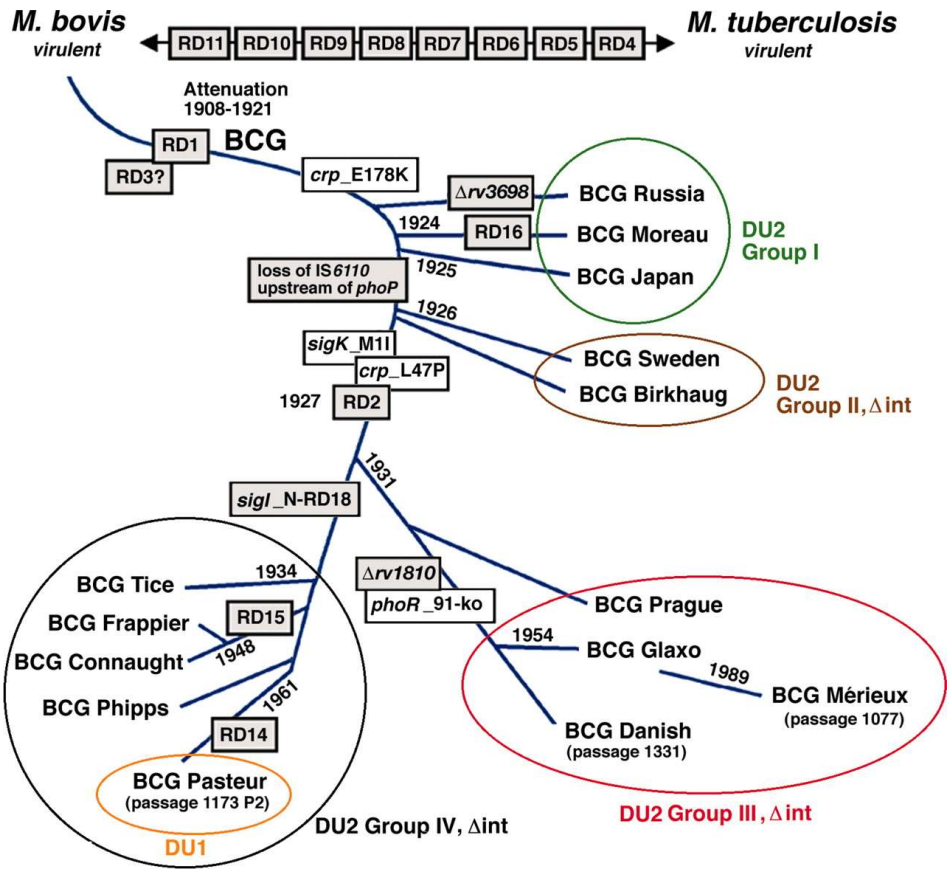


Figure 14. Genealogy of BCG vaccines [93]. The vaccines are distributed into four groups according to the position of genetic markers, region of difference (RD) markers and some strain-specific deletions. Strains in Group I could be considered more immunogenic and strains in Group III are considered more attenuated and less potent[94].

The main strains or seed-lots that are currently in international immunization programmes are: BCG Pasteur 1173 P2, BCG Danish 1331, BCG Glaxo 1077 (derived from the Danish strain), Tokyo 172-1, Russian BCG-I and BCG Moreau RDJ strains[85].

Comparing to wild-type (WT) Mtb, *M. bovis* contains many deleted regions in its genome (from RD4 to RD11) which may contain differential immunodominant antigens. In addition, during the *M. bovis* attenuation, over 100 genes were lost from BCG genome. The main reason for the BCG attenuation is the deletion of RD1, which includes nine genes. It leads to the loss of the protein secretion system ESX-1[95] and the loss of two small, secreted, immunodominant proteins: culture filtrate protein 10 (CFP-10) and the 6-kDa early secretory antigenic protein (ESAT-6). By its part, the system ESX-1 secretes both CFP10 and ESAT6, which mediates Mtb escape from the phagosome to the cytosol [96].

In spite of the massive coverage of BCG, TB remains an uncontrolled epidemic. The vaccine is able to confer protection against childhood disseminated TB including meningitis (70%-80% of the time) and miliary TB [97, 98]. However, its efficacy

against pulmonary TB in adults, has varied from no efficacy at all to as high as 80% protection[99].

Likely, this poorly protection is due to the fact that many of the genes absent in BCG are believed to be important to generate the human immune response: 23 % of human T cell epitopes present in known human bacilli are absent in the current BCG vaccine [100].

On the other hand, the geographic differences in BCG protection against pulmonary disease in adults could be explained with the fact that exposure to environmental mycobacteria either masks or blocks the protective effect of BCG. In addition, it could be because infection with intestinal helminths might modulate the protective efficacy of BCG through induction of a Th2 cytokine bias, or because of other immune regulatory effects [101].

With these discouraging protection data, in 2014 and 2015, all Member States of WHO and the United Nations committed to ending the TB epidemic and so to develop more efficacious vaccines. In this regard, two novel vaccination strategies are being considered. The first one consists on improving BCG protection by booster subunit vaccine and the other option aims at replacing the current BCG with a novel and better live attenuated strain (Figure 15)[102].

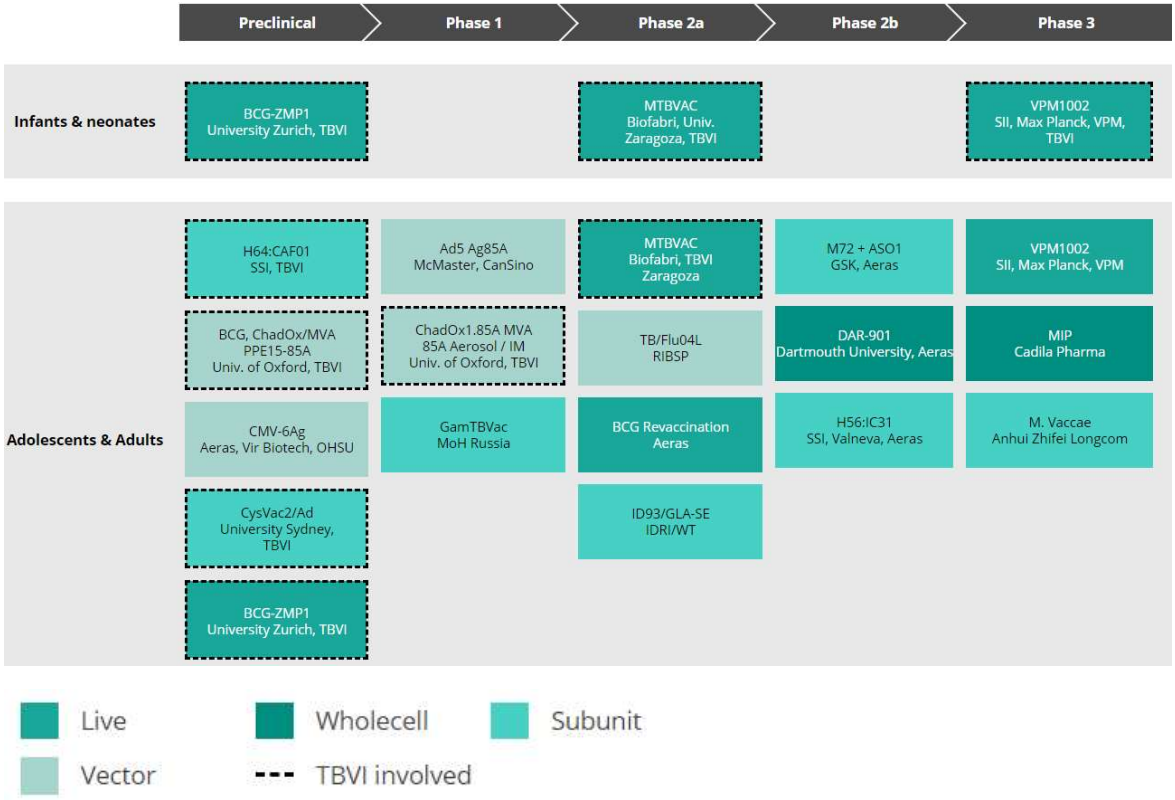


Figure 15. Pipeline of vaccines against TB that are now in preclinical or clinical studies. Adapted from [103].

1.2.5 MTBVAC

As previously mentioned, during the BCG attenuation, it lost a region of the genome called RD1, which codifies for ESAT6, produced by Mtb. In addition, when comparing *M. bovis* and Mtb genomes, *M. bovis* lacks of other regions of the genome, which probably contain important genes for the interaction of Mtb with its natural host, the human [100]. In this regard, the rational of Professor Carlos Martin to create a new TB vaccine was that a vaccine attenuated from Mtb would contain all these genes. That was the case of MTBVAC [104].

MTBVAC is the first live-attenuated Mtb vaccine that entered in clinical trials. Its prototype, SO2, was constructed by the laboratories of Prof. Carlos Martín and Prof. Brigitte Gicquel, attenuating the Mtb clinical isolate Mt103 strain by inactivation of the phoP gene, known as a virulence gene [105]. That isolate belonged to the modern Mtb Lineage 4, which together with Lineage 2 (Beijing strains) represent the most geographically widespread lineages of MTBC transmitted by the aerosol route between humans [106].

Later, following the Geneva consensus safety requirements for progressing new live attenuated mycobacterial vaccines to clinical trials [107], the vaccine had to have 2 non-reversible and independent deletions. In this context, MTBVAC was rationally constructed from SO2 by adding an extra deletion in the virulence gene fadD26, without antibiotic resistance markers (Figure 16), so the final product MTBVAC, has two independent deletion in *phoP* and *fadD26* genes [104].

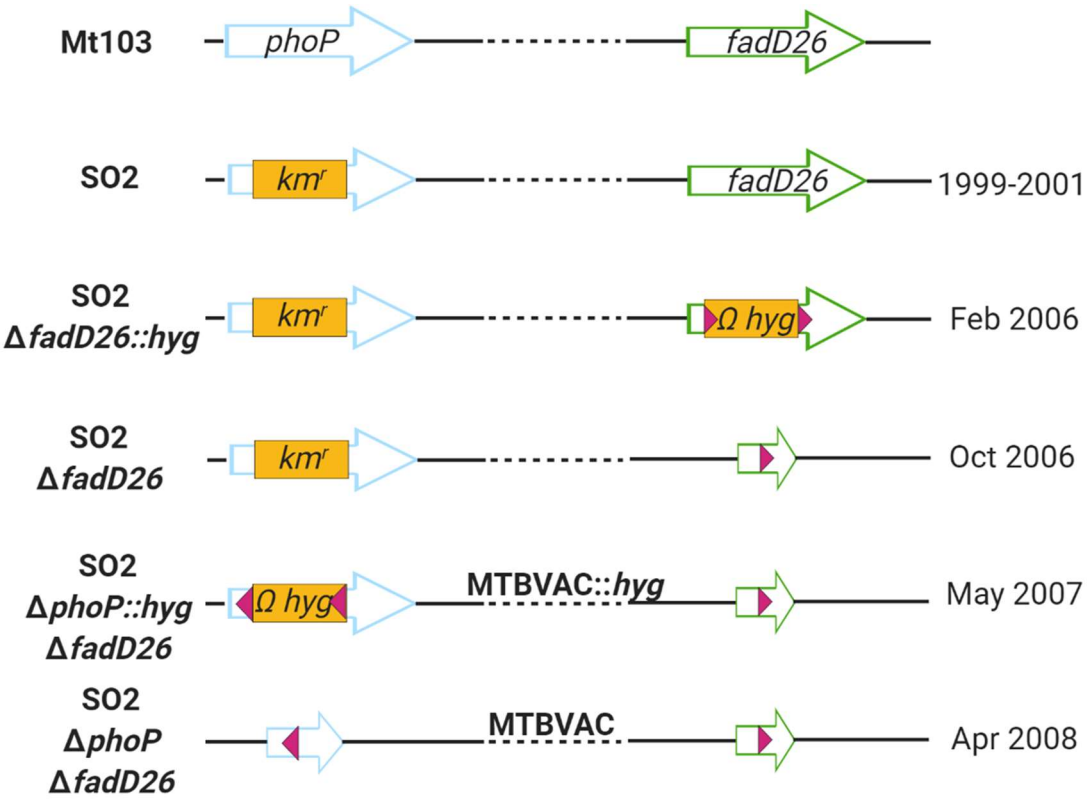


Figure 16. Construction of MTBVAC from Mt103. SO2 prototype vaccine has a single deletion in *phoP*. MTBVAC is the final candidate vaccine that presents a double genetic inactivation in *phoP* and *fadD26* in order to fulfil the Geneva consensus.

The gene *phoP* regulates more than 2% of Mtb genome. Importantly, it regulates genes within ESX-1 system, implicated in the secretion of the major antigen and virulence factor ESAT6, so that MTBVAC can produce but is unable to export nor secrete ESAT6 [108, 109]. The gene *fadD26* participates in the biosynthesis and export of phthiocerol dimycocerosates (PDIM), the main virulence associated cell-wall lipids of Mtb [110, 111].

Since 2001, SO2 and MTBVAC entered in preclinical trial in animal models such as BALB/c or C57BL/6 mice, guinea pigs, and nonhuman primates. Most MTBVAC and SO2 protection and immunogenicity studies showed comparable results to standard BCG, but in some cases, they demonstrated superior efficacy and immunogenicity [112]. For example, an independent study at Public Health England showed in guinea pigs a decrease in bacterial load in lungs and spleen in animals vaccinated with SO2, when compared to BCG. In adult and newborn C57BL/6 mice, MTBVAC conferred significantly greater protection than BCG SSI as measured by significant colony-forming units (CFU) reduction in lung bacterial burden [104, 113]. Also in newborn C57BL/6 mice, MTBVAC vaccination not only induced differential immune response to Ag85B compared to BCG vaccination, but also revealed greater immune response than BCG to stimulation with purified protein derivative (PPD) of secreted Mtb

antigens [114]. These positive differences observed in MTBVAC compared to BCG, may be explained because the former has more than five hundred human Tcell epitopes with respect to the latter (many of them within the RD1 region)[115].

Thanks to these encouraging data, MTBVAC entered into clinical trial in 2013 in Switzerland (phase Ia). At that point, a Spanish biopharmaceutical company, Biofabri, developed MTBVAC as a freeze-dried vaccine according to the current Good Manufacturing Practices (GMP).

This phase Ia consisted in studying the safety and local tolerance when comparing to BCG SSI in healthy adult volunteers who were BCG-naïve and HIV-negative. MTBVAC was at least as safe and immunogenic as BCG and what is more, at the same dose level as BCG, MTBVAC group showed greater frequency of polyfunctional CD4+ central memory T cells [116].

Following this successful phase, in 2015, MTBVAC was allowed to reach phase Ib. That phase consisted in clinical evaluation in newborns (clinical trial identifier: NCT02729571) and adolescents (NCT02933281) in TB-endemic countries, so it was carried out at SATVI site in Worcester, South Africa. The study goal was to evaluate the safety and immunogenicity of MTBVAC in comparison with BCG SSI in newborns, but first, a safety arm in healthy adults was accomplished with no adverse event, in December 2015. MTBVAC was safe in newborns[117] and it demonstrated to exert acceptable reactogenicity to progress to larger trials[118]. At the beginning of 2019, it started the phase IIa, a dose-Defining Safety and Immunogenicity Study of MTBVAC in South Africa, for neonates (NCT03536117) and adults (NCT02933281). Eventually, in the coming years, the phase II b will start to study the efficacy in adults with and without LTBI in South Africa (Figure 17).

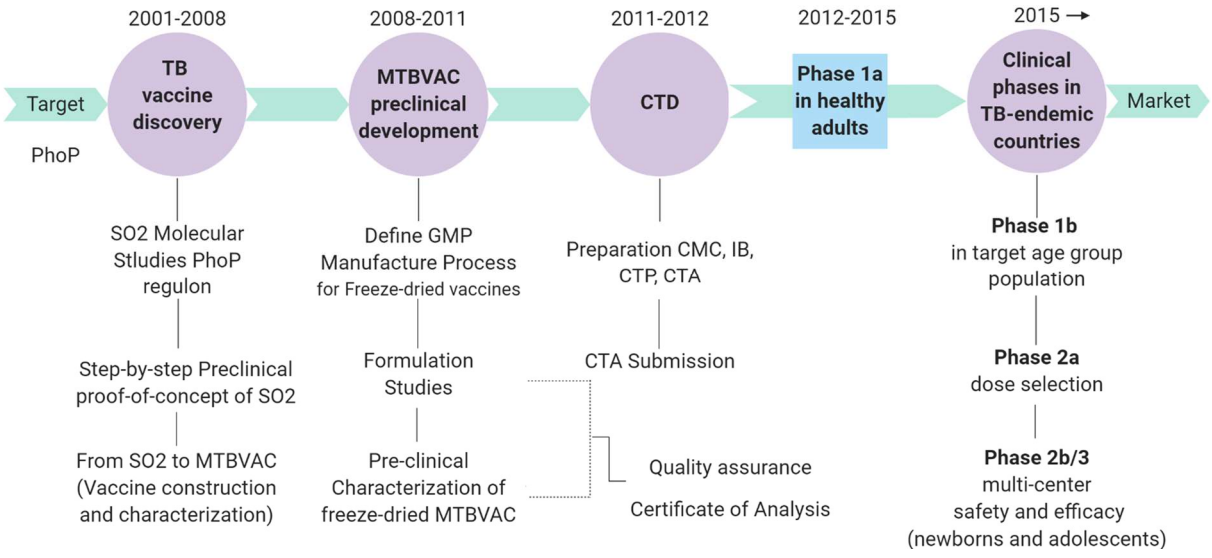


Figure 17. Development of MTBVAC from discovery to clinical development.

1.2.6 Interventional and observational studies using Mycobacteria against asthma

The hypothesis about inducing the Th1 response against T2-type asthma has been already explored in many animals' models and in observational or interventional studies in humans. However, the results concerning BCG are not robust and they still show controversial conclusions.

1.2.6.1 Evidences in mice

In mice, there are many experiments done about the impact of the Th1 response in asthma.

For example, the representative Th1 cytokine IFN-γ apparently suppresses the asthmatic airway response when administered for a short duration [119]. Moreover, different studies have demonstrated the preventive effects of BCG vaccination on asthma, in which BCG-infected mice showed a cytokine shift toward a Th1-like pattern and subsequent development of airway allergy was inhibited [120-122]. Specifically, a study showed that BCG reduced eosinophils and IL-5 in lungs, suggesting that IFN-γ produced during vaccination suppressed the local Th2 response in lungs [120].

Staying on this subject, other study used the rAg85B (one of the main antigen secreted by Mtb) as potential therapy against asthma. It is thought that this antigen induces a strong Th1 response and an increase in IL-22, which in part inhibited the eosinophilia [123].

On the other hand, others studies claim that BCG vaccination reduces allergic airway by inducing Treg cells production [124, 125].

1.2.6.2 Evidences in humans

In 1997, an epidemiological study reported an inverse association between tuberculin skin responses and atopic disorder[126]. Later in 2000, another study revealed less atopy (by allergen skin prick testing) in infants receiving BCG in Guinea-Bissau, particularly if BCG was administered in the first week of life vaccination[127]. Further epidemiologic studies revealed an association between BCG vaccination and less atopy [128, 129]. On the other hand, interventional studies in humans experimentally demonstrated that BCG vaccination improved lung function and reduced medication-use in adult asthmatics[130]. In subsequent studies, BCG revaccination further improved lung function[131].

Concerning TB, many observational studies showed a relation between the disease and a reduced prevalence of asthma or allergy. In fact, in 2000 von Mutius *et al*.

analysed data provided by the WHO between over 235000 people from different countries, and revealed that increasing TB notification rates were associated with a stepwise decrease in symptoms of asthma and rhinoconjunctivitis [12].

Controversially, there are others studies concluding that there is no relation between BCG vaccination and atopy or asthma. In 1997, an observational study reported that the rates of atopic disorder were similar in Norwegian children given BCG immunisation before six months of age to those in a selected group of children who had not received BCG [132]. In 2000, Omenaas *et al*. found no relationship between IgE levels and tuberculin responses in Norwegian adults vaccinated with BCG at 14 years of age[133]. In 2008, authors showed no correlation between BCG vaccination, and development of adult bronchial asthma, allergic rhinitis or atopy. That study suggested that the protection provided by intradermal BCG vaccination in infants to prevent atopic diseases may be limited in early childhood, when a substantial memory of cellular immune modulation still exists[134]. Eventually, a meta-analysis of 757 articles concluded that BCG vaccination is unlikely to be associated with protection against the risk of allergic sensitization and disease[135].

It seems that data about the protection conferred by Mtb against asthma are more robust than data concerning BCG vaccination. As example of it, an study revealed that the efficacy of the BCG vaccination in asthma prevention is lower than the one of active TB, and it lasts 2 years [136].

To sum up, it seems that mycobacterial infection may protect against asthma but there is still a lot of controversies to draw firm conclusions. Particularly it is difficult to know when the vaccination should be administered, as it seems that it may confer better protection when given early in life and that the protection cannot last in time. Another important fact is that the observational studies were carried out with data from intradermal vaccination, the route of approved BCG administration, whereas the natural infection by Mtb is through the airways.

1.3 TRAINED INNATE IMMUNITY

1.3.1 Innate versus Adaptive Immunity

In vertebrates, the immune system is typically divided into adaptive and innate system. It is widely accepted that adaptive immune system requires specialized cells such as T lymphocytes and B lymphocytes which specifically recognizes pathogens. In addition, it is dynamic and able to confer a better protection against reinfection through the development of memory features. Hence, upon a second encounter with the same pathogen, there is a rapid and more effective response thanks to the clonal expansion of T and B cells. In contrast, innate immune system has been classically described as static, pathogen- nonspecific and unable to adapt to an enhanced functional state, so incapable of building immunological memory. The defence leading by the innate system is executed by cell mediated phagocytosis (triggered by monocytes, macrophages and neutrophils), by natural killer (NK) cells or by cascades of constitutive proteins such as the complement system[137]. The adaptive response needs several days to be effective whereas the innate one is much more rapid, being able to act within minutes upon a pathogen stimuli[138].

However, this dichotomy proved not to be entirely true. Firstly, half a century ago, it was demonstrated the cross-protection between unrelated pathogens. It was mediated by the lymphocytes, which exerted beneficial collateral effects on the innate host defence to secondary infections through the release of cytokines such as IFN-γ, and therefore characterized as by-products of adaptive immunity [139, 140]. Last decade, pattern recognition receptors (PRRs) of innate immunity were identified, exposing that innate cells could specifically recognised different kinds of microorganisms having specific pathogen-associated molecular patterns (PAMPs)[141].

1.3.2 A new and revolutionary concept in immunology

Surprisingly, the classical features attributed to the innate immune system started to change and it seemed that the innate system displayed some adaptive features, including the protection to reinfection.

1.3.2.1 Non-specific effects of vaccines

Epidemiological studies concerning the protection mediated by different vaccines conferred the first evidences of non-specific effects beyond their target. In the 19th century, during the introduction of smallpox vaccine, the recipients showed an improvement in others conditions in addition to the smallpox, such as atopic diseases, measles, scarlet fever, and syphilis[142]. Moreover, in 1934, it was documented that

BCG improved survival of infants, and this effect widely exceeded the disease burden of TB in that age group[143].

Decades later, in 2003, an study showed that BCG vaccination in children in West Africa decreased morbidity due to infections other than TB[144]. Moreover, another study revealed a correlation between BCG vaccination and improvement in neonatal sepsis and respiratory tract diseases[145]. Others randomized trials with TB and measles vaccines showed their association with a reduction in overall child mortality, which cannot be explained by prevention of the target disease [146].

However, it must be taken into account that such epidemiological data do not discriminate between classical cross-protection mediated by T lymphocytes and innate immunological memory as the encountered in invertebrates (described below).

1.3.2.2 Innate immunological memory in plants and invertebrates

Nonspecific adaptation of innate immunity was firstly confirmed in non-vertebrate animals and in plants. This is easily observable as adaptive immunity appeared in the ancestors of cartilaginous fish 450 million years ago and is restricted to vertebrate species.

Plants lack mobile defender cells and somatic adaptive immune system. They take advantage of the innate immunity of each cell and of systemic signals emanating from infection sites[147]. The natural protection of plants includes constitutive and systemic acquired resistance mechanisms (SAR) firstly described in 1933[148]. Due to SAR, plants inoculated with attenuated microorganisms are protected for long periods against subsequent different infections including viruses, bacteria, fungi and oomycetes and this implies the existence of innate immune memory [149, 150].

Invertebrates also lack equivalents of T or B cells, so they protect only trough innate immune mechanisms[151]. A lot of studies reflect that priming invertebrates with a stimuli is protective against secondary contact with pathogens different from the priming stimulus [152]. In addition, the protection is related with hematocytes activation [153]. Moreover, this hypothesis is strengthened with the fact that in several classes of invertebrates was observed an allograft rejection after tissue transplantation, a phenomenon ascribed to immunological memory[152].

1.3.3 The appearance of the term trained immunity

1.3.3.1 Experimental evidences of innate immunity memory in vertebrates

First documented experiment in vertebrates showing that a priming stimulus could protect against a secondary infection, different from the first one, date back to the year

1978. It was demonstrated that BCG protected against secondary infections with *Candida albicans* or *Schistosoma mansoni* in nude mice. Therefore, this protection appeared to be mediated through T cell-independent mechanisms [154], and it involved activated tissue macrophages [155]. Later, CpG administration to mice conferred long-term protection against *Listeria monocytogenes* [156, 157]. Furthermore, another study demonstrated that the attenuated PCA-2 strain of *C. albicans*, induced protection toward *Staphylococcus aureus*[158]. Importantly, this protection was also induced in athymic mice, implying a T cell-independent mechanism [159] and what is more, the protection to reinfection was dependent on macrophages[158] and proinflammatory cytokine production[160]. More recently, it has been shown that latent herpesvirus increases resistance to *Listeria monocytogenes* and *Yersinia pestis* in mice [161]. Finally, a recent study demonstrated that NK cells also possess adaptive immune characteristics[162].

At the beginning of 2010, Mihai Netea's group was studying the mechanism by which Mtb and *C. albicans* were recognised by the innate immune system. Then, they assigned a project to a student in order to assess the impact of BCG on Toll-like receptor (TLR) expression. In that study, she used blood from students who were vaccinated with BCG before their departure for internships in developing countries. As a non-related stimulus, intended as a negative control not to be affected by BCG, she used *C. albicans*, the most common stimulus used at that time in the laboratory. Surprisingly, the response to *C. albicans* was strongly upregulated; even more, that was the case not only for the production of lymphocyte derived IFN-γ, but also for the monocyte-derived IL- 1β and TNF. After validating the results with more donors, they concluded that BCG vaccination had strong nonspecific effects that extended to the immune response against pathogens other than mycobacteria. At that point, they found in the literature that such non-specific effects of BGC had been already reported, as detailed above, in human epidemiological studies and in mice experimental models. On the other hand, the literature also suggested that these non-specific effects of BCG could be mediated trough non-specific memory within innate immunity [158] and related to plasticity of macrophages[163].

With the literature and with their own experimental data, Mihai's group concluded that not only classical adaptive immunity, but also the innate immunity is able to adapt after an infection or vaccination and to remain in such a state of enhanced function for a significant amount of time (Figure 18).

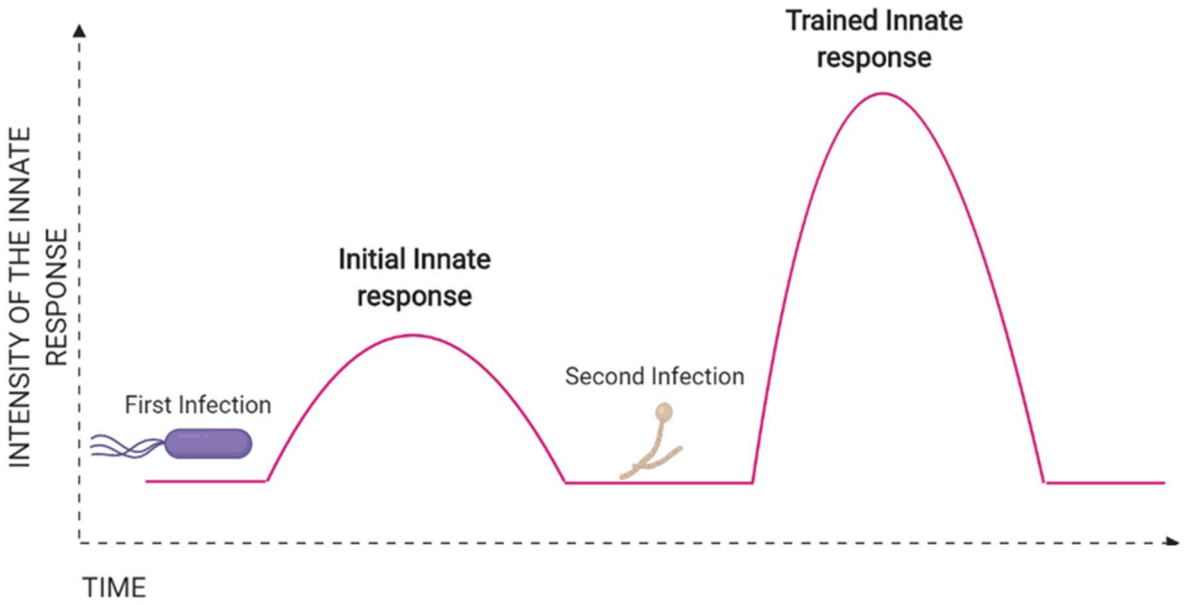


Figure 18. Implications of the innate system memory. Innate immune cells learn from an exposure to microorganisms or vaccines and it leads to an enhanced response upon a second unspecific reinfection.

This capacity of innate immunity to react in an adaptive manner to secondary infections implies memory characteristic of innate immunity, and therefore they proposed the term ‘‘trained immunity’’ for this biological process [164].

To confirm this new concept, they performed an experiment in deficient T and B cells mice. Mice were exposed to a low dose of *C. albicans* and reinfected with a high dose of *C. albicans* [165], and according with their hypothesis, mice were protected to the reinfection and this protection were dependent on an increased function of monocytes and macrophages. These cells became more capable of producing cytokines, phagocytosing and killing microorganisms, overall thanks to the *Candida* cell wall component β-1, 3-(D)-glucan (β-glucan) [165], and these effects were also noted in human primary monocytes [166]. Afterwards, they carried out another study in human volunteers[166], in which it was revealed a heightened *ex vivo* immune response to *C.albicans* in humans recently vaccinated with BCG, where they found monocyte derived TNF-α and IL-6 overproduced. Furthermore, in infants vaccinated with BCG, in collaboration with the group of Peter Aaby, it was confirmed again that monocytes were more responsive after vaccination [167, 168].

Finally, other groups demonstrated that trained immunity is not exclusive within the monocytic lineage but it is also attributable to other innate immune cell lineages such as NK cells [169].

1.3.3.2 Characteristics of trained immunity

The trained immunity is induced after a vaccination or infection and protects and increases resistance against secondary infection through mechanism independent of adaptive B/T lymphocytes. It is non-specific and thus might provide cross-protection to distinct infections, hence the innate immune system has memory. This type of immune response cannot be defined as either innate (as it is induced only secondarily in hosts that have previously encountered a primary infection) or adaptive (as this implies the specificity given by somatic diversification). In the midst of paradox, the term ‘‘trained immunity’’ is proposed to describe this type of immune reaction.

Furthermore, the heightened activation state involves macrophages and NK cells, and leads to an improved pathogen recognition by PRRs and a long-term enhanced protective inflammatory response, characterized by an increase in proinflammatory cytokine production (IL1-β, IL-6 and TNF-α). This state is dependent on epigenetic remodelling at the level of histone modifications and on the rewiring of intracellular metabolic pathways (Figure 19)[164].

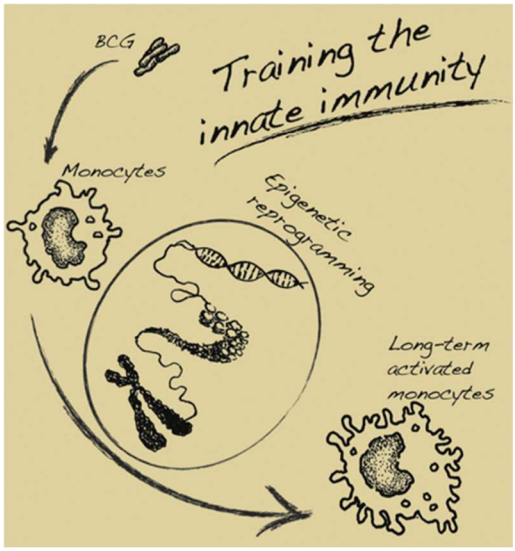


Figure 19. Model of trained immunity induced by BCG. BCG (as well as other infections or vaccines), induces Trained Immunity, a memory program in the innate immune system, which stay in a more active state protecting against non- specific infections[142].

1.3.4 Mechanisms under trained immunity

Trained immunity is not only a defense mechanism protecting after vaccination or infection, but also, when induced inappropriately, might mediate autoinflammatory or autoimmune diseases. So to explode its potential and to treat derived inflammatory disorders, it is crucial to understand the molecular mechanisms behind this immunity.

Trained immunity is characterized by a complex interplay between immunological, metabolic and epigenetic pathways. On the one hand, gene ontology analysis

predicted the involvement of cellular metabolism. On the other hand, changes in cellular metabolism affect the epigenetic program of innate immune cells, which in turn affects metabolic pathways and cytokine production (Figure 20) [170, 171].

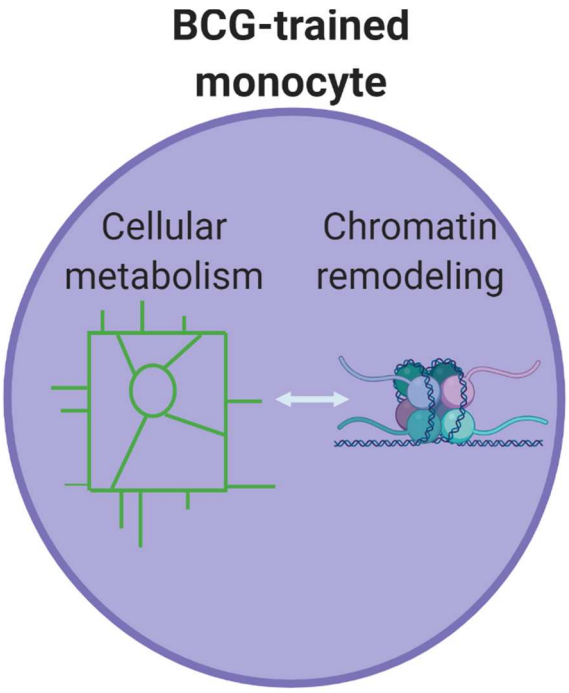


Figure 20. Reciprocal regulation between cellular metabolism and epigenetics changes in trained monocytes. Some stimuli, as BCG, induce changes in the metabolism, inducing epigenetics changes, which by its part produce other metabolic rewiring. Adapted from [172].

1.3.4.1 Metabolisms pathways implicated in trained immunity

There are evidences supporting that cellular metabolism correlates with the functional state of immune cells, as is the case for different subsets of lymphocytes [173]. For example, activated T cells increase both glycolysis and oxidative phosphorylation (OxPhos)[174, 175] while memory T cells depend on lipid synthesis[176]. To date, in the case of trained immunity many metabolic pathways seems to be implicated: glycolysis, tricarboxylic acid cycle (TCA), cholesterol synthesis, and glutamine and aspartate metabolism (Figure 21) [171].

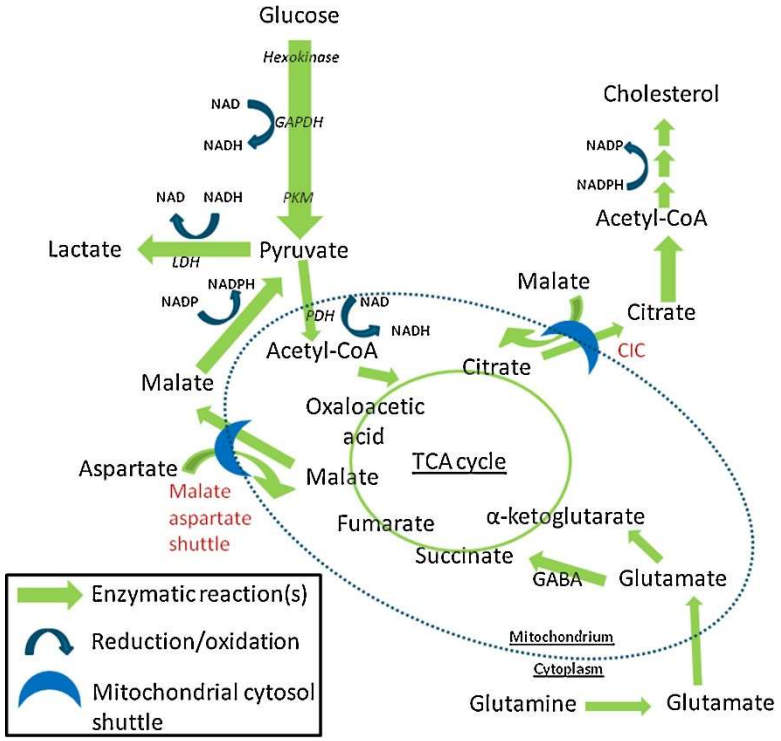


Figure 21. Metabolic pathways implicated in trained immunity. To date, it has been shown that glycolysis is upregulated and that glutamine metabolism, which fuels the TCA, it is also important for induction of trained immunity. Aspartate consumption from the medium is increased in trained cells and cholesterol synthesis pathway might be an essential metabolic pathway as well. Several metabolites produced by these pathways, which accumulate in trained monocytes and macrophages, could induce epigenetic modulators [171].

Glycolysis

Training mediated by β-glucan and BCG induces a switch from OXPHOS to glycolysis, resulting in more lactate production, as seen in the "Warburg effect". Therefore, trained monocytes show increased rates of glycolysis, which despite being less efficient producing ATP when comparing with OXPHOS, can be upregulated multiple folds and then results in a faster ATP production (Figure 22). Furthermore, these effects are mediated by activation of the Akt/mTOR/Hif1-α pathway [177].

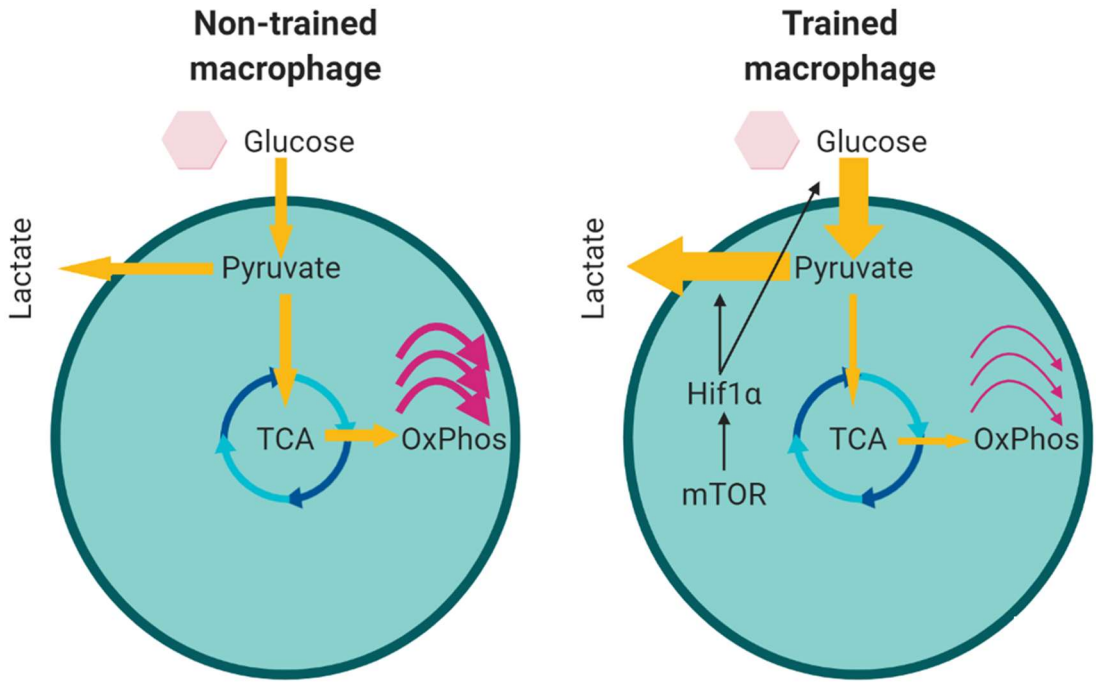


Figure 22. Comparative of the energetic metabolism between trained and non-trained macrophages. In trained macrophages, there is a shift to the glycolysis and so a reduction of OXPHOS.

TCA

Despite the fact that trained immunity decreases OXPHOS, there are some metabolites of the TCA that increase compared to non-trained macrophages, such as citrate, succinate and fumarate.

Citrate may serve as a source for fatty acid synthesis. For their part, fumarate and succinate stabilise Hif-1-α and therefore increase glycolysis and IL-1β transcription. Eventually, fumarate can also act as an antagonist of histone and DNA demethylases [170, 171].

Glutamine metabolism

Experiments with inhibitors of glutaminase-1, which catalyses the conversion of glutamine into glutamate, reveal that glutaminolysis is implicated in trained immunity. Thanks to this metabolism pathway, glutamine is incorporated to the TCA as glutamate, which in turns can be a source of succinate, fumarate and citrate. Hence, glutamate could be used for ATP production, acetyl-CoA production (necessary for epigenetics changes) or cholesterol and fatty acids production, both upregulated in trained macrophages [172].

Cholesterol synthesis

The cholesterol synthesis pathway is highly induced in training but its implication in the process is not known yet [172].

Aspartate metabolism

Aspartate may contribute to the induction of trained immunity, as its consumption is increase in trained macrophages. It could be used indirectly to upregulate cholesterol synthesis or citrate production. In addition, it may be used for purine production via pentose phosphate pathway, also upregulated in training, or to produce aminoacids required for histone methyltransferases [172].

1.3.5 Cellular reprogramming of innate immune cells

Gene transcription is controlled by interactions between regulatory events at gene promoters and distal genetic elements (enhancers). Accessibility of the DNA to transcription factor, enhancers and other proteins is essential for gene expression, and this accessibility depends on the chromatin structure, which is modulated by modifications in the nucleosomal histone proteins, also known as epigenetics changes [170, 178].

Recently, it has been discovered that some immunological signals induce rewiring of cellular metabolism, and that this rewiring influence the epigenetic changes underlying trained immunity. In training, for example, inhibitors of glycolysis interrupts the characteristic chromatin modifications and adapted phenotype of trained immunity, confirming a role for metabolic changes in epigenetic reprogramming. An explanation for this is that the enzymes modifying chromatin are partially regulated by concentrations of metabolic intermediates [170, 179].

These epigenetic changes lead to cellular activation, enhanced cytokine production and to a change in the metabolic state of the trained cell, so it causes a functional reprogramming of monocytes [180].

Nowadays, it is known that in mammals there are different pathways inducing epigenetics changes depending on the stimuli, for example BCG induces them via NOD2 signalling [166]and β-glucan and *C.albicans* via dectin-1[165] (Figure 23). In addition, there are different marks in the histones which change and influence trained immunity: both H3K4 trimethylation and H3K27 acetylation are associated with active chromatin whereas H3K9 trimethylation, is a repressive mark[181]. More specifically, stimulation with β-glucan and BCG vaccination produces the persistent enrichment of H3K4m3 at promoter genes encoding pro-inflammatory cytokines such as TNF-α, IL6 and IL-1β [166, 170].

These epigenetics marks were also observed in healthy human volunteers vaccinated with BCG, even 3 months after the vaccination. Moreover, these changes led to increased transcription and therefore to a nonspecific upregulation of *ex vivo* cytokines production [166].

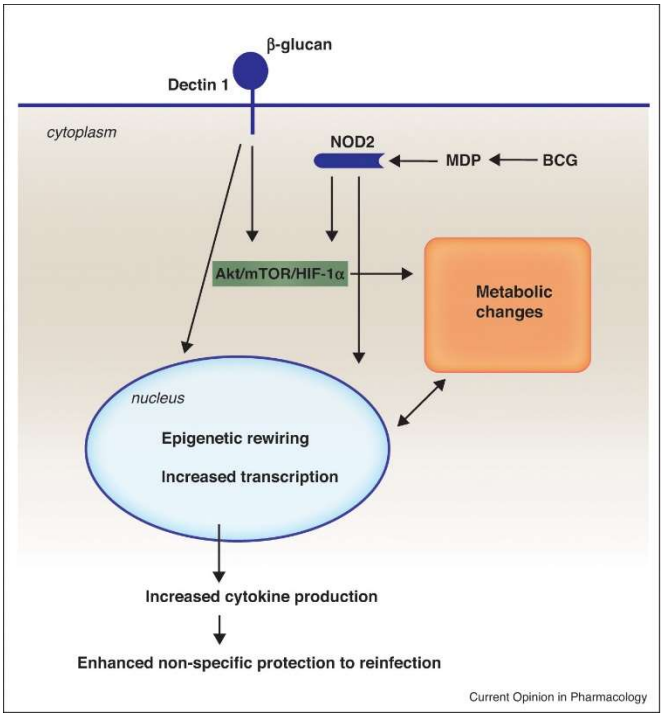


Figure 23. Overview of trained immunity. BCG and β-glucan induce training in monocytes through two different via, producing the upregulation of the Akt-mTOR-HIF-1α pathway, metabolic and epigenetic rewiring. The result is a higher cytokine production, which leads to enhanced unspecific protection to reinfection. [182]

1.3.6 The implication of trained immunity against tuberculosis

Around the world, only 5–10% of people infected with Mtb develop active TB. Others infected individuals (65-75%) develop latent tuberculosis infection (LTBI), a state where individuals do not display any clinical signs of disease [84, 183]. In all these individuals, after infection, there are established TB granulomas, where the innate immunity and adaptive immunity act together to control Mtb infection. This control is mediated through the activation of macrophages via pro-inflammatory cytokines released by Th1 cells, which therefore are able to more effectively kill the bacteria, and through the activity of CTLs as well [184].

In addition, a large proportion (20–25%) of individuals exposed to TB never develop any immunological memory against Mtb, suggesting that their innate immunity is able to finish with the infection by its own, without needing the adaptive immune system[185].

Nowadays, the precise mechanisms needed for protecting against Mtb remain unclear, although the IFN-γ action seems to be crucial [186]. The recent proposed checkpoint model for TB disease progression [187] suggests that the innate immune system constitutes the first 'checkpoint' or barrier that Mtb has to overcome to establish infection. The second checkpoint would be the adaptive immunity acting together with the innate system. Only by passing this point, the bacterium would be able to cause active disease and to infect other individuals.

On the other hand, the model suggests that before passing the first barrier, there is a chance for the innate immune system to eliminate the pathogen, a process referred as early clearance [185]. This is supported by different studies in which individuals who live under constant exposure, e.g. household contacts of TB cases, can clear the infection without the involvement of adaptive immunity, referred as early clearers. The most important evidence for the inherent resistance towards TB has been documented in a report of an outbreak on a US naval ship, where 13 exposed individuals did not develop any signs of exposure (they showed a negative tuberculin skin test reaction after 6 months) [188]. This implies that they were able to mount an effective innate immune response without the involvement of adaptive immunity.

There are different reasons that may explain this early clearance process. One of the most likely is that there exist genetic variations conferring enhanced resistance to TB (combined polymorphisms leading to increased production of IL-1β [189], heterozygosity for leukotriene A4 hydrolase polymorphisms, and a polymorphism of the vitamin D receptor) [190]. The other potential reason is the existence of an enhanced capacity to eliminate the pathogen by the trained innate immunity, established due to a previous exposure to microbial agents or vaccines[183].

With this background, it is reasonable to think that a TB vaccine able to induce trained immunity could be a better strategy to protect against the progression of TB disease.

Objectives

The most violent element in society is ignorance.

Emma Goldman

2. OBJECTIVES

The main objective of the present thesis is to elucidate unspecific effects of Mycobacteria in order to develop new potential applications of attenuated Mycobacteria for humans.

To reach this objective, several sub-objectives has been proposed:

- Establishment and characterization of allergen-driven asthma model in mice, induced by both experimental and human allergens in acute and chronic scenarios.
- Evaluation of the impact exerted by *Mycobacterium tuberculosis* infection in an OVA-induced asthma model in mice.
- Study of the asthma protection conferred by BCG and MTBVAC in acute and chronic asthma model in mice, with prophylactic and therapeutic approaches.
- Characterization of the clinical and immune response in asthma mice models upon BCG and MTBVAC vaccination.
- Comparison between the route, dose and timing for attenuated Mycobacteria delivery in order to find the best parameters for the translation to the clinic in humans.
- Study of the MTBVAC capacity to induce training of human monocytes *in vitro* and comparison of the training induced by BCG and MTBVAC *in vitro*.
- Elucidation of the metabolic and epigenetic mechanisms underlying the training induced *in vitro* by MTBVAC.
- Determination of the ability of MTBVAC to induce training *in vivo* in mice.

Material and Methods

Quand j'étais enfant, quand j'étais adolescent, les livres m'ont sauvé: cela m'a convaincu que la culture était la valeur la plus haute

Simone de Beauvoir

3. MATERIALS AND METHODS

3.1 BACTERIA AND CULTURE CONDITIONS

Attenuated mycobacterial strains were grown at 37°C in 7H9 broth (Difco Middlebrook) supplemented with albumin-dextrose-catalase (ADC) 10 % (0.2% dextrose, 0.5% BSA fraction V, 0.0003% beef catalase) (Difco) and 0.05 % (v/v) Tween-80 (Sigma). When grew on solid, it were used Difco Middlebrook 7H10 plates supplemented with 10% ADC. For plating homogenized organs from mice, it were used Difco Middlebrook 7H11 supplemented with 10 % ADC, polymixin B 50 U/ml, trimetroprim 0.02 mg/ml and amphotericin B 0.01mg/ml.

The main attenuated mycobacterial strains used in the present work were:

- BCG Danish, vaccine from the commercial sub-strain 1331. It was used for most of the asthma experiments.
- BCG Pasteur, vaccine from the strain 1173P2 which comes from Institut Pasteur Paris, France [191]. It was used only for training experiments.
- BCG InterVax, commercial vaccine from the Netherlands Vaccine Institute. It was used only for training experiments.
- BCG expressing GFP, it is a BCG Pasteur strain transformed with the replicative pJKD6 plasmid encoding green fluorescent protein (GFP). It is a kind gift from Luciana Leite, Butantan Insitute, Brazil. It was used for a single asthma experiment, included in Chapter 2.
- MTBVAC, a double mutant from the virulent strain MT103, in *phoP* and *fadD26,* constructed at the University of Zaragoza, Spain [110].
- MTBVACΔ*erp* (constructed by Dr. Solans) [192], MTBVACΔ*lys*A (constructed by Dr. Broset) [193], MTBVACΔ*esat6cfp10*[194] and MTBVAC heat-killed (obtained by boiling an MTBVAC culture at 100ºC for 15 minutes), all of them from the University of Zaragoza.

Mycobacterium tuberculosis H37Rv was grown at 37°C in Difco Middlebrook 7H9 broth supplemented with ADC 10 % (Difco) and 0.05 % (v/v) Tween-80 (Sigma), or on solid Middlebrook 7H10 plates supplemented with 10% ADC.

Attenuated mycobacterial strains manipulation was carried out in a biosafety level 1 (BSL1) laboratory or in a biosafety level 2 (BSL2) laboratory. Mtb manipulation was carried out in a biosafety level 3 (BSL3) laboratory

For experiments in mice, mycobacterial strains were growth until logarithmic phase. The collected pellet was resuspended in 0.1 volume of PBS-Tween 80 at 0.05 % respect to the initial culture volume. After eliminating the aggregates by centrifuging at 1400 rpm during 5min, supernatant was collected and it was added a final concentration of 5 % of glycerol. The preparation was aliquoted in vials of 0.5 ml and stored at -80ºC. Finally, CFUs present in the aliquots was counted by plating.

Bacterial suspensions for intranasal or subcutaneous vaccination were diluted, when necessary, in PBS from glycerol stocks described above. For oral vaccination, mycobacterial aliquots were centrifuged in order to eliminate the glycerol, and diluted in PBS prior to administration.

3.2 ANIMAL STUDIES AND ETHICAL PROCEDURE

3.2.1 Ethics

Experimental work was conducted in agreement with the National Directive for Animal Protection (RD53/2013) for safety of experimental animals, which meets the European Union Directive 2010/63, and with the approval from the Ethics Committee for Animal Experiments from the University of Zaragoza, under the project identification (PI) listed below:

- PI 46/14 for IgA-/- KO and pIgR -/- KO mice.
- PI22/15 for asthma studies in WT mice, TLR2, 4, or 9 -/- KO mice and TLR2,4,9-/- KO mice.
- PI46/18 for *in vivo* training experiments and for mice infected with H37Rv.

All animal procedures were performed by qualified researchers with the degree of "Personnel responsible for directing and design experimental animal procedures, Category C ", (Comunidad Autónoma de Aragón, Decreto 239/2008).

3.2.2 Mouse strains

The current work was carried out experimenting with the mouse strains mentioned below:

- C57BL/6JRj: immunocompetent inbred strain most widely used in mice experiments. In the present work, groups of 5-6 female, or smaller for the negative control, from 6 to 8 weeks old were used for the majority of asthma experiments and for the in *vivo* training experiment. They were supplied by Janvier Biolabs.

- IgA-/- KO: mice from strain C57BL/6. They were used in groups of 5-6 male or female from 6 to 8 weeks old (MMRRC repository).
- pIgR -/- KO: mice from strain C57BL/6. They were used in groups of 5-6 male or female from 6 to 8 weeks old (a kind gift from Gerard Eberl, Institut Pasteur, Paris).
- TLR 2-/- KO, TLR 4-/- KO, TLR 9-/- and triple KO TLR2,4,9 -/-: mice from strain C57BL/10. They were used in groups of 5-6 male or female from 6 to 8 weeks old (a kind gift from Julián Pardo).

3.2.3 Animal procedures

3.2.3.1 Development of allergic asthma in mice

Acute asthma

For induction of **OVA-specific asthma**, 6 to 8 weeks old female C57BL/6 (Janvier Biolabs) were sensitized with intraperitoneal injections of 50 µg chicken egg OVA (lyophilized powder, ≥98% (Sigma)) with 2 mg aluminium hydroxide (Sigma, St. Louis, MO), twice one week apart. Two weeks later, they were challenged to 100 µg OVA for three consecutive days, before being humanely sacrificed (Figure 24).

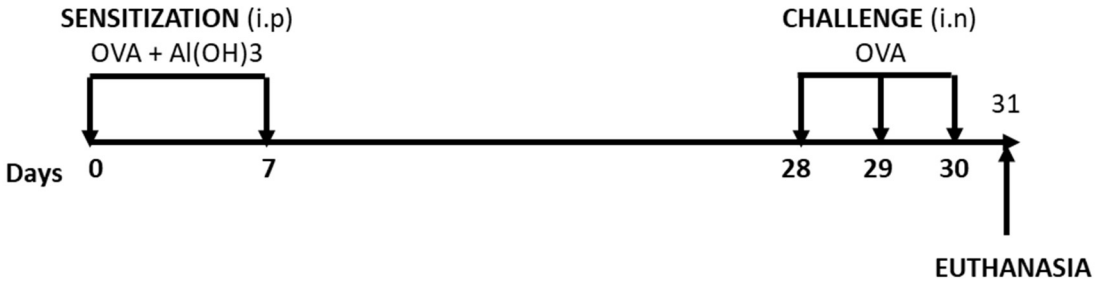


Figure 24. Model of allergic asthma induced by OVA in mice. It consisted in a sensitization phase where OVA was inoculated intraperitoneally together with aluminium hydroxide ($Al(OH)_3$), followed by a challenge phase, in which high doses of OVA were delivered intranasally during 3 consecutive days. i.p: intraperitoneal; i.n: intranasal.

In one experiment, as a proof of concept, in order to evaluate the preventive effect of the attenuated Mycobacteria in asthma, the bacteria were administered before the sensitization, intranasally (10^6 CFU) or subcutaneously (10^5 CFU) (Figure 25).

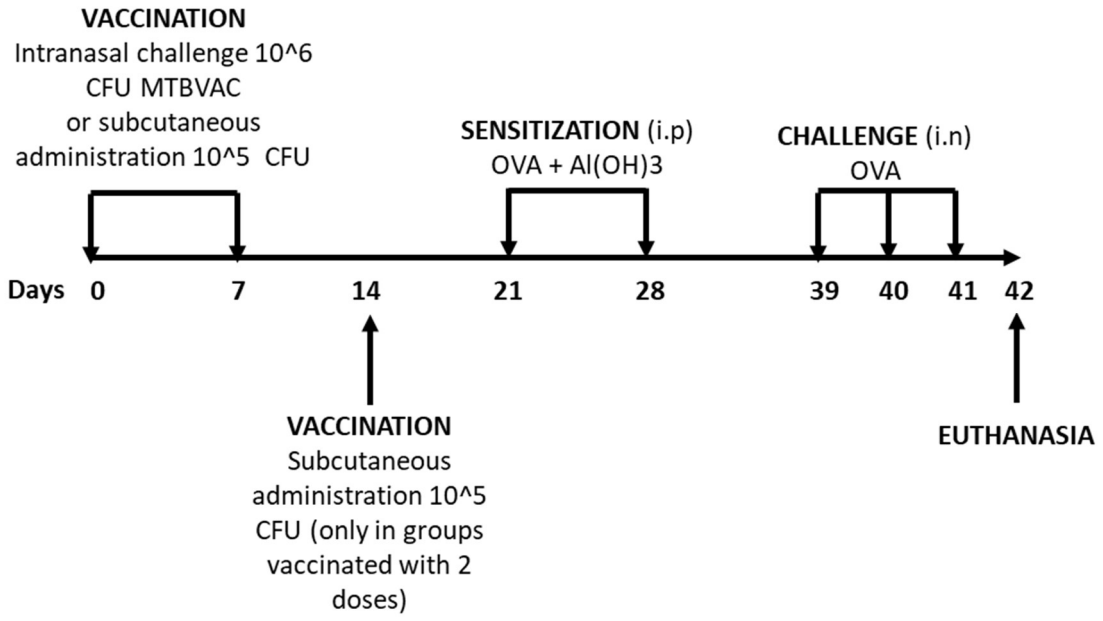


Figure 25. Model of OVA-induced acute asthma in mice when vaccinated prior to the sensitization phase. C57BL/6JRj mice were vaccinated by the intranasal route with 10^6 CFU or by the subcutaneous route once or twice, with 10^5 CFU. Finally, they were intranasally challenged to OVA for 3 consecutive days.

In most of the cases, in order to evaluate the prophylactic impact of the attenuated Mycobacteria in asthma, OVA sensitized mice were intranasally treated with 10^6 or 10^7 CFU, as indicated. One month later, they were challenged to 100 µg OVA for three consecutive days, before being humanely sacrificed (Figure 26).

Figure 26. Model of OVA-induced acute asthma in mice when vaccinated after the sensitization phase. C57BL/6JRj mice were sensitized and one week later, vaccinated by the intranasal route with 10^6 or 10^7 CFU. Finally, one month later, they were intranasally challenged to OVA for 3 consecutive days.

In the long-term protection model, mice were challenged to OVA 4 month after being intranasally treated with 10^6 CFU of BCG (Figure 27).

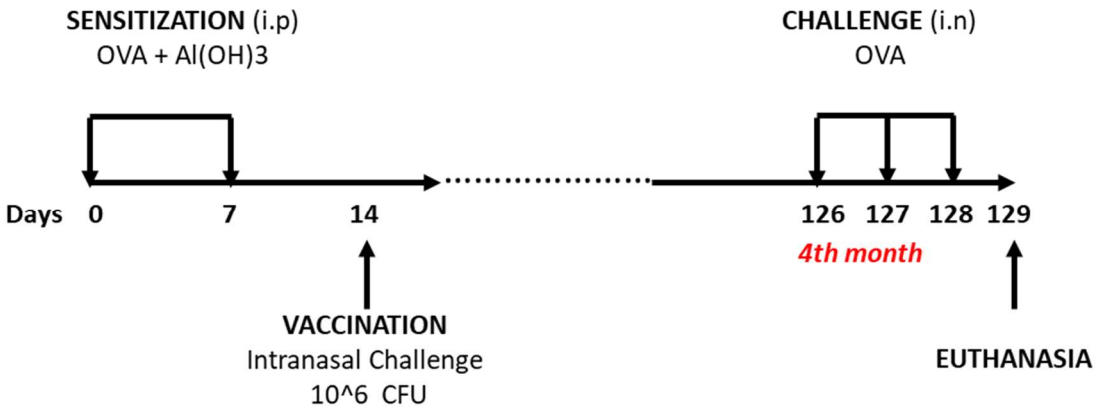


Figure 27. Model of OVA-induced asthma when challenging mice 4 months after the vaccination. Mice were sensitized to OVA and $Al(OH)_3$ by the i.p route, and then vaccinated by the in route with 10^6 CFU of BCG. Four months later, mice were challenged to 100 µg OVA by the intranasal route.

For **HDM-driven asthma**, 6 to 8 weeks old female C57BL/6 (Janvier Biolabs) mice were sensitized with a single intranasal administration of 10 µg HDM (Citeq). The following week, they were intranasally treated with 10^6 CFU of MTBVAC or BCG. Eventually, mice were challenged to 100 µg HDM for 3 consecutive days and then humanely sacrificed (Figure 28).

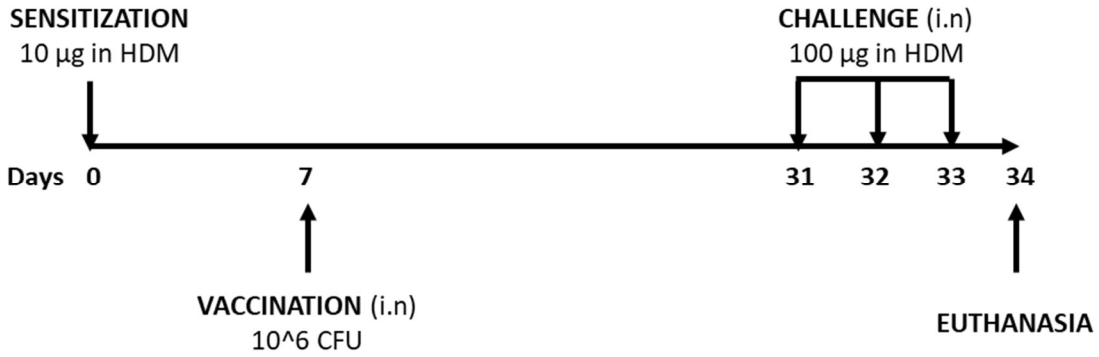


Figure 28. Time-course for testing attenuating Mycobacteria effect in an HDM-induced acute asthma model in mice. Mice were sensitized once to HDM by the i.n route, and then vaccinated by the i.n route with 10^6 CFU of BCG or MTBVAC. 25 days later, mice were challenged to 100 µg of HDM by the i.n route during 3 consecutive days.

Chronic asthma

For induction of **OVA-specific chronic** asthma, 6 to 8 weeks old female C57BL/6 (Janvier Biolabs) were sensitized with intraperitoneal injections of 50 µg chicken egg OVA (lyophilized powder, ≥98% (Sigma)) with 2 mg aluminium hydroxide (Sigma, St. Louis, MO), twice one week apart. Afterwards, to evaluate the prophylactic effect of the attenuated Mycobacteria, mice were intranasally vaccinated and then

challenged to 10 µg OVA twice per week, during 10 consecutive weeks, before being humanely sacrificed (Figure 29).

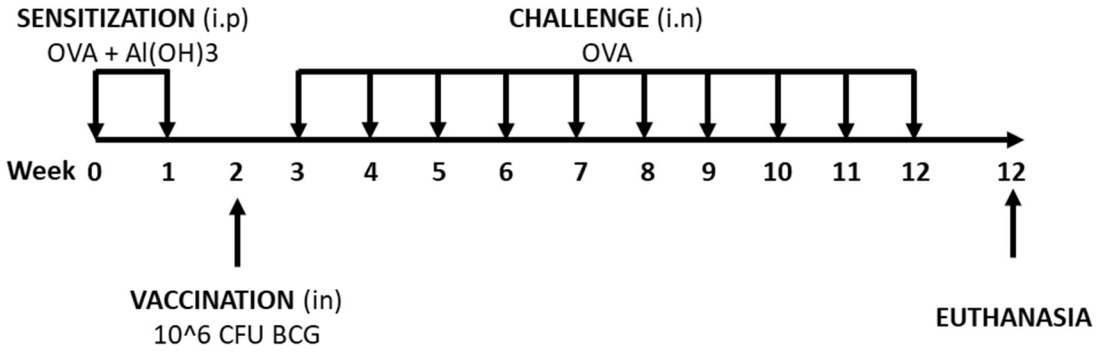


Figure 29. Time-course of a model of OVA-induced chronic asthma in mice when vaccinated after the sensitization. Mice were sensitized twice, one week apart. Then, they were intranasally vaccinated with 10^6 CFU of BCG, and the following week subjected to 10 µg of OVA intranasal challenge twice per week for 10 weeks.

In another experiment, to study the potential therapeutic effect of BCG against established asthma, the vaccine was delivered at week 8 of the procedure, in the half of the challenge phase (Figure 30).

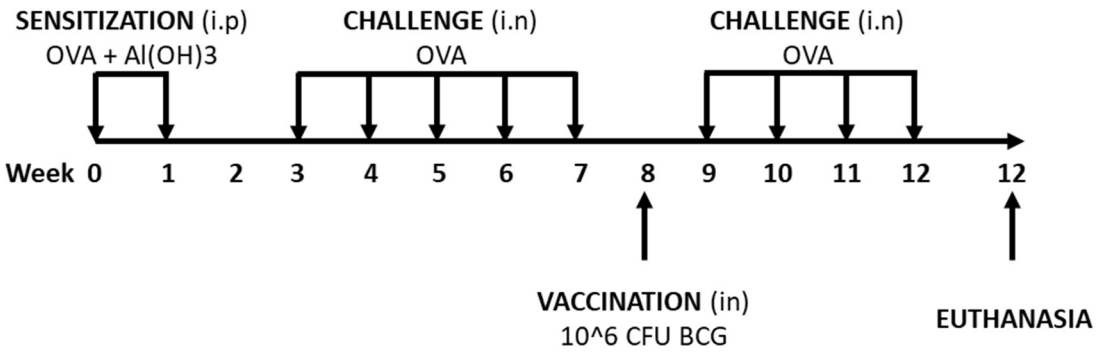


Figure 30. Model of OVA-induced chronic asthma in mice when vaccinated with BCG between OVA challenges. Mice were sensitized twice, one week apart. Afterwards, they were intranasally challenged to 10 µg of OVA twice per week for 5 weeks and then treated with 10^6 CFU of BCG before continuing with the OVA challenge phase for 4 extra weeks.

In this line, the therapeutic effect of MTBVAC in established asthma was also evaluated, following comparable experimental conditions (Figure 31).

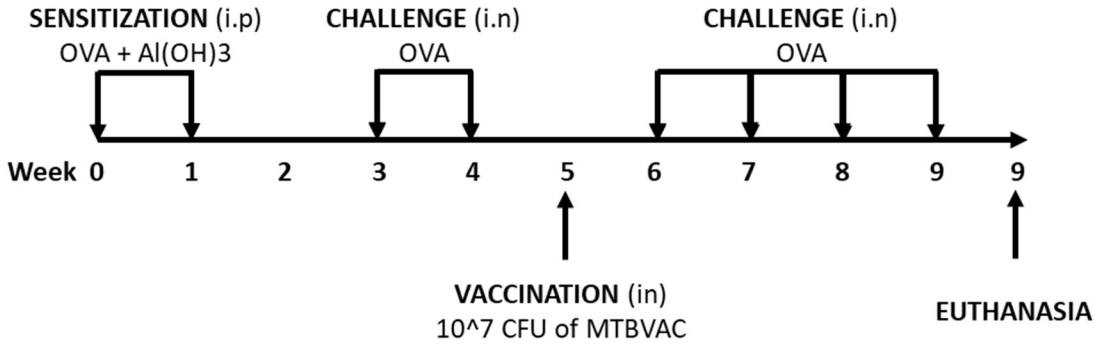


Figure 31. Model of OVA-induced chronic asthma in mice when vaccinated with MTBVAC between OVA challenges. Mice were sensitized twice, one week apart. Afterwards, they were intranasally challenged to 10 µg of OVA twice per week for 2 weeks, and then vaccinated with 10^7 CFU of MTBVAC before continuing with the OVA challenge phase for 4 extra weeks.

On the other hand, one, two or three doses of MTBVAC were administered by the oral route, between multiple challenges in a model of chronic asthma (Figure 32).

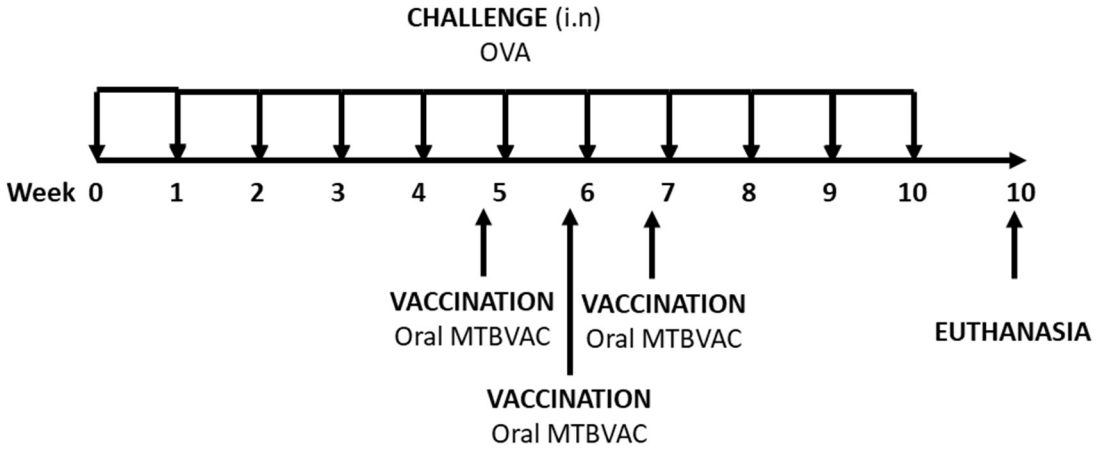


Figure 32. Oral vaccination in OVA-challenged mice in a model of established asthma. Mice were challenged to 10 µg OVA twice per week for ten consecutive weeks. The group vaccinated only once (MTBVAC 1X, described in section 6.20) was vaccinated the seventh week; MTBVAC 2x received the vaccination in both seventh and sixth week; MTBVAC 3x was vaccinated in the seventh, sixth and fifth week. The oral vaccination always consisted in introducing a cannula in the back of the mouth with 10^7 CFU per mice.

For induction of **HDM-specific chronic** asthma, 6 to 8 weeks old female C57BL/6 (Janvier Biolabs) mice were challenged to 10 µg HDM three times per week, during 3 weeks. Afterwards, mice were intranasally treated with 10^6 CFU of BCG or 10^7 CFU of MTBVAC. Three weeks later, mice were challenged to 10 µg of HDM three consecutive days, before being humanely sacrificed (Figure 33).

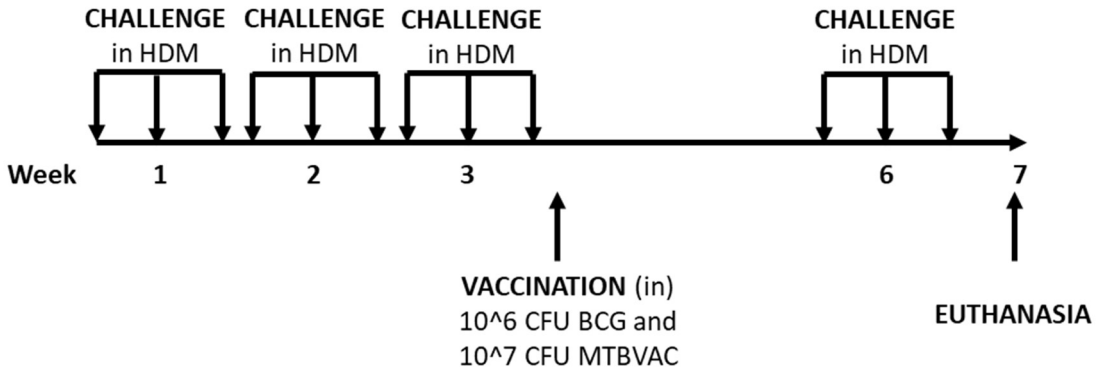


Figure 33. Time-course for a model of asthma triggered by HDM but with the vaccines delivered once the asthma was already established. Mice were challenged to 10 µg of HDM three times per week during 3 weeks. Then, they were intranasally vaccinated with 10^6 CFU of BCG or 10^7 CFU of MTBVAC. Three weeks later, mice were challenged to 10 µg of HDM three consecutive days and the following day they were sacrificed.

For ***Alternaria*-driven asthma** exacerbations, 6 to 8 weeks old female C57BL/6 (Janvier Biolabs) were challenged with 10 µg *Alternaria alternate* (a kind gift from DIATER) by multiple intranasal administrations. The vaccines were delivered between *Alternaria* challenges (Figure 34).

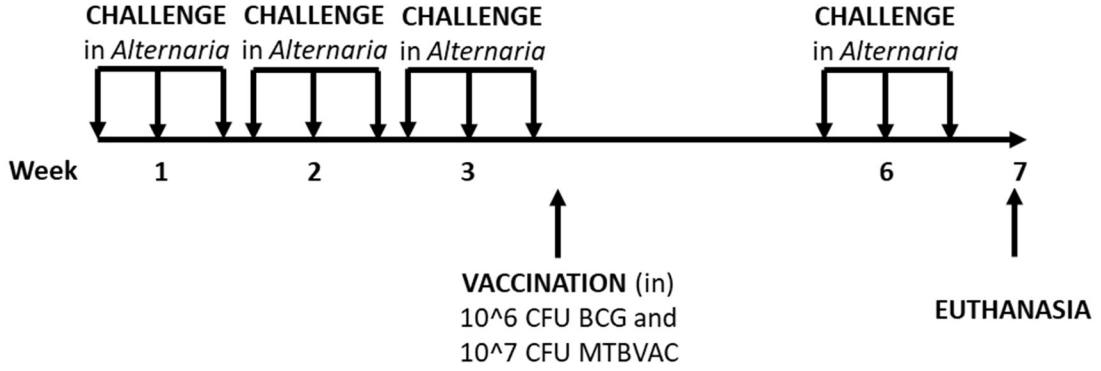


Figure 34. Time-course for a model of asthma exacerbations triggered by *Alternaria alternata* but with the vaccines delivered between challenges. Mice were challenged to 10 µg of *Alternaria alternata* three times per week during 3 weeks. Then, they were intranasally vaccinated with 10^6 CFU of BCG or 10^7 CFU of MTBVAC. Three weeks later, mice were challenged to 10 µg of *Alternaria alternata* three consecutive days and the following day they were sacrificed.

In all models, intranasal administrations were carried out in a final volume of 40 µl and administered in two subsequent drops placed on top of mouse's nose. The subcutaneous administration of the vaccines was carried out in a final volume of 100 µl and the intraperitoneal injections in a volume of 200 µl.

3.2.3.2 Infectious model with Mycobacterium tuberculosis

For the infectious model, OVA-sensitized mice were intranasally infected with a standard low-dose challenge (150 CFU) of the Mtb reference strain H37Rv (Figure 35). One month later, mice were challenged three times to OVA and the following day they were sacrificed.

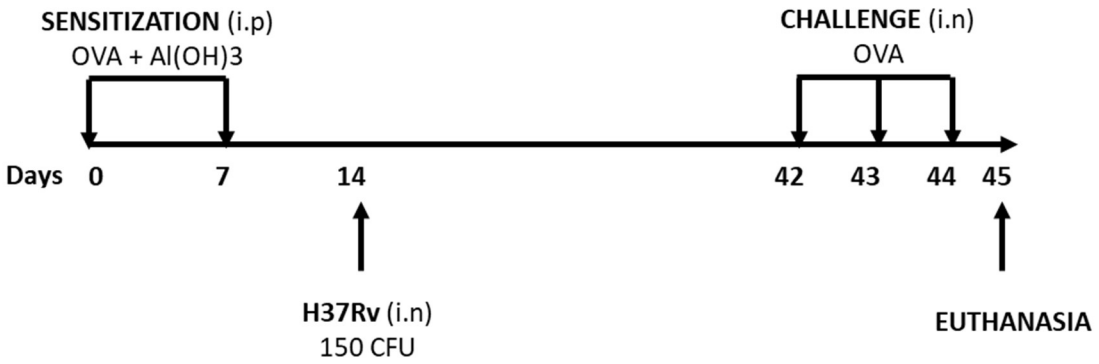


Figure 35. Mtb inmmunization in a model of allergic asthma in mice. Mice were firstly sensitized to OVA and then infected with H37Rv. One month later, they were challenged to 100 µg OVA for 3 consecutive days before being sacrificed.

3.2.3.3 Transfer

After obtaining lungs cell suspension, 10^8 cells were magnetic labelled with CD11c microbeads (CD11c Microbeads UltraPure mouse kit, Miltenyi). Then, the cell suspension was loaded onto a MACS Column placed in the magnetic field of a MACS Separator. So the magnetically labelled CD11c+ cells were retained within the column whereas the unlabelled cells run through. After removing the column from the magnetic field, the magnetically retained CD11c+ cells were able to be eluted. Eventually, cells were washed and resuspended in supplemented RPMI (RPMI 1640, SFB 10%, L-Glutamax 2mM, Penicillin/streptomycine y 2-mercaptoethanol 50 µM). After counting the CD11c+ cell suspension, a volume of 50 µl containing 5 x 10^5 cells was intratraqueally administered to the mice.

To corroborate that the CD11c+ isolation was properly done, cells were stained after the elution and eventually analysed by flux cytometry. The antibodies used were CD11c-VioBlue, CD11b-PercPvio700, SiglecF-PE and CD64-APC.

3.2.3.4 Anaesthesia

All mice were anesthetized when receiving intraperitoneal, subcutaneous or intranasal inoculations. Induction of anaesthesia was performed by inhalatory route with 5% of Isoflurane (Isoba Vet) using a vaporizer and the maintenance of the anaesthesia was performed at 1.5-2% of Isoflurane.

3.2.3.5 Euthanasia

In the study of asthma, at the end points of the experiments, mice were euthanized by cervical dislocation in order to not alter or damage the airways immunity and structure. For the *in vivo* training experiment, as blood collection was required, mice were sacrificed by CO_2 inhalation.

3.2.4 Recollection and preparation of samples

For BAL collection, trachea was cannulated and 0.8 ml of ice-cold PBS was introduced through the trachea. Immediately after, PBS was recollected. Supernatant was separated from cells by centrifugation 5 min at 4500 x g (Figure 36).

Figure 36. Recollection of the BALF. A cannula containing PBS was introduced trough the trachea for collecting cells and molecules present in the airways.

For cellular suspensions lungs were removed aseptically and 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). They were then incubated at 37ºC for 30 minutes, and homogenised using GentleMACS (Miltenyi Biotech) dissociator with the lung specific program according to manufacturer instructions. Afterwards, the homogenised was filtered and residual red blood cells were lysed using Red Blood Cells Lysing Buffer (Sigma).

Oesophagus were removed aseptically and crosswise cut. Then, they were added to 2 ml 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 37ºC for 30 minutes, and mechanically homogenized. Afterwards, the homogenized was filtered to eliminate tissue remnant.

Mediastinal lymph nodes were removed aseptically and mechanically disrupted for cell collection before being filtered to eliminate tissue remnant.

For bacterial burden determination and for lung stimulation with OVA, lungs were homogenized with the GentleMACS, in 1 ml of H_2O or PBS, respectively, using the RNA program according to manufacturer instructions. For CFU count, the preparation was plated onto agar medium 7H11 supplemented with 10 % ADC, polymixin B 50 U/ml, trimetroprim 0.02 mg/ml and amphotericin B 0.01mg/ml.

Lungs for cytokine analysis without any *ex vivo* stimulation were cut into small pieces with sterile scissor. Then, they were cultured overnight (ON) in 1 ml of medium Gibco AIM V serum free. Supernatant was recovered and stored at -80ºC for subsequent analysis.

Bone marrow cells were harvested from femurs and tibias by flushing cell culture media with a syringe. Red blood cells were lysed using Red Blood Cells Lysing Buffer (Sigma).

For histological analyses, left lung and/or oesophagus were fixed in formaldehyde 4% in PBS for 24 hours prior to subsequent hematoxilin-eosin or PAS staining. Images were obtained with a microscope Leica DM5000B.

Blood collection was carried out at the end point of some experiments, by cardiac puncture through the diaphragm using 25G needle, in order to collect 0.4-0.8 ml of blood.

3.2.5 Animal facilities

Animals for H37Rv studies, IgA-/- and pIgR -/- KO mice were maintained in the regulated “Centro de Investigación en Encefalopatías y Enfermedades Transmisibles Emergentes” (CIEETE, Zaragoza, Spain) facilities with reference number ES 50 297 0012 009.

Mice from all the other studies were maintained in the regulated “Centro de Investigaciones Biomédicas de Aragón” (CIBA, Zaragoza, Spain) facilities with reference number ES 50 297 0012 011.

In these facilities, mice were fed *ad libitum*. They were acclimatised to new conditions upon arrival during 3-7 days. Room temperature was 20-24ºC, humidity 50-70 % and light intensity 60 lux with the light-dark cycle of 12 hours.

3.3 FLOW CYTOMETRY ANALYSIS

Cells were acquired with a Gallios flow cytometer (Beckman Coulter) and analysed with the Weasel software. The laser configuration used was the one provided by the manufacturer. Three laser acquisition protocols were used in all cases: violet (405

nm), blue (488 nm) and red (638 nm). Flow panels were set up using isotype controls to assess fluorescent basal levels and compensation (data not shown).

3.3.1 Cells identified by extracellular staining

Cells for different organs were incubated for 15 minutes at 4ºC with Fc receptor blocking reagent (Miltenyi Biotech). Then, cells were washed and incubated for 20 min at 4ºC with the necessary antibodies, described below. Finally, cells were fixed with paraformaldehyde 4% in PBS for 15 min at room temperature, washed and then analysed by flux cytometry.

3.3.1.1 Myeloid cells staining

Eosinophils, neutrophils and macrophages from BAL, lungs and oesophagus were quantified by extracellular staining with the following antibodies: CD45-FITC or vioblue, siglecF-APC or PE, Ly-6G-Vioblue, MHCII-vioblue, CD64-APC and F4/80-FITC from Miltenyi Biotech; and CD11c-PE or VioBlue, CD11b-PerCP/Cy5.5, from BD Biosciences. Different cell populations were identified as described in Table 2.

	CD45	siglecF	Ly6G	CD11b	CD11c	MHCII	CD64	F4/80
Eosinophils	+	+	-	+	-	Not used	Not used	Not used
Neutrophils	+	-	+	+	-	Not used	Not used	Not used
Total Macrophages	+	Not used	-	Not used	+	Depend on activation degree	+	+
AM	+	+	-	Not used	+	Depend on activation degree	+	Not used
IM	+	-	-	Not used	+	Depend on activation degree	+	Not used

Table 2. Antibodies used for identification of myeloid cells in mouse lung by flux cytometry.

Eosinophils precursors at the bone marrow were characterized with the following antibodies: CD45-Vioblue (Clone REA737), CD34-FITC (Clone RAM34), c-Kit-APC(Clone REA791), IL5Rα-PerCP-Vio700 (Clone REA343), Sca1-PE (Clone D7), CD3-53 PE (Clone REA641), NKP46-PE (Clone REA815), B220-PE (Clone REA755), GR1-PE (Clone RB6-8C5), TER119-PE (Clone REA847), FcεR1alpha-PE (Clone MAR-1),from Miltenyi Biotech; and CD4-PE (Clone H120.19), CD8-PE (Clone 52-6.7) from BD Biosciences. Analysis was performed as shown in Figure 50. Eosinophil precursors were defined as Lineage- Sca1- CD34+ c-Kit^{low} IL5Rα+ cells,

using CD3, CD4, CD8, NKP46, B220, GR1, TER119 and FcεR1alpha markers to define Lineage cells.

3.3.1.2 Lymphocytes staining

For IgA secretory B cells staining, lung or BAL cells were determined by extracellular staining with the following antibodies: CD45R(B220)-VioBlue, IgA-FITC, CD3-vio700, CD38-APC, CD45-APC vio700 (Miltenyi). They were defined as CD45+ B220+ IgA+ CD3-.

3.3.2 Cells identified by intracellular staining

For intracellular staining (ICS), after labelling membrane proteins with extracellular antibodies, cells were fixed and permeabilized with the FoxP3 staining set (Miltenyi Biotech) or with the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences), according to manufacturer instructions.

3.3.2.1 Macrophages staining

For M1 and M2 macrophages identification, extracellular antibodies used were the mentioned in Table 2, in addition to CD206-APC and CD86-PE (BD Biosciences). Afterwards, intracellular antibodies were added: iNOS-APC or iNOS-PE (Miltenyi), and Arg1-APC (eBiosciences). M1 macrophages were identified as CD64+CD11c+iNOS+ or CD86high, and M2 as CD64+CD11c+Arg1+ or CD206+.

3.3.2.2 Lymphocytes staining

For Treg staining, extracellular antibodies were CD4-FITC (BD), CD25-PE (BD), CD44-VioBLue (Miltenyi) and the intracellular antibody used was FOXP3-APC (Miltenyi). Treg were defined as CD4+CD25+FoxP3+ cells.

For Breg identification, cells were incubated with 50 ng / ml PMA, 1 µg / ml iomycin, 10 µg / ml LPS, and 10 µg/ml Brefeldin A (Sigma) during 5 hours. For surface staining, cells were labelled with CD19-APCvio770, CD5-FITC, CD1d-PE, B220-vioblue, CD3-PerCPvio700 in culture medium with 10% FBS. Then, cells were fixed and permeabilized with the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) following manufacturer instructions, and stained with anti-IL10-APC. Breg cells were defined as B220+CD5+IL-10+.

For Th1 and Th2 cells identification, cells were incubated with 1mg/ml OVA or 1 µg/ml of anti CD3/CD28 (BD Biosciences) for 24 hours, and 10 µg/ml Brefeldin A (Sigma) was added during the last six hours. For surface staining, cells were labelled with anti-CD4-FITC (BD Biosciences) and anti-CD3-PerCPVio700 (Miltenyi Biotec), in culture medium with 10% FBS. Then, cells were fixed and permeabilized

with the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) following manufacturer instructions, and stained with anti-IFNγ-APC (BD Biosciences) and anti-IL5-PE (Miltenyi Biotech). Th1 cells were defined as CD4+CD3+IFN-γ+ and Th2 as CD4+CD3+IL-5+.

3.4 CYTOKINE ANALYSIS

Quantification of mouse IL-5, IL-4, IL-13 and IFN-γ (Mabtech Biotech), mouse IL-6, TNF-α and IL1-β (R&D Systems) and human IL-1β, IL-6, TNFα, IL-10, IFNγ, IL-17 and IL-22 (R&D Systems) was performed using specific commercial ELISA kits following manufacturer instructions.

Cytokine determination in the lungs was done from organ explants, prepared as described above, cytokines in BAL were measured in the supernatant of the BAL fluid.

To analyse OVA specific response in lymph nodes, $2x10^6$ cells from mediastinal lymph nodes suspensions were incubated with or without 1 mg/ml OVA for 7 days. Then, supernatant was collected to determine cytokine concentration. OVA-specific response for each cytokine was calculated as the difference between cytokine concentrations obtained following OVA stimulation minus the unstimulated control. To analyse OVA specific response in lungs, homogenized lung was incubated with or without OVA 1 mg/ml for 96 h. Further experimental steps were similar to those described for the OVA-specific response in the lymph nodes.

3.5 IGE AND IGA DETECTION

For total IgE and IgA, serum or BAL samples were added; for OVA-specific IgE or IgA, 100 µg OVA was added and incubated at 4ºC ON. Afterwards, plates were washed with PBS 0,05% Tween 20 and blocked with 1% BSA in PBS 0,05% Tween 20. Then, plates were washed and for specific antibodies, BAL or serum samples were added and incubated for 2h at 37ºC. After washing, the appropriated secondary antibody was added, anti Mouse IgE-HRP or anti Mouse IgA-HRP, and incubated 1h at 37ºC. Eventually, it was added TMB (3,3',5,5'-Tetramethylbenzidine) and the reaction was halted with H_2SO_4. Absorbance was measured at 450 nm.

3.6 IN VIVO RNA EXTRACTION

For RNA extraction, lungs were immersed into TRIzol reagent (Invitrogen) just upon harvesting, and frozen immediately with 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 4ºC. Aqueous upper phase containing eukaryotic RNA was recovered, added to 700 µl of isopropanol and centrifuged at 18,000 x g during 10 minutes at 4ºC. The resulting pellet was washed with 70 % EtOH and stored at -20ºC. Residual DNA was eliminated by DNAse treatment, RNA was purified with an extraction based on phenol-acid-chloroform and precipitated ON at -20ºC with isopropanol and sodium acetate. Eventually, RNA was resuspended in distilled water.

3.7 qRT-PCR

For asthma studies, cDNA libraries were constructed for gene expression analysis by quantitative real-time PCR (qRT-PCR) as followed. First, 0.5 or 1 µg of RNA was converted to cDNA using Prime Script RT Master Mix (Takara) according to the manufacturer's recommendations. Then, for the qRT-PCR the 10 µl of the reaction consisted of 1X SYBR Green PCR Master Mix (Applied Biosystems), 0.25 µM of each primer and appropriated diluted cDNA (usually 1/10). All PCR primers were designed using Primer Express software (Applied Biosystems) and they are described in Table 3:

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

Table 3. List of primers used for gene analysis by qRT-PCR for asthma experiments.

The genes expression was measured and normalized with respect to the levels of both actin and 18S mRNA by quantitative real-time PCR (qRT-PCR).

Reactions were carried out in triplicate in an Applied Biosystems StepOnePlusTM Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. The PCR program included an initial denaturation step for 10 min at 95ºC, followed by 40 cycles at 95ºC for 15 s and 60ºC for 1 min. The specificity of the PCR products was confirmed by the loss of fluorescence at a single temperature, when the double-strand DNA was melted to single-strand DNA.

On the other hand, for epigenetics studies the 10 µl PCR reaction consisted of 5 µl 1XSYBR Green PCR Master Mix (Applied Biosystems), 0.25 µM of each primer, 2 µl H_2O and 2 µl diluted DNA from immunoprecipitation reactions. Input samples were 1:25 diluted, and ChIP samples 1:3. Primers used are listed in Table 4.

Gene	5' sequence	3' sequence
***IL6* human**	5' TCGTGCATGACTTCAGCTTT	5' GCGCTAAGAAGCAGAACCAC
***IL6* human**	5'AGGGAGAGCCAGAACACAGA	5' GAGTTTCCTCTGACTCCATCG
***TNFA* human**	5' CAGGCAGGTTCTCTTCCTCT	5' GCTTTCAGTGCTCATGGTGT
***TNFA* human**	5' GTGCTTGTTCCTCAGCCTCT	5' ATCACTCCAAAGTGCAGCAG

Table 4. List of primers used for gene analysis by qRT-PCR for trained immunity experiments.

3.8 PERIPHERAL BLOOD MONONUCLEAR CELL AND MONOCYTE ISOLATION

Buffy coats from healthy donors were obtained from Sanquin Blood Bank, Nijmegen, the Netherlands, after written informed consent. Samples were anonymized to safeguard donor privacy.

Peripheral blood mononuclear cells (PBMCs) were isolated by dilution of blood in pyrogen-free PBS and differential density centrifugation over Ficoll-Paque (GE Healthcare) (Figure 37).

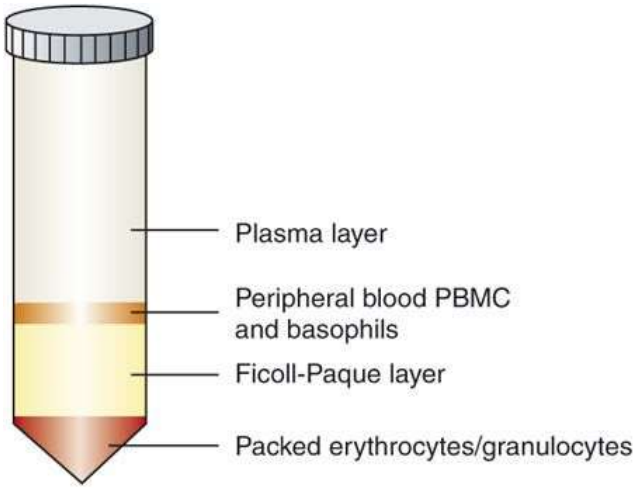


Figure 37. PBMCs isolation using density gradient over Ficoll-Paque. [195]

Cells were washed twice in saline and resuspended in culture medium (RPMI 1640 (Dutch modification)) supplemented with 50 µg /mL gentamicin, 2 mM glutamax, and 1 mM pyruvate. Cells were counted in a Coulter counter (Coulter Electronics), and the number was adjusted to 50×10^6 cells/ml. On the other hand, monocytes were isolated using Percoll separation. 150-200 x 10^6 PBMCs were layered on a hyper-osmotic Percoll solution, centrifuged at 580 x g for 15 min and then the interphase layer isolated and washed with iced PBS. Cells were resuspended in culture medium (RPMI 1640 (Dutch modification)) supplemented with 50 µg /mL gentamicin, 2 mM glutamax, and 1 mM pyruvate, and then counted. Monocytes were used after adherence for 1 h in polystyrene flat bottom 96-well plates (Corning).

3.9 STIMULATION OF PBMCS AND TRAINING OF MONOCYTES

For PBMCs stimulation, a total of 5×10^5 PBMCs in a 100-µL volume was added to round bottom 96-well plates (Greiner). For training experiments, 10^5 monocytes were added to flat bottom 96- well plates. Then, cells were stimulated with RPMI-as a negative control of stimulation-, 10 ng/ ml LPS (serotype O55:B5; Sigma-Aldrich, St. Louis, MO, USA), 1 µg/ml β-glucan, 5 µg/mL BCG InterVax (BCG vaccine Statens Serum Institut from the Netherlands Vaccine Institute), BCG Pasteur or MTBVAC (both laboratory strains), at different MOI, as indicated.

For PBMCs stimulation, after 48 h and 7 days, the supernatants were recollected after centrifuging. When took the supernatant at 48h, only a half of the volume was recollected and then the same volume of fresh medium was added. Cytokine concentrations were assessed in the supernatants using the ELISA technique. For monocytes training, it was followed the optimal conditions determined by the group of Mihai Netea. By 2016, they tested the three most used training stimuli from the literature: β-glucan, BCG vaccine, and oxLDL[196]. Moreover, they tried different

initial training periods, incubating each of these three stimuli for 2h, 4h or 24h. After washing the first stimuli, they also varied the resting time between 24 h, 3 days and 6 days, after which cells were restimulated with RPMI, LPS, or Pam3Cys for 24 h (Figure 38).

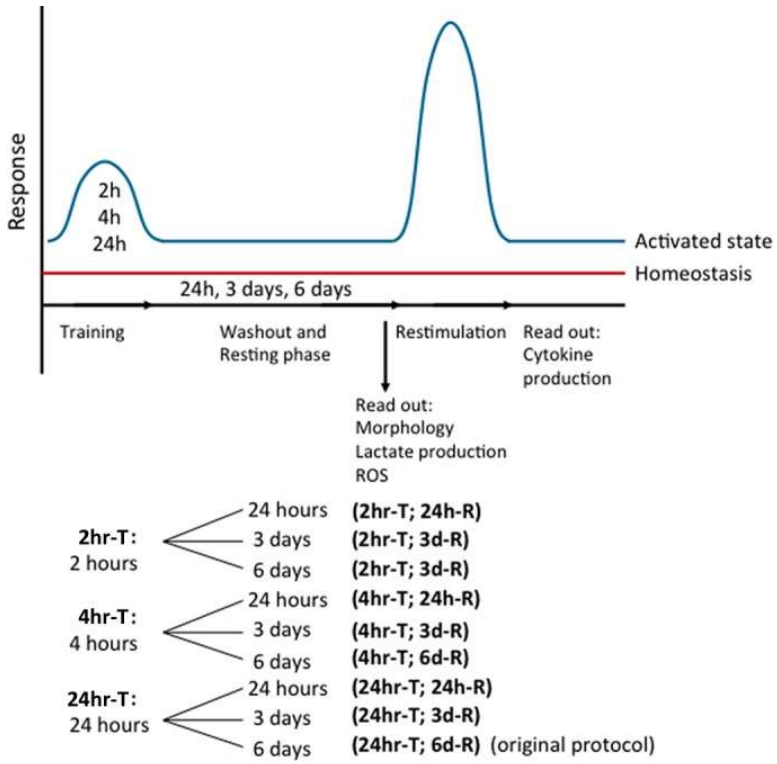


Figure 38. *In vitro* methodology to induce trained immunity in human monocytes. Cells were stimulated for 2 h (2 hr-T), 4 h (4 hr-T), or 24 h (24 hr-T). After the training stimulus was washed away, the cells rested for 24 h (24 h-R), 3 days (3d-R), or 6 days (6d-R), after which the cells were restimulated with RPMI, LPS, or Pam3Cys for 24 h [196].

Trained immunity was assessed in terms of the secondary cytokine response (increased with training), cell morphology -bigger cells when trained-, production of reactive oxygen species (ROS) and induction of glycolysis, as both paths are increased in trained cells. These effects were the most pronounced when using in combination β-glucan, with a training interval of 24 h and a resting time of 6 days [196].

As a result, in the present work, cells were incubated with the stimuli for 24 h at 37 °C. Thereafter, the supernatant was discarded, cells were washed and the medium replaced with fresh RPMI with 10% human serum. After 5 days at 37 °C, supernatant was discarded and cells stimulated with *E. coli* LPS (10 ng/mL), or RPMI as a control for an additional 24 h (Figure 39).

Subsequently, the supernatants were stored at −20 °C until ELISA was performed.

Figure 39. Time-course for training of human monocytes *in vitro*.

3.10 METABOLICS STUDIES FOR TRAINED IMMUNITY

3.10.1 Inhibitors

For the training experiments that used inhibitors, 50 µM BPTES (Sigma) or 1 µM oligomycin (Sigma) were added to the adherent monocytes together with the corresponding stimuli. The training time-line was the same as described above (Figure 39). After being restimulated with LPS, the supernatant was collected at day 7 for further ELISA analysis.

3.10.2 Seahorse Methodology

For the measurement of the oxygen consumption and for the assessment of glycolysis, a seahorse assay was carried out. 10^7 monocytes were trained with MTBVAC MOI 10, β-glucan or without stimulus in 10-cm Petri dishes (Greiner) in 10 ml of RPMI medium for 24 h. Then they were washed with warm PBS and after 5 days resting in RPMI culture medium, they were detached with Versene (ThermoFisher Scientific). 10^5 cells in triplicate were plated to 4 hours-calibrated cartridges in assay medium (DMEM with 0.6 mM glutamine, [pH adjusted to 7.4]; for oxygen consumption rate (OCR), 5 mM glucose and 1 mM pyruvate were also added) and incubated for 1 h in a non-CO2-corrected incubator at 37ºC. OCR and extracellular acidification rate (ECAR) were analyzed using a Cell Mito Stress Kit (for OCR) or a glycolysis stress test (for ECAR) kit in an XFp Analyzer (Seahorse Bioscience), with final concentrations of 1 mM oligomycin, 1 mM FCCP, 0.5 mM rotenone/antimycin A, 10 mM glucose, and 50 mM 2-DG.

3.10.3 Lactate fluorescent assay

Lactate in trained cell culture supernatants was measured using a coupled enzymatic assay in which lactate was oxidized and the resulting H_2O_2 was coupled to the conversion of Amplex Red reagent to fluorescent resorufin by horseradish peroxidase (HRP). Samples were first prepared by means of perchloric acid precipitation (as described below), as lactate dehydrogenase that is present in the serum can degrade the lactate. 30 µl of appropriated diluted samples and the standard curve samples were

added to a 96-well black plate. Then 30 µl of the reaction mix (28.5 µl PBS, 0.3 µl Amplex Red-Life Technologies-, 0.6 µl 10U / ml HRP-Thermo Scientific-, 0.6 µl 100 U / ml Lactate oxidase –Sigma-) were added to them. After 20 min of incubation in darkness, the fluorescence was measured.

3.10.4 Perchloric acid precipitation

Culture supernatant from training experiments (200 µl) were mixed with 35 µl of 13.7 % perchloric acid. After centrifuging, clear supernatant was recollected and neutralized with 9.8 µl of 4 N NaOH and eventually stored at -20 ºC for further analysis.

3.11 EPIGENETICS STUDIES IN TRAINED CELLS

For chromatin immunoprecipitation (ChIP) analysis, 10^7 monocytes were trained *in vitro* in 10 cm petri dishes (Greiner) in 10 ml medium, by adding the stimuli 24 h as described above. Thereafter, the supernatant was discarded, cells were washed and the medium replaced with fresh RPMI with 10% human serum. After resting for 5 days in culture medium, cells were detached from the plate with Versene and fixed in methanol-free 1% formaldehyde (Sigma). Cells were then sonicated by adding lysis buffer (20 mM Hepes pH 7.6; 1% SDS; 1 x PIC -Roche Complete mini tablet-; H_20) at a final concentration of 15 x 10^6 cells / ml. The sonication protocol was performed using a Diagenode Bioruptor UCD-300 at 4ºC for 3 x 10 min (30 seconds ON; 30 seconds OFF).

Afterwards, chromatin check for the preparation of the input was carried out. Briefly, chromatin from 500000 cells was incubated with elution Buffer (1% SDS, 0,1 M $NaHC0_3$ and H_20), 5 M NaCl and Proteinase K (Qiagen) for at least 1 hour at 65ºC, 1000 rpm shaking to drecrosslink chromatin from the histones. Then, chromatin was purified with MinElute PCR purifications columns (Qiagen) according to manufacturer instructions and DNA was quantified.

Then, ChIP was performed using antibodies against H3K4me3 (Diagenode). Briefly, chromatin from 500000 cells was incubated with 1 µg anti-H3K4me3 (Diagenode) and dilution Buffer (5% BSA, protease inhibitor cocktail (7x), H_20) at 4ºC rotating ON. Protein A/G magnetic beads were washed in dilution buffer with 0.15% SDS and 0.1% BSA, added to the chromatin/antibody mix and rotated for 60 min at 4°C. Beads were washed with 400 ml buffer for 5 min at 4ºC with 5 rounds of washes. After washing, chromatin was eluted using elution buffer for 20 min. Supernatant was collected, 8 ml 5 M NaCl and 3ml proteinase K were added and samples were incubated for 4 h at 65°C. Finally, samples were purified using Qiaquick MinElute PCR purification Kit (QIAGEN).

After ChIP, DNA was further processed for qPCR analysis using SYBR green (as described above). Primers pairs employed are listed in Table 4.

3.12 TRAINING *IN VIVO*

For the *in vivo* training experiment, 8 weeks old female C57BL/6 JRJ (Janvier Biolabs) mice were vaccinated by the subcutaneous route with MTBVAC 10^6 CFU and 4 weeks later, 10 µg / mice LPS was administered by intraperitoneal injection. Mice were humanely sacrificed 4 hours later to collect the blood, by CO_2 inhalation (Figure 40).

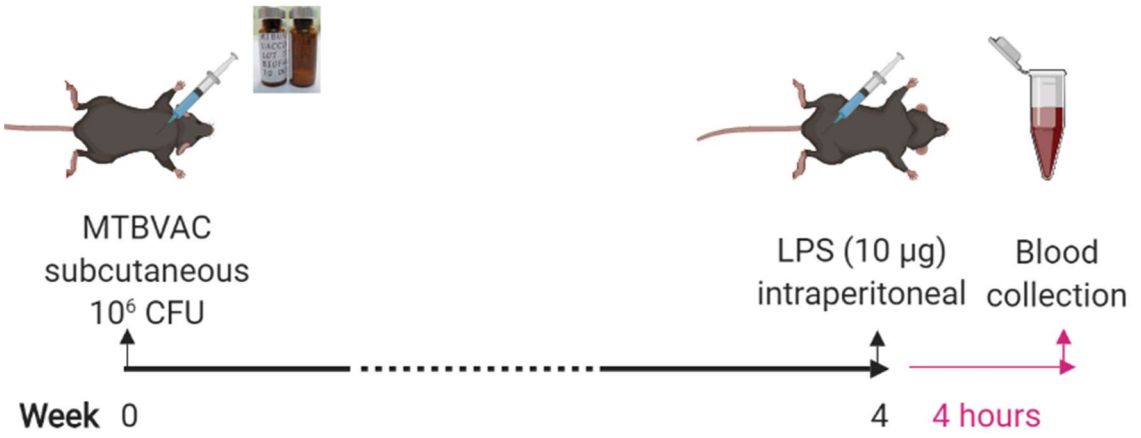


Figure 40. *In vivo* model of trained immunity induced by MTBVAC in mice. C57BL/6 mice were vaccinated with 10^6 CFU, by the subcutaneous route. Four weeks later, they were challenged to LPS, and after 4 hours, humanely sacrificed to collect the blood.

3.13 STATISTICS

Commercial mice were randomly distributed in groups of 5-6 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 $p<0.05$.

Chapter 1

STUDY OF THE IMPACT OF *MYCOBACTERIUM TUBERCULOSIS* INFECTION ON ASTHMA

Experiments from this Chapter were published in [197].

The paper is included in the Appendix

No art or learning is to be pursued halfheartedly...and any art worth learning will certainly reward more or less generously the effort made to study it.

Murasaki Shikibu

TB control is associated with a Th1 response, indeed individuals with polymorphisms in Th1-associated genes such as *ifng*, il12A/B or *stat*1 present a higher susceptibility to develop active TB [80]. On the other hand, many types of allergies, including most common type of asthma (T2-type asthma) are associated with a pathological and exacerbated Th2 cell response [1].

Due to the cross-regulation between these two immune profiles, which implies that the proliferation of one profile is opposed to the proliferation of the other, it has been speculated for years that mycobacterial infections could confer unspecific protection against asthma. Despite the fact that this hypothesis has been explored in humans in observational studies, the correlation between virulent Mtb infection and unspecific asthma protection has never been tested in animal models.

In this regard, in the present chapter, it will be evaluated for the first time whether the presence of pulmonary Mtb affects allergen-induced airway eosinophilia in an ovalbumin-driven asthma mouse model[197]. It could finally clarify the relation between TB and asthma, since animals models could allow us to characterize the mechanism underlying this process. Moreover, studies in animals allow conclusions to be drawn in a more controlled scenario, without other diseases or immune conditions that could falsify the results obtained in the epidemiological studies in humans.

4. RESULTS

4.1 Development of an ovalbumin-driven T2-type asthma in a mouse model

A model of allergic asthma in mice was set-up and characterised in order to demonstrate that it was able to reproduce the main features of clinical asthma in humans. Specifically, an acute model of allergic asthma induced by OVA was developed in mice (Figure 24). The OVA model is the most widely used and characterised asthma model in mice, so it seemed reasonable to start with this approximation. It allows to address the molecular basis of asthma and to study the potential protection conferred by Mycobacteria as well, in a more manageable manner than with a chronic model of asthma, which would take several months.

Firstly, as primary marker of asthmatic responsiveness, the eosinophil infiltration into the BAL (Figure 41) and lungs were quantified by flow cytometry, as these cells trigger the most of the symptomatology in asthma.

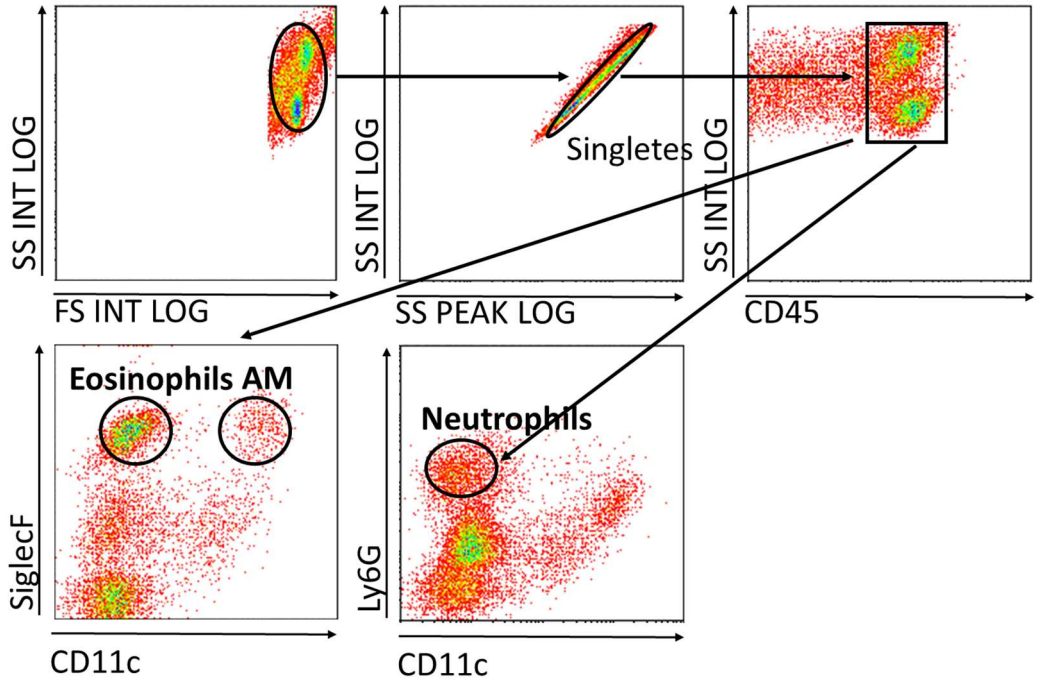


Figure 41. Gating strategy for determination of eosinophils by flow cytometry. Eosinophils are defined as CD45+SiglecF+Cd11c-, AM as CD45+SiglecF+Cd11c+, and neutrophils as CD45+Ly6G+Cd11c-. These are representative images from a mouse in the OVA control group.

The model clearly induced an eosinophilia in both BAL and lung, according to the clinic in humans (Figure 42). Further, massive lung eosinophil infiltration was observed by hematoxilin-eosin staining (Figure 42).

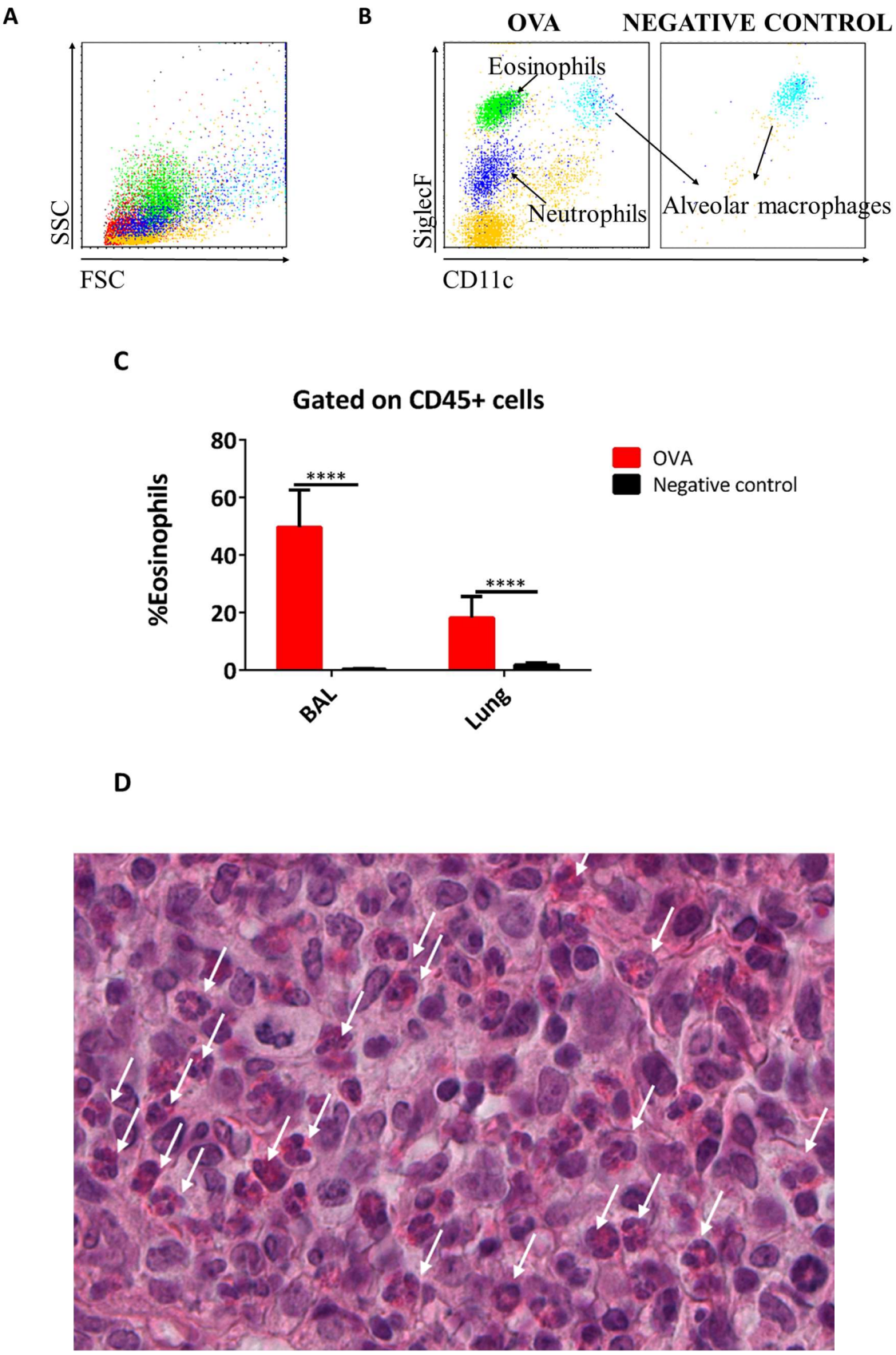


Figure 42. Eosinophilia in the airways in the model of acute asthma. Total cells (A) and myeloids cells (B) in the positive control (OVA) versus the negative control are identified by flow cytometry in the model of allergic asthma in mice. The negative control did not suffer any administration during the process. Eosinophils are coloured in green and identified as

CD45+SiglecF+CD11c-, neutrophils appear in dark blue and they are CD45+Ly6G+CD11c- and AM in light blue, are defined as CD45+SiglecF+CD11c+ (B). Myeloids cells were quantified by flow cytometry in BAL and lungs. Graph represents mean±SEM from pooled data of two experiments (n=12 mice for OVA-challenged and 9 for the negative groups). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA (C). Representative hematoxilin-eosin image from a lung amplified 40X from an OVA-treated mouse. White arrows indicate eosinophils (D).

Additionally, the Th1 and the Th2 responses were assessed by quantifying cytokines in BAL, lung explants, and lymph nodes, as it is shown below (Figure 48 and 49). In general, IL-4, IL-5 and IL-13, the typical cytokines highly produced in the Th2 response, were increased in the model. On the other hand, the IFN-γ, the hallmark of the Th1 response, did not show significant variations. Therefore, the immune profile induced by the allergen was clearly T2-type.

Other typical asthma hallmark is the remodelling 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 lungs after staining using Periodic Acid Schiff (PAS) technique. In the OVA model, different layers of epithelial cells could be distinguished in the alveolar walls, with an important proportion of PAS-positive cells, which correspond to goblet cells. In addition, mucosubstances were found in the airways (Figure 43). Therefore, the model is able to reproduce not only the molecular signs of asthma but also the clinical features.

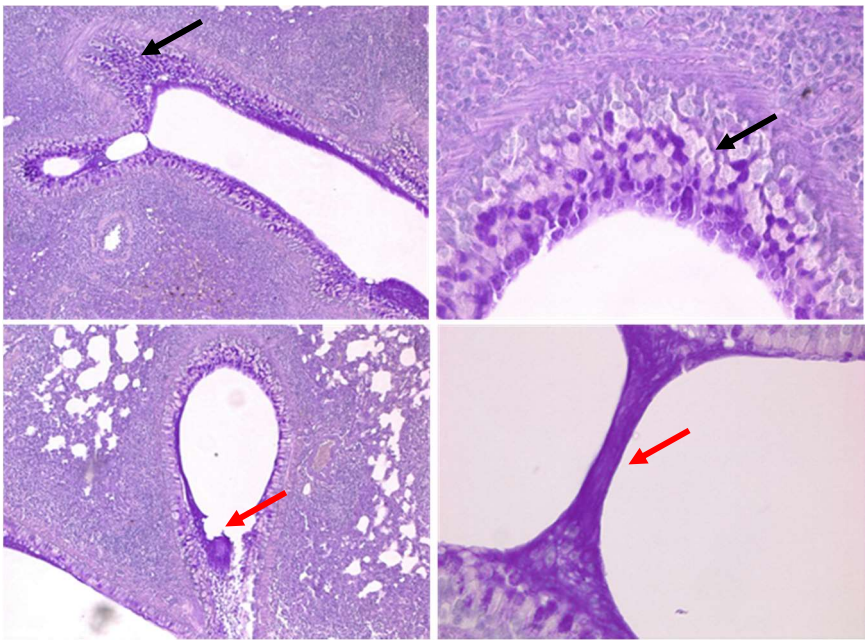


Figure 43. Representative images of PAS-stained fixed lungs from OVA-challenged mice. Mucosubstances are stained in purple, showing many region with mucus (red arrow) and goblet cells in a multilayer disposition producing the mucus (black arrows). Images in the left are taken with a lens of 10 magnification and images on the right, with a lens of 40 magnification.

4.2 The induction of an acute asthma model in mice requires sensitization and challenge phases

To establish the best and fastest allergic asthma model with which work throughout the research, two different models were evaluated: a model with sensitization and challenge phase and a model with no sensitization phase (Figure 44 A and 44 B, respectively). The first one is the model explained above, and the second one was chosen as a manner to ease and accelerate the research.

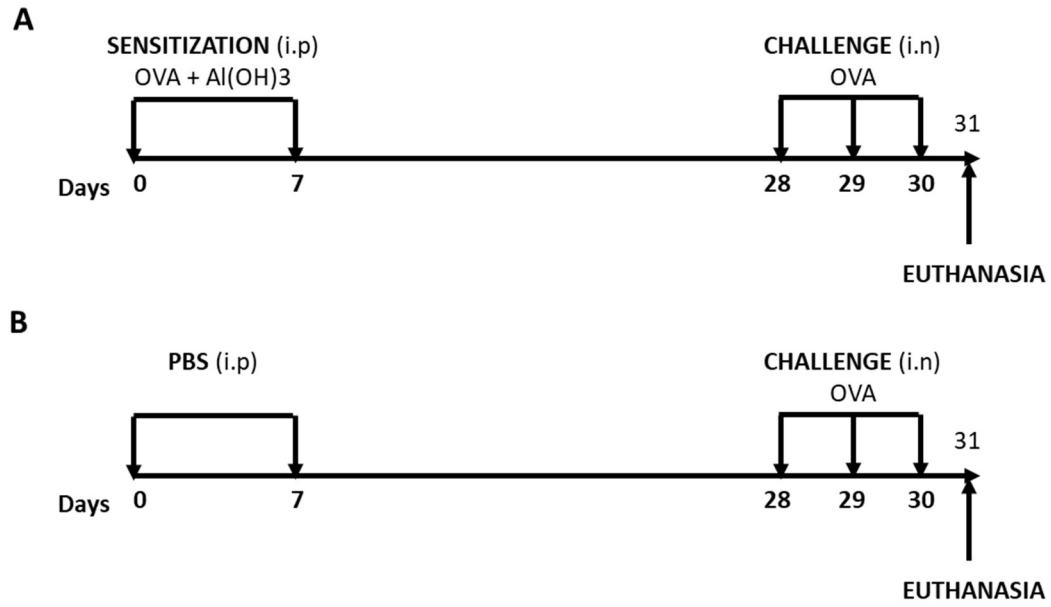


Figure 44. Comparison between two potential asthma OVA-induced models in mice. Sensitization phase consisted of two intraperitoneal (i.p) administration of OVA and $Al(OH)_3$, followed by an intranasal challenge phase (A). In the other model, there was no sensitization phase, only PBS i.p administered as a control, and there was only a positive challenge phase with intranasal OVA (B).

IgE, IL-5 and eosinophils were analysed in parallel in both models, since they are produced in response to the allergic cascade and they are clinically relevant in asthma in humans (Figure 45).

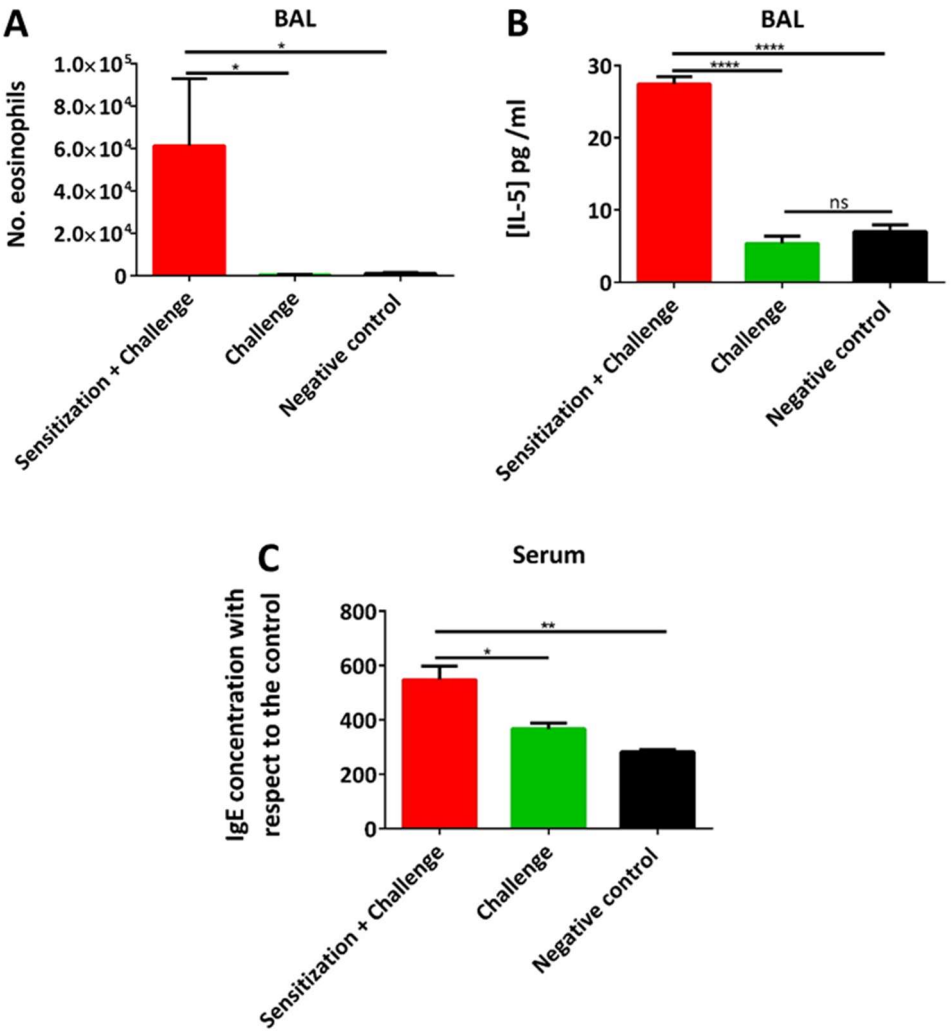


Figure 45. Allergic asthma hallmarks in the model of OVA-induced asthma in mice. Total eosinophils in BAL quantified by flow cytometry. The eosinophilia was clearly higher (about 140 times) in the model with sensitization and challenge than in the model with only the OVA challenge phase (A). IL-5 in BAL supernatant was significantly higher in the first group with respect to the only challenge group (B) and the same patron was observed for the IgE in serum, where values are calculated with respect to the blank with PBS (C). Data represent mean±SEM (n= 3 mice).*p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

The three measured parameters were more accentuated in the model with sensitization and challenge phases than in the model with only the latter phase, as is occurred in humans, where only sensitized individuals develop the allergic pathology.

As a result, thereafter the model chosen for the research was the one with sensitization and challenge phases.

4.3 Mtb infection prevents airway eosinophilia in an OVA-driven asthma mouse model

Appearance of asthmatic symptoms in humans comes preceded by an asymptomatic phase of allergen sensitization, which is likely to occur at an early life stage, likely in

childhood [198]. Thus, to evaluate strictly the ability of intranasal Mtb to prevent asthma, mice were infected once finalized the sensitization phase.

C57BL/6 mice OVA-sensitized were intranasally challenged with a low-dose (150 CFU) of the Mtb reference strain H37Rv. Four weeks later, lung eosinophilia was induced by intranasal OVA administration during three consecutive days (Figure 35). At this timepoint after infection, lung bacterial replication is efficiently controlled by host immune system in immunocompetent mice [199] and therefore this scenario would be comparable to a latent infection.

The results demonstrated that OVA-driven eosinophilia was abrogated following Mtb infection, both in BAL and lungs (Figure 46).

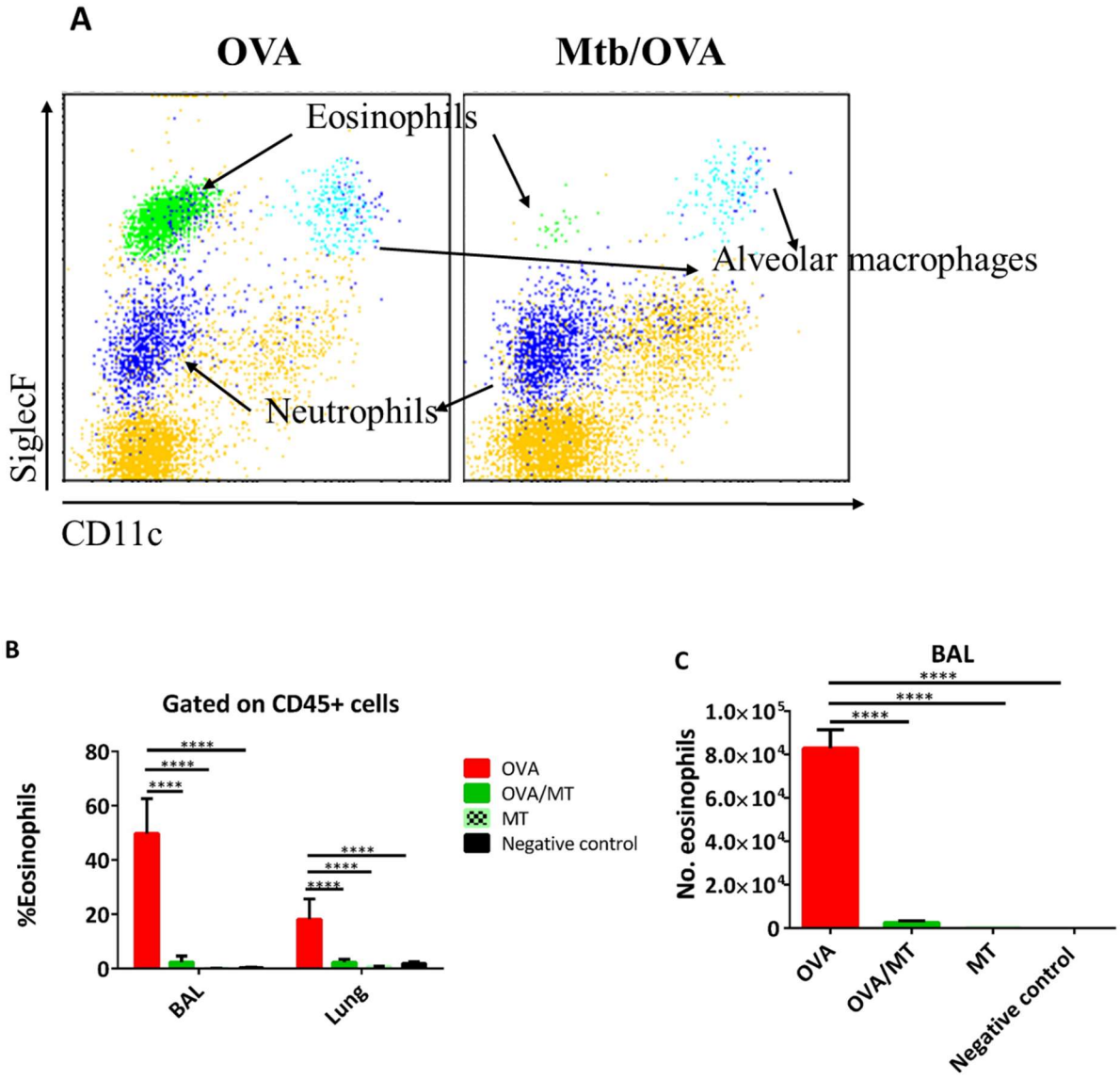


Figure 46. Eosinophilia after Mtb infection in a model of allergic asthma. Myeloid cells identification by flow cytometry in BAL (cells after gating on singlet cells and CD45+ cells) (A). Percent eosinophils in BAL and lung at day 45 quantified by flow cytometry (B). Total

eosinophils in BAL (C) at day 45 quantified by flow cytometry. Graphs represent mean±SEM from pooled data of two experiments (n=12 mice for OVA-challenged and OVA/MT and 9 for the MT and negative groups). MT: infected by *Mycobacterium tuberculosis*. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

Moreover, Mtb infection specifically prevented eosinophil infiltration, without modifying significantly other myeloid populations as neutrophils, AM or T lymphocytes (Figure 47).

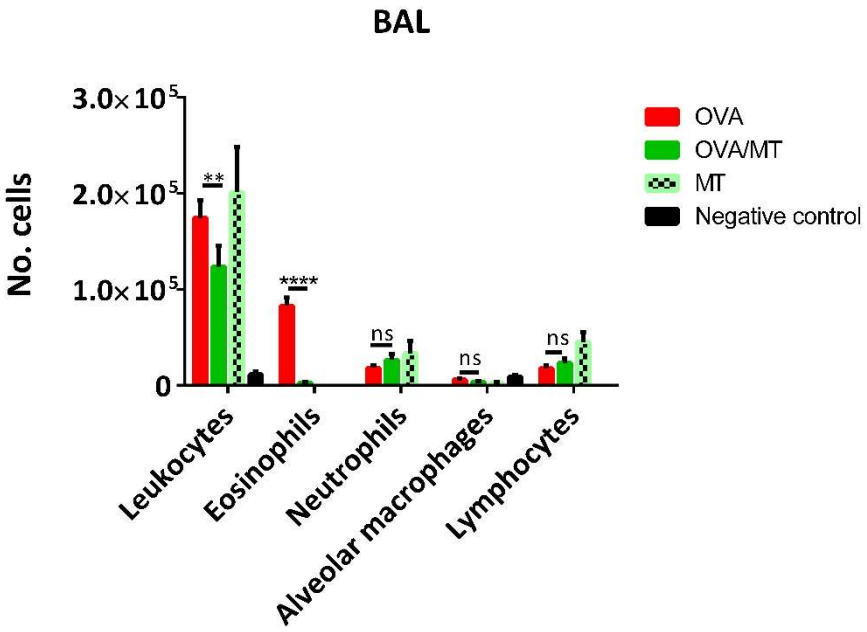


Figure 47. Total number of BAL cells in a model of allergic asthma. Number of leukocytes, eosinophils, neutrophils, AM and T lymphocytes in BAL at day 45, determined by flow cytometry. Graph represents mean±SEM from pooled data of two experiments (n=12 mice for OVA-challenged and OVA/MT groups and mice=9 for the MT and negative groups). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

4.4 The Th2 response driven by OVA, turns into a Th1 profile upon Mtb immunization

Next, Th1 and Th2 cytokine profiles were assessed. Data demonstrated a substantial reduction of Th2 cytokines IL-5, IL-4 and IL-13 in BAL (Figure 48 A) and lungs (Figure 48 B) from OVA-challenged mice following H37Rv infection, concomitantly with an increase of IFN-γ.

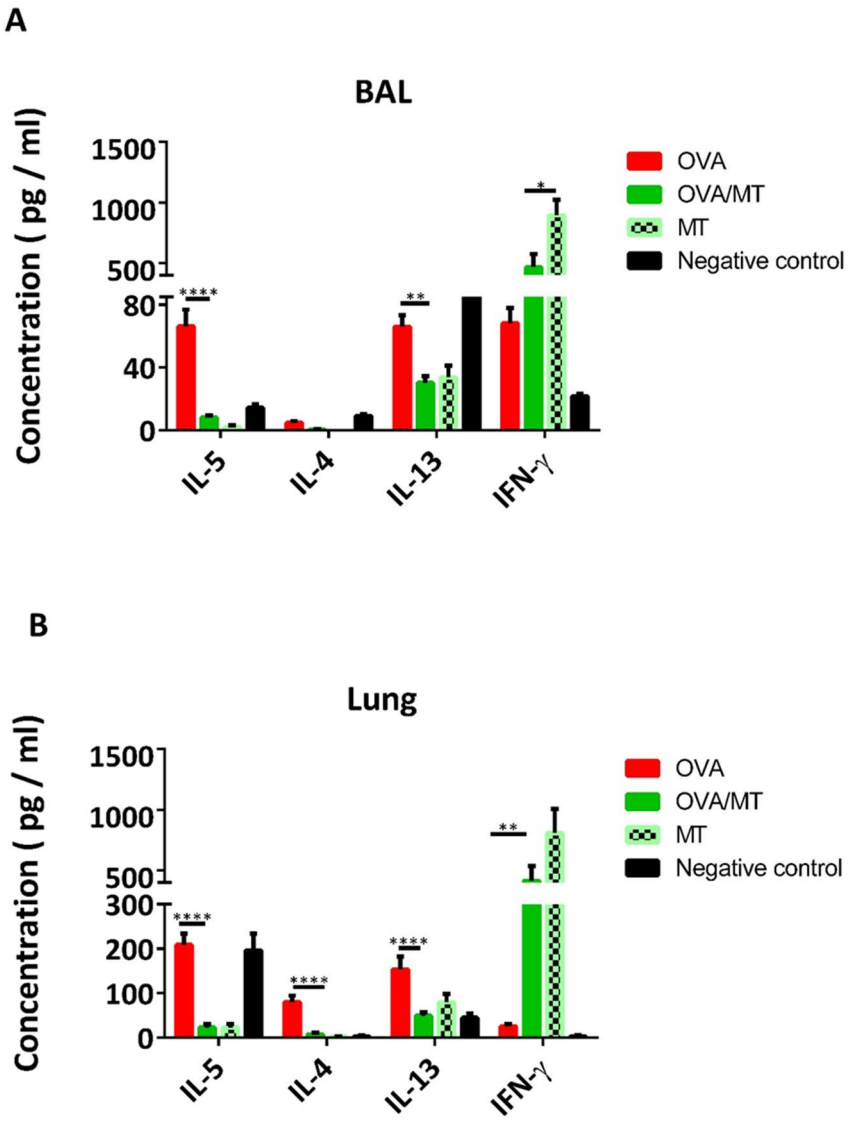


Figure 48. Cytokine concentration in BAL and in lung upon H37Rv infection in a model of acute asthma. IL-5, IL-4, IL-13 and IFN-γ were quantified in BAL (A) and lungs (B) by the ELISA technique. Graph represents mean±SEM from pooled data of two experiments (n=12 mice for OVA-challenged and OVA/MT groups and mice= 9 for the MT and negative groups). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

Allergen-specific response was also studied following *ex vivo* OVA stimulation of mediastinal lymph node cells. There was a substantial production of OVA-induced IL-5 and IL-13 in the OVA-challenged group, which should be produced by OVA-specific T cells, in the absence of IFN-γ response. Conversely, Mtb infection drove to an inversion of this profile, with a reduction of IL-5 and IL-13 levels, and a dramatic increase of OVA-specific IFN-γ response (Figure 49).

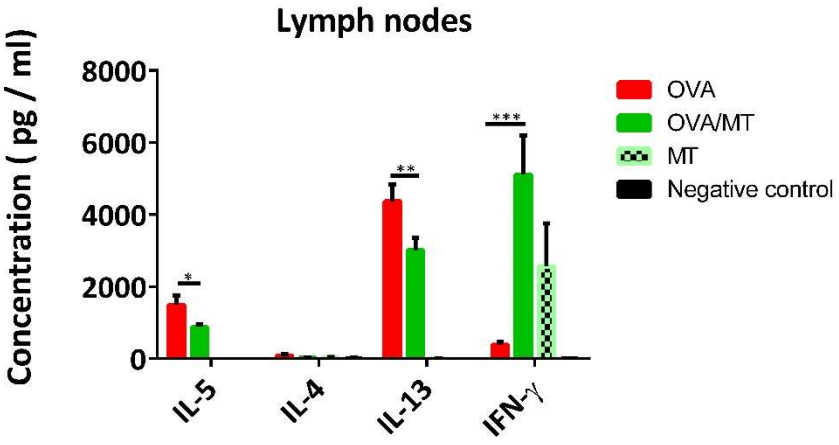


Figure 49. Cytokine concentration in supernatants from mediastinal lymph node cells incubated with OVA upon H37Rv infection in a model of acute asthma. Data are shown after subtracting baseline values obtained from unstimulated controls. Graph represents mean±SEM from pooled data of two experiments (n=12 mice for OVA-challenged and OVA/MT groups and mice=9 for the MT and negative groups). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

4.5 Mtb infection abrogates bone marrow eosinophil precursors

Previous studies demonstrated that newly produced eosinophils in the bone marrow substantially contribute to lung eosinophilia in OVA-induced asthma in mice [200]. Moreover, another study revealed that T cell-derived IFN-γ abrogated eosinophil production in the bone marrow [201]. Based on these results, it was evaluated whether eosinophilopoiesis was affected by Mtb infection under the experimental settings used in the present work (Figure 50).

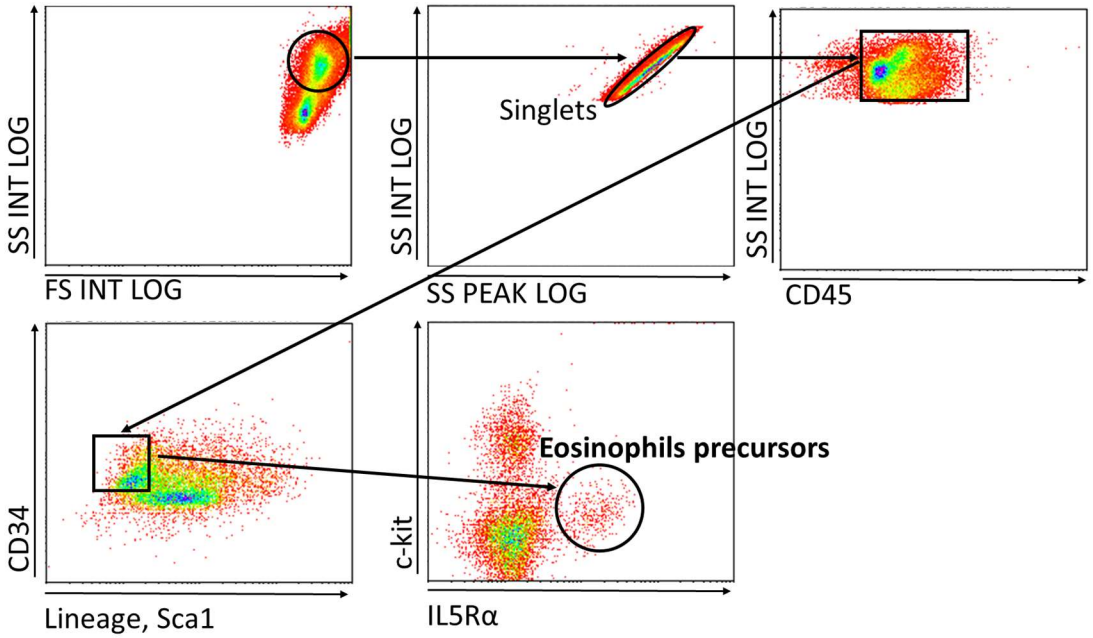


Figure 50. Gating strategy for determination of eosinophil progenitors in the bone marrow by flow cytometry. Eosinophils precursors in bone marrow, identified as Lin-Sca1-CD34+IL5Rα+cKit^{low}.

The results obtained in the present study indicated an increase of eosinophil precursors in the bone marrow following OVA challenge, as it had been previously described [200]. Remarkably, eosinophil progenitors were dramatically reduced in the H37Rv-infected mice, suggesting that immune response induced by TB infection profoundly affects eosinophilopoiesis in the bone marrow (Figure 51).

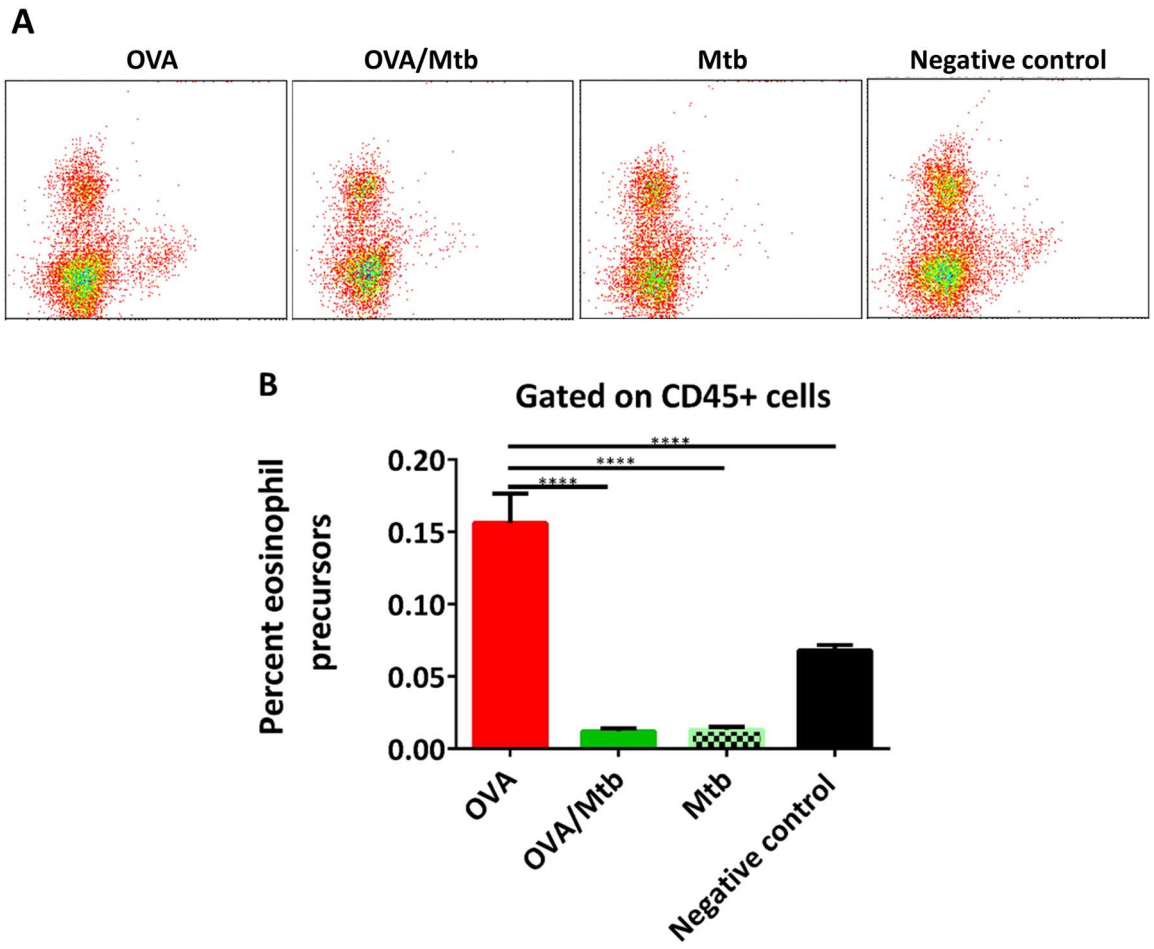


Figure 51. Mtb immunization abolished bone marrow eosinophil precursors. Eosinophil precursor comparative between groups identified by flow cytometry (A). Percentage of eosinophil precursors in bone marrow at day 45, quantified by flow cytometry (B). Graph represents mean±SEM from one experiment (n=5 mice). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

5. DISCUSSION

The present study reveals that the decrease in bone marrow eosinophil precursors induced by Mtb in OVA-challenged mice directly reduces the pathogenesis of asthma. Further, Mtb is able to reduce eosinophils in BAL, equivalent to a reduction in airways or sputum in humans. Clinically, it implies a reduction in the severity and in the number or exacerbations in asthmatics patients [202]. Moreover, it implies a reduction of the asthma symptomatology, as AHR and remodelling are consequences of eosinophils infiltration[203].

Following on from this, present data suggest that T cell-driven IFN-γ production in Mtb-infected OVA-challenged mice is not only generated by expanded lymphocytes that recognize Mtb antigens, but also by allergen-specific T cells. These cells seem to be re-educated from a Th2 to a Th1 profile due to the inflammatory environment associated with Mtb infection, as it is shown in the lymph nodes cytokines profile [197]. In this regard, switch from Th2 to Th1 profile has been previously described, and it is mediated by IL12 and interferons, cytokines classically associated with TB infection [204].

To sum up, the results exposed here provide the first evidence about the capacity of Mtb infection to impair eosinophil production at a bone marrow level. Studies in the literature indicate that Mtb can colonize bone marrow stem cells during LTBI [205]. Thus, Mtb could alter local inflammatory response in the host bone marrow, enhancing IFN-γ production, and impairing IL-5. It would have an impact on generation and release of eosinophil progenitors and therefore in restricting production of mature eosinophils that could be recruited to peripheral tissues upon allergen exposure in asthmatic individuals, which depend on IL-4, IL-13 and IL-5 [206].

These conclusions may explain the significant inverse correlation between asthma prevalence and TB notification cases observed in some epidemiological studies in humans. Regarding LTBI, a study performed in South Africa demonstrated a strong association between tuberculosis skin test (TST) positivity and lower prevalence of different types of allergy. Importantly, in the case of asthma, authors found a substantial reduction in asthma incidence in the segment of population with the highest TST value analysed in the study (>20mm), suggesting a correlation between magnitude of the TB-specific immune response and degree of unspecific protection [207]. More interestingly, the present study may be extrapolated to a clinical case, in which a patient of severe asthma, who was in hospital many times, evolved to a well-

controlled asthma diagnosis following pulmonary infection with Mtb [208]. Therefore, we could even hypothesise that Mtb may influence on established asthma.

The route of entry of Mtb is via the respiratory tract. After inhalation, the bacillus encounters and infects AM. If this first line of defense fails to eliminate the bacteria, there is a recruitment of immune cells to form a granuloma [186]. At this point, if the infected person is immune-competent, its immune system will be able to contain the infection, causing LTBI. At this latent state, TB is not transmissible but the tubercle bacilli are able to survive for years within the host [186]. If the granuloma fails to contain the infection, bacteria will disseminate to other organs and eventually they will re-enter the respiratory tract to be released. Therefore, the host become infectious and symptomatic, suffering from an active TB. Nowadays, it is estimated that 1,7 billion people suffer from LTBI, with only a small proportion (5-10%) expected to develop active TB over their lifetime[84]. Hence, here is the dual role of Mtb: it could be considered both as a pathogen in active TB, or a symbiont in 90% of individuals with LTBI. It could be speculated that selection of tolerance to latent TB throughout human evolution could have been favored due to unspecific protection conferred by Mtb-mediated Th1 response against certain Th2 cytokine-driven allergies, including asthma.

TB is an infectious disease that has co-evolved with humanity from early hominids in East Africa[209]. In fact, MTBC was originated in Africa and it underwent genetic diversification that corresponds to patterns of human migration [210]. Specifically, an early progenitor of Mtb was present in East Africa 3 million years ago, and it may have infected early hominids at that time[209].

Therefore, this coevolution already occurred when societies were rudimentarily composed of small groups of people dedicated to hunting and gathering [211]. In this regard, considering lifestyle of these ancient societies, asthma protection conferred by asymptomatic LTBI could have resulted advantageous to humans, particularly if we consider asthma-associated symptoms, which should be especially detrimental in individuals whose survival crucially depended on physical activity[197].

At present, multiple clinical trials in TB endemic countries are on-course to develop novel tools against this disease. Based on these results and the observational studies in the literature, asthma detection could be included as an additional exploratory endpoint in TB clinical trials providing highly valuable information. These trials represent an excellent opportunity to study in depth the relation between asthma and LTBI, and could help to elucidate the immunological bases of asthma protection induced by Mtb infection[197].

Chapter 2

PROPHYLACT AND THERAPEUTIC EFFICACY OF LIVE ATTENUATED MYCOBACTERIA AGAINST ASTHMA

La instrucción y cultura racional que la mujer adquiera, adquiéralas en primer término para sí, para desarrollo de su razón y natural ejercicio de su entendimiento.

Emilia Pardo Bazán

In the previous chapter, it was demonstrated that Mtb infection protects against allergic asthma in mice, through the rewiring of the pathological Th2 response responsible for asthma. In this sense, and supported by the literature, it was hypothesised that non-pathological Mycobacteria could also exert positive results against asthma. The clinical repercussion is clear, as attenuated Mycobacteria, already used as TB vaccines, could be also used against this allergic disease.

BCG, the current vaccine against TB, triggers a strong but not pathological Th1 response. In this regard, benefits of intradermal BCG vaccination for asthma have been studied for years [212], although with controversial results [133, 135]. BCG study has been extended to animal models, but testing only the vaccination prior or concurrently to allergen sensitization [120, 121]. MTBVAC, the first live attenuated Mtb vaccine that enter in clinical trials, also triggers a strong and controlled Th1 response, so it could also exert beneficial effects against asthma [113].

In the present study, it is evaluated the prophylactic and more importantly, for the first time, the therapeutic efficacy of BCG and MTBVAC, as the capacity of BCG to revert established asthma has never been addressed. In addition, it is compared the bacteria impact when administered the vaccine by different routes. Specifically, the intranasal route is studied in depth, since in Chapter I it has been proved to be efficient against asthma. In addition, in the present work it is evaluated for the first time the role of certain macrophages in the protection against asthma when using attenuated Mycobacteria.

6. RESULTS

6.1 Pre-sensitization administration of MTBVAC by the intranasal route confers better protection against asthma than the administration by the subcutaneous route

In the Chapter 1, it was demonstrated that Mtb is able to prevent asthma in mice. In this line, the next objective consisted in determinate whether attenuated Mycobacteria were able to prevent asthma, using the previously described OVA asthma model in mice. In this regard, in a pilot experiment, two reasonable routes of administration for the attenuated Mycobacteria were compared. On the one hand, the intranasal route was used, since it allowed the bacteria to reach the lung, where asthma was developing. On the other hand, the canonical subcutaneous route was used, as it is comparable to the intradermal route, which is used in clinic and so in epidemiological studies using BCG. Moreover, in order to enhance the immune response triggered by Mycobacteria administered by the subcutaneous route, which is systemic and it is not focused in the lungs, one or two doses were administered. The selected dose for the subcutaneous treatment was 10^5 CFU as it is similar to the clinical dose of intradermal BCG vaccination ($5x10^5$ CFU). For the intranasal treatment, it was used a dose of 10^6 CFU as it is closed to the clinical dose of BCG, and because in our laboratory, it has been demonstrated that intranasal BCG protection against TB is dose-dependent, being the dose of 10^6 CFU efficient.

Moreover, in this proof-of-concept experiment, the vaccine was administered before the sensitization, similar to what was done in experiments described in the literature using BCG as a potential asthma therapy (Figure 25).

The results revealed that the intranasal route was more effective than the subcutaneous in reducing the hallmarks of T2-asthma (eosinophilia and IL-5 in BAL), and so in preventing asthma (Figure 52).

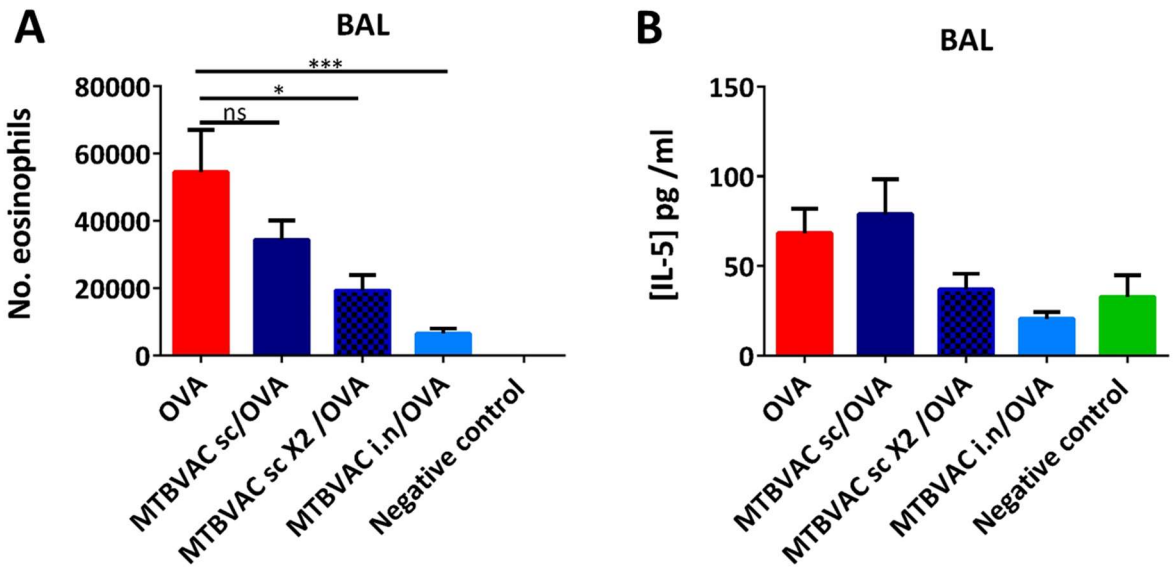


Figure 52. Comparative between intranasal and subcutaneous administration of MTBVAC prior to sensitization. Eosinophils were quantified in BAL by flow cytometry. There was no significant decrease in the group treated with one dose of subcutaneous vaccine when compared with the positive control and the biggest reduction occurred in the group treated with MTBVAC i.n (A). IL-5 was measured in BAL by the ELISA technique. Again, the biggest reduction comparing with the positive control was observed in the i.n-treated mice (B). sc: subcutaneous; i.n : intranasal; sc X2: two separated doses of subcutaneous MTBVAC. Data represent mean±SEM (n= 5 mice/group).*p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

6.2 Post-sensitization intranasal vaccination with MTBVAC or BCG prevents allergic asthma in mice

In individuals predisposed to asthma, the sensitization phase mostly occurred early in life, likely in new-borns. Therefore, it would be more reasonable to administer attenuated Mycobacteria, as prophylactic treatment against asthma, after the sensitization phase, so in children or adults. In this regard, the effect of MTBVAC and BCG in asthma was studied when administered after the sensitization phase (Figure 26) and one month later, mice were subjected to the challenge phase. The choice of vaccinating one month before the allergen challenge is because it is a standard period for the vaccine to induce a T cell response. As previously demonstrated, the intranasal route was more effective than the subcutaneous, so both vaccines were administered this way.

Many experiments were carried out to examine the prophylactic effect of these two vaccines in asthma, and the mainly markers for the follow-up of the protection were the eosinophilia and the Th2/Th1 cytokines. However, other significant asthma hallmarks were studied, such as the airway remodelling through the detection of

goblet cells and mucosubstances, the IgE concentration and the quantification of the Th1 and Th2 cells. All these data will be explained along this Chapter.

Eosinophils measurement by flow cytometry revealed a significant decrease in both group treated with BCG or MTBVAC, with respect to the OVA control group, and with values comparable to those in the negative control. Further, this patron occurred in BAL, as well as in lung (Figure 53). It implies that the vaccines are efficient in preventing eosinophilic asthma, a feature observed in all T2-type asthma and associated not only with the symptomatology of asthma but also with severity and exacerbations episodes. On the other hand, neutrophils and AM did not suffer significant variation in the vaccinated groups with respect to the positive group, suggesting that the vaccines do not disturb the other cell populations in the airways (Figure 53 A).

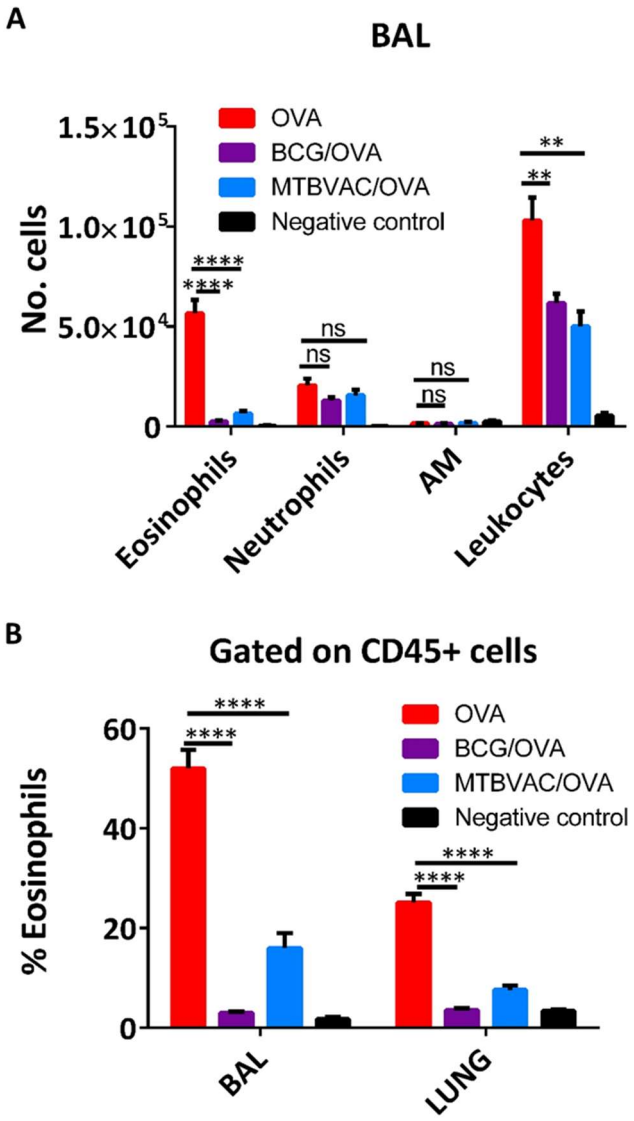


Figure 53. Myeloid cells in the airways in a model of acute asthma when vaccinated after the sensitization. Cells were quantified by flow cytometry in a model of OVA-induced acute

asthma, when vaccinated after the sensitization with a dose of 10^6 CFU, in BAL (A) and lungs (B). The positive control was compared with mice treated with MTBVAC or BCG and with the negative control. The eosinophilia was reduced with the vaccines, and the rest of cell populations showed similar values when compared positive and treated groups (A). Graph represents mean±SEM from pooled data of six experiments (n= 31 mice for OVA-challenged, 22 for MTBVAC/OVA, 33 for BCG/OVA, and 15 for negative groups). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

When comparing BCG and MTBVAC-treated groups, eosinophils were quite similar but still the BCG group showed a bigger decrease in the cell count than MTBVAC (Figure 54). Later in this chapter this aspect will be discussed.

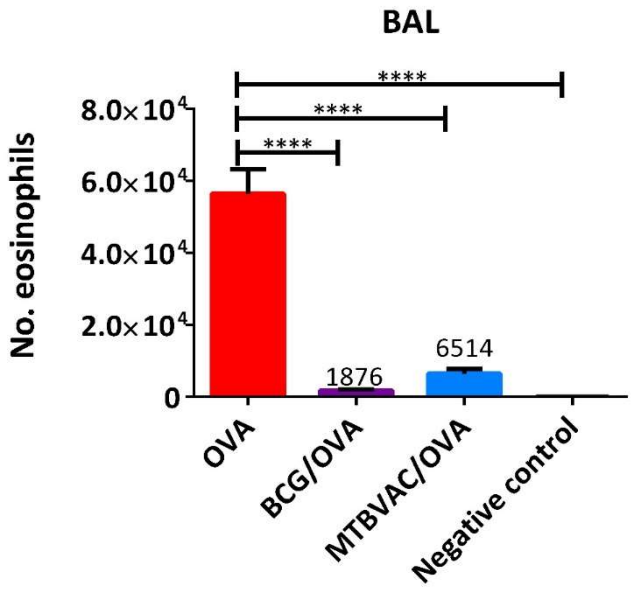


Figure 54. Comparison in the eosinophilia in BAL between BCG and MTBVAC treated groups. Cells were quantified by flow cytometry in a model of OVA-induced acute asthma, when vaccinated after the sensitization with a dose of 10^6 CFU. Eosinophils mean count is showed in the graph: there were an average of 1876 eosinophils in the group treated with BCG and an average of 6514 in the group treated with MTBVAC. Graph represents mean±SEM from pooled data of six experiments (n= 31 mice for OVA-challenged, 22 for MTBVAC/OVA, 33 for BCG/OVA, and 15 for negative groups). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

6.3 Th2 response induced by allergen exposure repolarizes to Th1 following intranasal MTBVAC or BCG administration

In order to better characterise the immune response underlying the allergic asthma model and the protective response conferred by the attenuated Mycobacteria, cytokines were quantified in BAL and lung explants (Figures 55 and 58). Moreover, allergen-specific response was also studied following *ex vivo* OVA stimulation of mediastinal lymph node cells when treated with BCG (Figures 55, 56 and 57) or MTBVAC (Figure 58).

Concerning BCG administration, the vaccine reduced the production of Th2 cytokine (IL-5, IL-13 and IL-4) with respect to the OVA control, and in contrast, it increased the production of the most important Th1 cytokine, the IFN-γ, in BAL, lungs and lymph nodes (Figure 55).

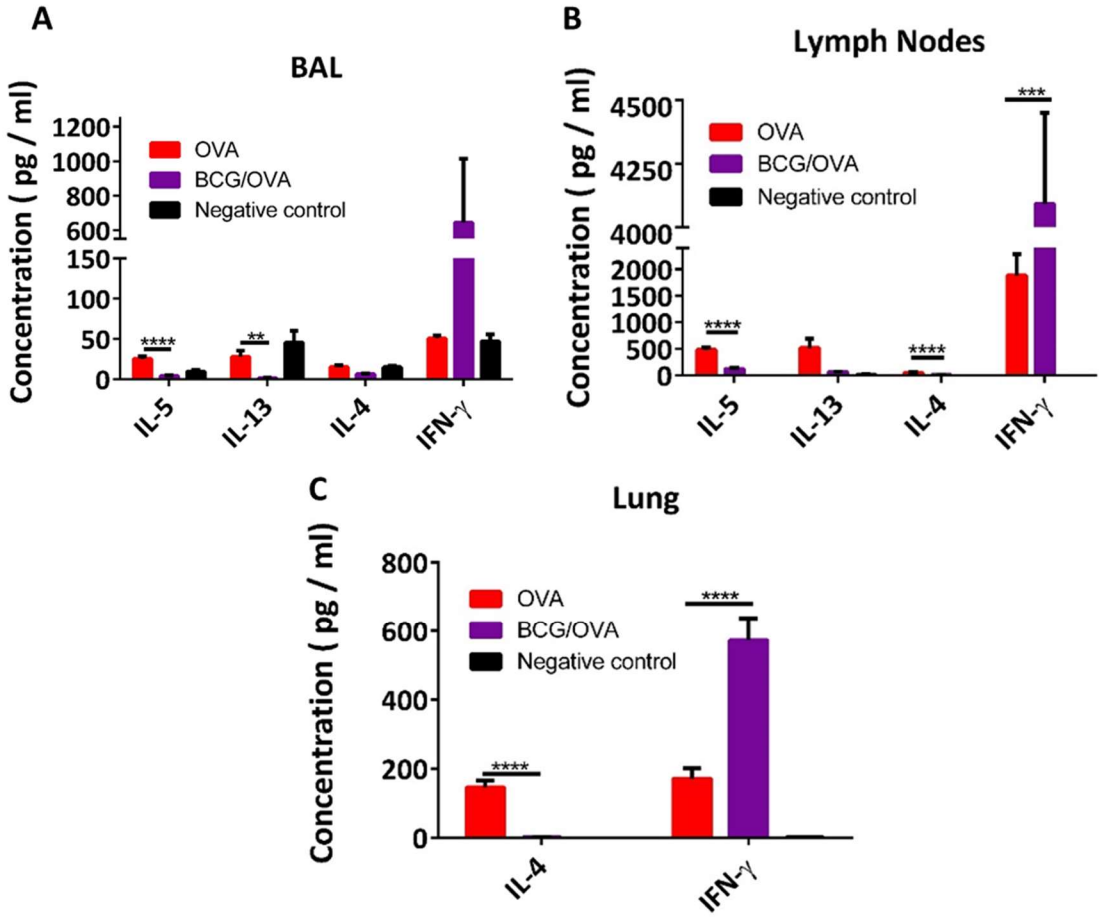


Figure 55. Cytokines production in BAL, lymph nodes and lung in a model of acute asthma when vaccinated with BCG. Cytokines were quantified by ELISA in a model of OVA-induced acute asthma, when vaccinated after the sensitization with a dose of 10^6 CFU. Cytokines in BAL were measured in the supernatant of the BALF(A). In the lymph nodes, cytokines were quantified after cell stimulation with OVA for 7 days. Data are shown after subtracting baseline values obtained from unstimulated controls (B). In lung explants, cytokines were analysed in the supernatant after 24 h of incubation (C). Graph represents mean±SEM from pooled data of different experiments. In BAL, data came from at least 11 mice for IL-5 and from at least 5 mice for the IL-13 and for the IL-4. In the lymph nodes, data came from 17 mice per group for IL-5, from 5 mice per group for the IL-13, and from 22 mice per group for the IL-4 and the IFN-γ. In lung explants, data came from 17 mice per group. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

Further, in the mediastinal lymph nodes, using the memory marker CD44, CD4+ lymphocytes producing IL-5 or IFN-γ after OVA or CD3 CD28 stimulation were quantified, so the former are supposed to be Th2 cells and the latter Th1 cells (Figures 56). After both stimulations, IL-5 producing cells decreased in the treated group with

respect to the positive control, whereas IFN-γ producer cells increased in the BCG-treated group (Figure 57 A and 57 B).

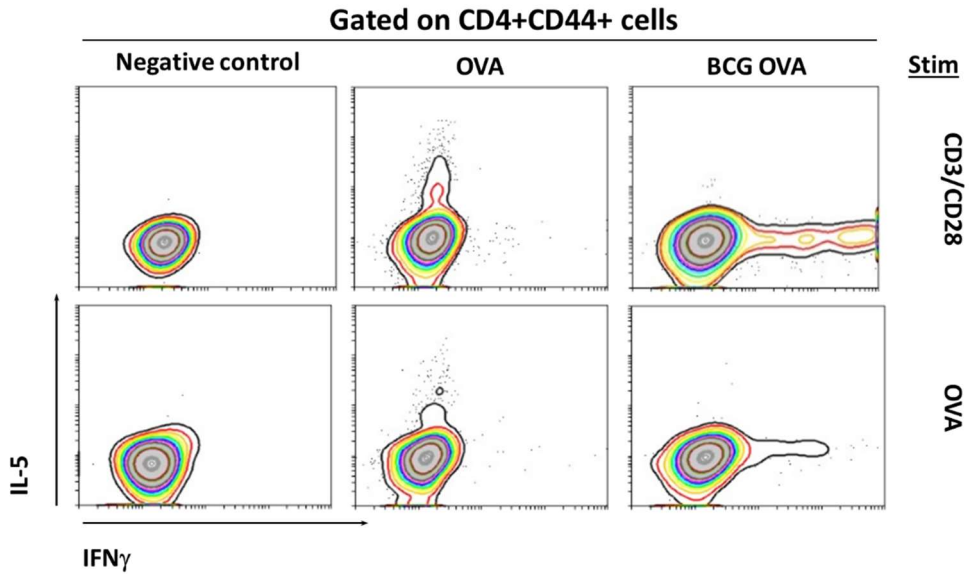


Figure 56. Gating strategy of IL-5 and IFN-γ producer cells in mediastinal lymph nodes in a model of acute asthma. Cells were analysed in lymph nodes by flow cytometry after ICS with IL-5 and INF-γ, characterising the Th2 and Th1 cells, respectively.

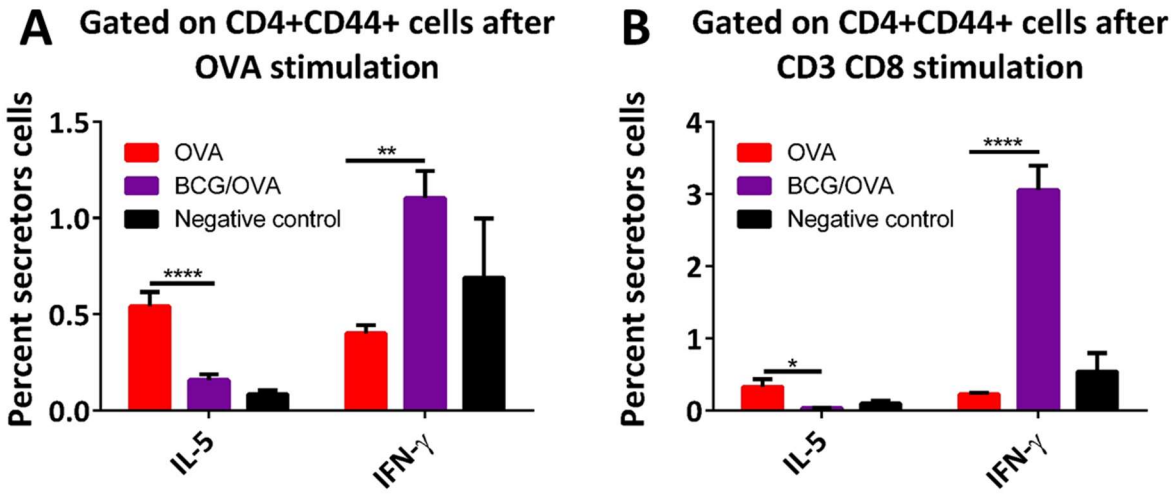


Figure 57. Mechanism of asthma protection conferred by BCG in a model of acute asthma. Percent of CD4+ lymphocytes producing IL-5 or IFN-γ after OVA (A) or CD3 CD28 (B) stimulation relative to T CD4+ CD44+ lymphocytes in mediastinal lymph nodes. Data from a model of acute asthma, when vaccinated after the sensitization with a dose of 10^6 CFU. Graph represents mean±SEM from pooled data of 1 (A) or 2 experiments (B) (n= 5-10 mice for OVA-challenged, 5-11 for BCG/OVA, and 3-6 for negative groups). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

On the other hand, concerning the experiments using MTBVAC, the OVA-challenged group revealed a substantial production of Th2 cytokines in BAL and in lymph nodes (where cytokines were OVA-specific and so produced by OVA-specific T cells), in

the absence of IFN-γ response. Conversely, MTBVAC drove to an inversion of this profile, with a reduction of IL-5, IL-4 and IL-13 levels, and a dramatic increase of IFN-γ response (Figure 58).

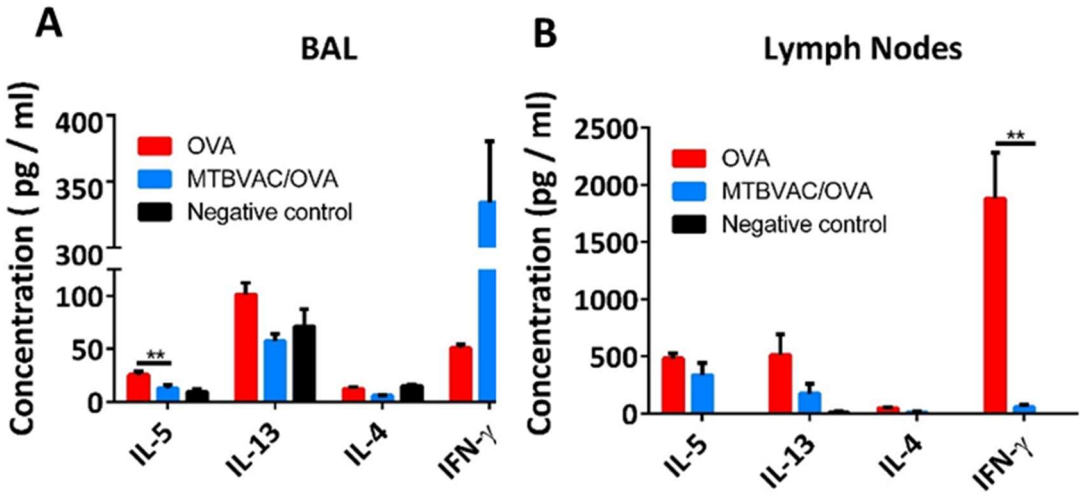


Figure 58. Mechanism of asthma protection conferred by MTBVAC in a model of acute asthma. Cytokines were quantified by ELISA in a model of OVA-induced acute asthma, when vaccinated after the sensitization with a dose of 10^6 CFU. Cytokines in BAL were measured in the supernatant of the BALF, IL-5 graph represents mean±SEM from pooled data of 3-5 experiments (n= 27 mice for OVA-challenged, 17 for MTBVAC/OVA, and 11 for negative groups), the other cytokine graphs represent data for 1 experiment (n= 5 mice per group) (A). In the lymph nodes, cytokines were quantified after cell stimulation with OVA for 7 days, IL-4 graph represents mean±SEM from pooled data of 3-5 experiments (n= 27 mice for OVA-challenged, 17 for MTBVAC/OVA, and 11 for negative groups), the other cytokine graphs represent data for 1 experiment (n= 5 mice per group) (B). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

Next, it was studied the balance of pulmonary Th1 and Th2 responses in the lung by analysing the RNA. The aims were to confirm the results obtained by ELISA and to analyse the expression of genes that would encode proteins that cannot be detected nor measured by ELISA. Gene expression analysis revealed different profiles depending on BCG immunization. In consonance with the previous results, vaccination clearly induced Th1-associated genes as *Ifng, Il12a*, or the transcription factor T-bet. Conversely, BCG down modulated genes codifying for typical Th2 cytokines and chemokines, such as IL-5, IL-4, IL-13 or CCL-1 (Figure 59).

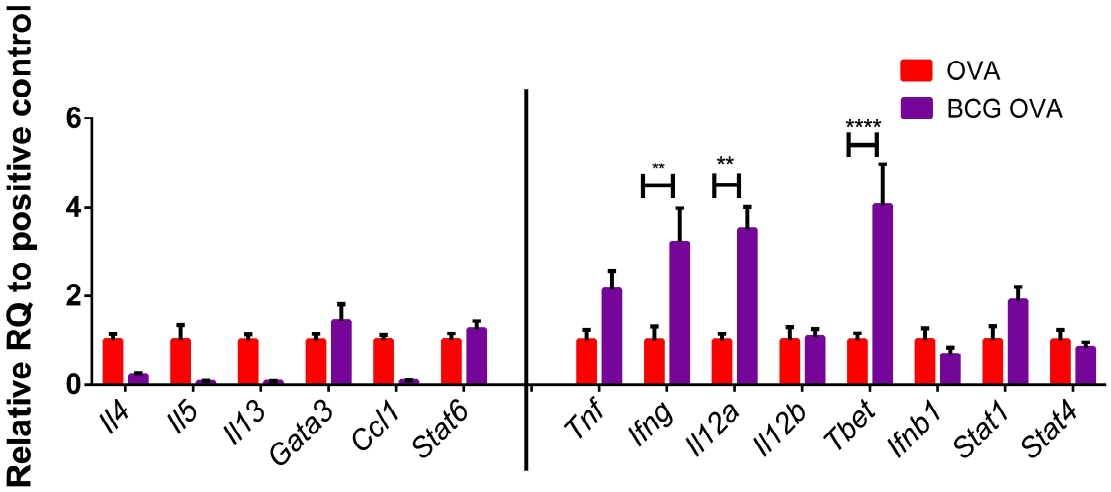


Figure 59. Th1 and Th2 activation markers measured by qRT-PCR upon vaccination in a model of acute asthma. RNA from lungs was analysed in OVA-challenged mice, untreated or treated with BCG one week after sensitization. Common Th2 genes are represented on the left and typical Th1 genes on the right. Data represent means±SEM from 1 experiment with 6 mice per group. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, by multiple t-student test.

To sum up, the positive control is characterized by an exacerbation of the Th2 immune profile that switches to a Th1 profile after the vaccination with attenuated Mycobacteria, BCG or MTBVAC. It implies that the vaccination re educates the immune system to a non-allergic profile. Likely, T cell-driven IFN-γ production in vaccinated OVA-challenged mice is not only generated by expanded lymphocytes that recognize vaccines antigens, but also by allergen-specific T cells, that seem to be re-educated from a Th2 to a Th1 profile due to the inflammatory environment associated with the vaccination.

6.4 BCG and MTBVAC reduce the airway remodelling in a model of OVA induced-acute asthma

Another point consisted in characterizing the airways remodelling. Lungs were stained with the PAS technique, showing in dark purple the mucosubstances (Figure 60). In the control with the allergen, there were many regions with mucus, overall in the bronchioles, and many regions with goblet cells in a multilayer disposition. With the treatment with BCG or MTBVAC, the goblet cells showed a single layer disposition and so the mucus production was considerably reduced.

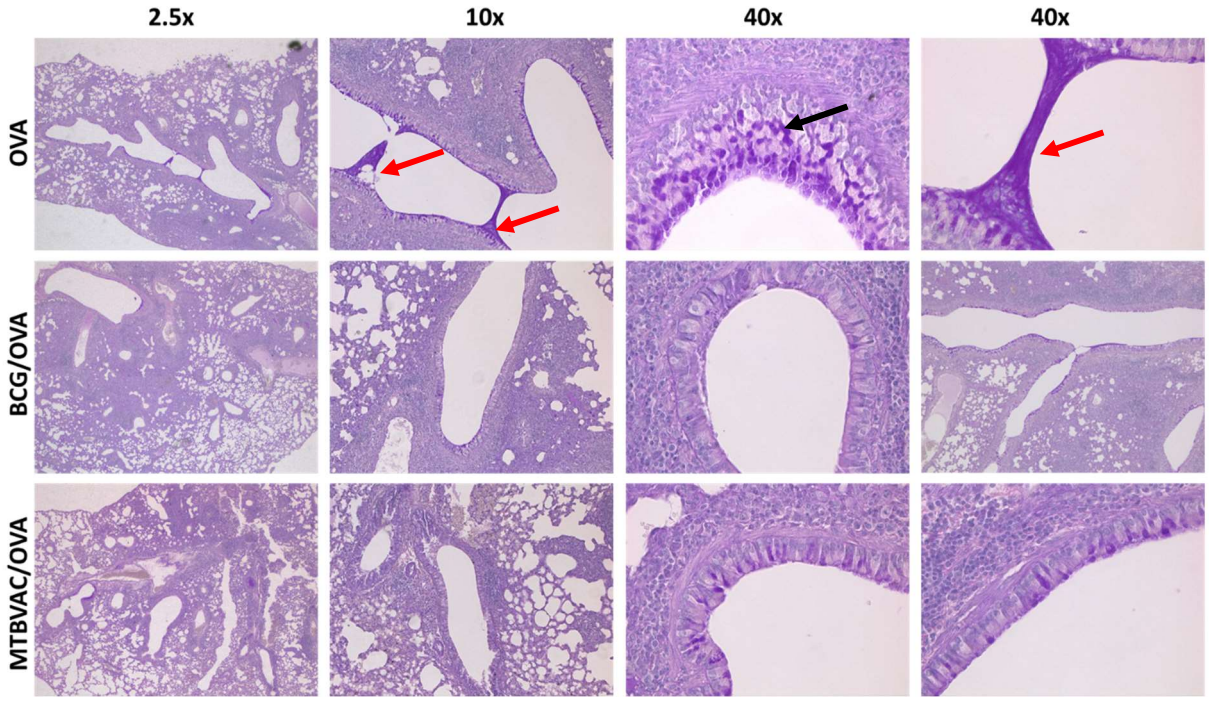


Figure 60. Representative images of PAS-stained fixed lungs from OVA-challenged mice and vaccinated OVA-challenged mice. Lungs were histologically studied in a model of OVA-induced acute asthma, when vaccinated after the sensitization with a dose of 10^6 CFU. Mucosubstances are stained in purple, showing many region with mucus (red arrow) and goblet cells in a multilayer disposition producing the mucus (black arrows). Images are taken with lens of 2.5, 10 and 40 magnification.

Briefly, both BCG and MTBVAC are able to revert asthma features, such as eosinophilia, Th2 cytokines and airway remodelling. However, the effect against asthma exerted by BCG was slightly stronger than the exerted by MTBVAC. Later, it will be deeply explained and discussed.

6.5 IgE production is not induced in the acute asthma model neither it is reduced with the vaccination

IgE production was also studied, when treated after the sensitization, and there was no differences between the positive control and the treated groups, either not a clear induction in OVA-challenged mice, so no more IgE detection was carried out later in this study (Figure 61). The lack of differences between groups could be due to different causes: the model is not able to induce a high IgE production or the kit available in the laboratory is not good enough to identify small IgE differences. Another explanation for the fact that there were no differences between positive and treated groups is that the vaccine is acting after the sensitization phase, as it is administered then, and the IgE production occurs during the sensitization.

However, the lack of IgE reduction with the treatment does not seem to be important. The treatment is a strong suppressor of eosinophilia, airway remodelling and Th2

response, which are down-stream the production of IgE in the allergic cascade and direct causes of the symptomatology. Therefore, the reduction of these allergic markers implies that even if IgE were produced, it would not cause allergic effects as the steps after it are modulated.

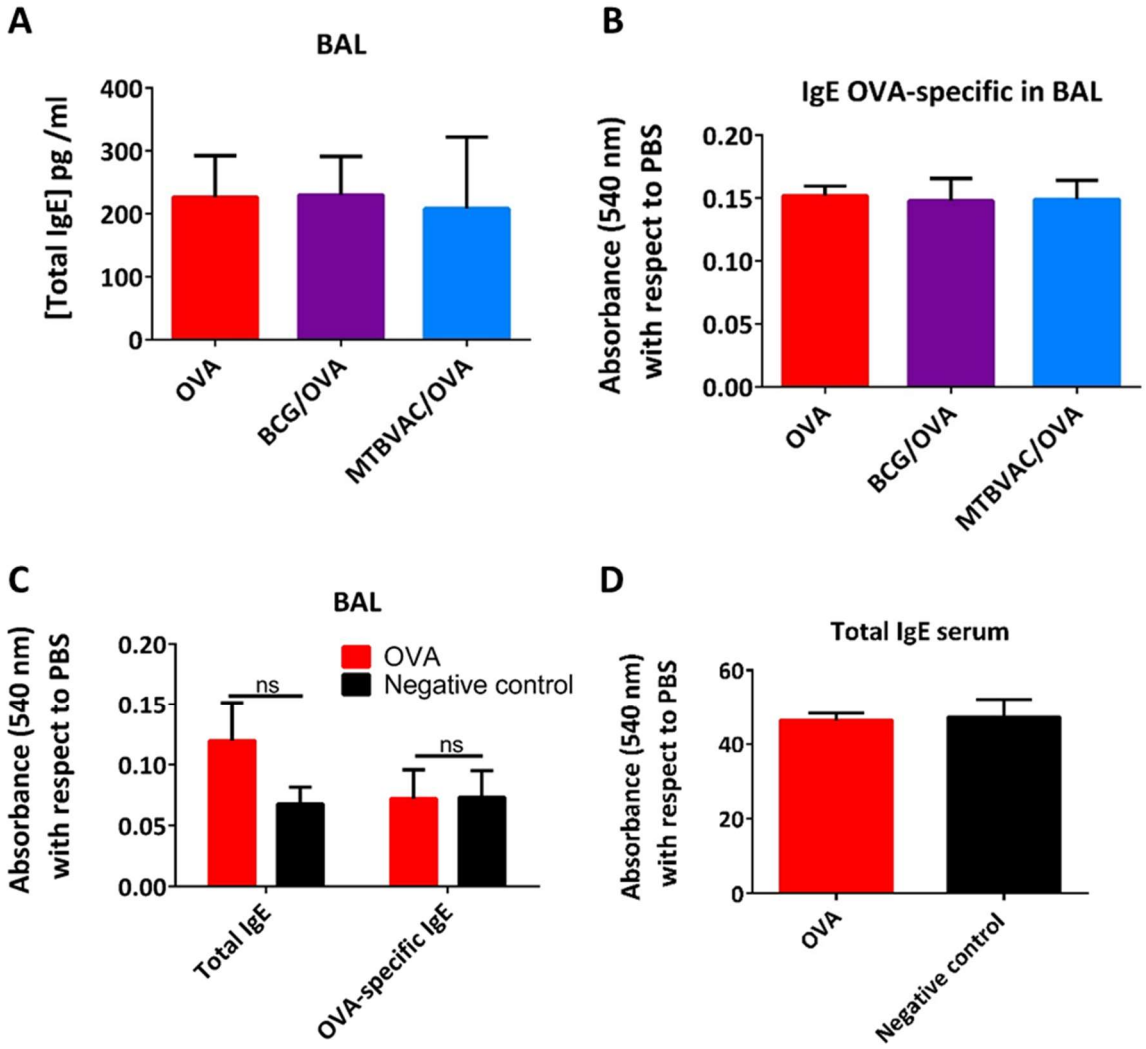


Figure 61. IgE in BAL and serum in a model of acute asthma. Total IgE in BAL (A,C) and OVA-specific IgE in BAL (B,C) were measured by ELISA, and total IgE were measured in serum (D) in a model of OVA-induced acute asthma, when vaccinated after the sensitization with a dose of 10^6 CFU. There were no differences in IgE production between groups. Data represent mean±SEM from 5 mice per group, and they were analysed by unpaired single t-student test.

6.6 Treg cell number are not altered by attenuated Mycobacteria

The role of Treg cells is not clear in asthma. There are evidences claiming that a lack of Treg is associated with high risk of asthma whereas other studies show that Treg cells are more elevated in asthmatic than in healthy children[45].

In the present work, Treg cells were analysed in BAL, lymph nodes and lungs (Figure 62) to elucidate their role in the protection against asthma conferred by BCG and MTBVAC.

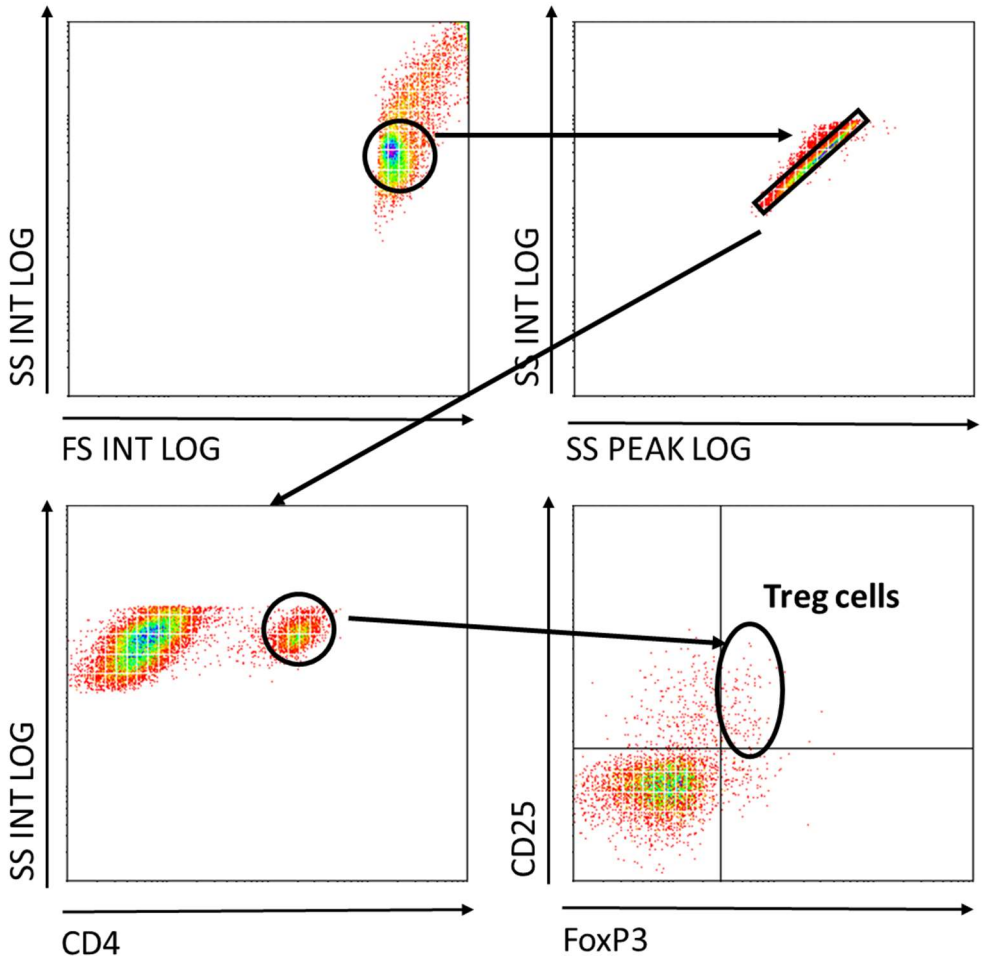


Figure 62. Gating strategy for identification of Treg cells by flow cytometry. Treg are defined as CD4+CD25+FoxP3+ cells.

The percent of Treg in lymph nodes (Figure 63 A) and lungs (Figure 63 B) did not show differences between the positive control and the vaccinated groups. Moreover, in BAL there was a very low number of Treg cells detected (between 0-10) to consider an effect of this population (data not shown). These data suggest that attenuated Mycobacteria do not trigger their protective effect through the induction of these cells.

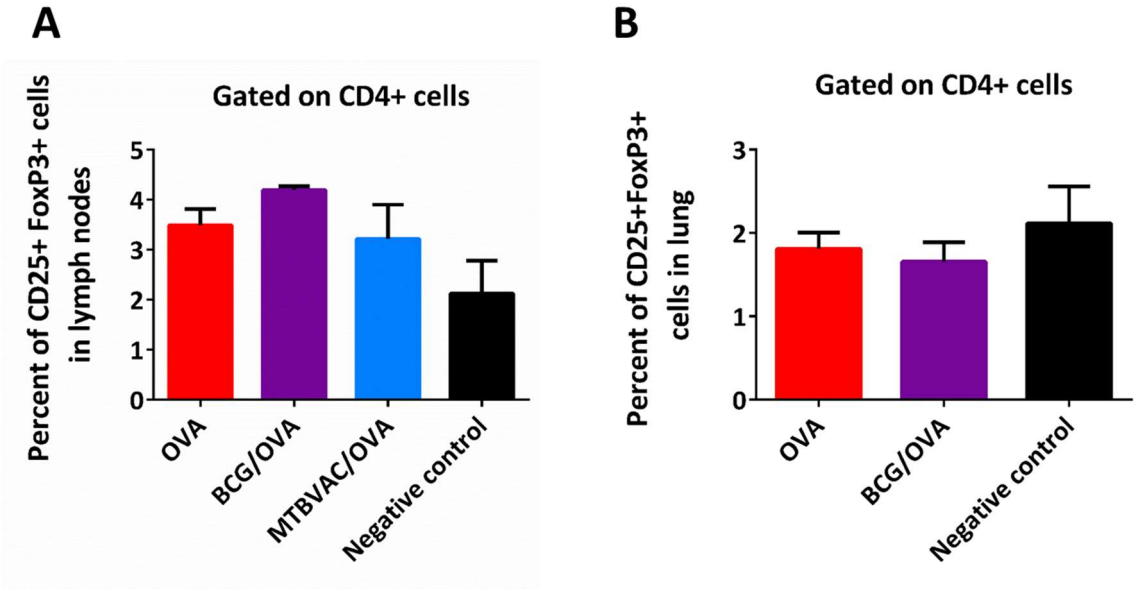


Figure 63. Treg cells are not implicated in the protection conferred by attenuated Mycobacteria against asthma. Treg cells in lymph nodes (A) and Treg cells in lungs (B) were quantified in a model of OVA-induced acute asthma, when vaccinated after the sensitization with a dose of 10^6 CFU. There were no differences in the percent of Treg between groups. Data represent mean±SEM from 5 mice per group.

6.7 B regulatory cells secreting IL-10 are not implicated in the protection against allergic asthma conferred by attenuated Mycobacteria

During the last decade, it has been hypothesized that a subset of B cells, referred as B regulatory cells (Breg), could modulate the immune response through the production of anti-inflammatory cytokines, often IL-10, and through the expression of inhibitory molecules. Specifically, the best-described Breg cell is the IL-10 secreting B10 cell (CD5 + and CD1d hi). Their modulatory effects have been demonstrated in a variety of murine models of chronic inflammation, including asthma [213], where Breg cells appear to be involved in mediating allergen tolerance [214].

In the present work, Breg cells were analysed to clarify their role in the protection exerted by the vaccines against allergic asthma. They were quantified by flow cytometry in both BAL and lung (Figure 64).

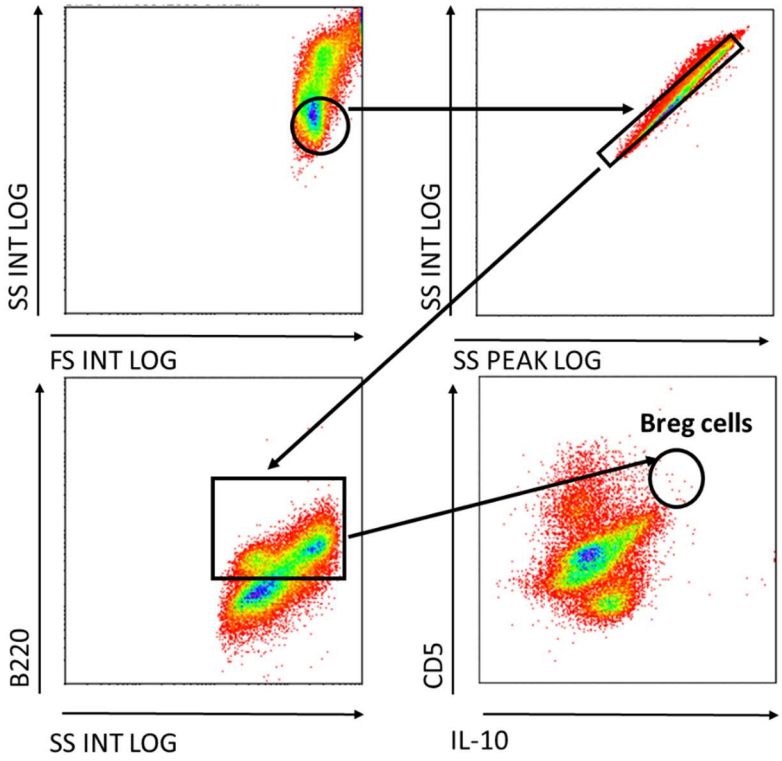


Figure 64. Gating strategy for the identification of Breg cells in lung by flow cytometry. Breg are defined as B220+CD5+IL-10+ cells.

In BAL, there was a very low amount of Breg, between 0 and 10 cells (data not shown) so they do not infiltrate in OVA-challenged mice nor in vaccinated OVA-challenged mice. On the other hand, in lung, there were very few cells and the percent of Breg cell did not show significant variation when compared the positive and the vaccinated groups (Figure 65).

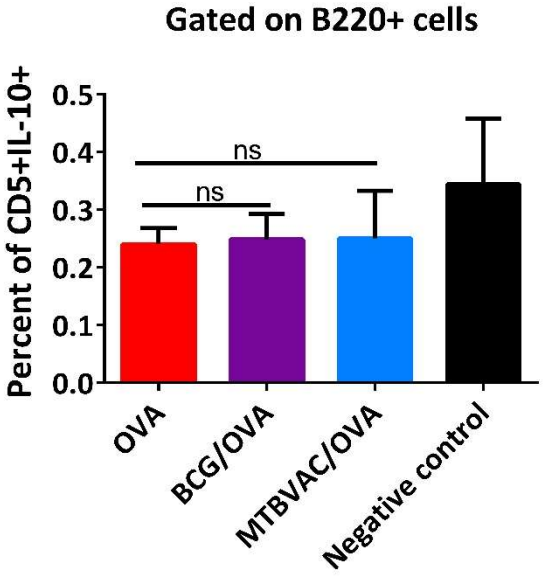


Figure 65. Study of the implication of Breg cells in the model of allergic asthma and in the protection conferred by attenuated Mycobacteria. Breg cells were analysed at day 45 by flow cytometry in lung, in a model of OVA-induced acute allergic asthma, when vaccinated

after sensitization with 10^6 CFU. Breg cells were defined as B220+CD5+IL-10+. Data represent mean±SEM from 5 mice per group. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

6.8 IgA may play a partial role in the protective effect exerted by attenuated Mycobactetia against allergic asthma

The antibody IgA can occur as a monomeric, dimeric or polymeric form. All these different forms are mainly found in the circulation, while secretory IgA (sIgA) is only found at mucosal surfaces. The sIgA is generated by the binding of dimeric IgA to the polymeric immunoglobulin receptor (pIgR) at the basolateral side of the epithelium, which results in the IgA release at the mucosal surface (lumen) by cleavage from the pIgR [215].

IgA is classically known for neutralizing toxins and bacteria (viruses) at mucosal surfaces [216] but it may be important in asthma too, as allergic disorders appear to be more common among patients with IgA deficiency [217, 218]. Indeed, there are lower IgA levels in BAL of severe asthmatics than in healthy subjects, which correlates with lung function and asthma symptoms. In mice, some experiments demonstrated that treatment with IgA protected mice against the development of eosinophilic airway inflammation [219]. As a conclusion, there may exist an inverse relation between IgA and allergy development, suggesting a protective role for IgA in allergic diseases such as asthma.

IgA could act different ways to protect against asthma. The main role would consist in the involvement of sIgA in the establishment of local interactions with bronchial mucus, thereby contributing to the "trapping" and removal of the antigen or allergen ("immune exclusion"). Furthermore, when IgA complexes are internalised and presented by monocytes, DCs mature and produce the regulatory IL-10. Finally, shifting the allergen-specific antibody response from IgE to IgA2 would result in the neutralization of the allergen in the mucosal lumen, before it could interact with IgE [220].

However, it has been also hypothesized that IgA may aggravate the inflammation, since eosinophils express receptors for IgA that can activate the cells upon binding of IgA immune complexes [221], resulting in the eosinophil degranulation [222].

Taken this into account, in the present work it was studied the involvement of IgA in the protection triggered by MTBVAC and BCG against allergic asthma. First, IgA levels were quantified in BAL, revealing an increase in the vaccinated OVA-challenged mice when compared with the OVA control (Figure 66). It suggests that IgA may be implicated in the protection of asthma upon Mycobacteria.

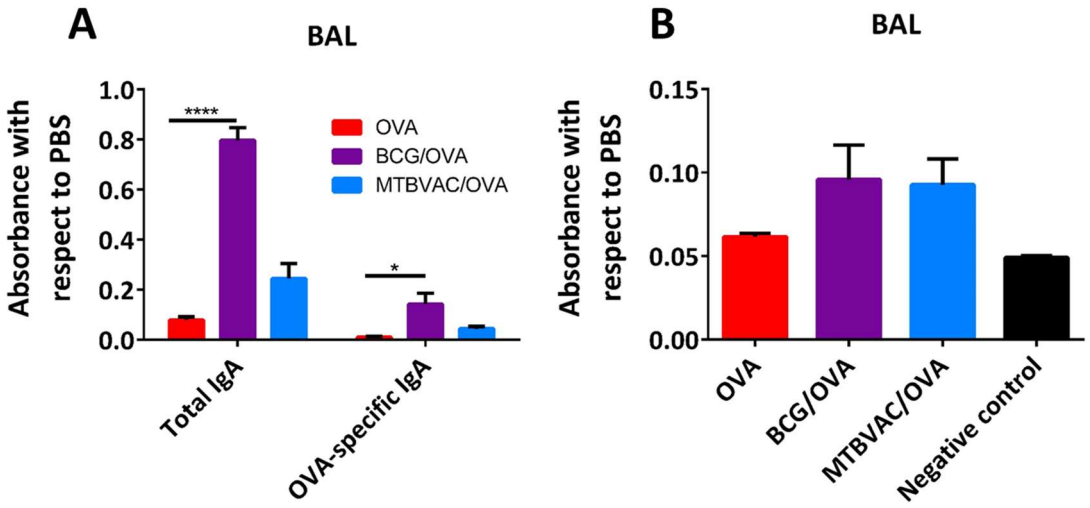


Figure 66. Implication of IgA in the protection of allergic asthma mediated by attenuated Mycobacteria, in two independent experiments. Each graph represents an independent experiment but both were carried out in an OVA-induced acute asthma model in mice, when vaccinated after the sensitization with 10^6 CFU. Total or OVA-specific IgA in BAL were measured by ELISA, data indicate the relative absorbance with respect to a PBS sample. Data represent mean±SEM from 5 mice / group (A). In another experiment, total IgA in BAL were measured by ELISA, data indicate the relative absorbance with respect to a PBS sample. Data represent mean±SEM from 4 mice in the OVA control, 6 mice in the BCG or MTBVAC-treated groups, and 2 mice in the negative control (B). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

Further, IgA secretory B cells were quantified in BAL by flow cytometry. In a consistent manner to what was explained above, these cells were increased in vaccinated OVA-challenged mice (Figure 67), and it correlated with the decrease in the eosinophil infiltration.

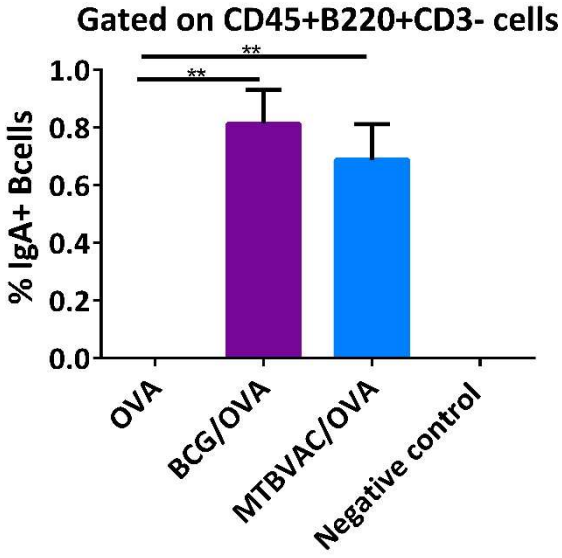


Figure 67. Increase in IgA+ cells upon attenuated Mycobacteria vaccination in a model of acute asthma. IgA secretory B cells were identified in lung by flow cytometry as CD45+ B220+ IgA+ CD3-. Data represent mean±SEM from 4 mice in the OVA control, 6 mice in the BCG or MTBVAC-treated groups, and 2 mice in the negative control (B). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

Since these results suggested a potential implication of IgA in the protection of asthma by Mycobacteria, it seemed reasonable to continue with an experiment in IgA-/- KO or pIgR-/- KO mice to confirm the role of IgA in the beneficial effects against asthma.

Firstly, immune markers were characterised in a model of OVA-induced acute asthma in IgA-/- KO mice. Eosinophils (Figure 68), IL-5 (Figure 69A) and IFN-γ (Figure 69B) were analysed as main allergic markers in the model. However, the eosinophilia and the IL-5 concentration still decreased and the IFN-γ increased as well upon treatment. These data suggest that the presence of IgA is not crucial for the protection against asthma mediated by attenuated Mycobacteria.

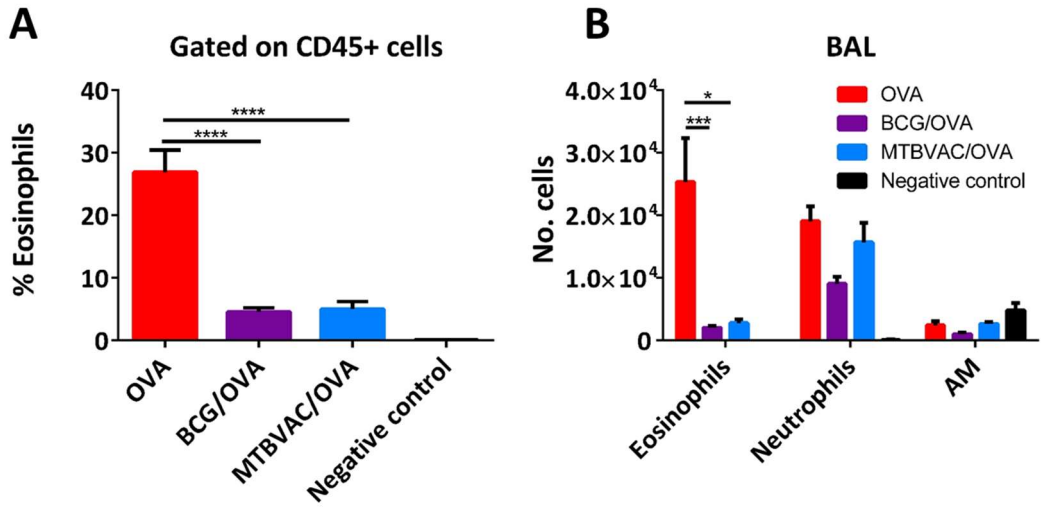


Figure 68. Myeloid cells in BAL suggested that IgA were not crucial for vaccines-mediated protection against asthma. The experiment was carried out in an OVA-induced acute asthma model in IgA-/- KO mice, when vaccinated after the sensitization with 10^6 CFU. Percent of eosinophils in BAL was measured by flow cytometry. Data showed a clear eosinophilia in the positive control in BAL, which was significantly reduced when treated (A). Eosinophils, neutrophils, and AM were quantified in BAL by flow cytometry. The eosinophilia was reduced with the vaccines, and the rest of cell populations remained similar when compared positive and treated groups (B). Graph represents mean±SEM from pooled data of two experiments (n= 12 mice for OVA-challenged, 12 for BCG/OVA, 4 for MTBVAC/OVA, and 7 for the negative group). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

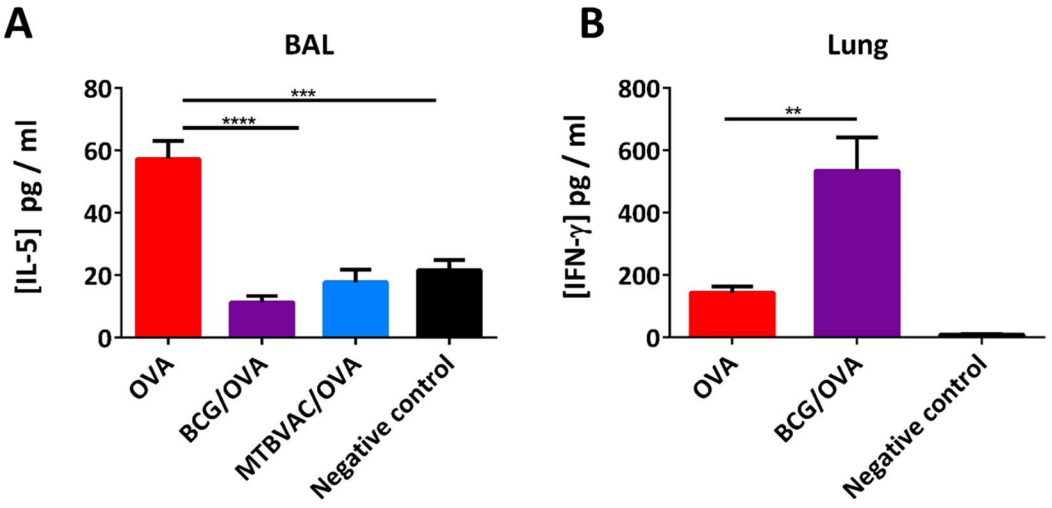


Figure 69. Cytokine production in a model of OVA-induced acute asthma in IgA-/- KO mice. The experiment was carried out in an OVA-induced acute asthma model in IgA-/- KO mice, when vaccinated after the sensitization with 10^6 CFU. IL-5 in BAL was measured by ELISA. Graph represents mean±SEM from pool data of 2 experiments (12 mice in OVA control and BCG treated groups, 4 mice in MTBVAC group and 7 mice in the negative control) (A). IFN-γ was measured in lung explant. Data represent mean±SEM from 6 mice in the OVA control and BCG-treated groups and 3 mice in the negative control (B). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

However, as previously seen, IgA levels in WT mice were elevated in the treated groups. It may imply that IgA antibodies play a partial role in the protection but without being essential for it or it could mean that the lack of IgA may be compensated with an increase on IgM. By its part, IgM could act as IgA "trapping" the allergen and reducing the allergic reaction. In this regard, IgM was analysed in BAL (Figure 70). Interestingly, both total IgM and OVA-specific IgM increased in BAL in the treated groups with respect to the positive control, suggesting that they could be replacing the IgA. At that point, the role of IgA in the protection against asthma was not clear, but it could be hypothesized that it was playing a partial role.

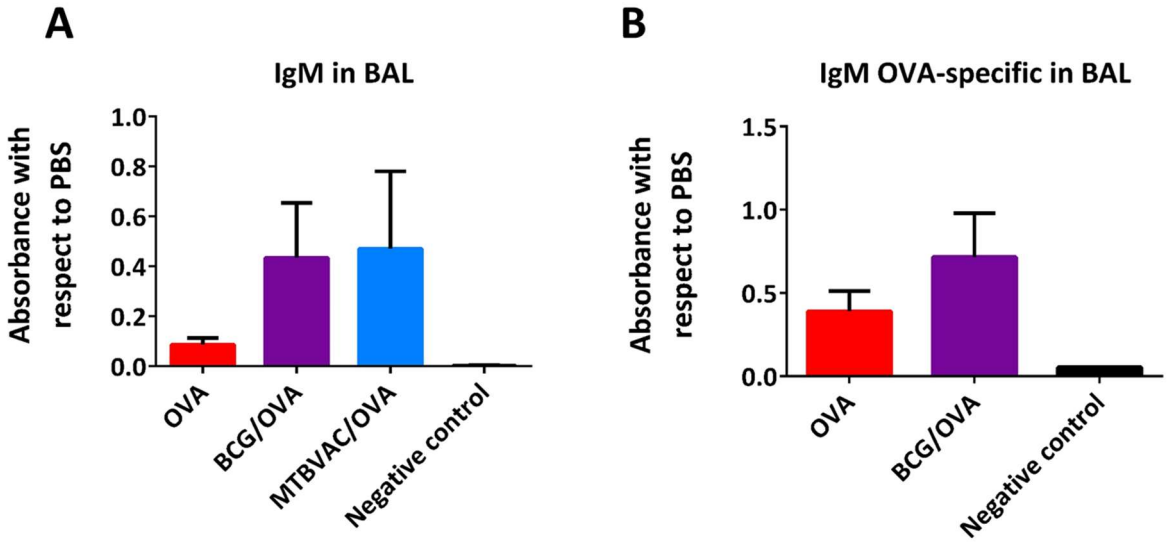


Figure 70. IgM may be compensating the lack of IgA in the partial protection against asthma. The experiment was carried out in an OVA-induced acute asthma model in IgA-/- KO mice, when vaccinated after the sensitization with 10^6 CFU. Total IgM in BAL was measured by ELISA. Data represent mean±SEM from 6 mice in the OVA-challenged and BCG treated groups and 4 mice for MTBVAC/OVA and negative group (A). OVA-specific IgM in BAL was measured by ELISA. Data represent mean±SEM from 6 mice in the OVA-challenged and BCG treated groups and 3 mice for the negative group (B) *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

Trying to elucidate the role of IgA, pIgR-/- KO mice were subjected to a model of OVA-induced allergic asthma, and vaccinated after the sensitization with 10^6 CFU. pIgR are important for the effects mediated by IgA in lungs as they actively translocate the dimeric IgA from the lamina propria to the lung lumen (where IgA can exert the "trapping" to the allergen). Importantly, these IgA can be allergen-specific as plasmatic cells resident in the lamina propria produce them. Eosinophilia and IL-5 (Figure 71) were measured in BAL and again they were reduced with the treatment, revealing that pIgR are not essential for attenuated Mycobacteria mediated protection.

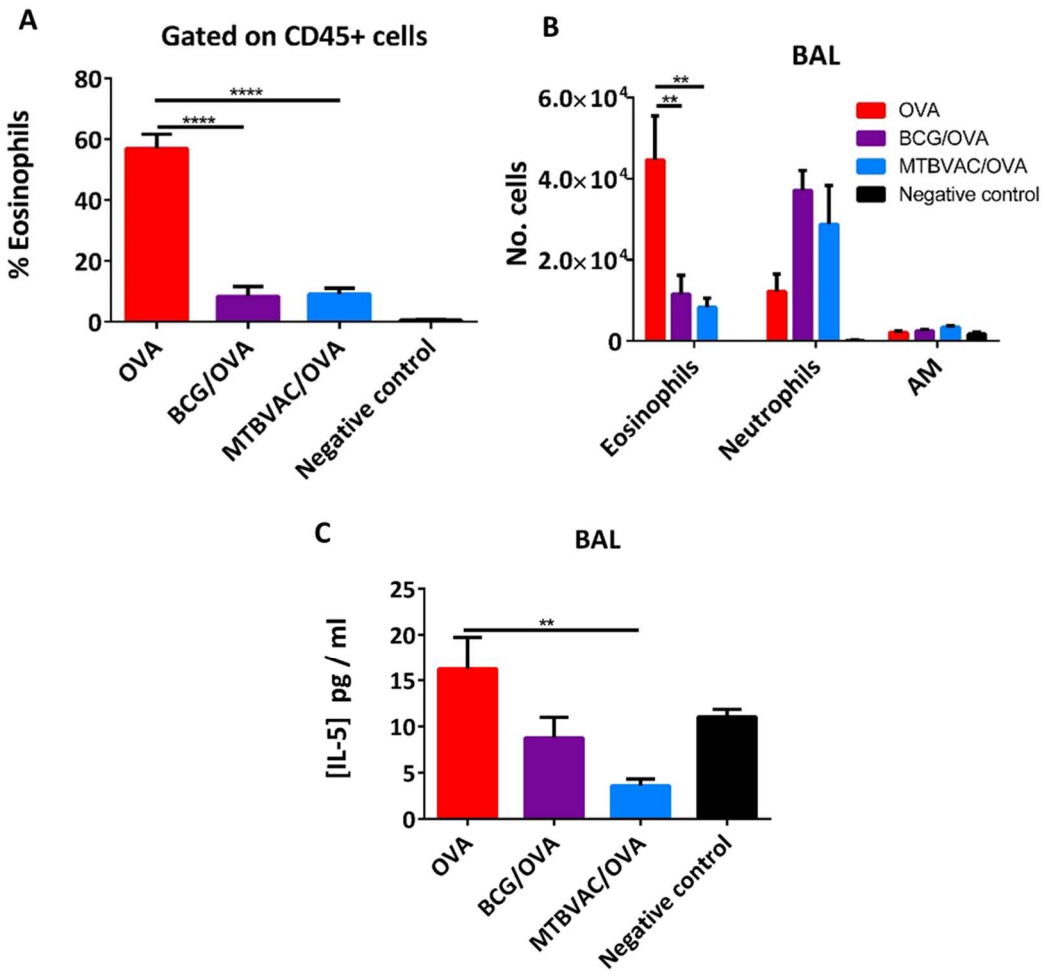


Figure 71. Myeloid cells and IL-5 in BAL suggested that pIgR are not crucial for vaccines-mediated protection against asthma. The experiment was carried out in an OVA-induced acute asthma model in pIgR-/- KO mice, when vaccinated after the sensitization with 10^6 CFU. Percent of eosinophils in BAL measured by flow cytometry. Data showed a clear eosinophilia in the positive control in BAL, which was significantly reduced when vaccinated (A). Eosinophils, neutrophils and AM were quantified by flow cytometry. The eosinophilia was reduced upon vaccination (B). IL-5 in BAL was measured by ELISA (C). Data represent mean±SEM from 8 mice for OVA-challenged, BCG-treated and MTBVAC-treated groups and 2 mice for the negative control. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

The role of IgM was also analysed in BAL, showing such a slight increase in the vaccinated groups that did not seem to be relevant (Figure 72).

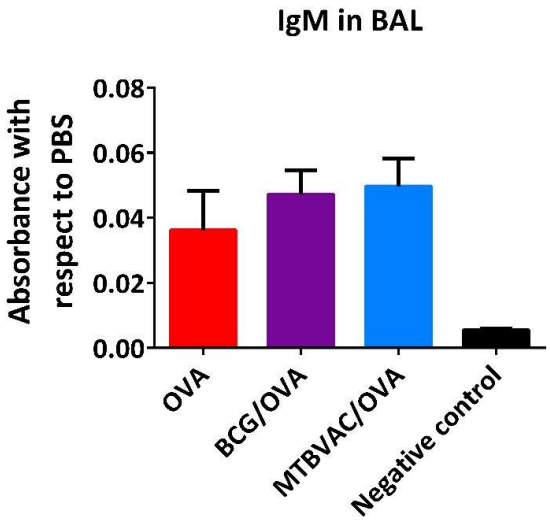


Figure 72. IgM detection in pIgR -/- mice in a model of acute asthma. The experiment was carried out in an OVA-induced acute asthma model in pIgR-/- KO mice, when vaccinated after the sensitization with 10^6 CFU. Total IgM in BAL was measured by ELISA, showing the absorbance with respect to a PBS sample. Data represent mean±SEM from 8 mice for OVA-challenged, BCG-treated and MTBVAC-treated groups and 2 mice for the negative control. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

Finally, IgA levels in BAL were also quantified in pIgR -/-KO mice, revealing a slight increase on these antibodies in BAL upon vaccination (Figure 73).

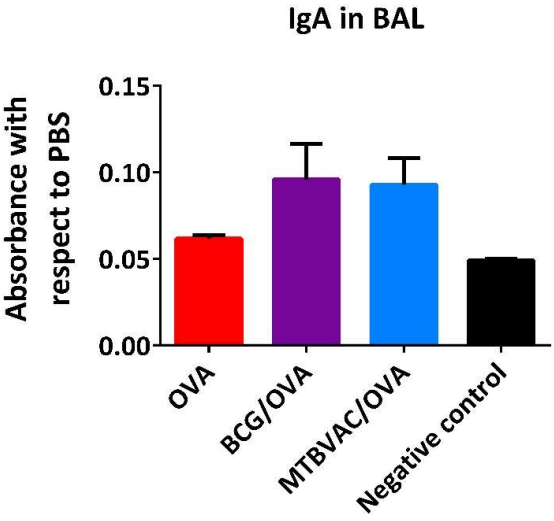


Figure 73. IgA in BAL in pIgR-/- KO mice. The experiment was carried out in an OVA-induced acute asthma model in pIgR -/- KO mice, when vaccinated after the sensitization with 10^6 CFU. Total IgA in BAL was measured by ELISA, data indicate the relative absorbance with respect to a PBS sample. Data represent mean±SEM from 4 mice in the OVA control, 6 mice in the BCG or MTBVAC-treated groups, and 2 mice in the negative control. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

Despite the lack of pIgR, monomeric IgA most of the time, and pIgA less frequently, can reach the airways from blood by passive transport [215]. In this case, they could

be OVA-specific IgA as well, as there is an OVA-specific response in the lymph nodes (as seen above) that could be transferred systemically. Therefore, the presence of these IgA in BAL (Figure 73), as it increases with the vaccination, could be important for asthma protection.

Considering all these data, it is clear that IgA and pIgR are not essential for the protection against asthma in the present model. However, there are evidences to hypothesise that IgA antibodies are playing a partial role on it. On the one hand, IgA and IgA+ B cells are increased in the vaccinated groups with respect to the positive control in wild type mice, and on the other hand, their absence could be partially compensate with an increase in IgM in IgA-/- KO mice.

6.9 TLR 2, 4 and 9 are not implicated in the induction of OVA-induced acute asthma either not in the protection conferred by attenuated Mycobacteria against T2- asthma.

Thanks to specific PRRs such as the TLRs, innate immune system can recognize some antigenic molecules from microbes, known as PAMPs [223]. Most TLRs are expressed in airway epithelial cells and in the respiratory track, playing an important role in regulating the innate and adaptive immunity in the airways [141]. There is still a lot of controversy about the role of TLR in inducing asthma and in its protection. It is widely known that TLRs are involved in the development of allergic asthma and that they can shift immune response towards pro- or anti-allergy responses after successfully recognizing microbial antigens depending on the stimulus and on the type of TLR activated [141, 224].

On the one hand, allergic studies suggest that activated TLR2 initiates Th2-immune responses [225, 226] and that polymorphisms in the TLR2 gene are associated to asthma. Thus, TLR2 seems to be associated to asthma induction [227]. However, TLR2 signalling can also induce the expansion of Treg cells, reducing asthma exacerbations [228]. TLR4 signalling increases IL-5 secretion by mast cells, causing the generation of allergic T2-type cytokines by the airway epithelial cells and the survival of eosinophils [229]. Contrary, TLR9 is a powerful inducer of Th1 immune responses [230]. For example, several studies have demonstrated that TLR9 activation by CpG ODN prevents allergic airway inflammation, bronchial hypersensitivity in asthma [231] and eosinophilic inflammation in the airways [230, 232].

On the other hand, TLR2, TLR4, TLR9 and possibly TLR8 are the key receptors that are involved in the recognition of Mtb. In this context, it is known that TLR-2 activated through Mycobacteria induces IFN-γ production; some TLR4 agonists, enhance the polarization of T effector cells toward a Th1 phenotype and TLR9 induce

a strong Th1 response as well. Actually, each one alone can activate different components of the immune system and reinforce anti-TB responses. Further, TLRs can cooperate between them. For example, TLR2 forms heterodimers with TLR4 to activate the macrophages in response to Mtb and it also has some cooperation with TLR9 [233].

With these backgrounds, it was contemplated to study the role of TLR2, 4 and 9 in the induction of the allergic asthma model and in the protection mediated by attenuated Mycobacteria.

In TLR2-/- KO, TLR4-/- KO and TLR-/- 9 KO mice, the induction of the allergic model was triggered similar as in WT mice. Moreover, attenuated Mycobacteria still prevented the development of asthma features: they reduce the eosinophilia (Figure 74) and induce the impairment of the Th2 response through the polarization to the Th1 profile (Figure 75).

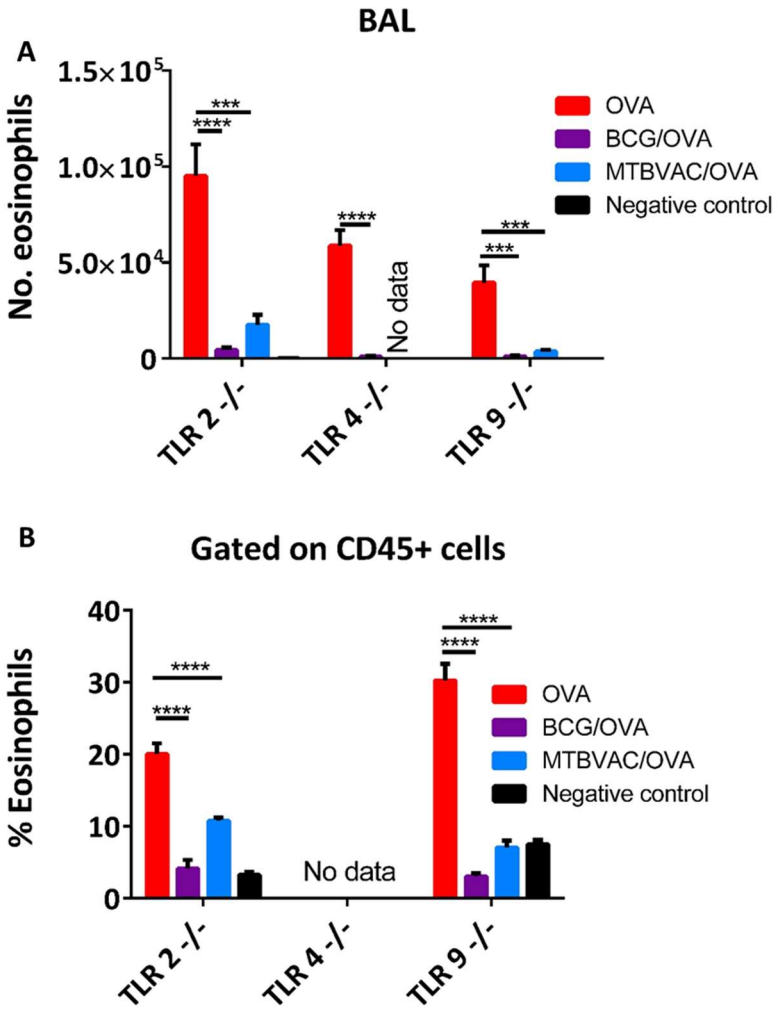


Figure 74. Eosinophilia in the airways suggests that TLR2, 4 and 9 are not crucial for the induction of asthma nor for the vaccines-mediated protection against asthma. Eosinophils were quantified by flow cytometry in a model of acute asthma when vaccinated with 10^6 CFU after sensitization TLR 2 -/- KO, TLR4 -/- KO or TLR9 -/- KO mice. In BAL, the eosinophilia

presented in the positive control was reduced with the vaccines (A). Percent of eosinophils in lung was measured by flow cytometry. Data showed a clear eosinophilia in the positive control in lung, which was significantly reduced when vaccinated (B). Data in TLR2 -/- KO represents mean±SEM from 5 mice for OVA-challenged, 4 mice for BCG-treated, 5 mice for MTBVAC-treated and 4 mice for the negative control. In TLR4 -/- KO, there are 6 mice in the OVA, BCG treated and negative group (there are no mice treated with MTBVAC). Finally, in TLR9-/- KO, there are 6 mice in the OVA group, 7 in both BCG and MTBVAC treated, and 2 in the negative control. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

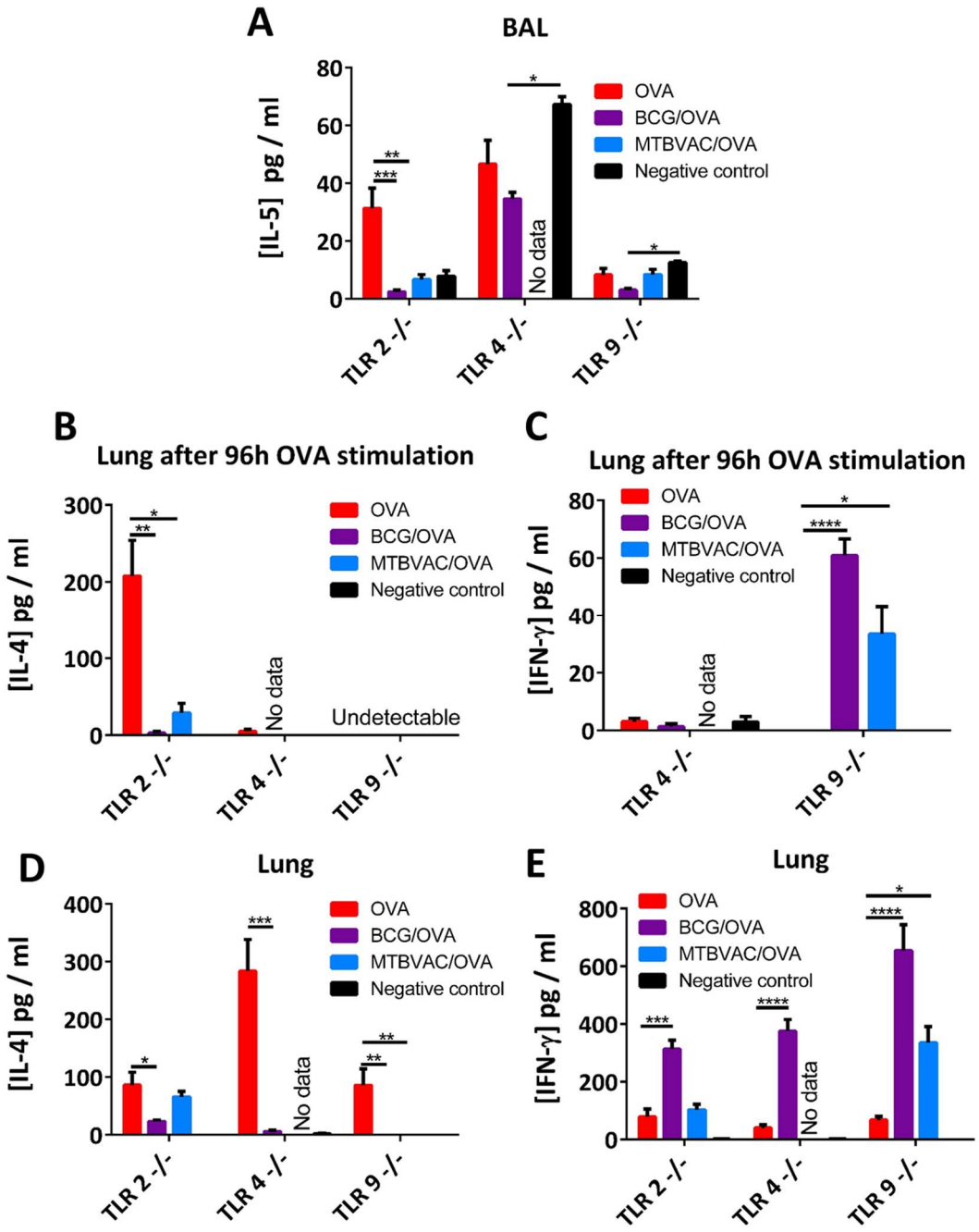


Figure 75. The initial Th2 response triggered by the allergen repolarized to a Th1 response upon MTBVAC or BCG in TLR 2, 4 and 9 -/- KO mice suggesting that these TLR are not essential for asthma nor for its protection mediated by attenuated Mycobacteria. Cytokines were measured in different samples in a model of acute asthma when vaccinated after

sensitization with 10^6 CFU TLR 2 -/- KO, or TLR4 -/- KO or TLR9 -/- KO mice. The IL-5 was reduced in BAL with the vaccines (A). The IL-4 and the IFN- γ were measured in lung supernatant after stimulating the organ 96 hours with OVA. The IL-4 decreased when vaccinating (B) and in contrast, the IFN-γ increased (C). IL-4 and IFN-γ were measured in lung explants. The IL-4 decreased with the vaccines (D) and consistently the IFN-γ increased (E). Data in TLR2 -/- KO represents mean±SEM from 5 mice for OVA-challenged, 4 mice for BCG-treated, 5 mice for MTBVAC-treated and 4 mice for the negative control. In TLR4 -/- KO, there are 6 mice in the OVA, BCG treated and negative group (there are no mice treated with MTBVAC). Finally, in TLR9-/- KO, there are 6 mice in the OVA group, 7 in both BCG and MTBVAC treated, and 2 in the negative control. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

The fact that the absence of a single TLR can be compensated with others TLR that act in collaboration, may explain the lack of implication observed for these three TLR in these processes. To avoid this, the study was carried out in the triple KO (TLR 2, 4, 9 -/-), but the results were similar to those obtained for each single KO. The eosinophilia was reduced upon treatment (Figure 76) and consistently Th2 cytokines decreased whereas the main Th1 cytokine (IFN-γ) increased (Figure 77). Eventually, these TLR are not essential for the protection mediated by Mycobacteria in allergic T2-asthma.

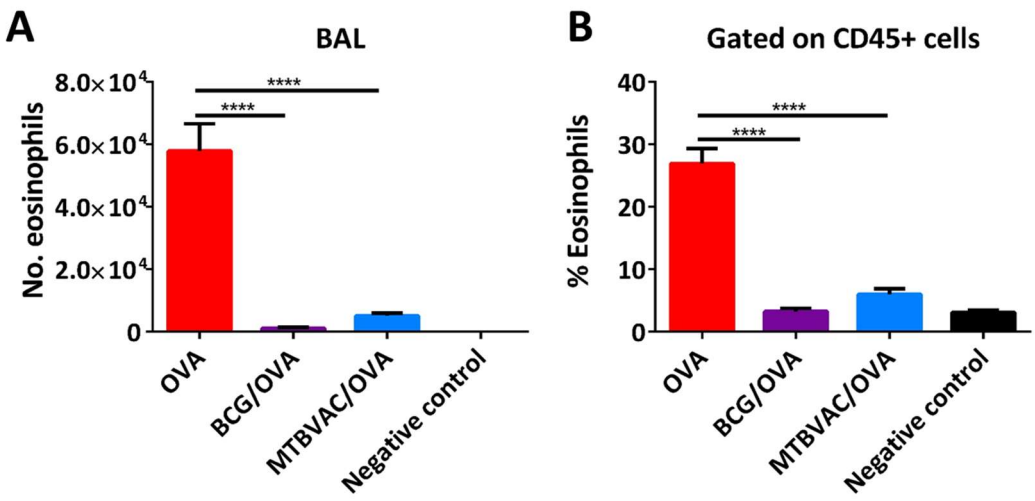


Figure 76. Eosinophilia in the airways did not vary in triple TLR2,4,9 -/- KO mice with respect to WT mice. Eosinophils were quantified by flow cytometry in a model of acute asthma when vaccinated after sensitization with 10^6 CFU, TLR 2,4,9 -/- KO mice. In BAL, the eosinophilia was reduced with the vaccines (A). There was a reduction in the percent of eosinophils in lung in vaccinated mice with respect to the positive control (B). Data represent mean±SEM from 7 mice for OVA-challenged, BCG-treated and MTBVAC-treated groups and 3 mice for the negative control. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

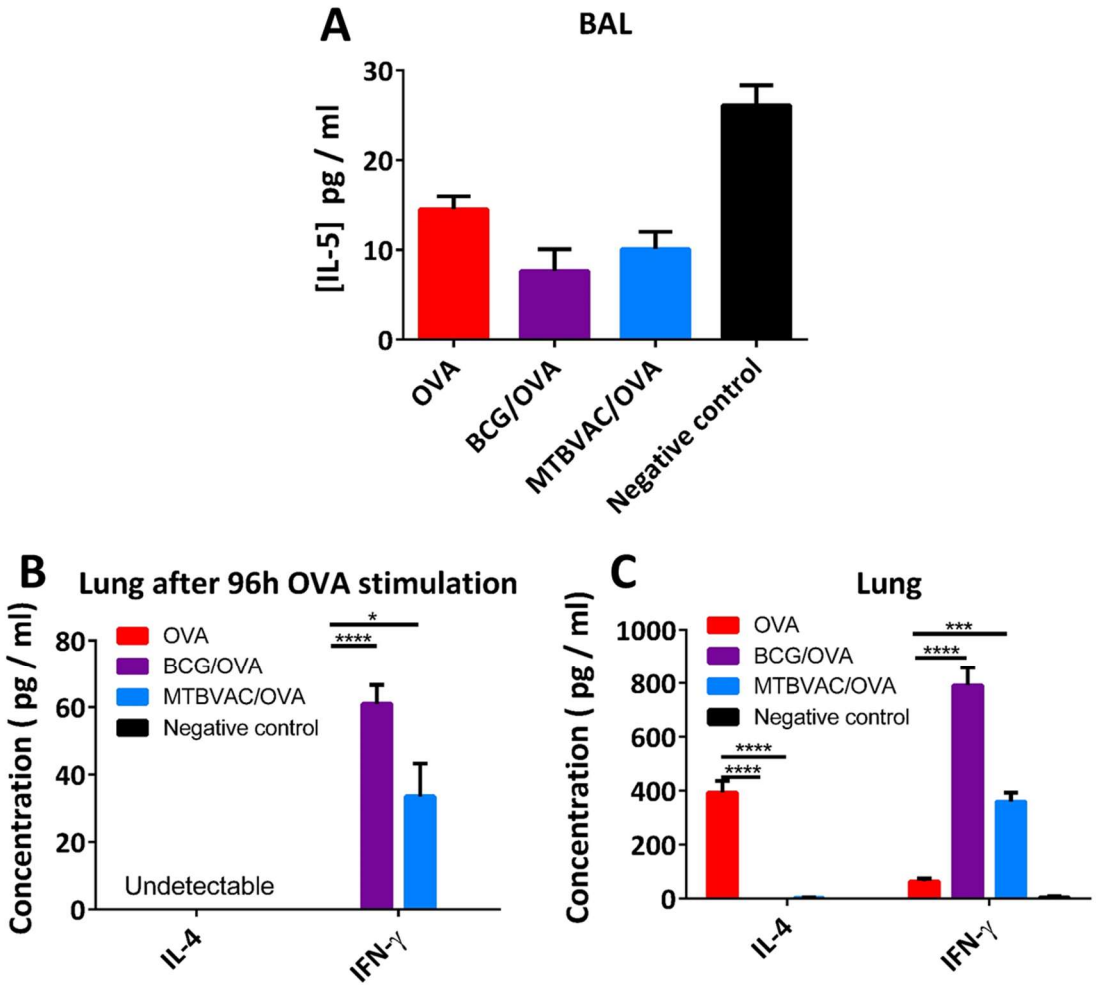


Figure 77. There was a re polarization from the Th2 response driven by the allergen to a Th1 profile upon vaccination in the triple TLR2,4,9 -/- KO. Cytokines were measured in different samples in a model of acute asthma when vaccinated after sensitization with 10^6 CFU, TLR 2,4,9 -/- KO mice. In BAL, the IL-5 was reduced with the vaccines comparing with the positive control (A). The IL-4 and the IFN- γ were measured in lung supernatant after stimulating the organ 96 hours with OVA, and comparing with a baseline without the allergen. The IL-4 was undetectable but the IFN-γ increased in the treated groups (B). IL-4 and IFN-γ were measured in lung explants. The IL-4 decreased with the vaccines and consistently the IFN-γ increased (C). Data represent mean±SEM from 7 mice for OVA-challenged, BCG-treated and MTBVAC-treated groups and 3 mice for the negative control. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

6.10 BCG and MTBVAC are able to re-educate M2 macrophages, induced by allergen administration, towards an M1 phenotype

Asthma has been linked with a pathological macrophage polarization towards an M2 phenotype[234], which triggers an immune modulatory environment that impairs Th1 response and favours expansion of Th2 cells[235]. Thus, exacerbated levels of M2 macrophages have been found in animal asthma models [236] as well as in samples from patients [234] and what is more, therapies targeting M2 macrophages alleviate allergic responsiveness[237]. In this context, it is relevant to know whether the

allergen model presented here is able to induce a M2 macrophage polarization and further, whether attenuated Mycobacteria are able to revert this polarization. Therefore, the present work analysed the macrophages phenotypes in OVA challenged mice and in Mycobacteria treated OVA-challenged mice.

Firstly, it has been hypothesized that the beneficial effects of intranasal attenuated Mycobacteria against asthma could be related with the ability of the vaccine to locally interact with the resident phagocytic cells in the lungs. Thus, to monitor populations infected in vivo following BCG immunization, a BCG strain that expressed the green fluorescent protein (GFP) was delivered. Data demonstrated that level of BCG infection was efficient enough to discern infected cells by flow cytometry. Noteworthy, using a panel of antibodies to detect myeloid cell markers, an important proportion of these macrophages expressed high levels of iNOS and CD86, two well-known markers of macrophage classical activation (or M1 phenotype) (Figure 78).

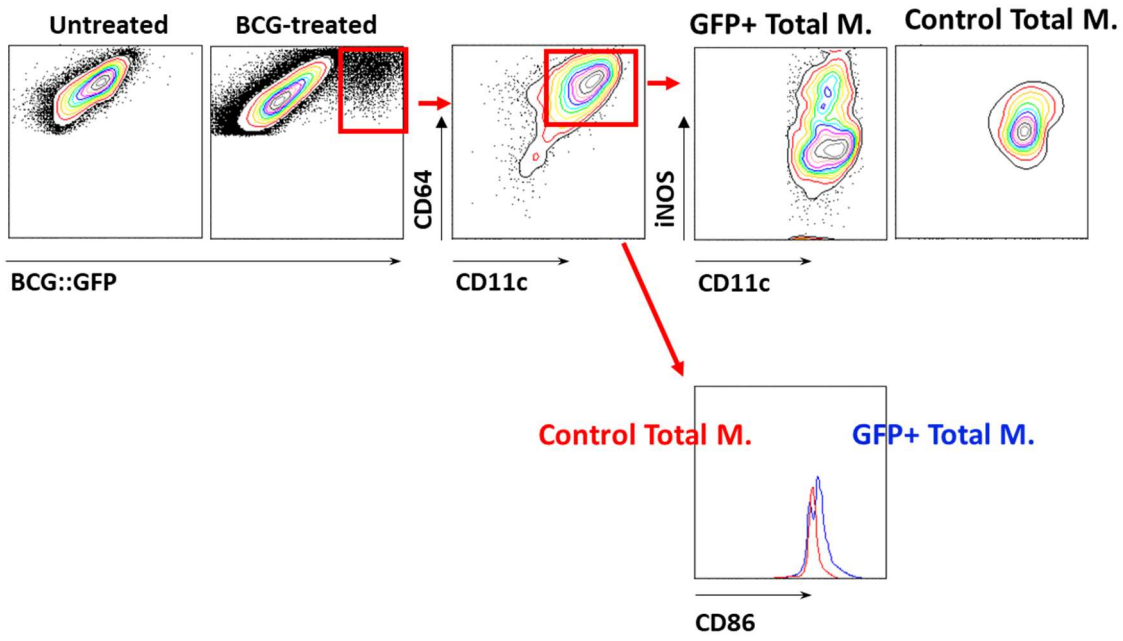


Figure 78. BCG administered by the intranasal route infects lung resident macrophages and induces classical activation. OVA-sensitized mice were immunized with 10^6 CFU of GFP-expressing BCG. One month later, infected cells were monitored and characterized by flow cytometry. Expression of M1-polarization markers iNOS and CD86 was analyzed in total macrophages (Total M).

Then, in this experiment it was compared the polarization of total lung macrophages in the OVA-challenged mice intranasally treated or not with BCG (with a dose of 10^6 CFU) and in another experiment it was evaluated the impact of MTBVAC, with a dose of 10^7 CFU. The reason why the dose of MTBVAC was increased will be later deeply explained, but it was basically to increase its persistence to similar level to that of BCG.

When administered BCG, percentage of iNOS- and CD86- positive macrophages was substantially higher in the treated group than in the OVA control (Figure 79 A, B). However, no difference was found in the case of MHC-II, an indicator of general activation, with most of the macrophages positive for this marker in the positive and vaccinated group (Figure 79 C).

In the experiment with MTBVAC, percent of iNOS-positive CD11c+ cells was higher in the MTBVAC-treated group as well (Figure 79 D). Moreover, in another experiment with MTBVAC-treated OVA induced mice, the percentage of iNOS+ AM was higher in the vaccinated group when compared with the OVA group (Figure 79 E). The expression of CD86+ in AMs was also higher in treated mice (Figure 79 F) but de expression of MHCII+ in AM was similar between both groups (Figure 79 G). AM represent the main lung resident phagocytic cells, so it leads to hypothesise that they are phagocyting BCG and thus polarizing to M1.

Importantly, expression of CD206, a classical marker associated with M2 macrophages, was greater in macrophages from OVA group compared to those from BCG treated mice, which expressed similar levels of CD206 to those from the naïve macrophages (Figure 79 H,I).

To complement these data, RNA was isolated from lungs of OVA-challenged mice. Expression analysis of genes linked with macrophage polarization clearly demonstrated that BCG immunization led to an upregulation of M1 profile-associated genes, such as *Nos*2 and *Il1b*. In contrast, it inhibited the expression of M2 activation markers such as *Ym*1, *Arg*1 and *Retln*a (Figure 79 J).

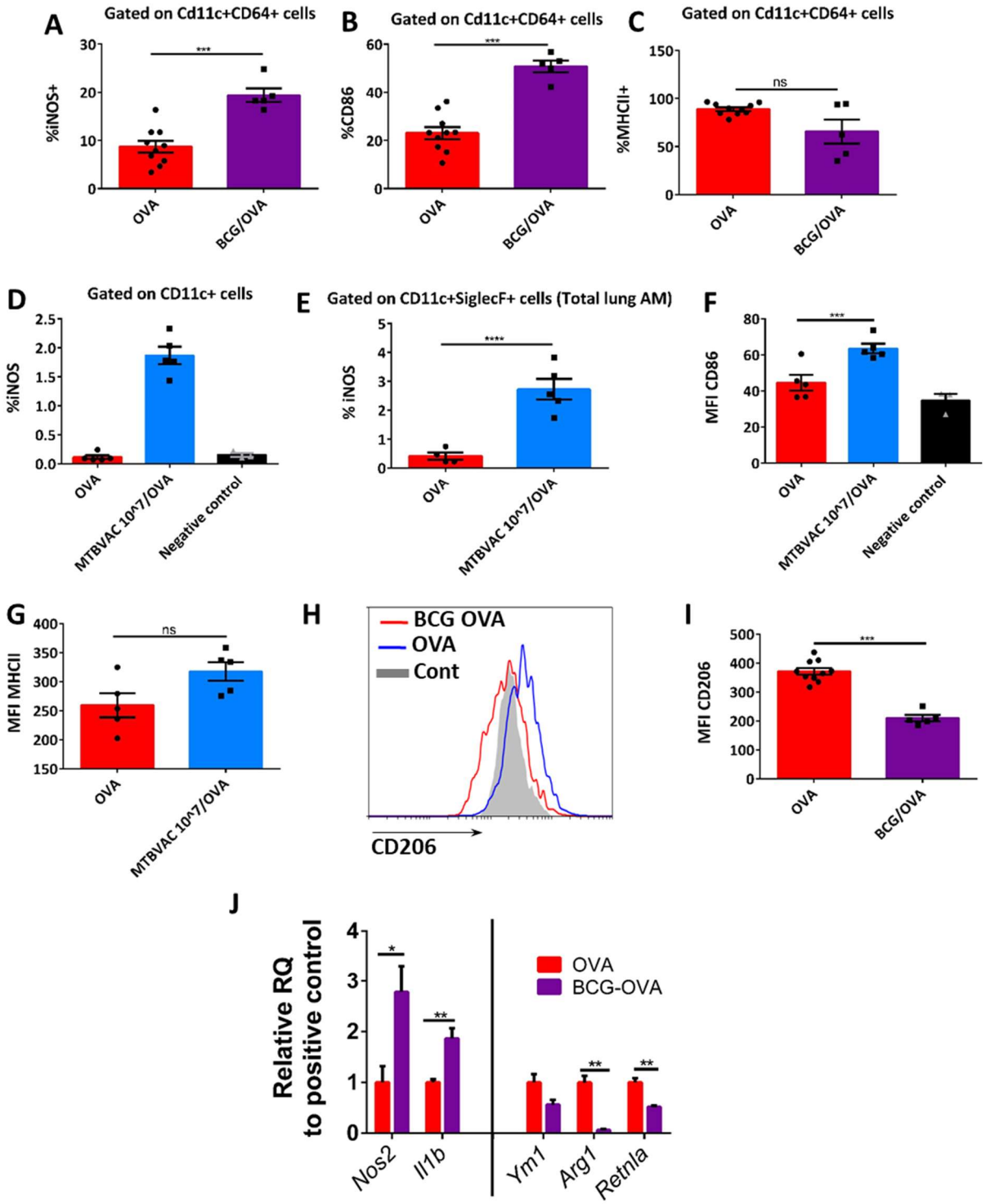


Figure 79. BCG and MTBVAC administered by the intranasal route induced M1 polarization. OVA-sensitized mice were immunized with 10^6 CFU of BCG or 10^7 CFU of MTBVAC. One month later, cells were characterized by flow cytometry. Expression of M1-polarization markers iNOS and CD86, of the activation marker MHCII and of the M2-marker CD206 was analyzed. Representative diagrams are shown in the figure. Percentage of iNOS-, CD86, -MHCII- macrophages was measured in OVA-challenged untreated or BCG-treated mice over total lung macrophages (A, B and C, respectively). Percentage of iNOS over CD11c+ cells (D) or over AM in lung (E) in MTBVAC-treated OVA induced mice was quantified. Comparison of Mean Fluorescence Intensity (MFI) corresponding to CD86 (F) and MHCII (G)

level of expression in OVA-challenged untreated or MTBVAC-treated. Representative overlay histogram showing CD206 surface expression of indicated experimental groups (H). Graph shows comparison of Mean Fluorescence Intensity (MFI) corresponding to CD206 level of expression from OVA-challenged mice, untreated or BCG-treated (I). M1 and M2 activation markers measured by qRT-PCR in lungs from OVA-challenged mice, untreated or BCG-treated (J). Data in the graphs are representative mean±SEM from a minimum of 5 mice per group. *p<0.05; **p<0.01; ***p<0.001; by unpaired single t-student test.

Altogether, these data demonstrated the enrichment of M2-like macrophages during asthmatic responsiveness, and suggested that the therapeutic efficacy of attenuated Mycobacteria could rest on the modulation of this response by inducing classical activation of total and alveolar lung macrophages.

6.11 Macrophages transference from BCG-treated OVA-challenged mice are unable to protect against asthma

Following with the macrophages study, an adoptive transfer of macrophages was carried out in order to confirm their implication in the mechanism of protection mediated by attenuated Mycobacteria against asthma.

One month and a half after intranasal BCG administration, CD11c+ cells (mainly macrophages), harvested from lungs of these vaccinated donors, were adoptively transferred to mice previously sensitized to OVA. The following week, receptors mice were intranasally challenged to OVA for 3 consecutive days and eventually, the next day, humanely sacrificed (Figure 80).

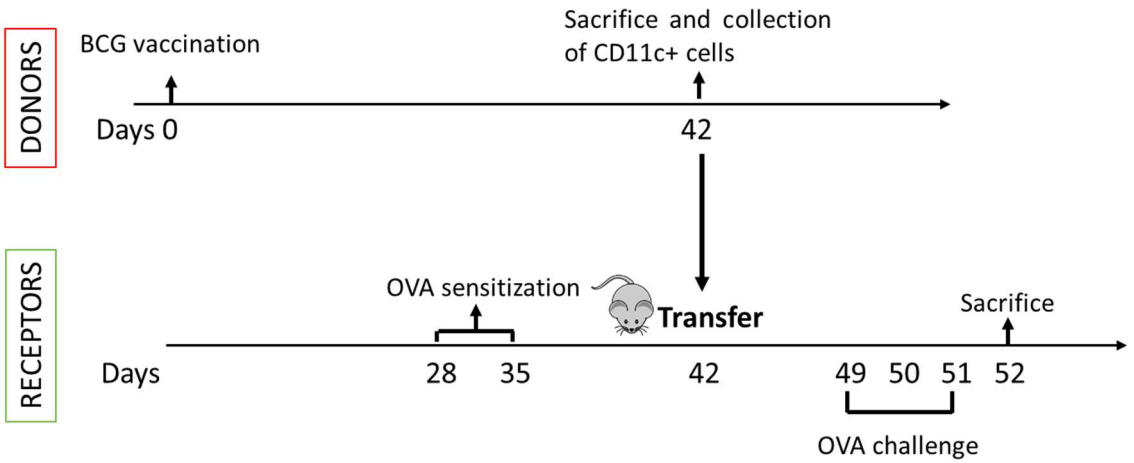


Figure 80. Model of adoptive transfer of CD11c+ cells from vaccinated mice. Donors were vaccinated with 10^6 CFU of BCG by the intranasal route. Month and a half later, they were sacrificed, and their CD11c+ cells (a half million cells) from lungs were transferred by the intratraqueal route to OVA sensitized mice. Next week, these receptors were OVA challenged for 3 days, before being sacrificed.

In order to obtain a more pure positive CD11c fraction to transfer, the separation was repeated through a second column. There was an initial 5% of CD11c+ cells in lungs,

which turned to a 75-80% in the positive CD11c fraction after the separation. Therefore, the efficiency of CD11c+ selection during the magnetic isolation was high enough to be considered a sufficient separation. In addition, the cell viability was analysed with Trypan Blue and it was higher than 90% percent.

However, at day 52 there was an eosinophilia in the receptor group similar to those in the OVA control group (Figure 81), so the transfer of CD11c+ positive cells was not enough to protect against allergic asthma.

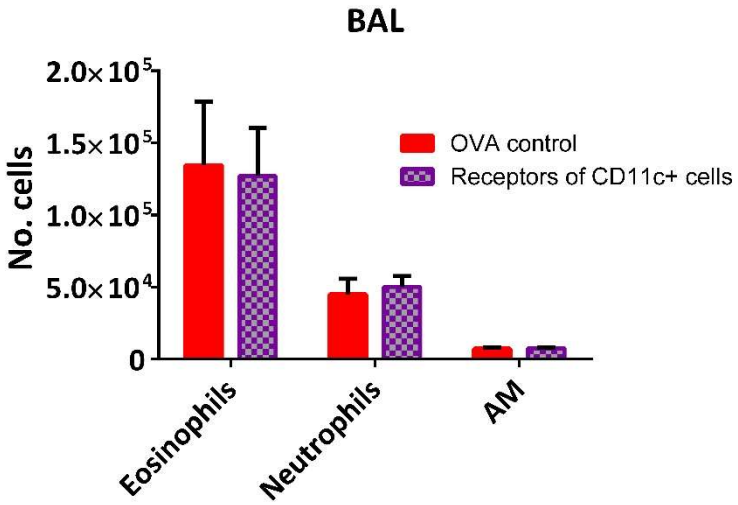


Figure 81. Eosinophilia remained in receptors of CD11c+ cells from vaccinated mice. Total myeloid cells in BAL was quantified by flow cytometry, revealing a similar increase in eosinophils in the positive control and in CD11c+ cell receptors mice. Data represent mean±SEM from 6 mice for OVA-challenged, and 5 mice for the receptors. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

The fact that transferring macrophages from BCG vaccinated mice is not protecting, could be explained different ways. Macrophages are important but not enough for asthma protection; the time between the reception of macrophages and the OVA challenge is too short to subverts lung immune environment associated with asthma; the number of transferred cells are not enough to modulate the entire lung environment, or the viability of cells when transferring them is low. Therefore, it could be interesting to explore new macrophages transfer protocols in the coming future.

6.12 Vaccination with MTBVACΔ*esat6/cfp10* conferred similar protection to that obtained with MTBVAC

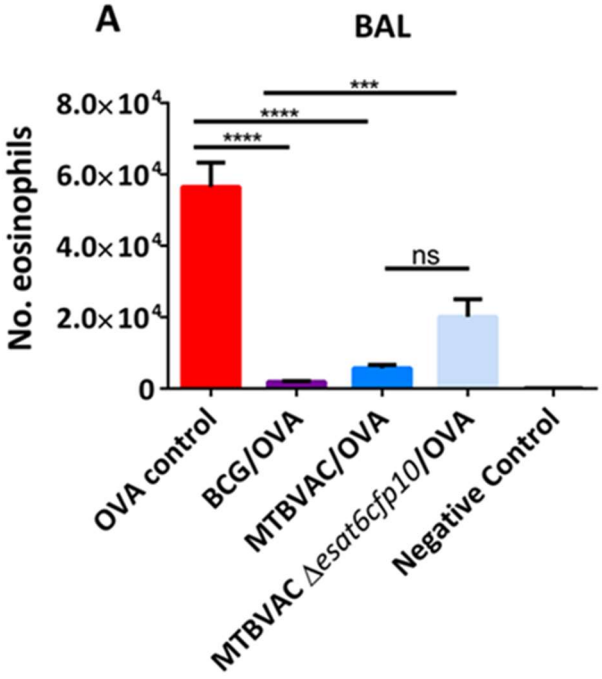
IL-1β is a key pro-inflammatory cytokine that initiates and maintains the inflammatory response. In response to certain stimuli, it is synthesized as a biologically inactive proIL-1β in monocytes and macrophages. Conversion to its biologically active form requires caspase-1 (casp-1), which mediates the proteolysis

of proIL-1β, which is then released as a mature cytokine [238, 239]. However, casp-1 activation is controlled by a family of multiprotein complexes known as 'inflammasome'.

It is known that Mtb infection induces IL-1β secretion. Further, recently it has been demonstrated that the Mtb protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome and importantly, the presence of ESAT-6 in Mtb is sufficient for caspase-1 activation and IL-1β secretion [240].

Some studies relate severe asthma and inflammation of the airways with the activation of the inflammasome [241]. In this regard, in the present work it has been hypothesised that the slight better protection against asthma conferred by BCG when compared to MTBVAC may be due to the fact that MTBVAC expresses ESAT-6. Therefore, contrary to BCG, MTBVAC is able to activate the inflammasome through this way. To validate this hypothesis, it was compared the efficacy of BCG, MTBVAC and MTBVACΔ*esat6cfp10*, a MTBVAC substrain created in the present laboratory [194], in a mice model of OVA-induced acute asthma. All the vaccines were administered by the intranasal route at a dose of 10^6 CFU/ mice, and the dose was corroborated and demonstrated to be similar between the three vaccines by plating the inoculums.

However, the results revealed that the lack of ESAT6/CFP10 does not let MTBVAC reach the protection level conferred by BCG (Figure 82).



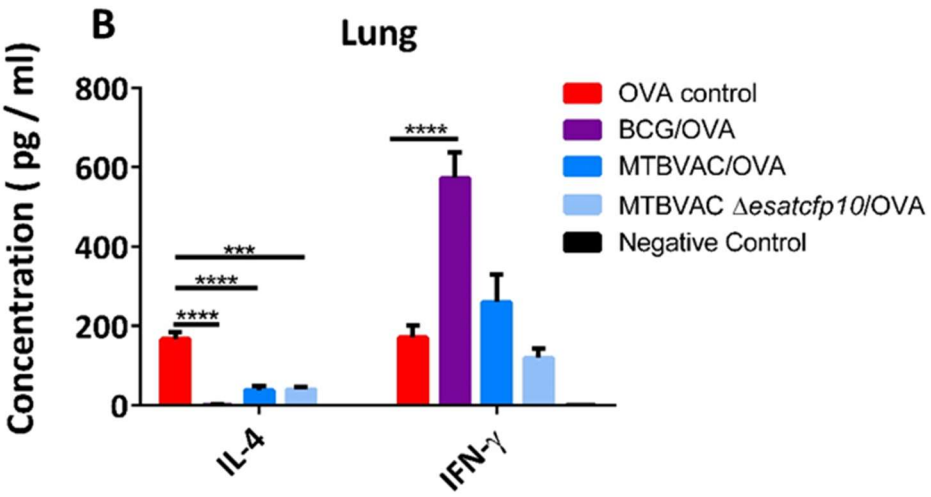


Figure 82. MTBVACΔ*esat6cfp10* does not confer better protection against asthma than MTBVAC. Total eosinophils in BAL were quantified by flow cytometry, revealing a slight increase in eosinophils in the MTBVACΔ*esat6cfp10*-treated group when compared to the MTBVAC-treated group (A). IL-4 and IFN-γ were measured in lung explants demonstrating that the polarization from the Th2 response to the Th1 response upon the vaccination was similar between MTBVAC and MTBVACΔ*esat6cfp10* and lower than with BCG (B). Data represent mean±SEM from 6 mice for MTBVACΔ*esat6cfp10*-treated, and at least 15 mice for the rest of groups. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

As a conclusion, another difference between BCG and MTBVAC should explain this distinction, as it will be discussed below.

6.13 The protection against asthma decreases as the attenuation of the administered Mycobacteria increases

The administration of BCG to people at risk of immune suppression, as people suffering from HIV, is not recommended. For this reason, it is important to develop new TB vaccines, more attenuated and so safer than BCG. With this purpose, different attenuated Mycobacteria were constructed in the present laboratory from MTBVAC: MTBVACΔ*erp*, MTBVACΔ*lysA*, and MTBVAC Heat Killed.

MTBVAC Δ*erp* is a live highly attenuated MTBVAC-based vaccine, created by an extra inactivation in the gene *erp* of MTBVAC, a virulence factor of Mtb. It is much more attenuated than BCG and MTBVAC [192] so it persists less in lungs than BCG and MTBVAC (Figure 83).

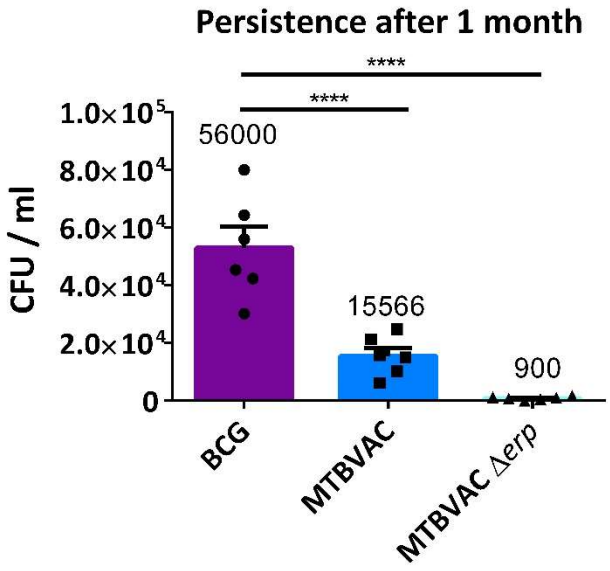


Figure 83. MTBVAC Δ*erp* persists less in the lungs than MTBVAC or BCG. 10^6 CFU/ mice of BCG, MTBVAC or MTBVAC Δ*erp* were administered by the intranasal route to C57/Bl6 mice. One month after the vaccination, lungs were recollected and CFU counted. Data represent mean±SEM from 6 mice per group. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA

MTBVAC Δ*lysA* is an auxotrophic MTBVAC strain that without Lys supplementation cannot grow, and it has been demonstrated to be highly attenuated, more than MTBVAC [193]. Finally, MTBVAC Heat Killed is the wild type strain of MTBVAC but it is not alive, so it is the most attenuated MTBVAC substrain and the one that less persists in the lungs, as it cannot replicate and it is more easily eliminated (paper in preparation by the present laboratory).

In the present work, it has been demonstrated that at the same dose, BCG protects slightly better than MTBVAC against asthma. It could be hypothesised that it is because MTBVAC is more attenuated than BCG, as demonstrated in Figure 83 and in [192], and so the former persists less in the lung than BCG does. To support this, it was performed an experiment comparing the protection conferred by different attenuated Mycobacteria. OVA-challenged mice were vaccinated with different attenuated Mycobacteria by the intranasal route with 10^6 CFU, and the eosinophilia was measured in order to compare the protection against T2-asthma (Figure 84).

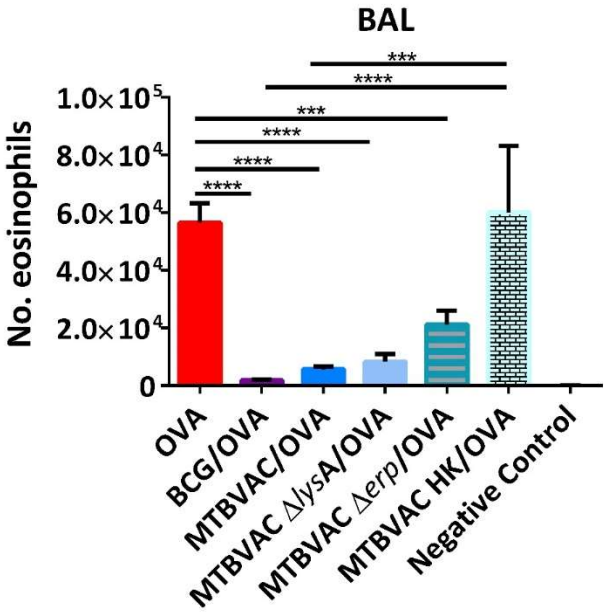


Figure 84. Eosinophilia between different attenuated Mycobacteria in OVA-challenged mice. Eosinophils were quantified in BAL. When the vaccine attenuation increased, eosinophilia in BAL increased as well. Graphs represents mean±SEM from pool data: OVA control, BCG and MTBVAC recollect data from 5 experiments, with 22-32 mice per group, the rest of the groups represent data for at least 6 mice. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

It is clear that when the vaccine attenuation increases, the eosinophilia increases as well, revealing the importance of the persistence in the asthma protection. The fact that the vaccination with MTBVAC Δ*lys*A protected only a slight worse than MTBVAC may be explained because mice contain Lys that could be being used by the Mycobacteria.

Moreover, IL-5 was also measured to characterise the Th2 profile, and again the impairment of the exacerbated Th2 profile was less evident with the increase in the attenuation of the vaccines (Figure 85).

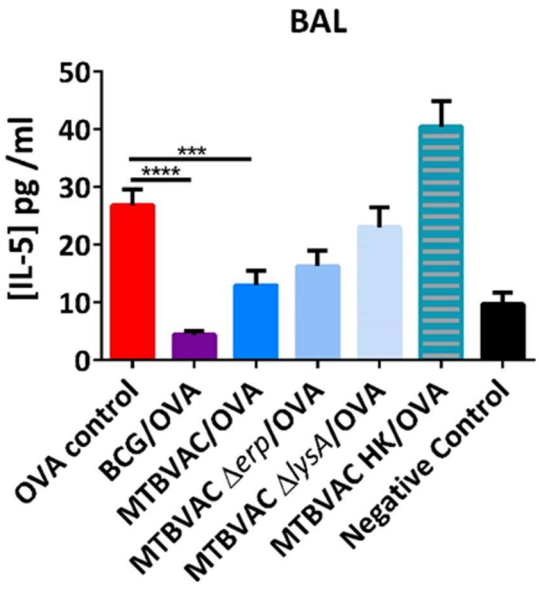


Figure 85. IL-5 profile between different potential TB vaccines. IL-5 was measured in BAL. It is seen how as the vaccine attenuation increased, IL-5 in BAL increased. Graphs represents mean±SEM from pool data: OVA control, BCG and MTBVAC recollect data from 5 experiments, with 22-32 mice per group, the rest of the groups represent data for at least 6 mice. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA

On the other hand, as persistence was so important, the vaccine dose administered was corroborated by plating a sample of the vaccine preparation before administered it. The range of counted inoculums was from $8x10^5$ CFU/mice for an experiment with BCG and 2.5 x 10^6 for a single experiment with MTBVAC, but on the other experiments, 10^6-$2x10^6$ CFU/ mice were administered. Therefore, the dose were similar enough between all the different vaccines to affirm that the results obtained are due to differences in attenuation and not to differences in doses.

Finally, taking into account that bacteria attenuation is so important in asthma protection, it is hypothesised that MTBVACΔ*esat6cfp10* may be attenuated with respect to MTBVAC, and it could explain why the substrain protected a slight worse than MTBVAC against asthma. However, its attenuation has not been analysed as MTBVACΔ*esat6cfp10* was constructed only for studying the role of ESAT6 in the protection against TB, but it could be analysed in the future.

6.14 The protection conferred by BCG and MTBVAC against asthma is dose dependent

Above, it has been described that MTBVAC persists less than BCG in the lungs when administered by the intranasal route (Figure 83) and, what is more, it has been demonstrated that more attenuated- and so less persistent- the vaccine was, lower the

protection against asthma was. In this regard, it has been hypothesised that the vaccine dose could influence the protection.

To demonstrate it, different vaccines doses, created by serial dilutions, were tested in a model of OVA-induced acute asthma. The vaccine dose was corroborated by plating and counting CFU and it was shown to be correct in all cases. Both eosinophilia (Figure 86) and cytokines concentration (Figure 87) varied with the doses, showing that the eosinophilia reduction and the polarization from a Th2 profile to a Th1 profile decreased with the reduction of the vaccine dose.

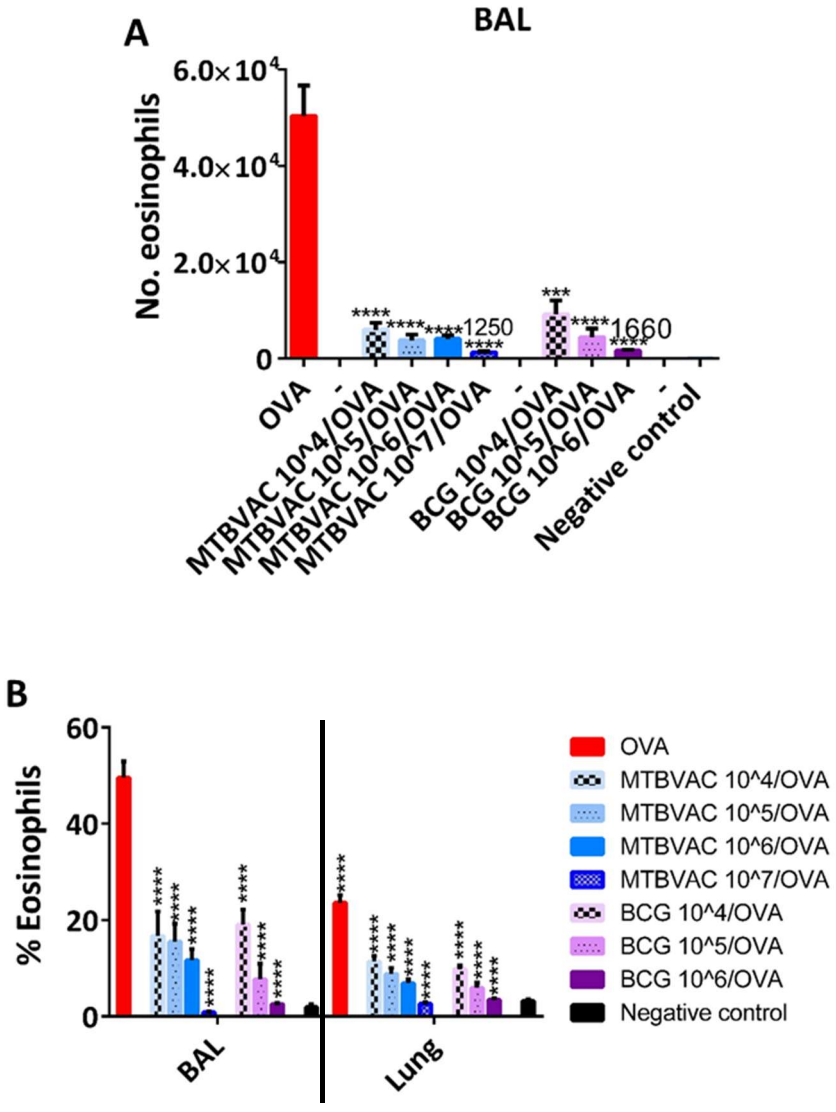


Figure 86. Asthma protection correlates with the vaccine dose. Total eosinophils in BAL (A) or percentage of eosinophils in BAL and lung (B) were quantified by flow cytometry, revealing that as the vaccine dose increased, the eosinophilia decreased. Data represent mean±SEM from 6 mice for doses of 10^4 and 10^5, from 2 experiments (12 mice) in the 10^7 dose of MTBVAC and in the negative control, and from 5 experiments (at least 27 mice) for the dose of 10^6 and the OVA group. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

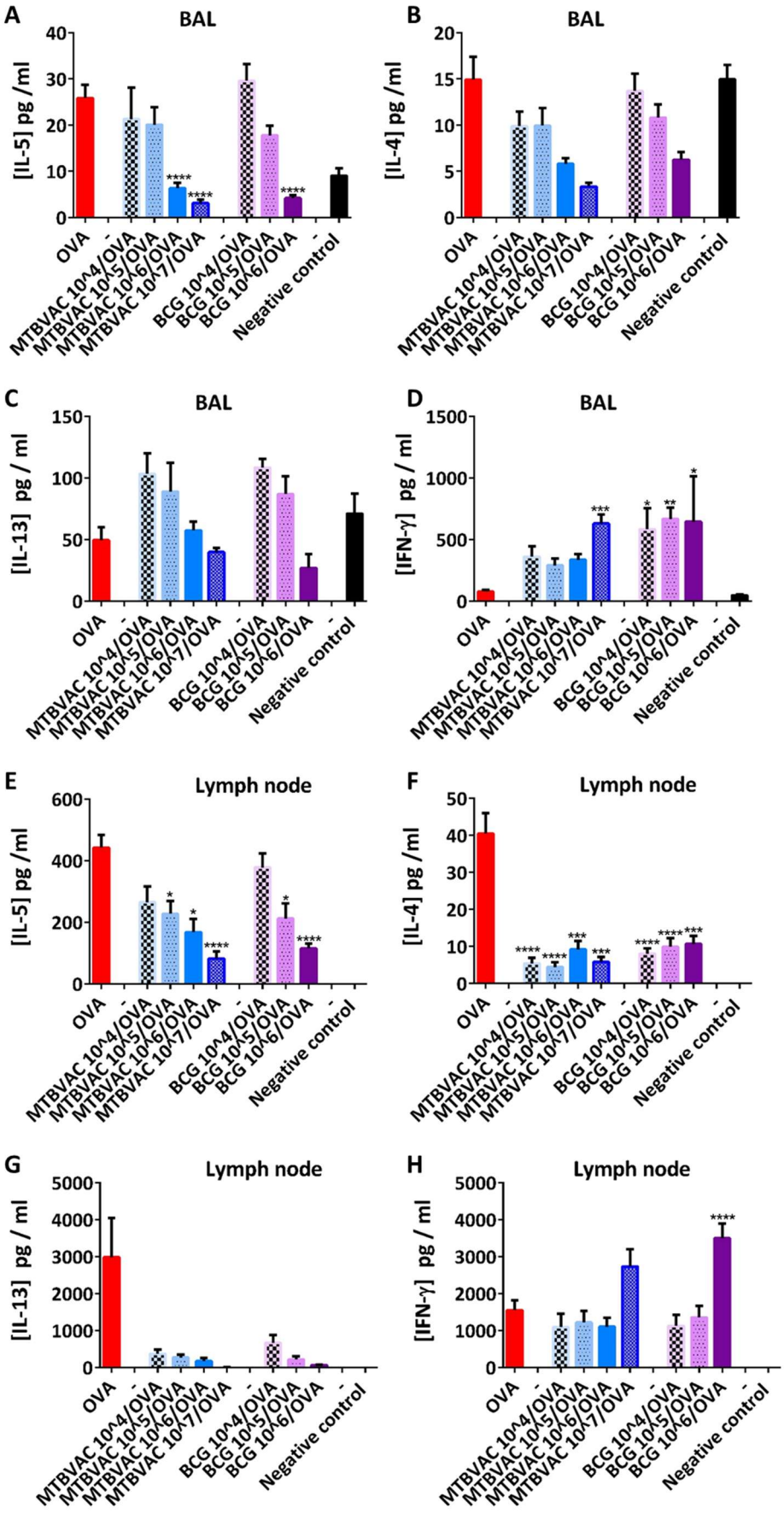

A
BAL
[IL-5] pg /ml
B
BAL
[IL-4] pg /ml
C
BAL
[IL-13] pg / ml
D
BAL
[IFN-γ] pg / ml
E
Lymph node
[IL-5] pg /ml
F
Lymph node
[IL-4] pg /ml
G
Lymph node
[IL-13] pg / ml
H
Lymph node
[IFN-γ] pg / ml
OVA
-
MTBVAC 10^4/OVA
MTBVAC 10^5/OVA
MTBVAC 10^6/OVA
MTBVAC 10^7/OVA
BCG 10^4/OVA
BCG 10^5/OVA
BCG 10^6/OVA
Negative control

Figure 87. Immune response re-polarization inversely correlates with the vaccine dose. IL-5 was measured in BAL (A) and in lymph nodes (specific for OVA) (E). IL-4 was measured in BAL (B) and in lymph nodes (specific for OVA) (F). IL-13 was measured in BAL (C) and in lymph nodes (specific for OVA) (G). The decrease of these three Th2 cytokines was generally more pronounced when increased the vaccine dose. IFN-γ was measured in BAL (D) and lymph nodes (specific for OVA) (H) and it increased with the vaccine dose. Data represent mean±SEM from 6 mice for doses of 10^4, 10^5 and from 2-5 experiments (at least 12 mice) in the 10^6, 10^7 dose and in the OVA groups. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

Moreover, macrophages in lung were also analysed. In MTBVAC-treated mice, only in the group treated with 10^7 CFU the percent of iNOS+ cells over AM was higher than in the OVA control (Figure 88 A). The expression level of CD86 in AM cells was more elevated only when treated with the highest dose of MTBVAC or BCG (Figure 88 B). Eventually, the MHCII marker was similarly expressed between groups (Figure 88 C).

In conclusion, there exists a positive correlation between the appearance of the M1-profile and the vaccine-mediated protection, and what is more, the dose has to be the highest tested to be fully efficient against asthma.

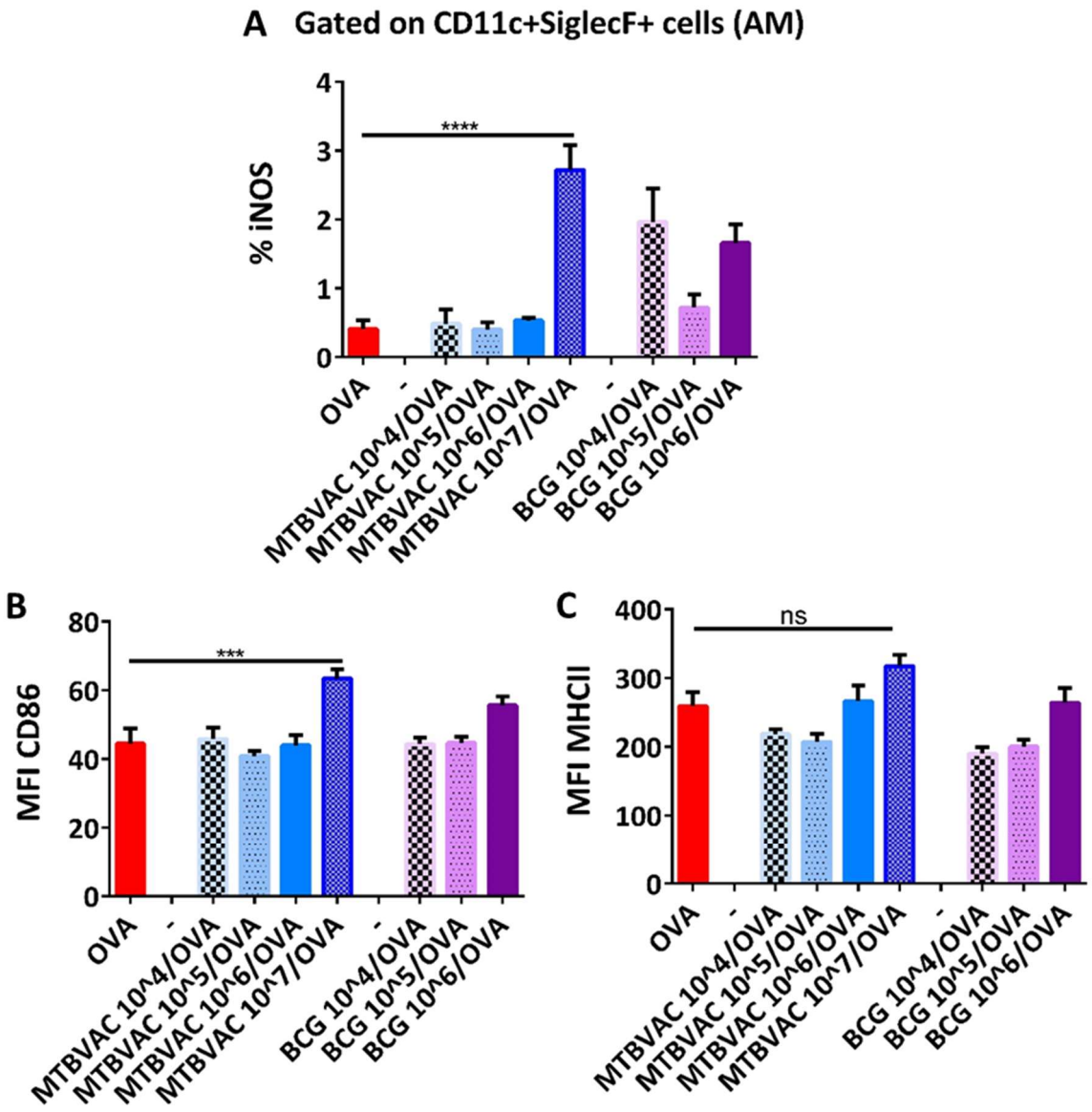


Figure 88. Macrophages re-polarization correlates with the vaccine dose. OVA-sensitized mice were immunized with different doses of MTBVAC and BCG. One month later, cells were characterized by flow cytometry. Percentage of iNOS+ cells in AM was quantified (A). Comparison of Mean Fluorescence Intensity (MFI) corresponding to CD86 (B) and MHCII (C) level of expression was analyzed. Data represent mean±SEM from 6 mice per group. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

Interestingly, one month after mice vaccination with MTBVAC 10^7 or BCG 10^6 there were the same CFU in lungs in both cases (Figure 89). Therefore, it could explain why at that doses, both vaccines modulated the response against asthma in a similar way, conferring an equivalent protection.

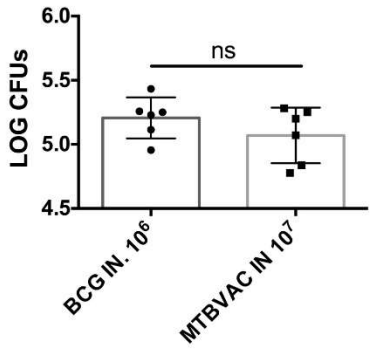


Figure 89. Persistence in lungs one month after intranasal vaccination was equivalent when using MTBVAC 10^7 and BCG 10^6. Count of CFU in lungs in mice after being vaccinated by the intranasal route. Data represent mean±SEM from 6 mice per group. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA. Unpublished data from our laboratory [242].

6.15 BCG beneficial effect for acute asthma is maintained four months after vaccination

Until now, in the present work it has been demonstrated that BCG prevents asthma when administered by the intranasal route after the sensitization one month before the exposure to the allergen (the challenge phase). However, as a potential prophylactic or even therapy for asthmatic humans, it would be better if its beneficial effects lasted more than one month, since people could be protected for long periods.

In this regard, it was done an experiment vaccinating mice after the sensitization but challenging them to OVA four months later (Figure 27).

The vaccine exerted a long-term protection against asthma, as its prophylactic effect was maintained four months after vaccination (Figure 90).

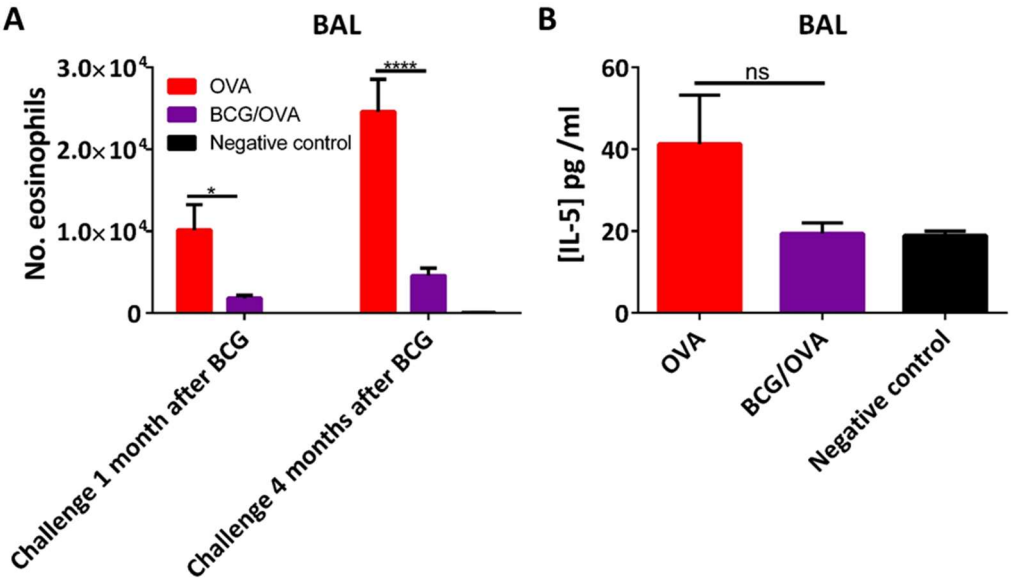


Figure 90. BCG prophylactic effect is maintained for at least 4 months. Total eosinophils were quantified in BAL by flow cytometry, when challenged 4 months after BCG vaccination

or 1 month after BCG (as a control and as it had been done until now). In both cases, eosinophilia was reduced in the BCG-treated group (A). IL-5 was analysed in BAL by the ELISA technique when challenged 4 months after the vaccination, and there was a reduction in the BCG-treated group with respect to the positive control (B). Graphs represent mean±SEM from pool data of two experiments when challenged 4 months after BCG (12 mice in the positive control and in the treated group and 6 mice in the negative control). When challenged 1 month after BCG, data represent one experiment (6 mice in the positive control and in the treated group and 3 mice in the negative control). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

Likely, this protection is due to memory OVA-specific T cells, because at that moment there was almost no Mycobacteria in the lungs (Table 5).

	BCG in lungs (CFUs)
Intranasal vaccination	10^6
1 month after the vaccination	2500
4 months after the vaccination	100

Table 5. Count of CFU in lungs from mice 1 or 4 months after the vaccination.

6.16 MTBVAC and BCG protects against HDM-driven asthma

Once the asthma model triggered by OVA had been deeply characterized, it was clinically relevant to know whether Mycobacteria protected also against HDM, one of the most common allergen for humans. With this purpose, in the present study, it was set up a model of acute asthma induced by this aeroallergen (Figure 28). At that moment, we did not know that the dose of 10^7 for MTBVAC was the equivalent to that of 10^6 for BCG, so the standard dose of 10^6 CFU was used for both vaccines.

Firstly, it is remarkable that HDM was able to induce the hallmarks of T2-type asthma, including eosinophilia, and Th2 cytokines. However, these features were less pronounced than they were in the OVA-driven asthma model. Importantly, attenuated Mycobacteria were able to protect against this allergic asthma since they reduced the eosinophilia and impaired the Th2 response, through the Th1 polarization (Figure 91).

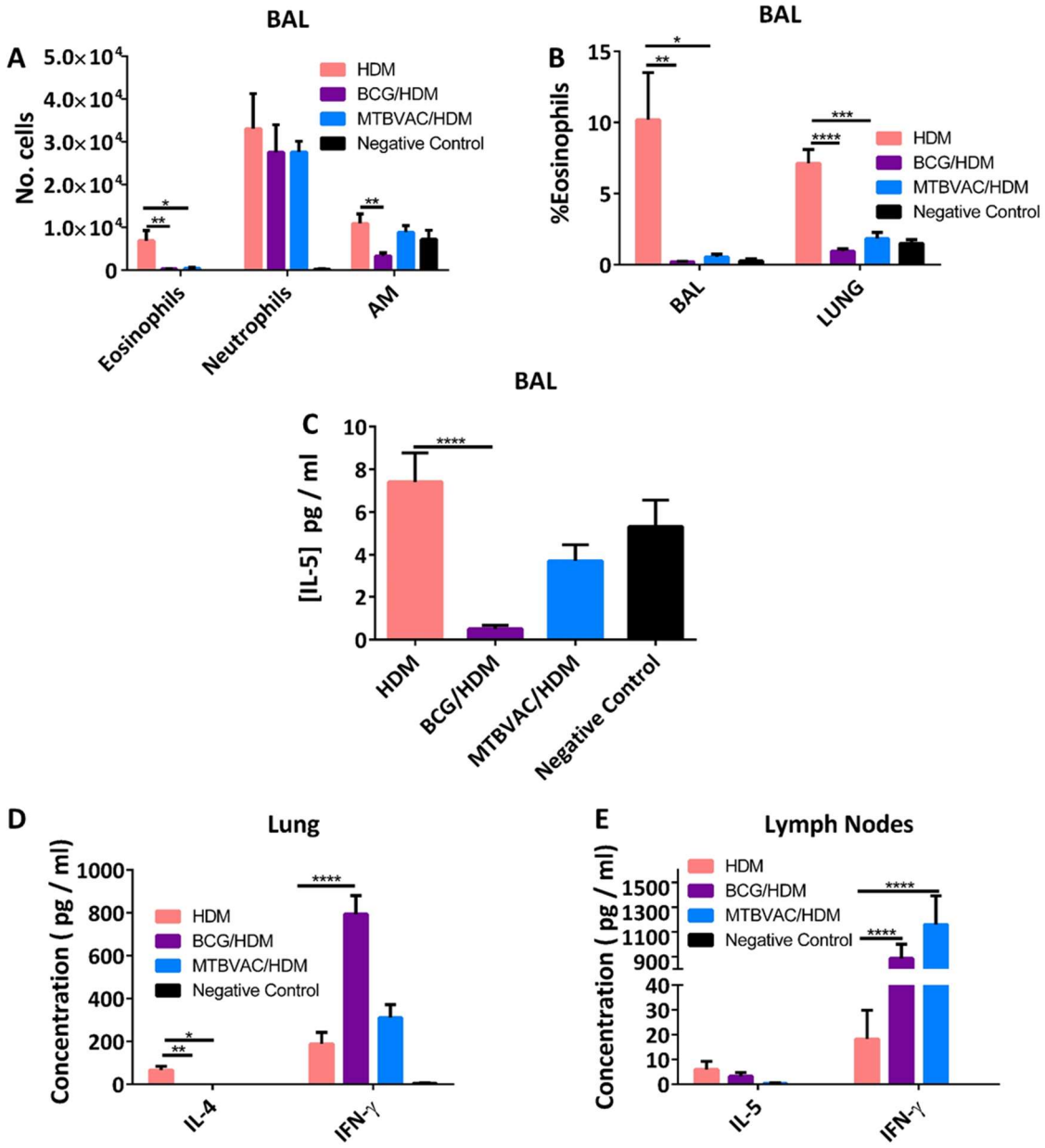


Figure 91. Attenuated Mycobacteria protect against HDM-induced acute asthma. Total myeloid cells were quantified in BAL by flow cytometry (A). When compared with the positive control, the eosinophilia was dramatically reduced with the vaccination and the neutrophilia remains stable with respect to the positive control. The percent of eosinophils in BAL and lung measured by flow cytometry also revealed a clear decrease with the vaccines (B). IL-5 was analysed in BAL (C), the IL-4 in lung explants (D) and the OVA-specific IL-5 in lymph nodes (E) by the ELISA technique, and there was a reduction for these cytokines in the vaccine-treated groups with respect to the positive control. Finally, the IFN-γ was measured in lung and in lymph nodes (here as OVA-specific cytokine), and in both cases there was a significant increase upon treatment (D and E). Graphs represent mean±SEM from pool data of two experiments (12 mice in the positive control and in BCG-treated groups and 6 mice in MTBVAC-treated and negative control groups). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

6.17 BCG prevents chronic asthma in mice

Asthma is a chronic disease of the lungs, so it is clinically more relevant to evaluate the impact of attenuated Mycobacteria in a model of chronic asthma than in an acute model. Therefore, once the acute asthma model was deeply characterised, it was set up a model of OVA-induced chronic asthma to study the role of BCG preventing the chronic development (Figure 29). Here, BCG was administered prior to multiple challenges. Importantly, even in that scenario, BCG was able to prevent the eosinophilia and to impair the Th2 response increasing the IFN-γ production (Figure 92).

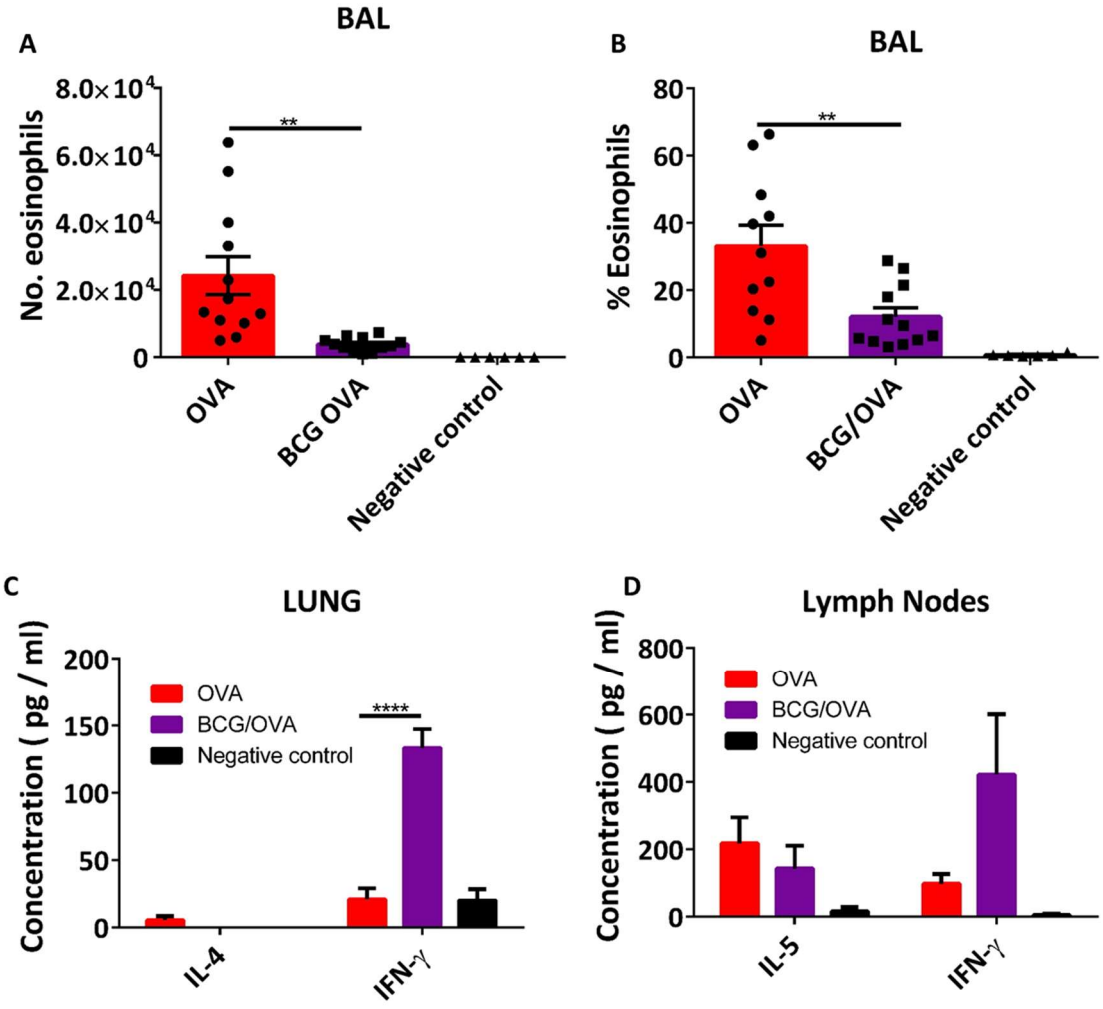


Figure 92. BCG prevents eosinophilia and impairs the Th2 response in chronic asthma in mice. Total eosinophisl (A) and percent of eosinophils (B) were quantified in BAL by flow cytometry. When compared with the positive control, the eosinophilia was significantly reduced upon BCG. IL-4 and IFN-γ were analysed in lung explants (C) and OVA-specific IL-5 and IFN-γ in lymph nodes (D) by the ELISA technique. There was a Th2 cytokine reduction in the BCG-treated group with respect to the positive control. Finally, the IFN-γ increased when treated (C and D). Graphs represent mean±SEM from pool data of two experiments (12 mice in the positive control and in the BCG-treated groups and 6 mice in the negative control). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

6.18 Attenuated Mycobacteria inhibit lung remodelling and eosinophilia in a scenario of established asthma

In order to elucidate the therapeutic potential of BCG against established asthma, experiments of chronic asthma model were carried out, but delivering the vaccine (10^6 CFU) at week 8 of the procedure, in the half of the challenge phase (Figure 30).

Eosinophils were quantified in BAL in a control group sacrificed at week 8, corroborating the existence of eosinophilia at the time when BCG was administered (Figure 93).

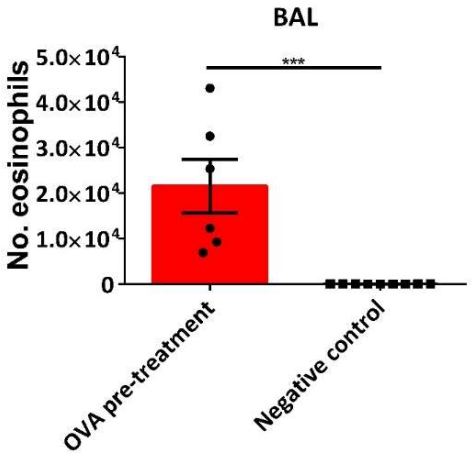


Figure 93. Eosinophilia was already established when BCG was administered in a model of chronic asthma. Eosinophils were quantified by flow cytometry in BAL just before the vaccination in a control group. Data represent mean±SEM from 6 mice in the OVA group and 9 mice in the negative control, analysed by an Unpaired T-test.

Interestingly, eosinophils at week 12 were clearly reduced in the BCG-treated group, indicating the capacity of BCG to overcome established allergen-induced eosinophilia (Figure 94).

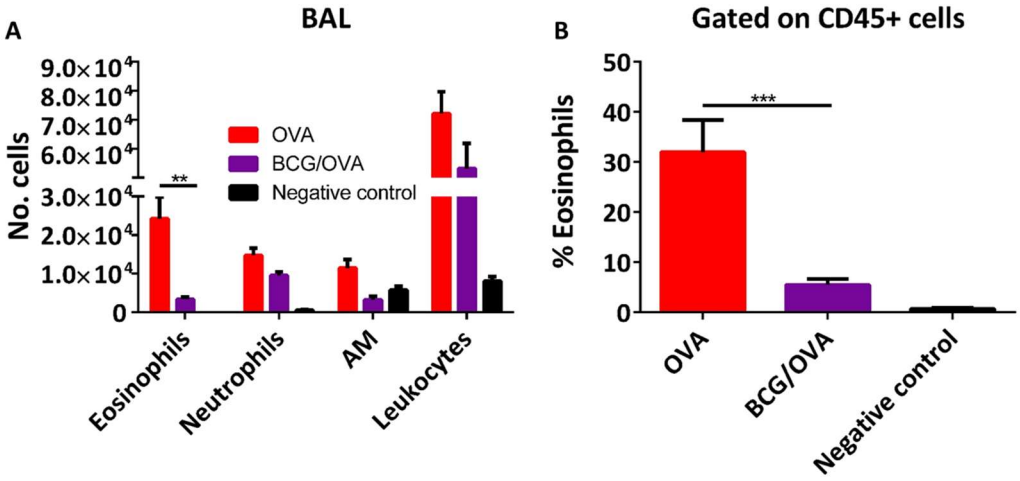


Figure 94. BCG therapeutic effect against chronic asthma in mice. Total myeloids cells (A) and percent of eosinophils (B) were quantified in BAL by flow cytometry. When compared

with the positive control, the eosinophilia was significantly reduced upon BCG. Neutrophils and leukocytes did not suffered significant changes with respect to the positive control and AM remains at comparable levels to those of the negative control. Graphs represent mean±SEM from pool data of two experiments (12 mice in the positive control and in BCG-treated group and 6 mice in the negative control). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

The reduction in eosinophil infiltration came accompanied with an increase in the level of IFN-γ in the BAL and lung from BCG-treated mice. In addition, there was a re polarization of the OVA specific Th2 response to OVA specific Th1 response in lymph nodes (Figure 95).

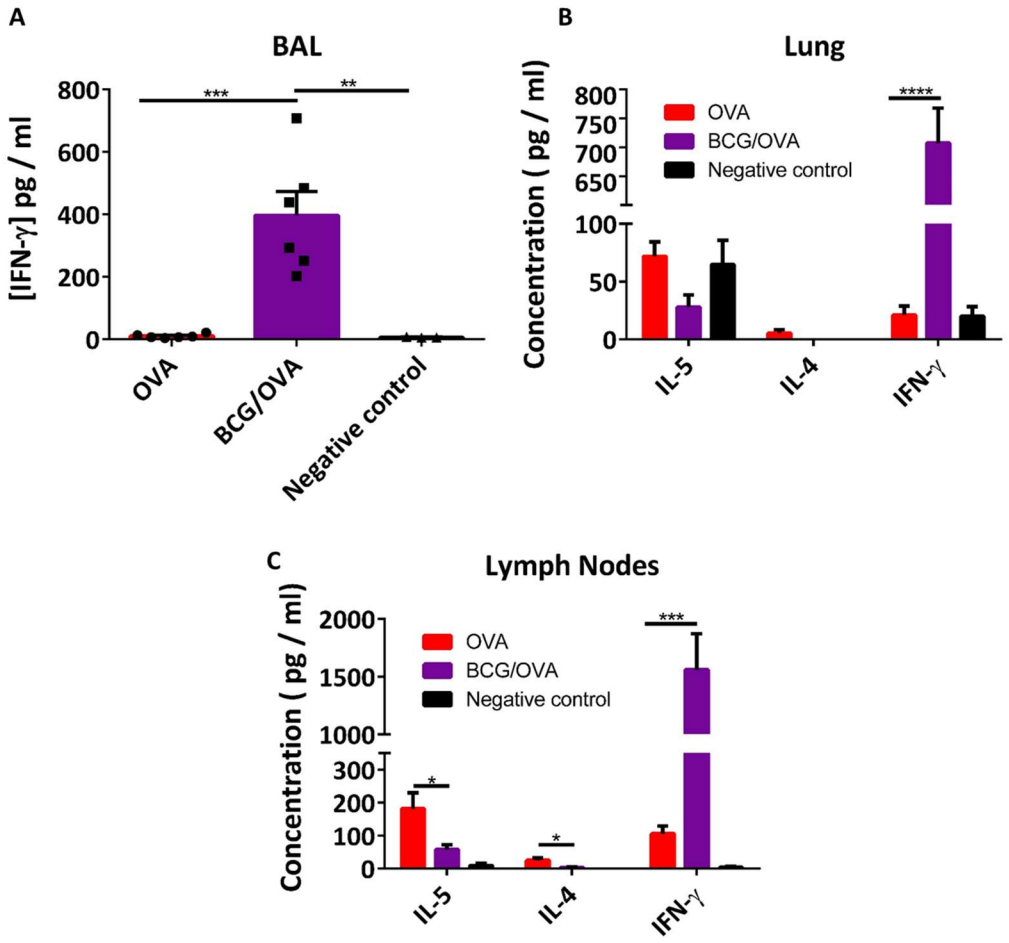


Figure 95. Mechanism of therapeutic effect of BCG in chronic asthma. IFN- γ was measured in BAL (A); IL-5, IL-4 and IFN-γ were analysed in lung explants (B) and OVA-specific IL-5, IL-4 and IFN-γ in lymph nodes (C) by the ELISA technique. Graphs represent mean±SEM from pool data of two experiments (12 mice in the positive control and in BCG-treated group and 6 mice in the negative control). *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

The impact of MTBVAC in established asthma was also examined in another experiment of chronic asthma (Figure 31).

MTBVAC dose was increased ten times with respect to BCG in order to increase its persistence to similar levels of those of BCG (Figure 89). An additional group

sacrificed at week 5, confirmed that there was an eosinophilia at the time when MTBVAC was administered (Figure 96).

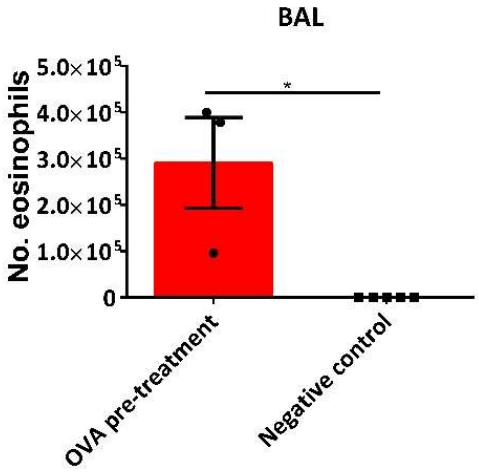


Figure 96. Eosinophilia was already established when MTBVAC was administered. Eosinophils were quantified by flow cytometry in BAL just before the vaccination in a control group. Data represent mean±SEM from 3 mice in the OVA group and 5 mice in the negative control, analysed by an Unpaired T-test.

Importantly, data from week 9 revealed that MTBVAC was able to revert OVA-induced eosinophilia in the airways when administered in a scenario of established asthma (Figure 97).

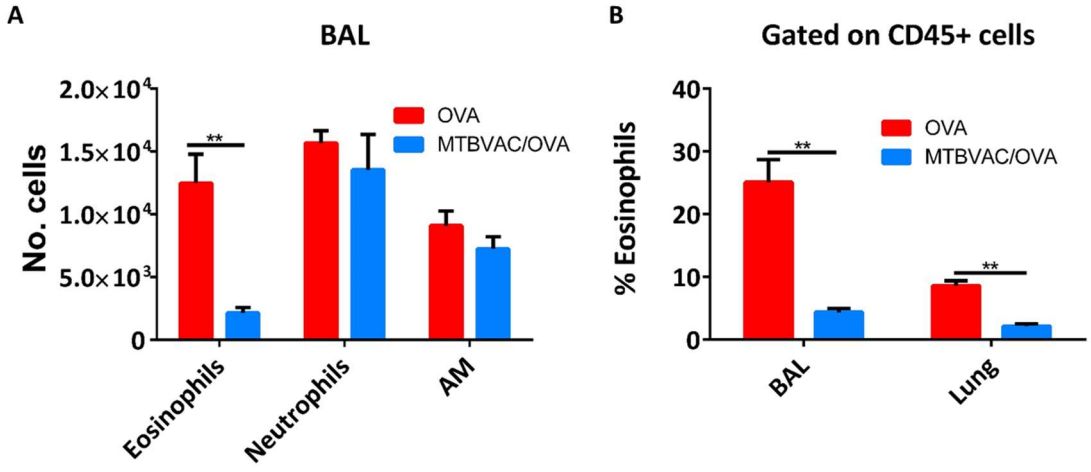


Figure 97. MTBVAC reduces eosinophilia in the airways in established chronic asthma in mice. Total myeloid cells in BAL (A) and percent of eosinophils in BAL and lungs (B) were quantified by flow cytometry. When compared with the positive control, the eosinophilia was significantly reduced upon MTBVAC. Neutrophils and AM did not suffered significant changes with respect to the positive control. Data represent mean±SEM from 6 mice in the OVA group and 7 mice in the MTBVAC-treated group. Analysed by Mann- Whitney test.

In addition, M2- or M1 polarized lungs macrophages were evaluated by analysing expression of Arg-1 or iNOS markers, respectively. A substantial proportion or Arg-

1-positive macrophages was found in the OVA group that was absent in the MTBVAC-treated mice. Further, iNOS-positive macrophages were more frequent in the MTBVAC OVA group, suggesting that the vaccine may repolarize asthma-associated macrophages towards a pro inflammatory M1 profile (Figure 98).

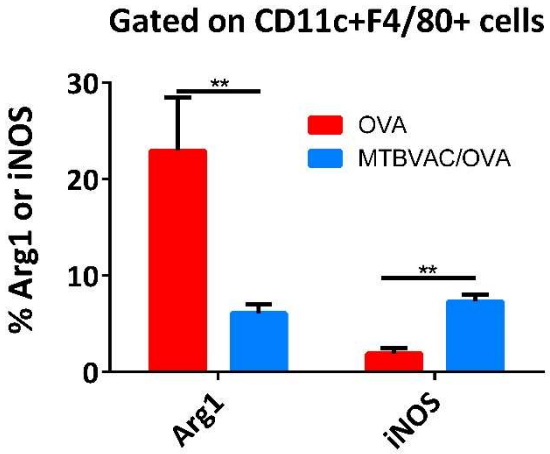


Figure 98. MTBVAC re-polarizes lung macrophages from M2 to M1 in a model of chronic asthma. Intracellular expression of iNOS and Arg-1 in lung macrophages at week 9. Data represent means±SEM, analysed by Mann- Whitney test.

Eventually, it was assessed lung remodelling by PAS staining and histological evaluation, revealing a substantial presence of goblet cells in the OVA group, which were almost absent upon treatment with MTBVAC (Figure 99). Moreover, in the OVA control, there was a thickening of the cell wall in the airways, specifically in the smooth muscle layer, which correlates with the severity of the disease. Finally, there also occurred a disorganization of the epithelial layer, also a typical sign of asthmatic patients [243]. Importantly, this thickening and disorganization were less pronounced in the MTBVAC-treated group.

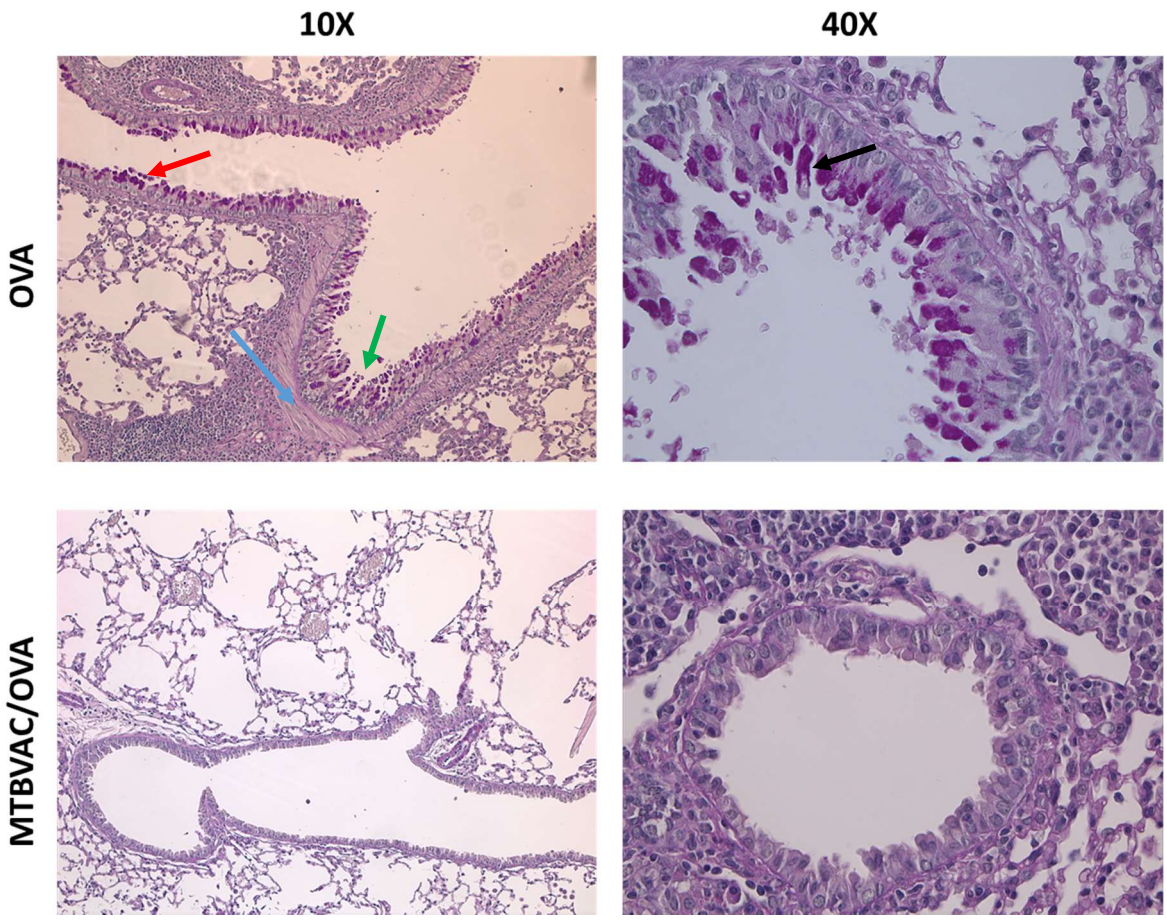


Figure 99. Representative images of PAS-stained fixed lungs from OVA-challenged mice and vaccinated OVA-challenged mice in a model of chronic asthma. Mucosubstances are stained in purple, showing many region with mucus (red arrow) and goblet cells in a multilayer disposition producing the mucus (black arrows). On the other hand, only in the OVA group there was a thickening of the smooth muscle layer (blue arrow). Finally, again in the OVA group, there occurred a destructuring of the epithelial layer (green arrow). Images were taken with lens of 10 and 40 magnification.

6.19 MTBVAC and BCG inhibit eosinophilia and lung remodelling in a model of HDM-driven established asthma

Following with the aim to elucidate the therapeutic effect of attenuated Mycobacteria against established asthma, it was conducted an experiment using another chronic asthma model triggered by the human allergen HDM (Figure 33). In this case, the vaccine (10^6 CFU of BCG or 10^7 CFU of MTBVAC) was delivered once the eosinophilia was already established (Figure 100).

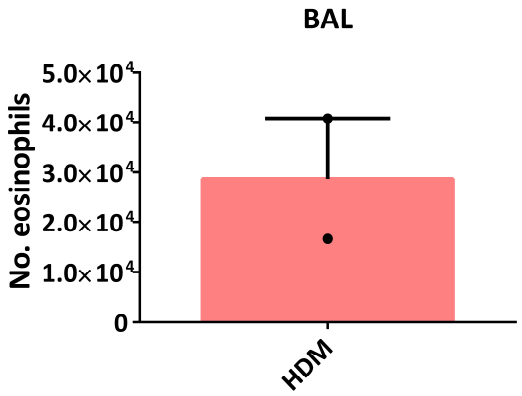


Figure 100. Eosinophilia at the point of vaccines administration in a chronic model induced by HDM. A control group, which suffered from HDM challenges during 3 weeks, was sacrificed the same day in which other groups received the vaccination (week 3). The eosinophilia observed in this control confirmed that vaccines were delivered in a scenario of established asthma. Graphs represent mean±SEM from 2 mice.

In the seventh week, eosinophils were determined in BAL and the Th2/Th1 response was characterized in BAL, lung and lymph nodes. Importantly, data revealed that eosinophils were clearly reduced in the treated groups (even to similar values to those in the negative control), indicating the capacity of MTBVAC and BCG to overcome established HDM-induced eosinophilia (Figure 101).

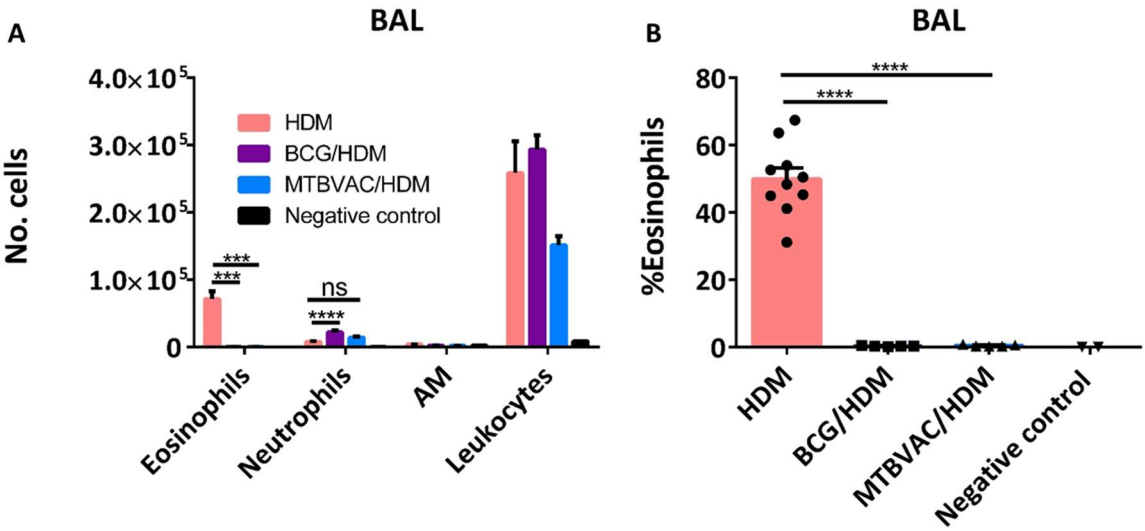


Figure 101. Eosinophilia is reduced by attenuated Mycobacteria in a scenario of established asthma induced by HDM. Total myeloid cells in BAL (A) and percentage of eosinophils in BAL (B) were quantified by flow cytometry, revealing that the eosinophilia decreased upon BCG or MTBVAC treatment. Data represent mean±SEM from 12 mice for the HDM group, 5 mice for the treated groups and 2 mice for the negative control. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

This reduction came accompanied with a re polarization from a Th2 cytokine profile to a Th1 profile led by an increase of IFN-γ in BAL and lungs. Moreover, the same re

polarization appeared in OVA-specific cytokines from the mediastinal lymph nodes (Figure 102).

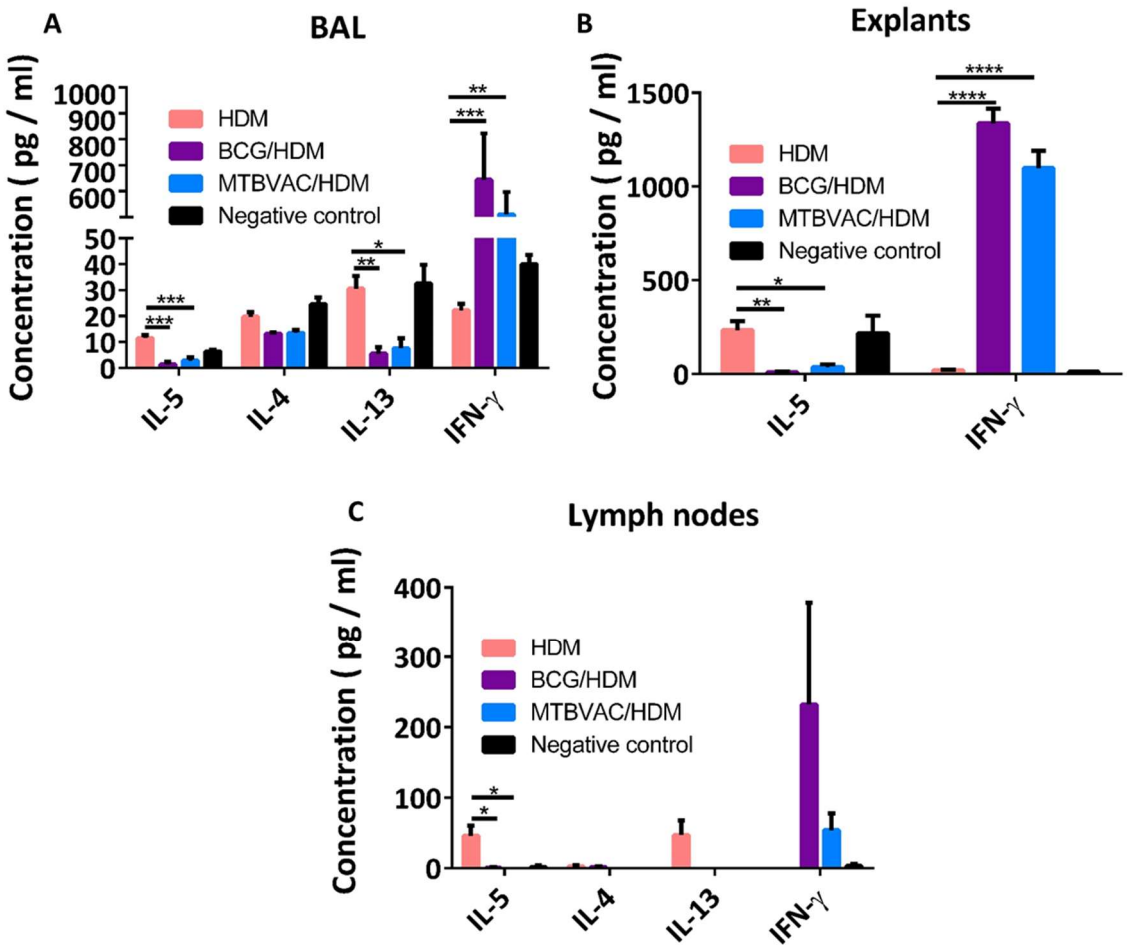


Figure 102. Re polarization from Th2 to Th1 profile upon treatment with attenuated Mycobacteria in a scenario of established asthma induced by HDM. Th2 cytokines (IL-5, IL-4 and IL-13) and IFN-γ were analysed in BAL (A) and in lung explants (B). OVA-specific Th2 cytokines and IFN-γ were measured in lymph nodes (C) by the ELISA technique. In the three situations, Th2 cytokines were reduced with respect to the positive control upon treatment. Contrary, the IFN-γ increased when vaccinated. Data represent mean±SEM from 12 mice for the HDM group, 5 mice for the treated groups and 2 mice for the negative control. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

Moreover, lung remodelling was assessed by histological evaluation of PAS staining. Data demonstrated a substantial presence of goblet cell producing mucus in the HDM group, which were almost absent upon treatment with BCG or MTBVAC (Figure 103).

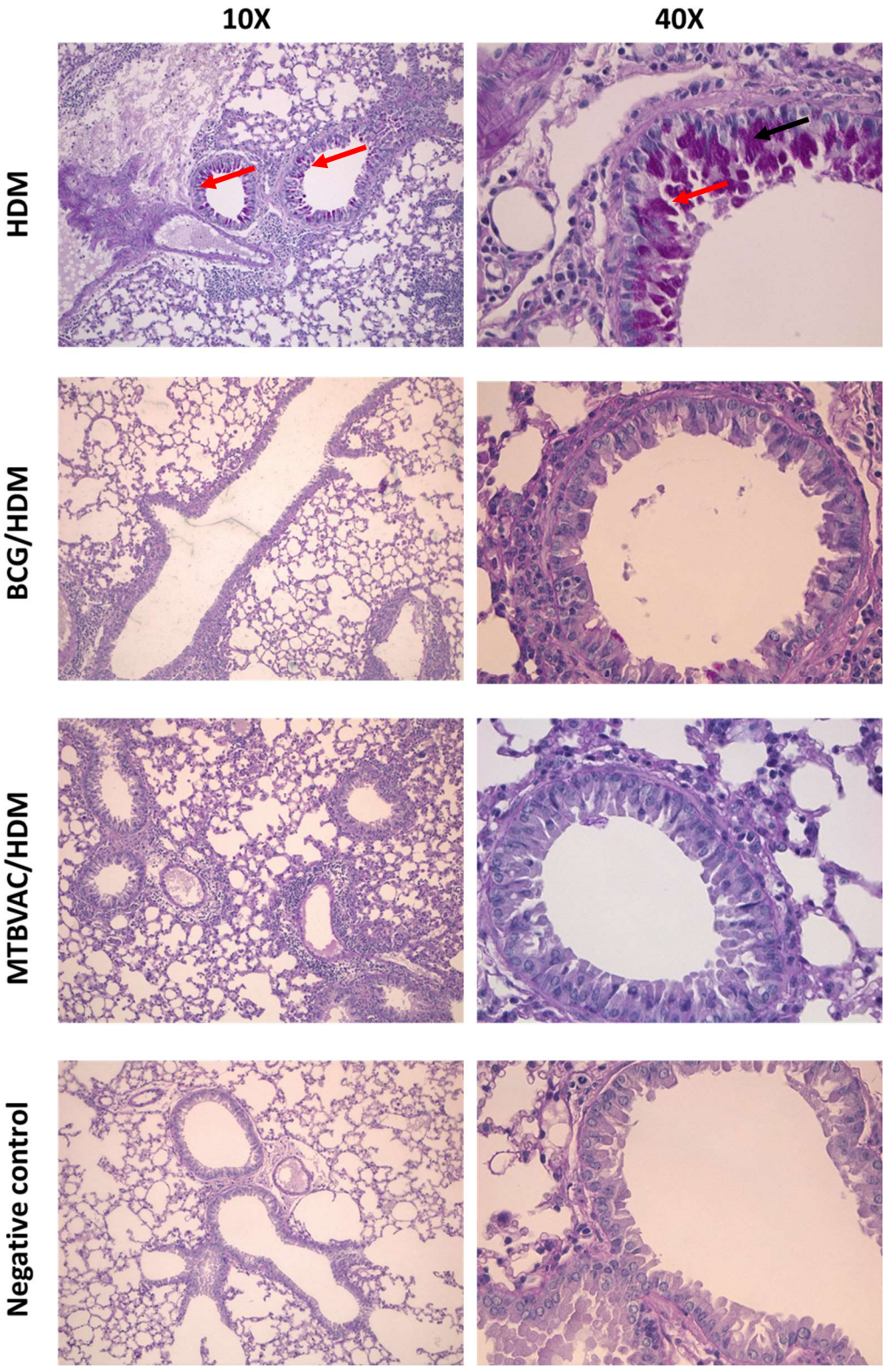


Figure 103. Representative images of PAS-stained fixed lungs in a scenario of established asthma induced by HDM. Mucosubstances are stained in purple, showing many region with mucus (red arrow) and goblet cells in a multilayer disposition producing the mucus (black arrows). Images are taken with lens of 10 and 40 magnification.

6.20 MTBVAC administration by the oral route slightly protects against asthma

The oral route is considered safer for humans when administered attenuated Mycobacteria than the intranasal route, which could be connected with the brain. Further, the oral route has already been used for administrating human life vaccines as it is the case for the vaccine against rotavirus [244] and so the use of this route would be easily transferred to humans.

The intranasal route is much better than the subcutaneous route protecting against asthma (Figure 52). For this and for other reasons described above, it has been hypothesised that the vaccine has to be in contact with the lung immune system to exert its beneficial effects in asthma. To give stronger support to it and for the clinical relevance of the oral treatment, it was carried out an experiment testing 1, 2 or 3 MTBVAC administrations by the oral route in a model of OVA-induced established asthma (Figure 32).

The oral vaccination was administered once the asthma was already established (Figure 104).

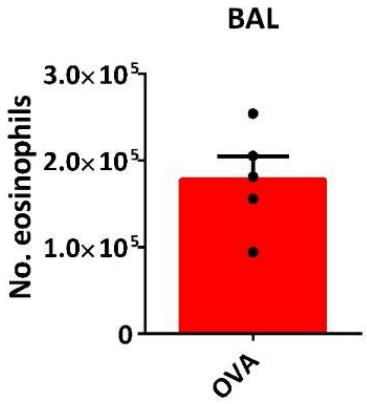


Figure 104. Eosinophilia was already established a day before the first oral vaccination. Eosinophils were quantified by flow cytometry in BAL just before the first oral vaccination. Data represent mean±SEM from 4 mice.

At the end of the experiment, in the tenth week, the eosinophilia and the cytokine profile revealed a partial protection against asthma when used 3 doses of the vaccine (the eosinophilia was reduced by half) (Figure 105). Therefore, the oral route was not as efficient as the intranasal route protecting against asthma and so that fact gives stronger support to the hypothesis that the Mycobacteria has to be in contact with lung cells to exert his action.

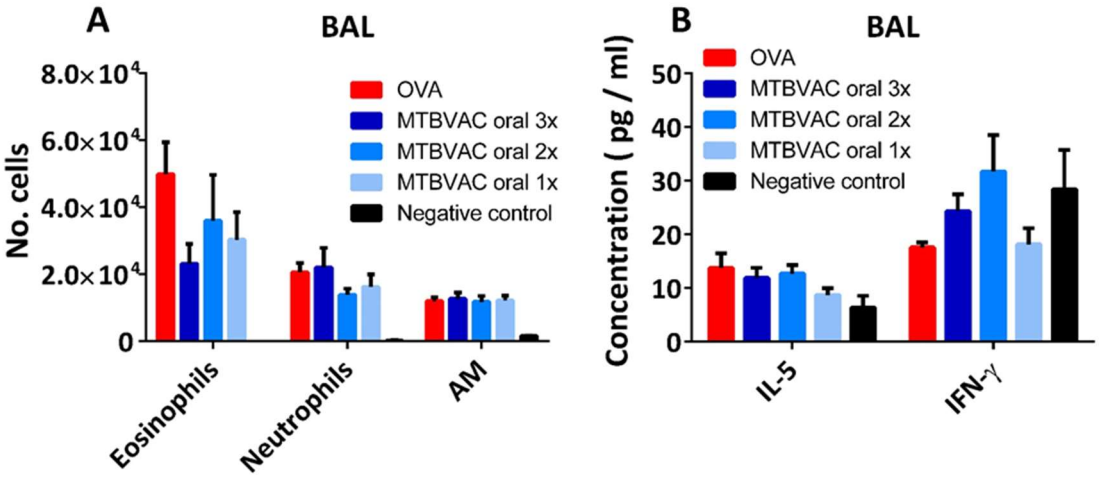


Figure 105. Three doses of oral MTBVAC conferred a partial protection against asthma. Myeloid cells (A) were quantified in BAL by flow cytometry. The group treated with 3 doses of MTBVAC showed a partial reduction in the eosinophilia, but still not as high as in previous experiment with the intranasal administration. IL-5 and IFN-γ were analysed in BAL by the ELISA technique (B), and there were no important differences between groups. Data represent mean±SEM from 6 mice in the positive control, 8 in the treated groups and 3 mice in the negative control. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

6.21 BCG and MTBVAC reduce eosinophils exacerbations triggered by *Alternaria alternata*

The fungus *Alternaria alternata* is a major allergen in many parts of the world though, the precise prevalence of *Alternaria* sensitivity is unknown. Clinically, sensitivity to this fungus is especially relevant since it has been linked to asthma development, and overall with severe and life-threatening episodes of asthma leading to hospital admission and even fatal asthma exacerbations. These exacerbated episodes usually occur during thunderstorms in late summer and early autumn, because of the dispersion of spore, which leads to increased morbidity and mortality. Unfortunately, diagnostic and therapeutic reagents for *Alternaria* sensitivity are not standardized [245].

In addition to allergenic proteins, *Alternaria*, but not other aeroallergens such as HDM, secretes proteases that contribute to airway inflammation and tissue damage. Interestingly, *Alternaria*-dependent protease activity *in vivo* leads to an increase of IL-33 in the airways. The rapid IL-33 release, which underlies the development of a robust Th2 inflammation, leads to the specific subsequent and rapid asthma exacerbations seen in *Alternaria* sensitized individuals. In addition, in these episodes of exacerbation there is a rapid inflow of ILC2 cells and eosinophils, and a mucus release into the airways, potentially contingent on IL-33 [246, 247].

In the present work, it was examined the potential impact of attenuated Mycobacteria impairing the robust Th2 response seen in *Alternaria*-driven exacerbated asthma episodes. Thus, mice were vaccinated by the intranasal route between *Alternaria* challenges in a model of asthma exacerbations triggered by multiple inoculations of the fungus (Figure 34).

First, eosinophils were determined in BAL in an additional group sacrificed at week 3, confirming presence of eosinophilia at the time when attenuated Mycobacteria were administered (Figure 106).

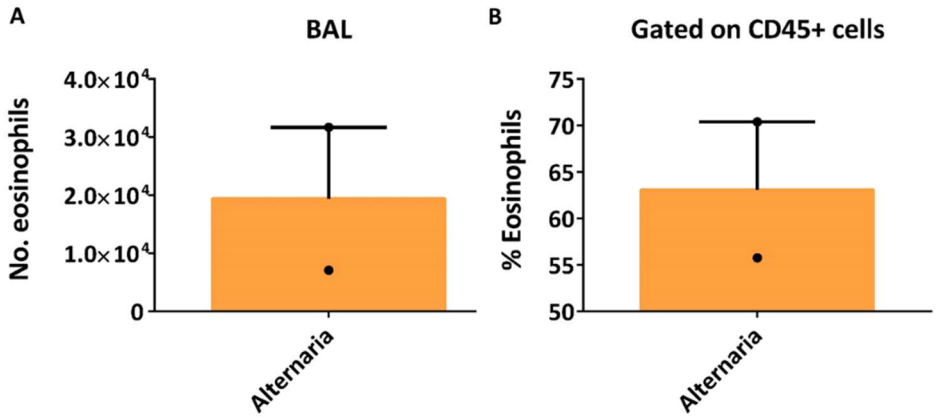


Figure 106. Eosinophilia at the point of vaccines administration in an exacerbation model triggered by *Alternaria alternata*. Total eosinophils (A) or percent of eosinophils (B) measured in BAL by flow cytometry. Both graphs represent a control group, which suffered from *Alternaria alternata* challenges during 3 weeks and which was sacrificed the same day in which other groups received the vaccination. The eosinophilia observed in this control confirmed that vaccines were delivered in a scenario of established asthma. Graphs represent mean±SEM from 2 mice.

Interestingly, at week 7, percent of eosinophils in the positive control was higher than in the OVA and HDM-driven asthma (both acute and chronic scenarios), revealing that a model of exacerbation was established. Importantly, eosinophilia was reduced in the treated groups, indicating the capacity of BCG and MTBVAC to mitigate *Alternaria* driven-exacerbations (Figure 107), as the level of eosinophilia is seemingly a biomarker for asthma exacerbations [248-250].

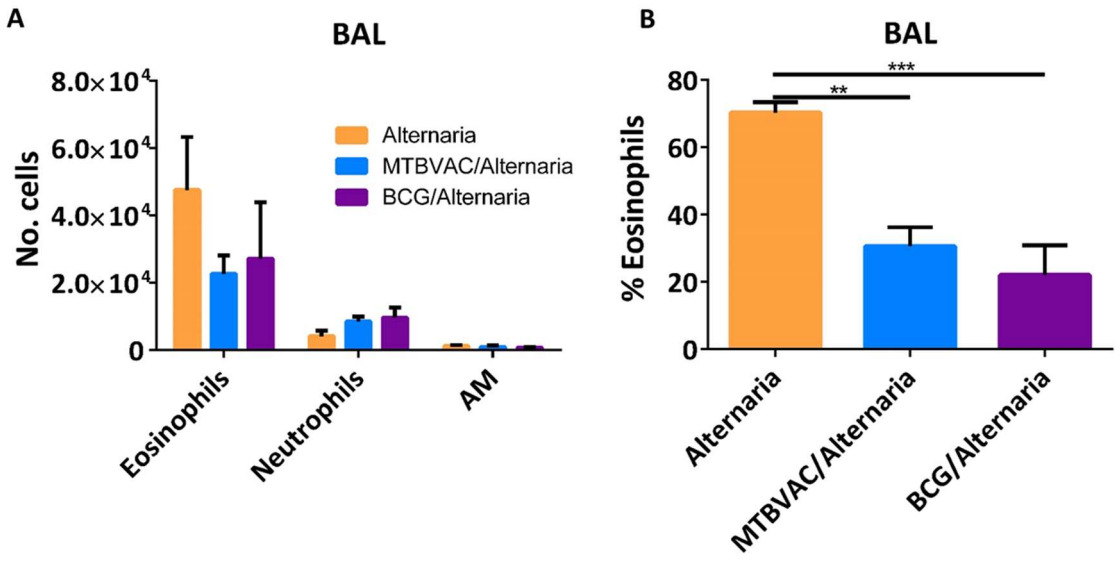


Figure 107. Attenuated Mycobacteria mitigate *Alternaria* driven-asthma exacerbations. Total myeloid cells were quantified in BAL by flow cytometry. When compared with the positive control, the eosinophilia was partially reduced with the vaccination and the neutrophilia remained stable (A). The percent of eosinophils in BAL measured by flow cytometry revealed a significant decrease with the vaccines. Moreover, in the positive control, the eosinophils percent was higher than the one obtained in the models of OVA or HDM-driven asthma (B). Data represent mean±SEM from 6 mice per group, *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

Moreover, the cytokines profile revealed a re polarization from the Th2 profile in *Alternaria*-induced mice to the Th1 profile upon treatment. However, this modulation is not as accentuated as with the other allergens, probably because *Alternaria* exerts its action through slight different mechanisms that imply tissue damage. Interestingly, IL-33 is significantly reduced upon treatment in lung explants. It is logical to think that IL-33 measurements are clinically relevant in these samples, as in *Alternaria*-driven asthma this cytokine is mainly produced by epithelial cells present in the airways (Figure 108).

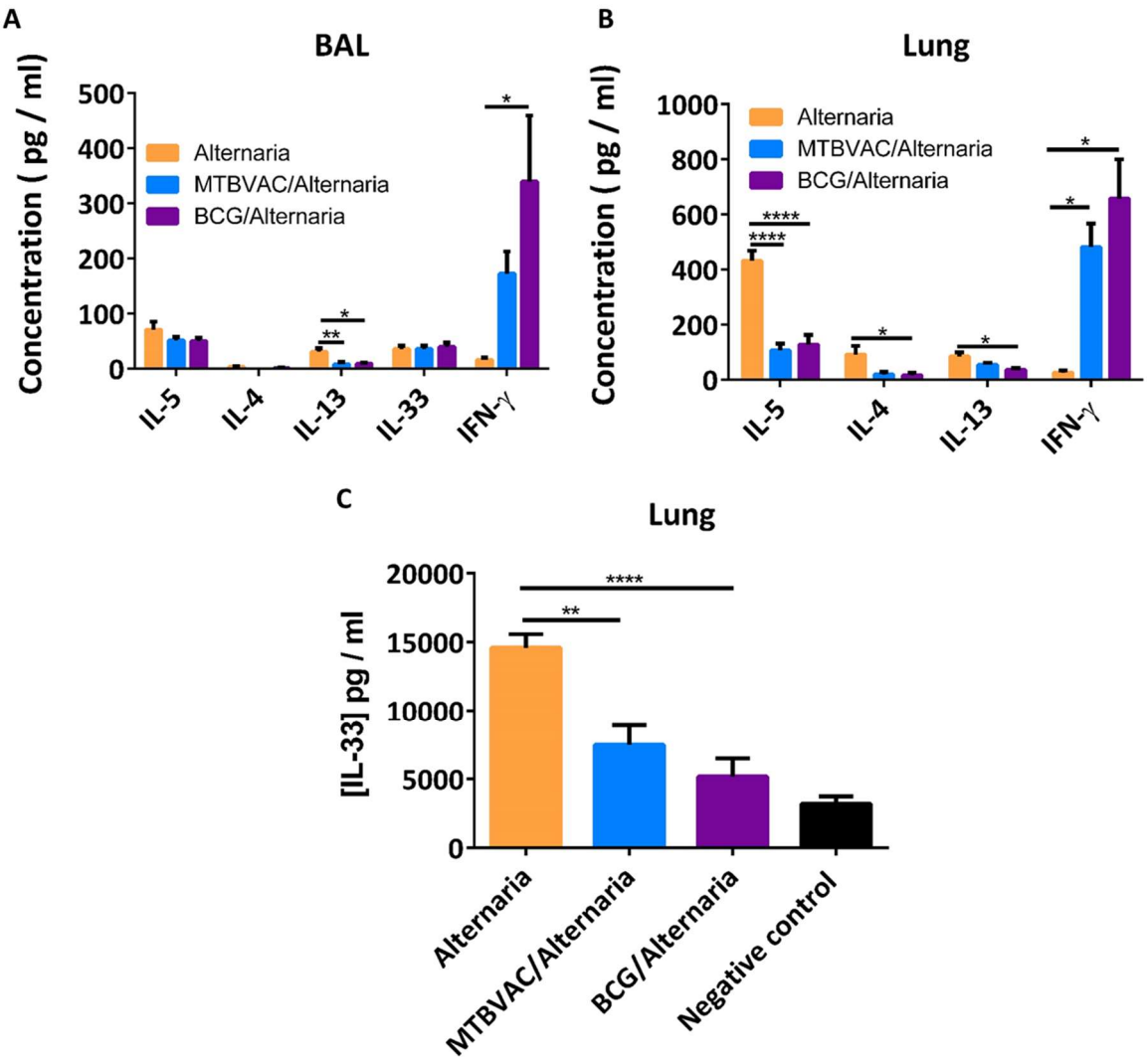


Figure 108. Cytokines profile in a model of exacerbated asthma induced by Alternaria when treated with attenuated Mycobacteria. IL-5, IL-13, IL-4, IL-33 and IFN-γ were measured in BAL (A), and lung explants (B). Data represent mean±SEM from 6 mice per group, *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

The intention is to continue studying in depth this new model, trying different concentrations of the allergen, studying the impact on epithelial tissue and selecting the optimal vaccine dose.

6.22 MTBVAC and BCG are able to prevent eosinophil infiltration in oesophagus in mice

Eosinophilic esophagitis (EoE) is a food allergy-associated chronic inflammatory disease of the oesophagus. However, not only food allergens play a role in EoE pathogenesis, aeroallergens may play an important role as well, as exposure to them is associated with the development of EoE in humans [251] and mouse models [252].

Clinically, it is characterized by organ dysfunction including dysphagia to food impaction, food refusal, vomiting, failure to thrive and regurgitation. Histologically, the main feature is an intense eosinophilic infiltration. The oesophagus is normally devoid of eosinophils, so any number of eosinophils on biopsy indicates an abnormality [253]. Eosinophils have been shown to have direct effects on immune function in the oesophagus, by acting as APCs and secreting cytokines, and on tissue damage, through the release of their granules content [254]. The presence of eosinophils, preclinical studies and the concomitance of EoE with other allergic diseases, indicate an allergic component and an implication of the Th2 response on the disease. In fact, a number of Th2 cytokines, such as IL-4, IL-5 and IL-13, are elevated in both patients with EoE [255] and in murine models of EoE [256].

In industrialized countries, the incidence of EoE has increased dramatically in the last decades to a prevalence rate of 1:1000, causing a considerable public health and economic burden. Specifically, in the USA the cost associated with EoE is about $1 billion per year [254]. In addition, current management strategies against the disease, such as swallowed topical steroids, are nonspecific and there are concerns regarding their long-term use. Therefore, there is a need to find new and more specific treatments [257].

It seemed logical to hypothesise that since attenuated Mycobacteria are able to impair the Th2 response exacerbated in asthma, they could also impair the Th2 response underlying the EoE. In addition, it has been demonstrated that Mycobacteria can suppress the eosinophilia in the airways, so it led to hypothesised that this reduction could be extended to the oesophagus. In this regard, oesophagus from OVA-challenged mice and from i.n. Mycobacteria-treated (with 10^6 CFU of BCG or 10^7 CFU of MTBVAC) were analysed.

First, oesophagus of OVA control mice from the model of acute asthma showed an eosinophilic infiltration (Figure 109). This likely occurred because while the intranasal inoculations were done, traces of the allergen passed to the digestive tract, sensitizing cells in the oesophagus. In addition, there were no neutrophils, as it occurs in human EoE. Importantly, this eosinophilia was reduced in groups treated with BCG or MTBVAC (Figure 109), suggesting that attenuated Mycobacteria could be considered a potential treatment against EoE.

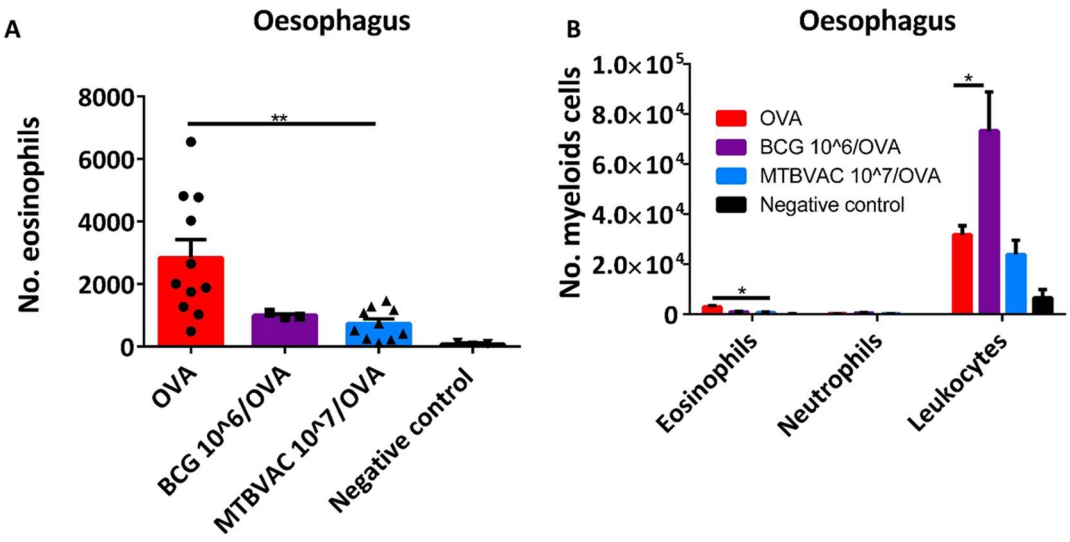


Figure 109. Mycobacteria prevent eosinophil infiltration in EoE in mice. Total eosinophils (A) and total neutrophils and leukocytes (B) were quantified by flow cytometry in the oesophagus, revealing that the eosinophilia decreased upon BCG or MTBVAC treatment. However, when treated with BCG, there was a leukocyte infiltration, not seen with MTBVAC. Data represent mean±SEM from 11 mice for the OVA and MTBVAC/OVA groups (data were pooled from two experiments), 4 mice for BCG- treated group and 3 mice for the negative control. *p<0.05; **p<0.01; ****p<0.0001, by two-way ANOVA.

The fact that intranasal administered Mycobacteria were able to induce an immunologic response in the oesophagus may be explained by two different ways. Leftovers of bacteria are passing to the digestive tract during the intranasal inoculation, or Th1 repolarized specific OVA cell present in lymph nodes (as demonstrated in the asthma model) are subverting the Th2 scenario established in the allergic oesophagus.

More cytokine and histological studies of oesophagus should be done to give additional protection data to those of the reduction of eosinophils. For example, histological studies of these oesophagus, did not reveal a clear epithelial damage, as they have been subjected to an acute model and likely there has not been enough time for it, and also because the model is not specific for EoE. Moreover, eosinophilia were not detected in the stained oesophagus. In fact, only one eosinophil was detected in the histological preparation. This is likely because the total eosinophils in the oesophagus (an average of 3000) is too low to be detected in a single histological section of the organ (Figure 110).

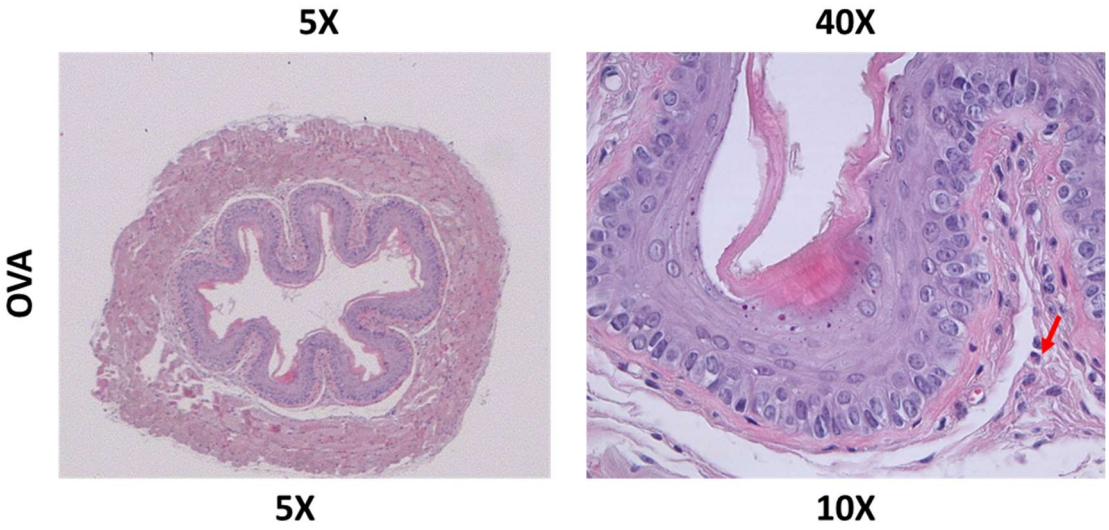


Figure 110. Representative images of HE-stained fixed oesophagus from OVA-challenged mice in an acute model of asthma. Eosinophil is indicated by a red arrow. Images are taken with lens of 10 (A) and 40 (B) magnification.

However, taking into account that BCG and MTBVAC have demonstrated to be able to reduce Th2 cytokines and subverts remodelling in the airways, it is plausible to think that these effects can be transferred to the oesophagus. Therefore, deeper experiments should be done, also in more specific EoE models, to confirm these promising results.

7. DISCUSSION

In the present study, we demonstrate that intranasal BCG and MTBVAC are able to prevent and revert asthma-associated responsiveness induced by different allergens. Therefore, they not only prevent T2-type asthma but also they are capable to treat established T2-asthma.

On the other hand, our data seem to indicate that attenuated Mycobacteria are able to prevent eosinophilia in EoE, a clinically relevant food allergy in humans. In this regard, we could hypothesise that Mycobacteria exert beneficial effects in other types of eosinophilia or T2-syndrome, different from asthma, such as food allergies. It would allow us to collaborate with clinicians in order to find new targets and to explore different routes of treatment administration, according to our hypothesis about the organ-dependent effect mediated the bacilli.

T2-type asthma is suffered in most than 80% of asthmatics children and in the majority of adults. It includes not only allergic asthma but also non-allergic and still eosinophilic asthma [1]. In this regard, BCG and MTBVAC are effective against T2-type asthma, and so against most of the cases. However, they are not effective against neutrophilic asthma, a much lower frequent disease, so hypothetically, in the clinic, asthma should be phenotyped before attenuated Mycobacteria administration, as occurs in the precision medicine approaches.

Further, severity of asthma and exacerbations correlates with the number of eosinophils in the sputum [35, 202]. Nevertheless, given the importance of eosinophils in lung defence mechanisms, one must consider that the best way to treat asthma should include not its complete elimination, but the partial control of eosinophilic response. Here, we demonstrated that these cells are reduced upon attenuated Mycobacteria treatment, but only to similar levels to those of the negative control, so they are not totally eliminated.

Moreover, we demonstrated that BCG and MTBVAC are effective against acute response in asthma and against chronic asthma as well, where repeated exposures to the allergen induce a chronic state of inflammation collectively known as airway remodeling, measured through goblet cell hyperplasia, epithelium destructuring and airways wall thickening.

Traditional drugs for asthmatics are only focused in mitigating the symptoms of the disease. However, novel biological therapies have emerged during the last decade focusing on the blockade of specific pathways underlying the development of asthma, such as IL-4, IL-5, IL-13 or IgE. Despite the fact that most of the clinical trials using

this immunotherapy drugs showed positive results, the efficacy achieved by inhibiting a single pathway resulted in general partial, since asthma is highly complex. For instance, blocking IL-5 inhibits eosinophilia, whereas outcomes for lung function are less favourable [67]. Therefore, we hypothesise that a more global approach as the obtained with attenuated Mycobacteria could be more efficient dealing with the different aspects of the pathology.

In allergic asthma, allergen-specific CD4+ T cells play a central role in asthma pathology. 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[258]. These long-lived T cells underlie the perpetuation of asthma throughout lifetime. In predisposed individuals, after sensitization and upon allergen exposure, specific memory CD4+T cells migrate to lungs and recognise allergen-derived peptides presented by APCs. Then, they start to secrete Th2-cytokines which will orchestrate the inflammatory response. Thereby, hampering allergen-specific memory Th2 cells should be the goal for a therapy against asthma. In this regard, immunotherapy based on low-level allergen epitope exposure induces anergy on allergen specific Th2 cells [259].

In the present work, analysis of mediastinal lymph nodes demonstrate that intranasal MTBVAC and BCG affect allergen-specific Th2 cells. Using flow cytometry, we found IL-5-producing T cells in the OVA control group that diminished upon BCG, whereas OVA-specific IFNγ-producing cells arised. It implies that Mycobacteria turn the Th2 cells involved in asthma to a Th1 non-pathological profile, what is additionally supported by cytokine data in BAL and lung.

Importantly, BCG-induced Th1 profile was also observed in our studies in the long-lasting model, where four months after vaccination, eosinophilia was still reduced even though bacteria was almost cleared. It suggests that BCG might be inducing allergen-specific memory Th1 cells that persisted even after Mycobacteria elimination, which could confer a long-term protection against allergen exposure. This is clinically relevant as patients potentially could take a single dose of the vaccine to mitigate asthma for a long period, such as spring.

Nevertheless, it remains unclear whether the Th1 cells seen after vaccination are generated *de novo* from naïve T cells and are cross-regulating the expansion of Th2 cells, or instead they are the same clones that were Th2 prior to vaccination and have been re-polarized to Th1 upon vaccination. In this regard, plasticity of Th2 cells has been demonstrated in some experiments where Th2 cells become Th1 in the presence of an appropriate environment with interferon and IL-12 [260].

It is important to highlight that the Th1 response induced by the treatment is not pathological, as it has been historically demonstrated in BCG vaccinated and LTBI individuals. The response is induced by the very nature of the mycobacteria and if they replicate, they do it very slowly, without producing a peak of antigen. Moreover, after a certain period – about 4 months in mice- the bacilli is cleared from the lungs, as we demonstrated in the long-lasting experiment.

The important role of alternatively activated macrophages or M2 macrophages in asthma has been already described in the literature. In fact, they are elevated in lungs from asthmatic individuals and their increase correlates with worse symptoms. However, there is not a clear explanation for this pathological macrophage polarization in asthma. It is hypothesised that allergens can directly cause damage in the alveolar epithelium, and as reaction against the injury, macrophages are alternatively activated to induce a wound-healing response [261]. M2 is a simplified terminology that encloses different subsets of macrophages with regulatory skills, including M2a, M2b and M2c. The particular presence of M2a macrophages has been linked with an induction of Th2 adaptive response [51]. In allergic asthma, at least two different pathways can explain this relation. On the one hand, activated macrophages express high levels of MHC-II molecules and so they are capable of presenting allergen-derived peptides to T lymphocytes. On the other hand, they secrete cytokines such as IL-4 or IL-13, which in its turn can trigger T cell polarisation to a Th2 profile [262].

In the present study, apart from Th2 cells, we provide evidences for the implication of distinct polarized macrophages in the pathology of asthma and in the Mycobacteria-mediated protection. Our data clearly indicate that BCG and MTBVAC impairs M2 macrophages associated to allergen exposure. This is likely due to the fact that these mycobacteria are able to activate macrophages in a classical way, referred as M1, probably because of their intracellular status. Thus, internalization of both BCG and MTBVAC by lung macrophages induce the expression of typical M1 markers such as iNOS (here confirmed by flow cytometry and RNA analysis) and the secretion of cytokines such as IL-1β, TNFα and IL-12 (here demonstrated by RNA quantification). This inflammatory response is antagonist to the generated by alternatively activated macrophages.

Interestingly, our data with GFP-expressing BCG indicate that not only infected macrophages adopt an M1 phenotype, but also uninfected cells. It implies that infected macrophages produce many signals that could activate their neighbours, triggering the expansion of a response that counterbalance asthma-associated environment.

Multiple observational and interventional studies has explored the beneficial effects of BCG in asthma. The former include epidemiological studies where comparing asthma prevalence in BCG vaccinated individuals [127] and the latter are based on the comparison between a vaccinated and a placebo arm concerning the development of asthma symptomatology [130]. However, the conclusions are unclear since data showed opposite findings: some studies manifest a relation between BCG vaccination and the decrease in asthma prevalence and other epidemiological studies revealed not association between BCG intradermal vaccination and low risk to develop asthma [132, 133, 135].

This discordance between epidemiological studies and our data may be explained by the route of BCG administration. BCG has been historically administered by the intradermal route, and our data show evidences that the beneficial effects of attenuated Mycobacteria is organ dependent. What is more, when vaccinated by the subcutaneous or the oral route, the efficiency was by far lower than when used the intranasal route. Therefore, bacteria need to be present in the lungs to induce the anti-asthma response. This should be logical if we consider that attenuated Mycobacteria must interact with lung resident macrophages to reshape their activation phenotype.

Following on this, our study exposed in the last chapter and robust studies showing lower prevalence of asthma among TB patients, support our hypothesis, since Mtb infection is usually through the respiratory route. These studies are more consistent than those of BCG. For example, a correlation between TB notification cases and lower prevalence of asthma was found [12]. Another study in latent-TB infected individuals (LTBI) demonstrated a correlation between tuberculosis skin test (TST) and lower prevalence of allergy [207, 208].

To conclude, our results with live attenuated vaccines pulmonary delivered are reflecting a natural unspecific protection that already occurs in the nature, as it was discussed in the previous chapter.

Interestingly, we found that the bacteria dose and persistence are crucial for asthma protection. First, we compared the protection between different attenuated Mycobacteria and we showed that as more attenuated the Mycobacteria were, less effective the protection was. Secondly, the dose is so important that until certain dose there is no protection. The mechanisms underlying the role of the persistence and dose in the protection were not elucidated in the present work. In the case of the dose, we could hypothesised that it is related to the fact that bacteria should be in contact with macrophages in the lungs. Hence, macrophages phagocyte the bacteria and start to produce signals to their neighbours which in turns polarize to an anti-asthma profile. In this regard, a minimum initial number of macrophages should be infected to

produce the necessary signals for the environment. On the other hand, concerning the persistence, likely a certain number of bacteria should be present for a certain period of time in order to be phagocyted for a high number of macrophages. In addition, likely the stimulus- Mycobacteria- should remain to reprogram the Th2 response to a Th1 response. In this regard, Mycobacteria derived peptides have to reach mediastinal lymph nodes to induce *de novo* Th1 expansion and simultaneously clear the Th2 cells, or they have to re-polarize the Th2 cells to Th1 cells. Anyway, it must take time and probably, Mycobacteria have to compete with OVA-driven response, so it will be definitely better if bacteria persist longer and in a high dose to compete with the response triggered by the allergen.

On the other hand, the slight lower protection observed in MTBVAC with respect to BCG at the same dose, is because the former persists less than the latter. In this regard, when increased the dose of MTBVAC 10 times to reach an equivalent persistence to those of BCG, the protection obtained is equivalent between both vaccines. Thus, pulmonary MTBVAC should be considered an attractive approach for T2-asthma, with some advantages with respect to BCG, not only in the safety but also in the administration to the lungs. In terms of safety, BCG is resistant to isoniazid and pyrazinamide, but MTBVAC is fully susceptible to current TB antibiotics [104]. In addition, in clinical trials intradermal MTBVAC showed a lower reactogenicity than BCG[116], which could be related with the fact that MTBVAC is more efficiently clear from the lungs, as we demonstrated here. In terms of delivery, MTBVAC is more efficiently nebulized than BCG, when used a clinical nebulizer and it counterbalance the increase in the MTBVAC dose needed to reach the BCG-protection level (Figures 111 and 112). Experiments carried out in our laboratory (data not published) with the clinical device OMROM U100, compared the nebulization efficacy between MTBVAC GMP produced and two different doses of BCG GMP produced. The nebulization efficacy refers to the percent of bacteria recovered in the nebulized fraction compared to the initially present.

Firstly, it was compared lots of BCG and MTBVAC GMP tested, containing $5x10^6$ and $1.1x10^7$ CFUs/ml respectively. In 5 minutes of nebulization, the volume recovered was 1.2 ml. Here, the amount of CFUs recovered was $1.2x10^5$ CFUs for BCG and $1.98x10^6$ CFUs for MTBVAC, so the nebulization efficacy was higher when used MTBVAC (Figure 111).

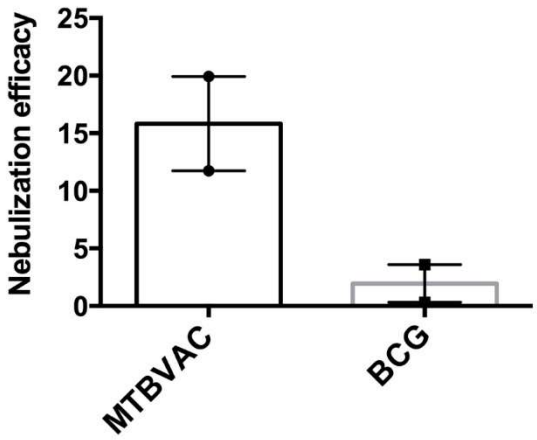


Figure 111. MTBVAC is more efficiently nebulized than BCG when used doses of 1.1x10^7 and 5x10^6 CFUs/ml respectively. Graph represent data from two experiments for each vaccine. Data not published[263].

Secondly, in our laboratory it was tested BCG and MTBVAC GMP lots containing 1.7x10^7 and 1.1x10^7 CFUs/ml respectively. In 5 minutes of nebulization, the volume recovered was 1.2 ml. The mean amount of CFUs recovered was 3.3x10^4 CFUs for BCG and 1.98x10^6 CFUs for MTBVAC (Figure 112). To sum up, a therapeutic dose of MTBVAC can be nebulised by a clinical device, whereas a BCG dose cannot.

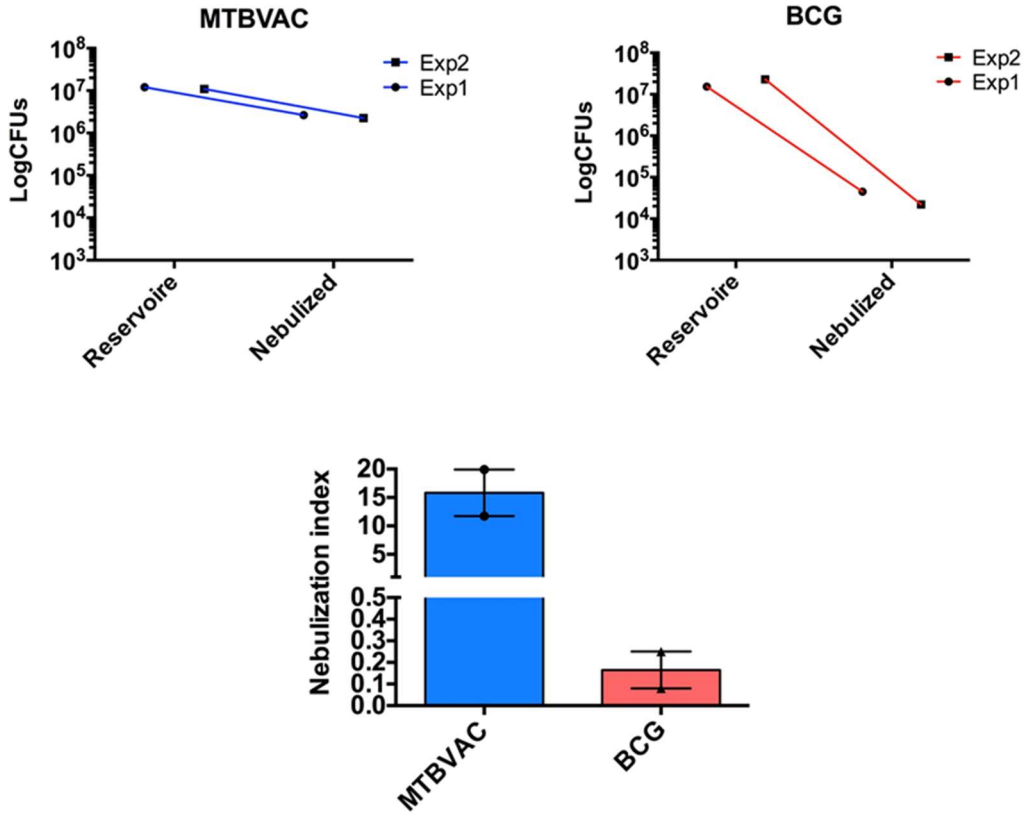


Figure 112. MTBVAC is more efficiently nebulized than BCG when used doses of 1.1x10^7 and 1.7x10^7 CFUs/ml respectively. Graph represent data from two experiments for each vaccine. Data not published[263].

The explanation for these differences in nebulization may lie in the nature of the bacteria. On the one hand, as demonstrated by members of our laboratory, MTBVAC bacillus is shorter than BCG, being 0.6 µm the length for MTBVAC and 2 µm the length for BCG (Figure 113). On the other hand, observations made in our laboratory showed that MTBVAC experiment less clumping than BCG. This is likely due to the lack of certain glucolipids in the surface of MTBVAC when compared to BCG, as is the case for PDIM. This hypothesis is supported by the literature, which revealed that selective sugars are implicated in cell aggregation or clumping [264]. In this regard, when used BCG the nebulizer pores plug, and they block faster when used higher doses of it.

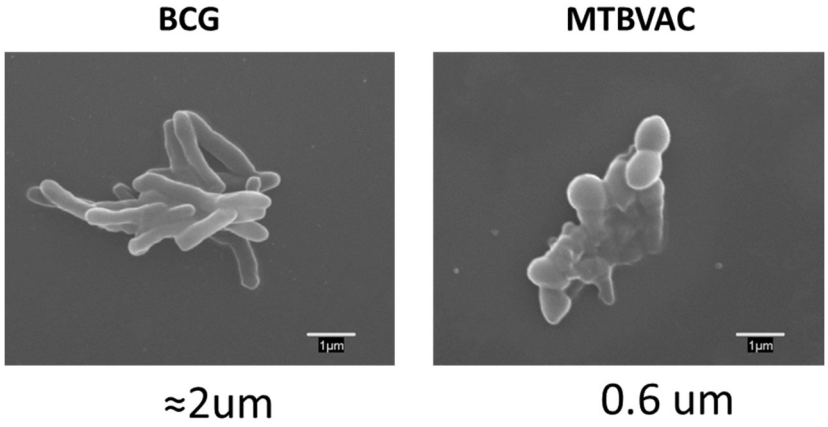


Figure 113. Electronic microscope images of BCG and MTBVAC. Representative images of electronic microscope revealed that MTBVAC bacterial length is shorter than BCG. Figures from our laboratory, unpublished.

Despite the huge potential benefits of attenuated Mycobacteria in asthma, we should be cautious in the translationality to humans, as currently there is no treatments in clinic based on pulmonary delivery of live organisms. However, we are still optimistic due to two main reasons. First, BCG has been already administered by the aerosol route to humans, as it is reported in a study in children from 1968 [265]. Further in the 70s, studies for lung cancer used aerosol inoculation of BCG containing high bacterial loads. In spite of not having found therapeutic effects, either no major toxicity issues appeared [266]. On the other hand, taking into account our results, administration of live attenuated Mycobacteria not only prevents but also reverts asthmatic inflammatory response and so asymptomatic people would not need to be vaccinated early in life but when developing the disease. This is clinically relevant as the application of preventive products must be extremely cautious with regard to safety, as they target healthy individuals. Conversely, concerning therapeutic products, the cost-benefit balance can change if the therapy is effective enough. In asthma, exacerbations and severe and uncontrolled cases cause about 0,4 million

deaths per year. In addition, in less severe cases, asthma profoundly affects the quality of life of patients. Eventually, being asthma a chronic disease without current cure, it causes huge economic burden for the national budgets.

In conclusion, the present work supports the use of attenuated Mycobacteria for the control of the asthmatic responsiveness in a long-term fashion, suggesting its further exploration even in clinical trials in the coming future. Moreover, it gives a chance for the application of attenuated Mycobacteria in the control of food allergies and other types of eosinophilia.

You have to act as if it were possible to radically transform the world. And you have to do it all the time

Angela Davis

Chapter 3

INDUCTION OF TRAINED IMMUNITY BY MTBVAC IN HUMAN MONOCYTES *IN VITRO*

Experiments in this chapter, with the exception of the *in vivo* experiment in mice, were carried out during a research stay in the Mihai Netea's laboratory at the Radboud University in Nijmegen, The Netherlands

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BCG is able to induce trained immunity both *in vitro* and *in vivo* scenarios, as it was demonstrated in human monocytes *in vitro* [166] and in interventional studies with infants vaccinated [167, 168]. This capacity is clinically relevant since it is related with the therapeutic efficacy of vesical BCG instillations in patients with bladder cancer [267], and with the decrease of respiratory infections in children vaccinated with BCG[144, 145]. Thus, the induction of trained immunity enables BCG to protect against other diseases different from TB.

Since MTBVAC is a live attenuated Mtb vaccine that could replace BCG, it is clinically important to demonstrate that it maintains the nonspecific effects exerted by the current TB vaccine. In this regard, in this chapter it was assessed for the first time the potential of MTBVAC to induce *in vitro* trained immunity in human monocytes. Moreover, the epigenetic and metabolic pathways underlying this process were also examined here.

8. RESULTS

8.1 MTBVAC exerts immunomodulatory effects in PBMCs comparable to BCG

First, it was studied the potential of MTBVAC to stimulate human PBMCs *in vitro*. PBMCs from healthy volunteers were stimulated *ex vivo* with MTBVAC, BCG Intervax, LPS, β-glucan, glycerol or RPMI.

Different MOI of MTBVAC were added, in order to examine the concentration that most stimulates the cells. BCG Intervax was used to be compared with MTBVAC, as it was the vaccine used for standard training protocols carried out in the Mihai Netea's laboratory. In addition, BCG Intervax came from a commercial vial, so the formulation was different from that of MTBVAC, as apart from live bacillus, it contained thousands of death bacteria not indicated in the prospectus. At the time of using BCG Intervax, bacteria were not alive (because it was stored at 4 ºC), but the dose used was equivalent to a MOI of 7.5 x 10^{-2}, taking into account the live bacteria in the initial vial.

On the other hand, β-glucan and LPS were used because they are always employed as positive and negative controls in training experiments [168]. Glycerol was used as a control, because MTBVAC was aliquoted on it, and it was added at the same concentration as the glycerol was in the wells with MTBVAC (0,05 % glycerol) . Finally, RPMI represented the unstimulated cells.

MTBVAC led to the production of IL-6, TNF-α, IL1-β, IL-10 and IFN-γ after 48 h of stimulation (Figure 114). Indeed, MTBVAC MOI 10 was the dose that induced the highest cytokine production, even to higher levels than those of BCG Intervax. All of these cytokines, with the exception of the IFN-γ, were mainly produced by monocytes, as after 48 h of stimulation, lymphocytes had not enough time to respond.

Moreover, after 7 days of stimulation, MTBVAC led to the production of IL-22, IL-17 and IFN-γ, mainly by activated T lymphocytes.

For its part, glycerol did not seem to stimulate cells except as regards the production of TNFα. Therefore, in further experiments glycerol was eliminated from vaccines vials before being added to the plates. On the other hand, β-glucan did not stimulate the production of cytokines by lymphocytes (data at 7^{th} day).

In summary, MTBVAC stimulates and activates human PBMCs *in vitro*, being the MOI of 10 the dose that stimulates the most, to at least similar levels than those of BCG.

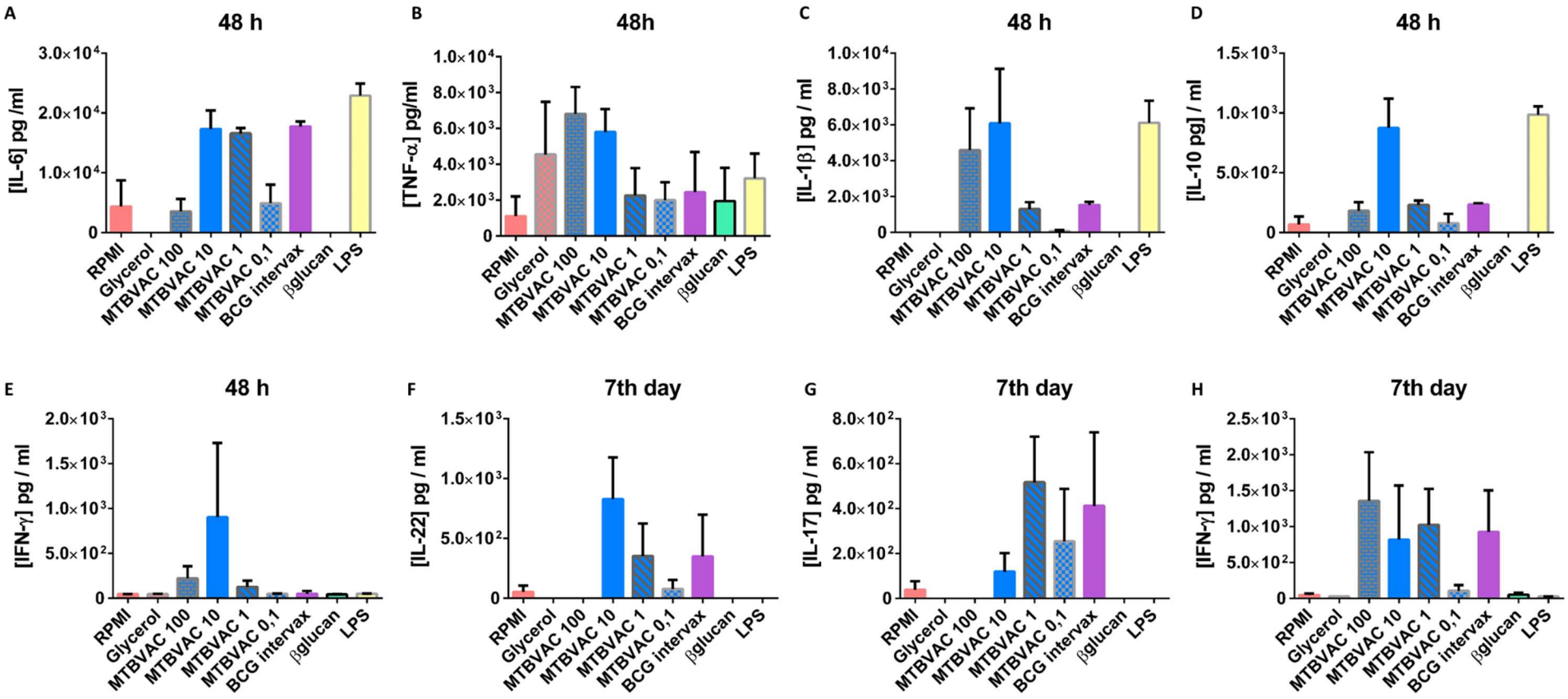

Figure 114. MTBVAC stimulates human PBMCs *in vitro.* IL-6, TNFα, IL-1β, IL-10, IFN-γ, IL-22 and IL-17 production by human PBMCs 48 h or 7 days after stimulation with different doses of MTBVAC, BCG Intervax, glycerol, β-glucan and LPS. Mean ± SEM, n = 3 (from 3 different individual donors).

8.2 MTBVAC induces acute stimulation in human monocytes *in vitro*

In another experiment, it was evaluated the impact of MTBVAC in human monocytes *in vitro*. Fist, the vaccine demonstrated to induce an acute stimulation of these cells. After 24h with MTBVAC, monocytes produced the pro-inflammatory cytokines IL-6, TNFα and IL-1β, and in a lower concentration, IL-10. Moreover, the effect mediated by MTBVAC in the production of cytokines was dose-dependent, being the MOI of 10 the best dose inducing the acute response (Figure 115).

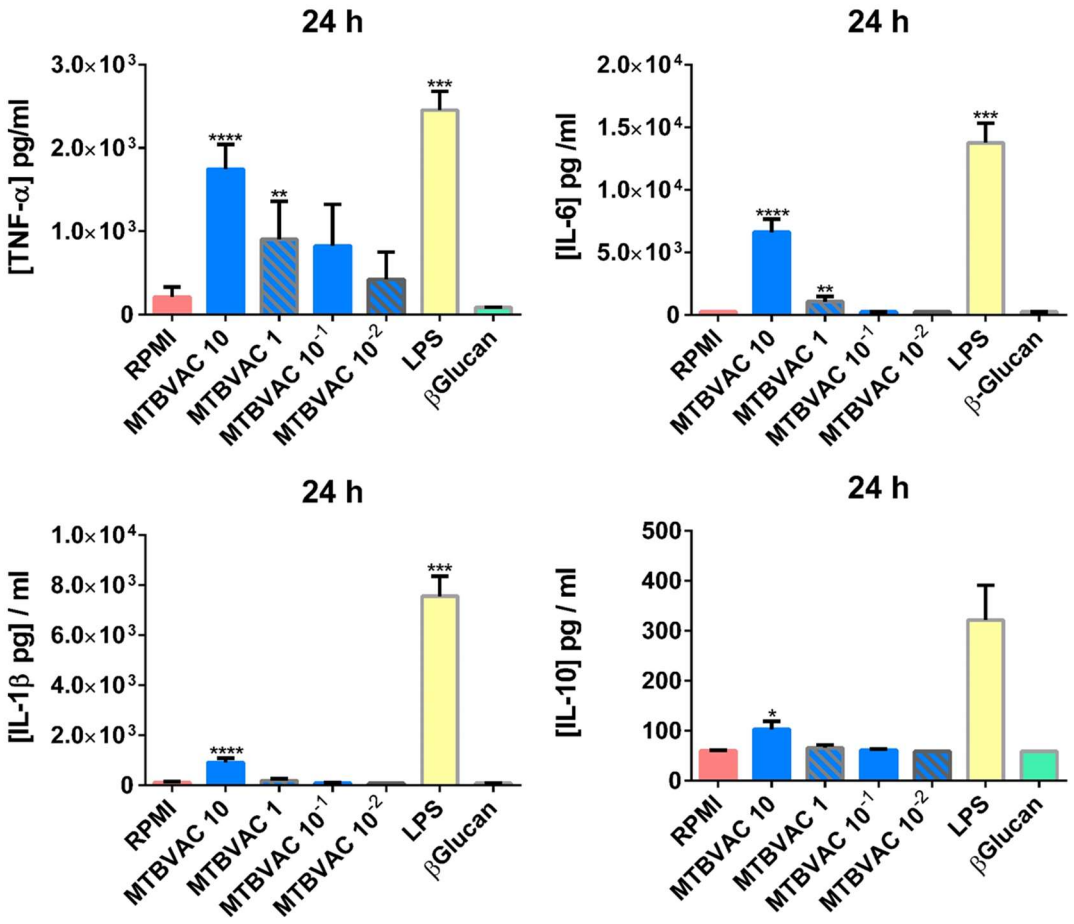

Figure 115. Cytokines produced by monocytes after 24 h stimulation with MTBVAC. TNFα, IL-6, IL-1β and IL-10 were quantified after 24 h of stimulation with different MOI of MTBVAC. The medium contained gentamicin. Mean ± SEM, n = 9-27; pooled from 3-9 independent experiments with 3 individual donors each. *p<0.05, Wilcoxon signed-rank test.

On the other hand, as MTBVAC is a live vaccine, it was evaluated the impact of the presence of the antibiotic gentamicin in the medium (Figure 116). In general, the production of cytokines was similar with and without the antibiotic (Figure118).

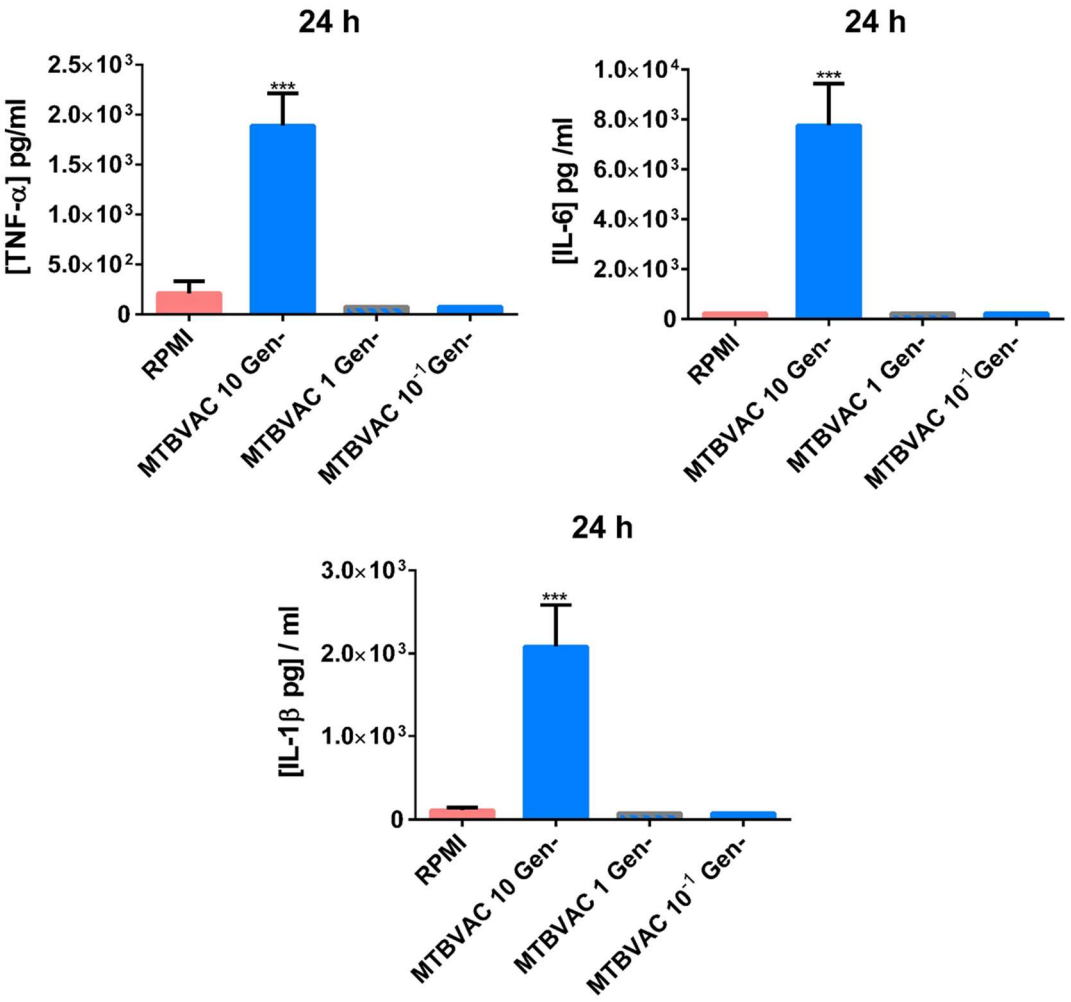

Figure 116 Cytokines produced by monocytes after 24 h stimulation with MTBVAC without antibiotic. TNFα, IL-6 and IL-1β were quantified after 24 h of stimulation with different MOI of MTBVAC. The medium did not contain gentamicin. Mean ± SEM. When used the MOI of 1 and 0.1, only one donor is shown; data from MOI of 10 represent 3 experiment (9 donors) and the RPMI control represent 27 donors. *p<0.05, Wilcoxon signed-rank test.

In parallel, experiments using BCG Pasteur or BCG Intervax were carried out, in order to compare the response exerted between BCG and MTBVAC. As BCG Pasteur is also a live vaccine, the influence of the antibiotic was evaluated (Figure 117). As occurred with MTBVAC, the MOI of 10 was the dose that induced the highest cytokines production. In addition, the antibiotic did not have a remarkable impact on the response. On the other hand, BCG Intervax induced a slight lower cytokine production when compared to BCG Pasteur MOI 10. As previously mentioned, BCG Intervax was not alive (because it was stored at 4ºC). In fact, the dose used for BCG Intervax was equivalent to a MOI of 7.5 x 10^{-2}, taking into account the live bacteria in the initial vial, which at the time of adding them they were no longer alive. However, as BCG Intervax came from a commercial badge, it contained thousands of death bacteria that cannot be quantified.

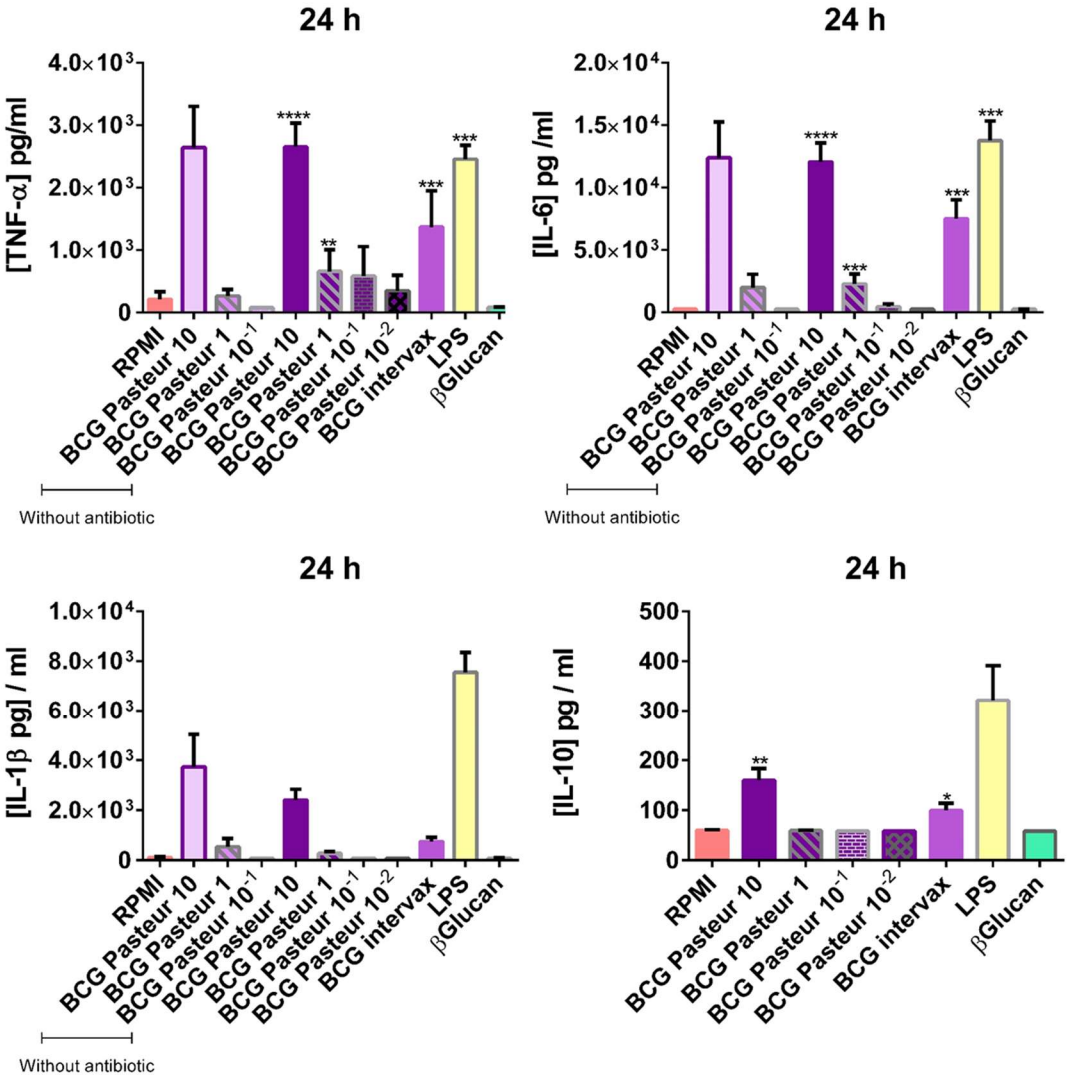

Figure 117. Cytokines produced by monocytes after 24 h stimulation with BCG with and without antibiotic. TNFα, IL-6, IL-1β and IL-10 were quantified after 24 h of stimulation with different MOI of BCG. The medium contained gentamicin except when indicated in the figure. Mean ± SEM, n = 9-27; pooled from 3-9 independent experiments with 3 individual donors each. *p<0.05, Wilcoxon signed-rank test.

To sum up, when compared the best doses for each vaccine, MTBVAC and BCG Pasteur induced a strong and similar acute response in human monocytes *in vitro*. Further, the MOI of 10 is the one that stimulated the highest cytokine production for both vaccines (Figure 118).

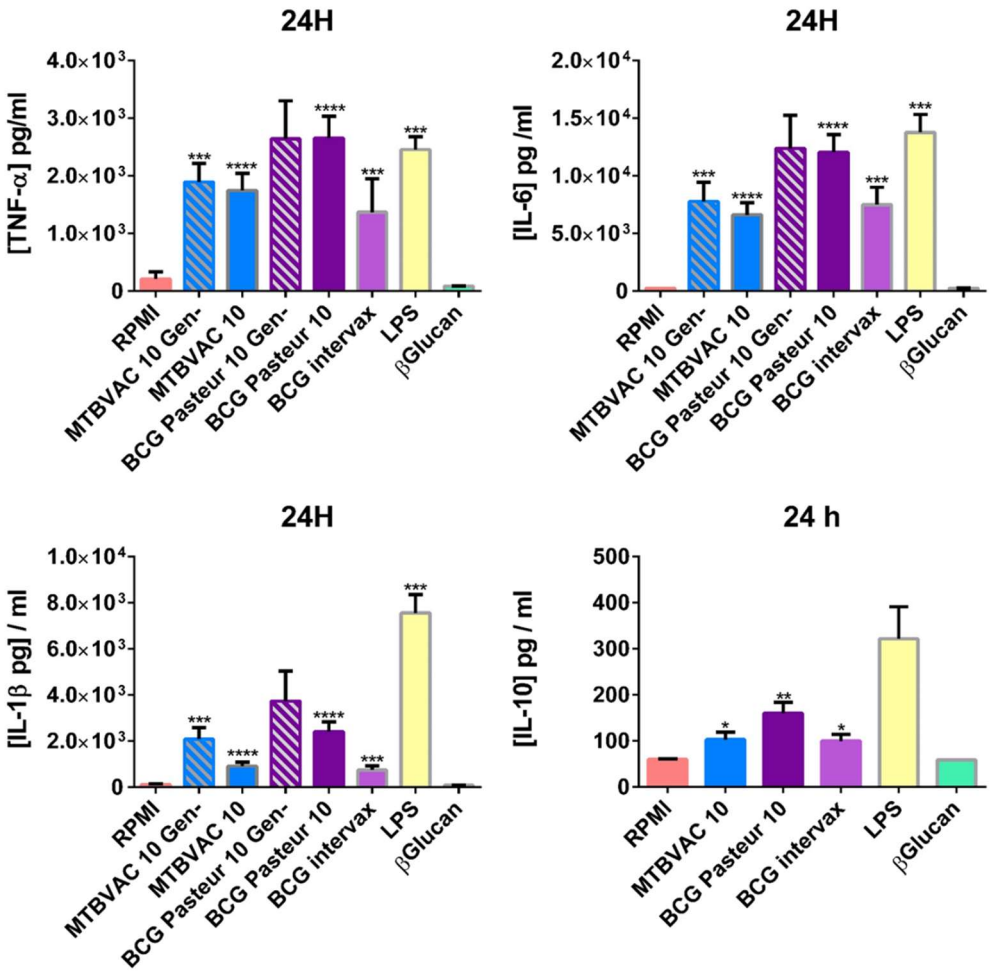

Figure 118. Comparison in the cytokines produced by monocytes after 24 h stimulation with the best doses of each vaccine and condition. TNFα, IL-6, IL-1β and IL-10 were quantified after 24 h of stimulation with different conditions. The medium contained gentamicin except when indicated in the figure (Gen-). Mean ± SEM, n = 9-27; pooled from 3-9 independent experiments with 3 individual donors each. *p<0.05, Wilcoxon signed-rank test.

8.3 MTBVAC induces trained immunity in human monocytes *in vitro*

For studying the potential of MTBVAC to induce innate immune memory *in vitro,* human monocytes were stimulated with different doses of MTBVAC, for 24 h. Then, the stimuli were washed and cells rested for 5 days before being re stimulated with LPS from *Escherichia coli.* IL-6 and TNFα concentration in the supernatant was assessed, because their overproduction after a second unspecific stimulus is a consequence of the trained phenotype of monocytes. The production of IL-1β was not measured, since the monocytes were already macrophages after being stimulated a week before, and these cells, contrary to monocytes-which need a single stimulation-, need a second signal, such as ATP provided by other cells, to produce the mature IL-1β [268, 269].

MTBVAC demonstrated to overproduce both TNFα and IL-6 upon second and unspecific stimulus, revealing that MTBVAC is able to induce trained immunity in human cells. Further, there was a dose-dependent effect of MTBVAC in the induction of training *in vitro* (Figure 119), as the MOI of 10 induced the highest production of cytokines. As expected, there was no cytokine production when used LPS as first stimulus, corroborating that it induces tolerance and not trained immunity[170]. In contrast, β-glucan was used as positive control of trained immunity, and according to that, it induced the highest cytokines concentration.

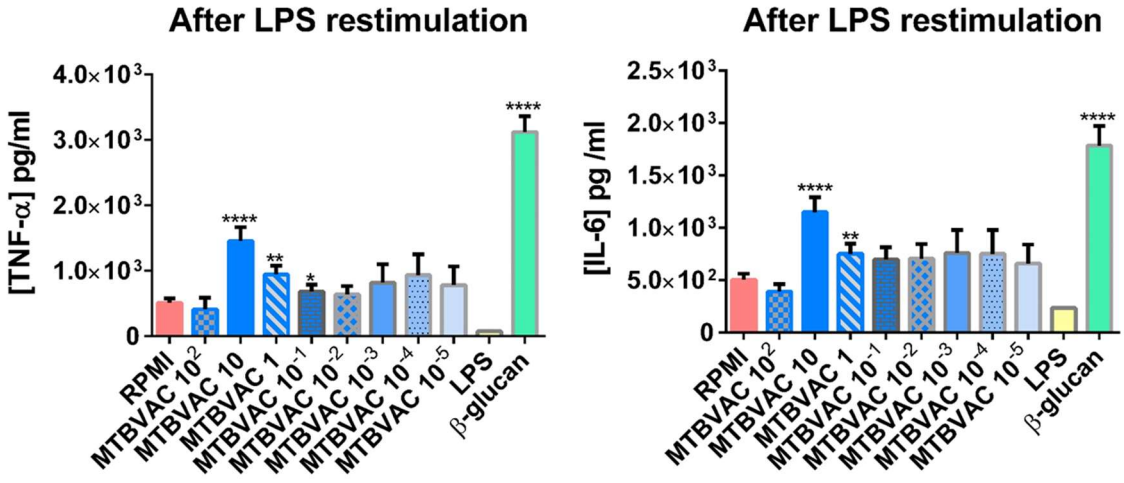

Figure 119. MTBVAC exerts a dose-dependent effect in the induction of trained immunity in human monocytes *in vitro*. Descending doses of MTBVAC were added as priming stimulus and after re stimulation with LPS, TNFα and IL-6 were quantified. Mean ± SEM, n = 6-27; pooled from 2-9 independent experiments with 3 individual donors each. *p<0.05, Wilcoxon signed-rank test.

As occurred in the acute stimulation, the presence of antibiotic in the medium did not affect the trained immunity mediated by MTBVAC, though the TNFα production was slightly higher when not used the antibiotic (Figure 120 and Figure 123).

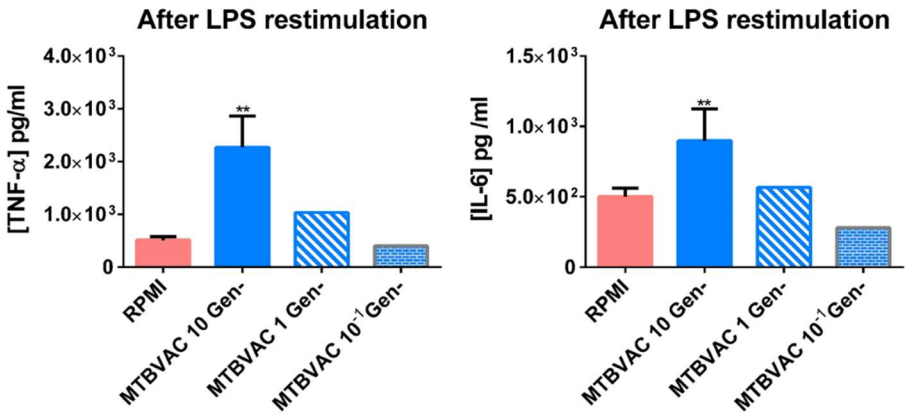

Figure 120. Without antibiotic, MTBVAC exerts a similar effect in the induction of trained immunity with respect to when used antibiotic. Descending doses of MTBVAC were added

as priming stimulus and after re stimulation with LPS, TNFα and IL-6 were quantified, in a medium without gentamicin (Gen-). Mean ± SEM, n = 12-27; pooled from 4-9 independent experiments with 3 individual donors each, in the case of MTBVAC 10 and RPMI, respectively; for MTBVAC 1 and 0.1, only 1 donor was analyzed. *p<0.05, Wilcoxon signed-rank test.

In this regard, in order to corroborate the importance of the bacteria being alive, MTBVAC HK, which is dead, was used as first stimulus in another experiment, and compared with MTBVAC alive. The results showed that the trained immunity induction was slightly stronger when used the live bacterium (Figure 121).

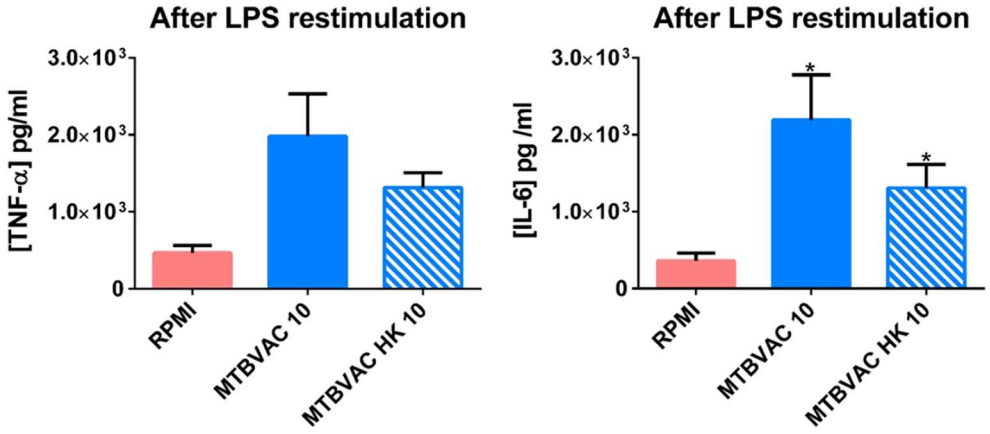

Figure 121. MTBVAC alive seems to induce highest cytokines production than MTBVAC dead. MTBVAC MOI 10 and MTBVAC HK MOI 10 were added as priming stimulus and after re stimulation with LPS, TNFα and IL-6 were quantified. Culture medium contained gentamicin. Mean ± SEM, n = 9; pooled from 3 independent experiments with 3 individual donors each. *p<0.05, Wilcoxon signed-rank test.

The capacity of BGC to induce training was also assessed with the aim of comparing with MTBVAC. As previously described in the literature, BCG Intervax induced trained immunity [196], and here it was demonstrated that BCG Pasteur too (Figure 122). Concerning the stimulation with BCG Pasteur, the MOI of 10 when not used antibiotic was likely toxic for monocytes, as revealed the shape of cells and the decrease in the number of cells observed in the microscope. It would explain why that condition did not induce a high cytokines concentration. On the other hand, the MOI of 10 and MOI 1, when used antibiotic, were the best inducing trained monocytes. Concerning BCG Intervax, the dose used at that time in Mihai Netea's group (MOI 7.5 x 10^{-2}), induced a high TNFα production, but the IL-6 concentration did not increase compared to the control with RPMI.

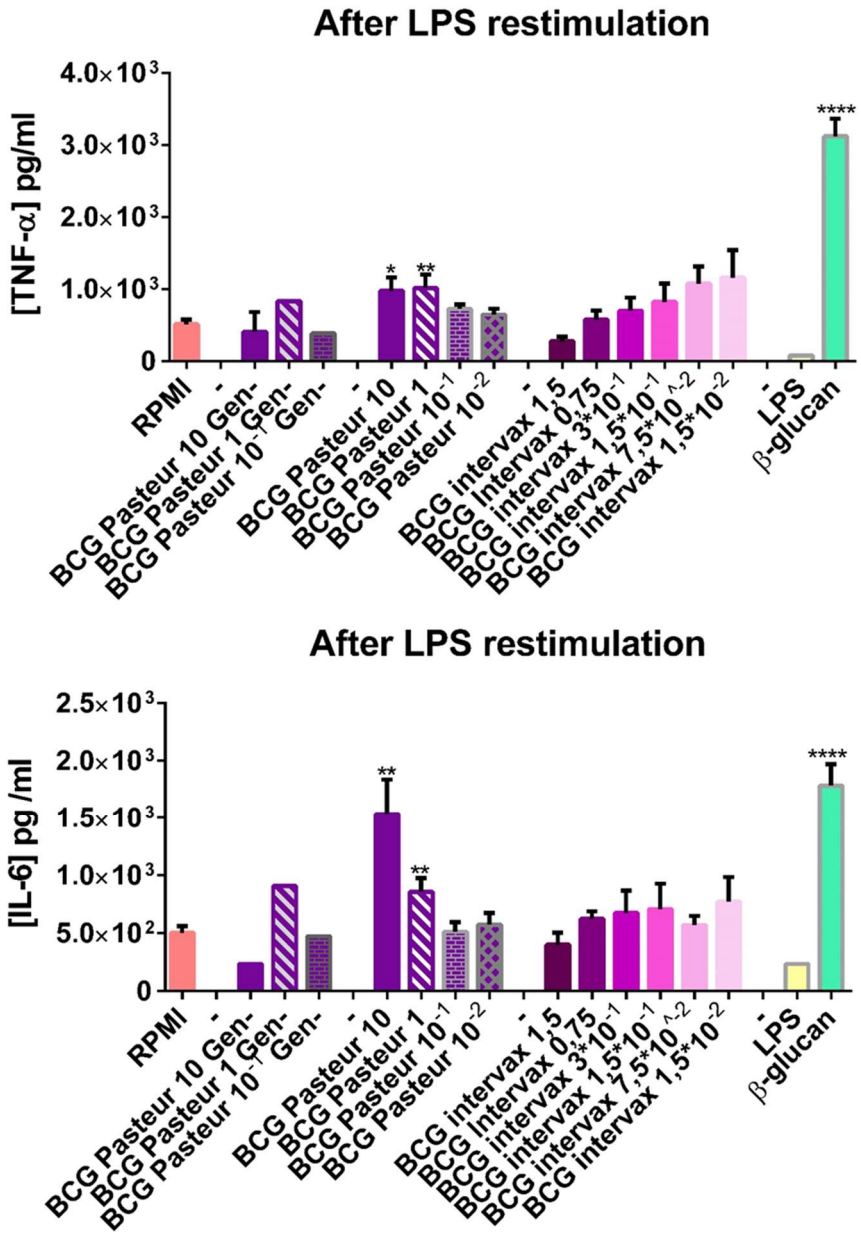

Figure 122. Cytokines produced by monocytes after re stimulation with LPS, when used BCG as first stimulus, with and without antibiotic. TNFα and IL-6 were quantified after re stimulation with LPS, when the first stimulus consisted in different MOI of BCG. The medium contained gentamicin except when indicated in the figure (Gen-). Mean ± SEM, n = 6-27; pooled from 2-9 independent experiments with 3 individual donors each. For BCG Pasteur Gen- MOI 10, 1 and 0.1 only 1 donor was analyzed. *p<0.05, Wilcoxon signed-rank test.

Comparing the optimal doses for each vaccine, the level of IL-6 and TNFα produced after second stimulation with LPS was similar between MTBVAC and BCG Pasteur and, concerning MTBVAC, even superior to BCG Intervax (Figure 123). The global comparison between all the conditions is detailed in Supplementary Figure 1.

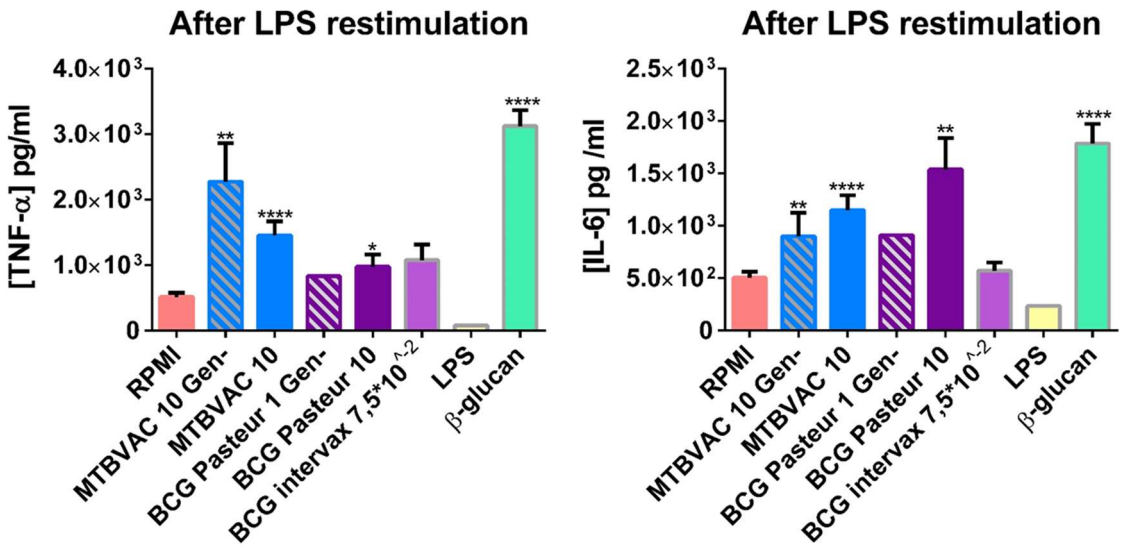

Figure 123. Comparison in the cytokines produced by monocytes after LPS re stimulation with the best doses of each vaccine and condition. TNFα and IL-6 were quantified after 24 h of stimulation plus 5 days of resting and finally 24 h with LPS. The medium contained gentamicin except when indicated in the figure (Gen-). Mean ± SEM, n = 12-27; pooled from 4-9 independent experiments with 3 individual donors each, except for BCG Pasteur MOI 1 Gen-, which corresponds to only 1 donor. *p<0.05, Wilcoxon signed-rank test.

In conclusion, MTBVAC triggers the induction of innate immune memory, referred as trained immunity, in human cells *in vitro*.

8.4 MTBVAC reprograms cellular metabolism in human monocytes *in vitro*

As previously described in the literature, induction of trained immunity is accompanied by a reprogramming in the metabolism of innate immune cells. It is known that BCG-mediated training triggers an upregulation of the glycolysis in human monocytes [172]. In this regard, here it was studied the impact of MTBVAC–mediated trained immunity on this metabolic pathway. Therefore, lactate secretion was measured because its production correlates with the activity of glycolysis, as it is the end product of the pathway and blocks further ATP production. The results showed that MTBVAC induced a high lactate production, indeed to similar levels to those induced by β-glucan, the best training inductor described in the literature, revealing that MTBVAC upregulates the aerobic glycolysis rate in trained monocytes (Figure 124).

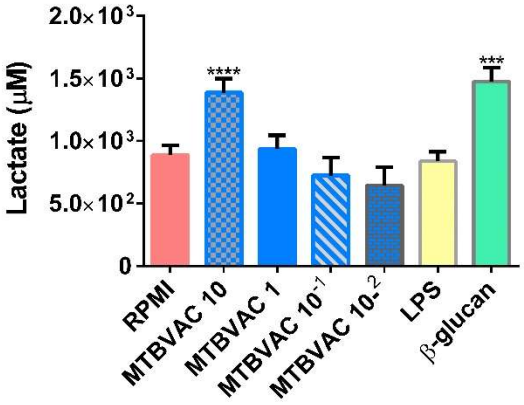

Figure 124. MTBVAC induces trained immunity through upregulation of aerobic glycolysis. Lactate production by human monocytes 5 days after 24h-stimulation with different MOI of MTBVAC. Mean ± SEM (n = 3-24; pooled from 1-8 independent experiments). *p<0.05, Wilcoxon test.

Moreover, the withdrawal of antibiotic from the medium did not show remarkable differences concerning the lactate production (Figure 125 and Figure 126 B).

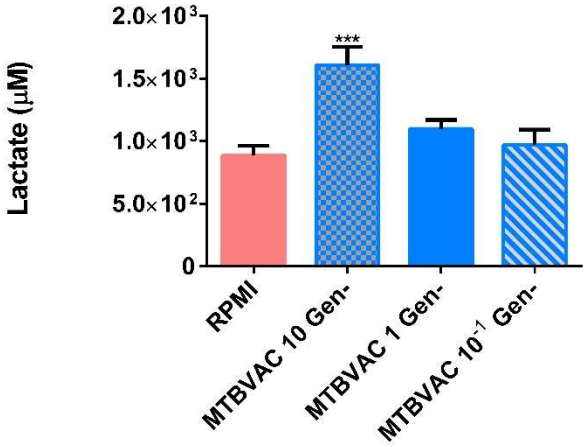

Figure 125. The lack of antibiotic does not affect the upregulation of glycolysis mediated by MTBVAC-trained immunity. Lactate production by human monocytes 6 days after 24h-stimulation with different MOI of MTBVAC, in the absence of gentamicin. Mean ± SEM (n = 3-24; pooled from 1-8 independent experiments). *p<0.05, Wilcoxon test.

On the other hand, lactate production was also measured with BCG as first stimulus (Figure 126 A), and it was comparable to that of MTBVAC (Figure 126 B).

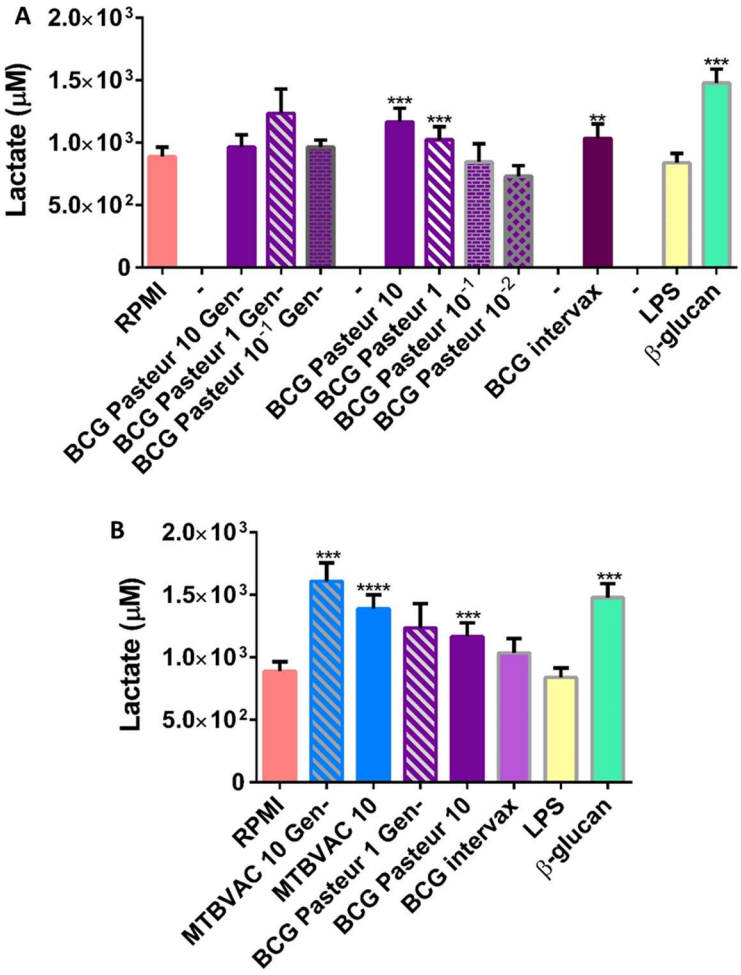

Figure 126. BCG induces comparable levels of lactate to those of MTBVAC, although slightly lower. Lactate production by human monocytes 5 days after 24h-stimulation with different MOI of BCG Pasteur and BCG Intervax, with or without antibiotic (Gen-) (A). Overview of lactate production between the most remarkable doses of each condition (B). Mean ± SEM, n = 3-24; pooled from 1-8 independent experiments. *p<0.05, Wilcoxon test.

Continuing with this, it was analyzed the oxygen consumption rate (OCR)-as a measured of oxidative phosphorylation-, and the extracellular acidification rate (ECAR) – as a measure of the glycolytic conversion of uncharged glucose to 2 lactate− plus 2 H+-. MTBVAC produced a moderate increase in the OCR (Figure 127 A) and ECAR (Figure 127 B), corroborating the increased metabolic activity in the human trained monocytes.

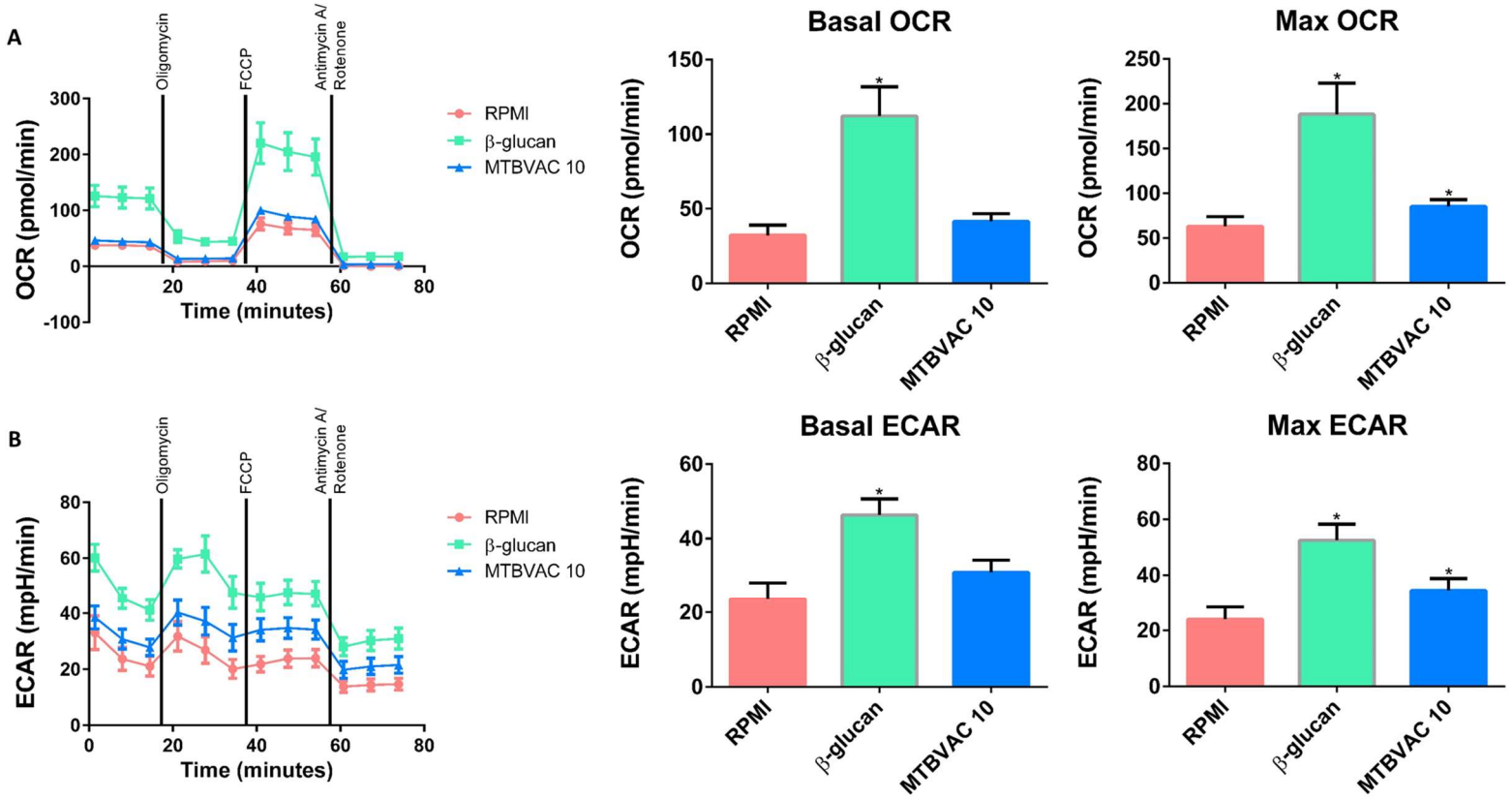

Figure 127. OCR and ECAR increase in MTBVAC-induced trained monocytes. Basal and Maximum (Max) OCR (A) and ECAR (B) of monocytes were determined 5 days after 24h-stimulation with β-glucan or MTBVAC by extracellular flux measurements. Mean ± SEM, n = 8; pooled from 3 independent experiments. *p<0.05, Wilcoxon test.

On the other hand, experiments with inhibitors of metabolic pathways confirmed the importance of these energetic pathways in the induction of trained immunity by MTBVAC. Oligomycin, which is an inhibitor of ATP synthase and blocks the oxidative phosphorylation, inhibited the production of cytokines after LPS re stimulation of monocytes (Figure 128). In addition, the same occurred when used BPTES, a glutaminase inhibitor (Figure 128), which suggests that the boosting with glutamate to the TCA is crucial for MTBVAC-mediated trained immunity.

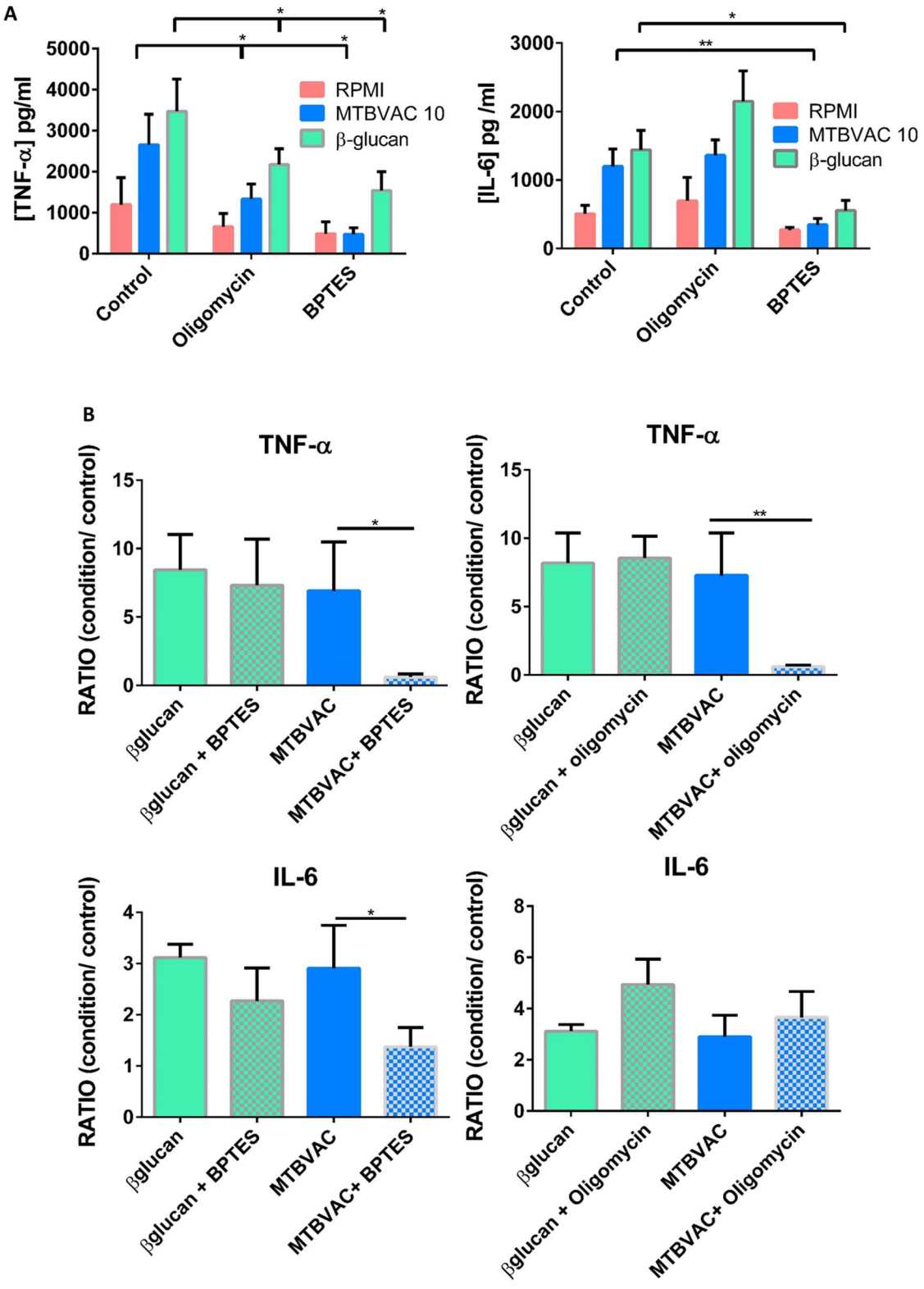

Figure 128. Glutaminolysis and oxidative phosphorylation are important for the induction of trained immunity mediated by MTBVAC. Human monocytes were treated with oligomycin or BPTES, and simultaneously incubated with MTBVAC or β-glucan for 24h, and re stimulated with LPS 6 days later. IL-6 and TNFα were measured. Total cytokine concentration is shown (A). Ratio calculated dividing cytokine concentration in each condition by RPMI with or without the inhibitor (B). Mean ± SEM, n = 8; pooled from 3 independent experiments with 3 or 2 individual donors each. *p<0.05, Wilcoxon test.

To conclude, it was demonstrated not only that MTBVAC induces an increase in the glycolytic rate, but also that the vaccine-mediated trained immunity depends on oxidative phosphorylation and indeed in a higher degree, in the glutaminolysis.

8.5 MTBVAC induces epigenetic reprograming in human monocytes *in vitro*

In addition to metabolic remodelling, epigenetic reprogramming is another hallmark of trained immunity. Some immunological signals induce metabolic changes, which in part affect the epigenetic program leading to modifications in cellular metabolic pathways [170, 171].

A typical mark for active chromatin is the trimethylation in the histone 3 lysine 4 (H3K4me3), and the literature described a persistent enrichment of this mark at promoter genes encoding IL-6, TNFα and IL-1β after trained immunity triggered by BCG *in vivo* [170]. In this regard, it was studied the deposition of the H3K4me3 in the promoter region of both *TNFA* and *IL6* genes, at day 6 after *in vitro* stimulation with MTBVAC. Importantly, MTBVAC led to an enrichment of the H3K4me3 at the promoter regions of *TNFA* and *IL6,* and what is more, the accumulation of the mark was comparable to that of gold training inductor β-glucan (Figure 129).

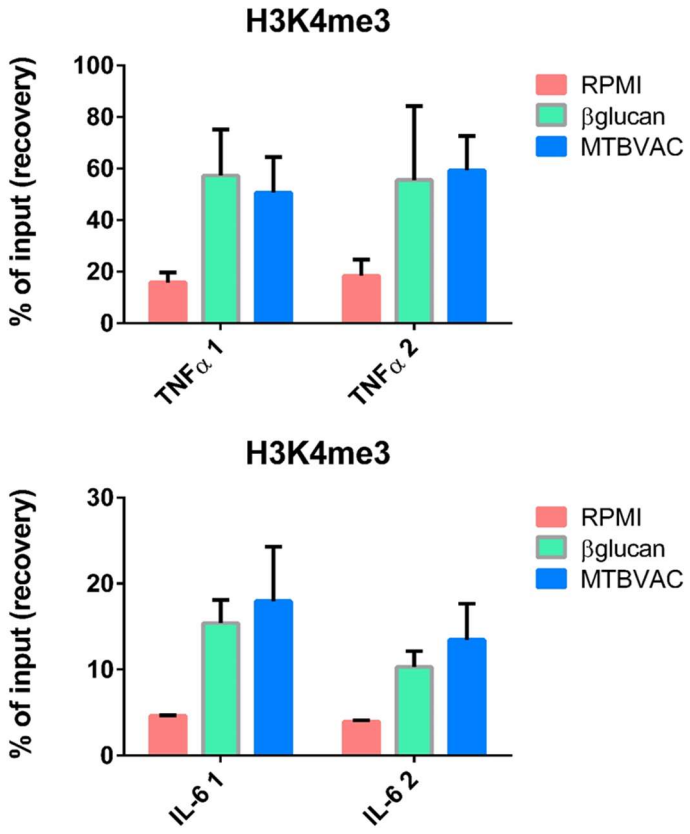

Figure 129. MTBVAC induces changes in the epigenetic program of human monocytes *in vitro*. Cells were stimulated with MTBVAC MOI 10 for 24 h, and after 5 days of culture with gentamicin, H3K4me3 mark was assessed at the level of promoters of *TNFA* and *IL6* with two different pairs of primers each. Mean ± SEM, n = 3.

8.6 MTBVAC is able to induce trained immunity *in vivo* in mice

With the aim of evaluating the ability of MTBVAC to induce innate memory *in vivo*, which would be more relevant than the *in vitro* capacity, C57BL/6 mice were subcutaneously vaccinated with 10^6 CFU of MTBVAC, a clinically relevant dose. Four weeks later, mice were challenged to 10 µg LPS, administered by the intraperitoneal route, and after 4 hours, pro-inflammatory cytokines were measured in serum (Figure 40).

According to the previous results, whereas TNFα, IL-6 and IL-1β were not detected in mice only vaccinated, cytokine concentration was higher in mice vaccinated with MTBVAC and subsequently challenged with LPS, than in mice which had not been vaccinated prior to LPS secondary challenge (Figure 130). Therefore, MTBVAC demonstrated to be capable of inducing trained immunity in innate cells from mice *in vivo*.

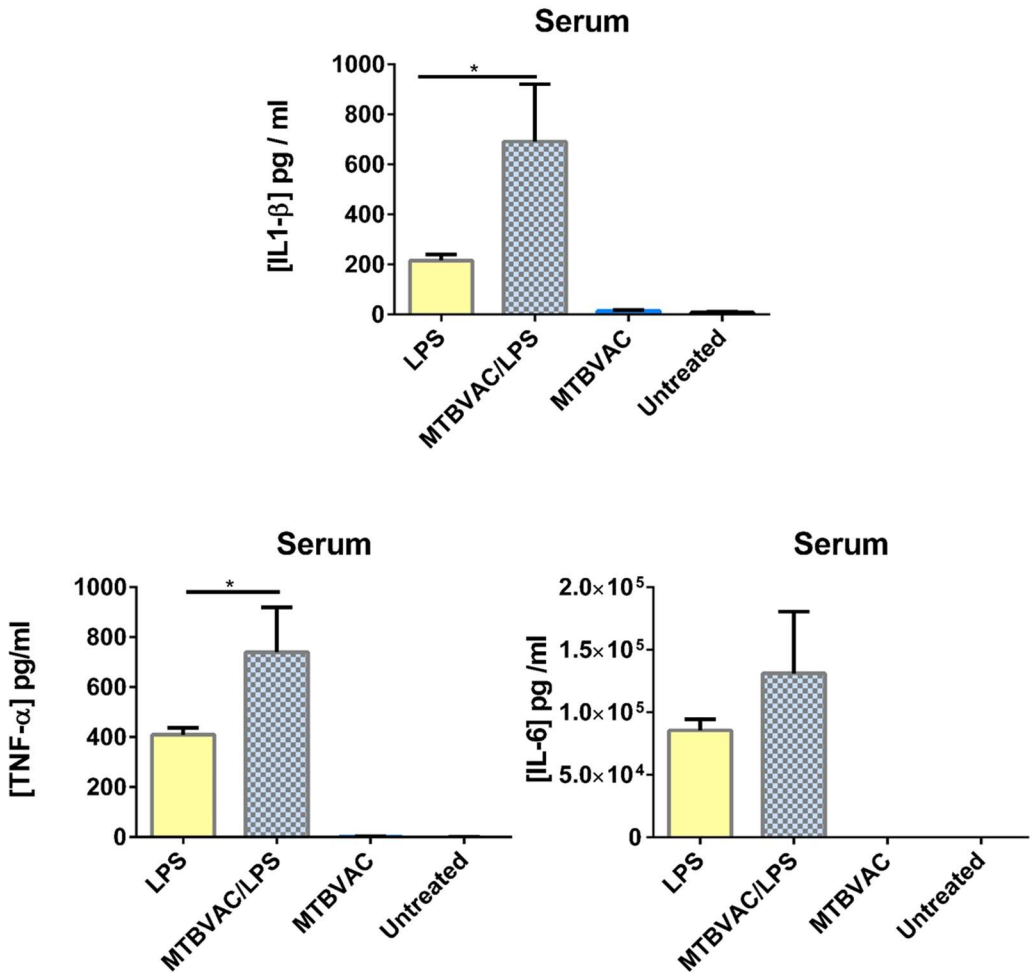

Figure 130. MTBVAC induces *in vivo* trained immunity in mice. IL-1β, TNFα and IL-6 were quantified in circulating serum from mice vaccinated with MTBVAC and 4 weeks later challenged to LPS (MTBVAC/LPS), or in mice only vaccinated (MTBVAC) or challenged (LPS). Mean ± SEM, n=6 mice / group. *p<0.05, Mann-Whitney test.

To recapitulate, the results exposed here demonstrate that the capacity of MTBVAC to induce trained immunity is similar to that of BCG strains usually used in vaccination programs.

9. DISCUSSION

Despite the high global coverage of BCG, TB is still the tenth cause of death worldwide, since the vaccine protects against disseminated forms of TB but not against respiratory forms.

With the aim of providing a more efficient TB vaccine, MTBVAC was constructed. Contrary to BCG, which is attenuated from *M.bovis,* MTBVAC is a new potential TB vaccine based on the rational attenuation of *M. tuberculosis.* It lets us to hypothesise that MTBVAC could be a better TB vaccine for humans, as it contents a number of epitopes recognised by human CD4+ T cells, absent in BCG[115].

Apart from TB, BGC has demonstrated to be efficient against other diseases, such as bladder cancer[270] or yellow fever[271]. These unspecific effects mediated by BCG seem rely on the induction of trained immunity.

Here, we demonstrate that MTBVAC is able to induce immune memory in human monocytes *in vitro,* and what is more, the induction of trained immunity extended to an *in vivo* scenario in mice. Since the vaccine is called to improve BCG protection, these results are of great relevance for the future clinical development of MTBVAC, since they demonstrated that MTBVAC could exert all the unspecific beneficial effects mediated by BCG against unspecific diseases. In addition, the induction of trained immunity by MTBVAC was dose-dependent. MOI 10 induced the highest responsiveness, so it leads us to hypothesise that monocytes need to phagocyte the bacillus for reprograming their cellular state to the trained profile.

More specifically, our results revealed that MTBVAC is capable to both induce acute responses in PBMCs and monocytes, and as previously mentioned, trigger long-term reprogramming of monocytes. In this regard, upon stimulation with MTBVAC, innate immune cells underwent metabolic and epigenetics changes which underlie an enhanced response after secondary unspecific re stimulation, at least to similar levels than those of commercial BCG strains.

Concerning metabolic remodelling, our work showed that innate immune memory triggered by MTBVAC produces an upregulation of the aerobic glycolysis, as it occurred for β-glucan and BCG [172]. It may implies that glycolysis favors the adaptive monocyte response by quickly producing energy during infection, where a swift and robust response is required. Moreover, it was observed an increase in the oxidative phosphorylation, as measured by OCR. Further, this pathway demonstrated to have at least a partial role in the induction of training mediated by MTBVAC, as revealed experiments with oligomycin. It was different to what happened with trained

immunity mediated by β-glucan or BCG, where oligomycin did not have an effect on the induction of trained immunity [172]. Therefore, more experiments should be done to confirm this distinct metabolic implication when used MTBVAC.

Moreover, the literature claims that glutaminolysis is important for the induction of trained immunity mediated by BCG [172]. In this line, glutaminolysis demonstrated to be crucial for the trained immunity mediated by MTBVAC, as demonstrated experiments with the BPTES inhibitor. Therefore, the entrance of glutamine to the TCA cycle is essential for the enhanced innate immune response triggered by MTBVAC.

In addition to metabolic changes, trained immunity relies on epigenetic remodelling. In this regard, MTBVAC led to an enrichment of the mark H3K4me3, which is associated with an increase and more robust gene transcription, specifically in the promoter regions of IL-6 and TNFα [171]. Importantly, it is in line with the increase in cytokines production or responsiveness seen in monocytes after stimulation with MTBVAC.

Following with this, experiments carried out in Mihai Netea's group, revealed that there was an interplay between metabolism and epigenetics upon stimulation with MTBVAC (paper in preparation). Experiments inhibiting the glutaminolysis, showed a loss of H3K4me3 accumulation in the promoter of *TNFA* and *IL6* genes, revealing that glutaminolysis is essential for the epigenetic remodelling underlying the enhanced cytokine production after re stimulation. It has clinical relevance, since the relation between metabolism and epigenetic is important for the beneficial unspecific effects mediated by different stimuli, but it is also linked with the development of diabetes [272], atherosclerosis [273] or other inflammatory diseases.

Our results in MTBVAC vaccinated mice showed that after an unspecific re stimulation, there was an enhanced pro inflammatory response. It gives stronger support to the functional relevance of trained immunity mediated by MTBVAC. Likely, the vaccine exerts its effect through the reprogramming of the hematopoietic cells, as it has been described for BCG [274]. On the other hand, in order to discard the influence of the adaptive system in the responsiveness upon MTBVAC and re stimulation, experiments in SCID mice could be carried out in the future, under similar experimental settings.

Interestingly, in a recent experiment performed in collaboration between our laboratory and José Yuste's group, MTBVAC protected against lethal infection with *S. pneumoniae* in mice (paper in preparation). Even though mechanisms underlying

this protection have not been totally elucidated, the results presented here let us to hypothesise that the protection is mediated through the induction of trained immunity.

On the other hand, MTBVAC-induced trained immunity may have an impact also in the protection against TB. In this regard, the vaccine would favor the early clearance of the bacillus, by increasing the pro inflammatory activity of macrophages, and it would enable the individual to eliminate quickly the infection.

To sum up, in this chapter we demonstrate that MTBVAC induces trained immunity by metabolic and epigenetic cell reprogramming, through similar mechanisms to those of BCG. In this regard, MTBVAC would be able to exert the unspecific effects mediated by BCG and potentially, it could improve the protection against TB, as it is a Mtb based vaccine. Therefore, our results give strong support for the acceleration in the clinical trials with MTBVAC and also a chance for the use of MTBVAC against bladder cancer or pneumonia.

Conclusions

Rien dans la vie n'est à craindre, tout est à comprendre. C'est maintenant le moment de comprendre davantage, afin de craindre moins

Marie Curie

10. CONCLUSIONS

1. *Mycobacterium tuberculosis* prevents asthma in a global strategy. It reduces the infiltration of eosinophils in lungs and abolishes the eosinophils production in the bone marrow. Mtb re-educates allergen-specific T cells from a Th2 to a Th1 profile upon infection, leading by the INF-γ production and so inducing an anti-asthma inflammatory response.

2. Latent tuberculosis infection could have conferred physical advantages in ancient societies by preventing asthma symptoms more than 70000 years ago, when it was crucial as survival depended on hunting and gathering.

3. MTBVAC and BCG prevent acute and chronic allergic asthma in mice and further, they exert a therapeutic effect against established asthma, when administered by the intranasal route. They are efficient against asthma driven by different allergens such as OVA and HDM.

4. MTBVAC and BCG beneficial clinical effects in asthma include the reduction of eosinophils infiltration in the airways, the impairment of Th2 cytokines through the induction of a Th1 profile, and the reduction in the airways remodelling.

5. Mechanistically, the protection conferred by MTBVAC and BCG vaccines against asthma mostly depends on the change from Th2 T cells to Th1 cells and on the macrophages re-polarization from a M2 profile to a M1 profile.

6. The protection provided by MTBVAC and BCG against asthma is organ-dependant, as they must be administered by the intranasal route in order to be efficient.

7. The protection conferred by MTBVAC and BCG is dose dependent and increase with the bacterium persistence. That is why BCG protects slightly better than MTBVAC at the same dose.

8. The protection conferred by MTBVAC against asthma is equivalent to that of BCG, when it is administered at a dose 10 times higher than that of BCG -10^7 CFU-. Further, MTBVAC exhibits better safety profile than BCG and it is much more efficiently delivered to the lungs by clinical nebulizer. This allows a therapeutic dose of MTBVAC to be administered, while a suitable dose of BCG does not.

9. **To sum up, attenuated Mycobacteria are demonstrated to be efficient against allergic asthma in mice in both prophylactic and therapeutic**

approaches when administered by the intranasal route. Therefore, we give strong support for their clinical study against asthma in humans.

10. MTBVAC induces trained immunity in human monocytes *in vitro,* in at least similar levels to those of BCG.

11. MTBVAC-induced innate memory produces changes in the metabolism and epigenetic reprogramming. Specifically, the vaccine upregulates the glycolysis, and needs glutamine metabolism and oxidative phosphorilation to mediate the enhanced responsiveness after second unspecific re stimulation.

12. MTBVAC induces trained immunity *in vivo* in mice.

13. **To conclude, the ability of MTBVAC to induce trained immunity is clinically relevant as it could exert the same unspecific effects than BCG in other diseases different from TB, such as bladder cancer or some respiratory diseases.**

11. CONCLUSIONES

1. *Mycobacterium tuberculosis* previene el asma desde una estrategia global. Reduce la infiltración de eosinófilos en los pulmones y acaba con la producción de eosinófilos en la médula ósea. La infección con Mtb reeduca a las células T específicas de alérgeno que pasan de un perfil Th2 a uno Th1, con la consiguiente producción de IFN-γ, induciendo finalmente una respuesta inflamatoria contraria al asma.

2. La infección latente con tuberculosis pudo haber conferido en sociedades antiguas de hace más de 70000 años, ciertas ventajas físicas, como la prevención del asma. Esto pudo ser crucial para la supervivencia, que entonces dependía de la caza y recolección.

3. MTBVAC y BCG previenen el asma aguda y crónica de tipo T2 en ratones; además, ejercen un efecto terapéutico contra el asma que ya está establecido, cuando se administran por la vía intranasal. Ambas vacunas son efectivas frente al asma inducido por diferentes alérgenos como OVA o HDM.

4. Los beneficios clínicos de MTBVAC y BCG frente al asma incluyen la reducción de la infiltración de eosinófilos en las vías aéreas, la disfunción en la producción de citoquinas típicas de la respuesta Th2 a través de la inducción del perfil Th1, y una menor remodelación de las vías aéreas.

5. En cuanto al mecanismo, la protección conferida por MTBVAC y BCG frente al asma depende principalmente de la reconducción de la respuesta Th2 hacia una respuesta Th1, y de la repolarización de los macrófagos M2 a los macrófagos M1.

6. La protección llevada a cabo por MTBVAC y BCG frente al asma es dependiente del órgano, ya que tienen que ser administradas por la vía intranasal para ser efectivas.

7. La protección frente al asma que ejercen MTBVAC y BCG es dosis dependiente, y aumenta con la persistencia de la bacteria. Esto explica por qué BCG protege ligeramente mejor que MTBVAC cuando se administran a la misma dosis.

8. La protección conferida por MTBVAC frente al asma es equivalente a la de BCG, cuando se administra con una dosis 10 veces superior a la de BCG -10^7 CFU-. Además, MTBVAC presenta un mejor perfil de seguridad que BCG y se administra a los pulmones de una manera mucho más eficiente que BCG,

usando un nebulizador clínico. Esto permite que una dosis terapéutica de MTBVAC pueda ser administrada, mientras que una dosis apta de BCG no.

9. **En resumen, se ha demostrado que las micobacterias atenuadas son eficientes frente al asma alérgica en ratones, tanto como tratamientos profilácticos como terapéuticos, cuando se administran por la vía intranasal. Todo esto apoya su futuro estudio clínico frente al asma en humanos.**
10. MTBVAC induce "trained immunity" o inmunidad entrenada, en monocitos humanos *in vitro*, al menos a niveles similares a los inducidos por BCG.
11. La memoria innata inducida por MTBVAC produce cambios en el metabolismo y reprogramación epigenética. Concretamente, la vacuna aumenta la tasa glucolítica, y requiere del metabolismo de la glutamina y de la fosforilación oxidativa para responder de una manera más potente y eficaz frente a una segunda estimulación, incluso inespecífica.
12. MTBVAC induce "trained immunity" *in vivo* en ratones.
13. **Como conclusión, la capacidad de MTBVAC para inducir "trained immunity" es de gran relevancia clínica, ya que la vacuna podría ejercer los mismos efectos inespecíficos que BCG ejerce en otras enfermedades diferentes a tuberculosis, como son el cáncer de vejiga u otras enfermedades respiratorias.**

The excitement of learning separates youth from old age.

As long as you're learning you're not old.

Rosalyn Sussman Yalow

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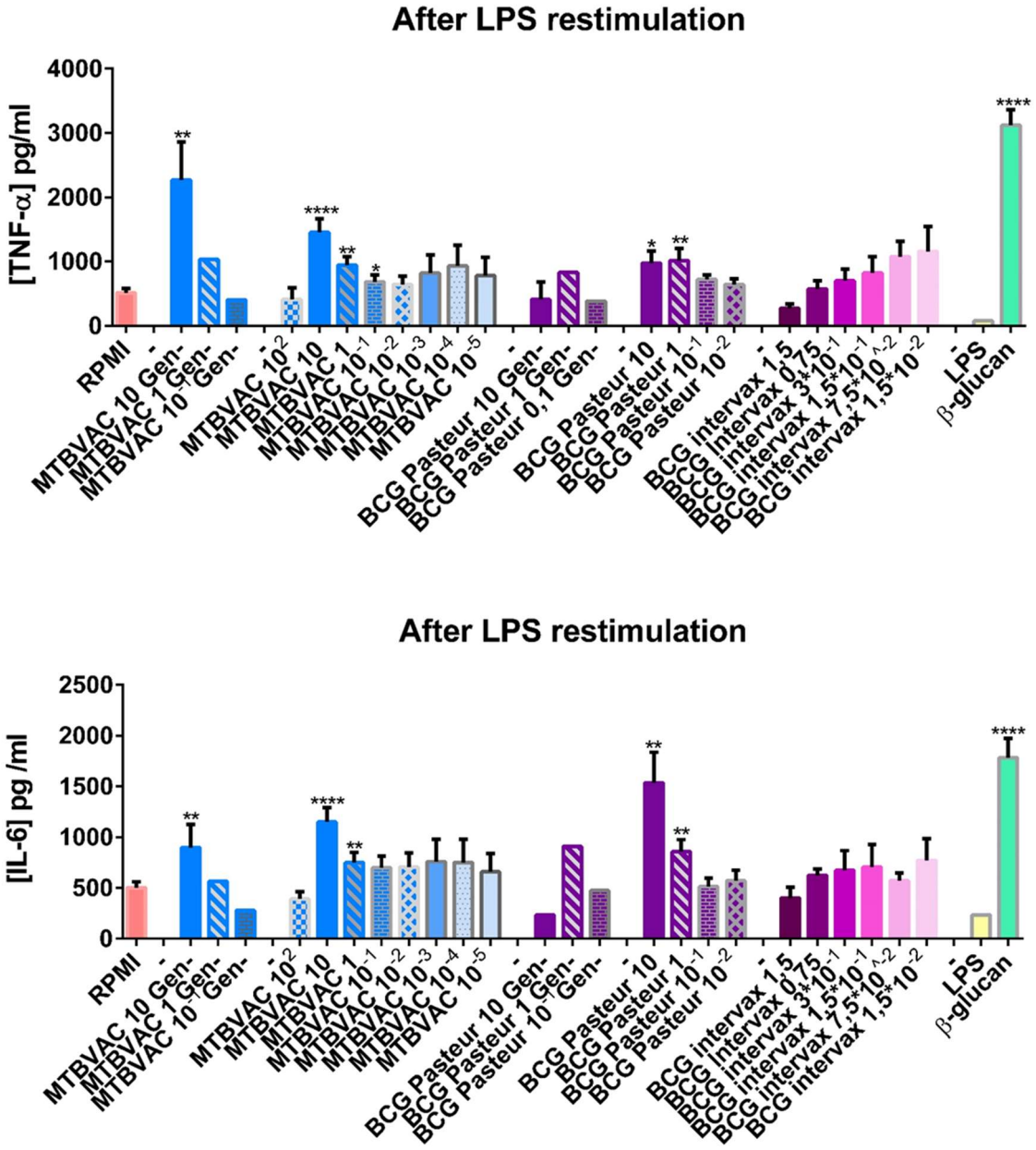
13. SUPPLEMENTARY FIGURES



Supplementary figure 1. Comparison of trained immunity triggered by all the different conditions after re stimulation with LPS. Graphs indicate the conditions used for the first stimulus. Then, cells were in all cases re stimulated with LPS. TNFα and IL-6 were quantified. Mean ± SEM, n = 6-27; pooled from 2-9 independent experiments with 3 individual donors each, except for MTBVAC and BCG Pasteur MOI 1 and 0.1 Gen-, which corresponds to only 1 donor. *p<0.05, Wilcoxon signed-rank test.

14. APPENDIX

The paper derived from the work presented in chapter 1 is attached below (Tarancon, R., et al., ***Mycobacterium tuberculosis*** **infection prevents asthma and abrogates eosinophilopoiesis in an experimental mode**l. Allergy, 2019).

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LETTER TO THE EDITOR

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Mycobacterium tuberculosis infection prevents asthma and abrogates eosinophilopoiesis in an experimental model

To the Editor,

Tuberculosis (TB) is one of the top 10 causes of death worldwide, with 10 million new cases and 1.5 million associated deaths according to WHO TB Report 2018. TB in humans is mostly caused by *Mycobacterium tuberculosis* (Mtb). Following Mtb entry within the host by the respiratory airways, the pathogen colonizes and multiplies inside lung macrophages. In 90% of the cases, infection is controlled, although not eliminated, when TB-specific T cells reach the lungs and produce proinflammatory cytokines that trigger macrophage activation. TB control is associated with a Th1 response, and indeed, individuals with polymorphisms in Th1-associated genes such as *IFNG*, *IL12A/B*, or *STAT1* present a higher susceptibility to develop active TB. Equilibrium between human immune system and bacterial virulence factors drives to formation of granulomas, structures conformed by a nucleus of infected macrophages surrounded by a barrier of lymphocytes that restrict bacterial spread. At this state, Mtb infection is in a latent stage (LTBI), and it is asymptomatic[1].

Since many types of allergies, including asthma, have been typically linked with a Th2 response, it has been speculated for years that mycobacterial infections could confer unspecific protection against this type of pathologies. This has been widely revised in the context of BCG vaccination, with controversial studies showing opposite conclusions[2]. With respect to TB disease, some epidemiological studies have identified a significant inverse correlation between asthma prevalence and TB notification cases in countries where standardized data are available[3]. Regarding LTBI, a study performed in South Africa demonstrated a strong association between tuberculosis skin test (TST) positivity and lower prevalence of different types of allergy. Interestingly, in the case of asthma, authors found a substantial reduction in asthma incidence in the segment of population with the highest TST value analyzed in the study (>20 mm), suggesting a correlation between magnitude of the TB-specific immune response and degree of unspecific protection[4]. Beyond these observational studies, to our knowledge correlation between virulent Mtb infection and unspecific asthma protection has never been tested in animal models. In the present study, we evaluated whether presence of pulmonary Mtb affects allergen-induced airway eosinophilia in an ovalbumin-driven asthma mouse model.

Inbred mice C57BL/6 are considered resistant to TB[5]. They tolerate high lung bacterial burdens during long periods of time without presenting any symptomatology. We intranasally challenged OVA-sensitized C57BL/6 mice with a low dose (150 CFU) of the Mtb reference strain H37Rv. Four weeks later, lung eosinophilia was induced by intranasal OVA administration during three consecutive days (Figure 1A). At this time point after infection, lung bacterial replication is efficiently controlled by host immune system in immunocompetent mice (Figure S1), and therefore, this scenario would be comparable to a latent infection. Massive lung eosinophil infiltration was observed by hematoxylin-eosin staining (Figure 1B). Eosinophils were quantified in bronchoalveolar lavages (BAL) and lungs by flow cytometry (Figures 1C, S2). Our results demonstrated that OVA-driven eosinophilia was abrogated following Mtb infection, both in BAL and in lungs (Figures 1C, 1D, S3). Moreover, Mtb infection specifically prevented eosinophil infiltration, without reducing significantly other myeloid populations as neutrophils, alveolar macrophages, or T lymphocytes (Figure S3).

We next assessed Th1 and Th2 cytokine profiles. Our data demonstrated a substantial reduction of Th2 cytokines IL-5, IL-4, and IL-13 in BAL and lungs from OVA-challenged mice following H37Rv infection, concomitantly with an increase of IFNγ (Figure 1E, 1F). We also evaluated allergen-specific response following ex vivo OVA stimulation of mediastinal lymph node cells. We showed a substantial production of OVA-induced IL-5 and IL-13 in the OVA-challenged group, in the absence of IFNγ response. Conversely, Mtb infection drove to an inversion of this profile, with a reduction of IL-5 and IL-13 levels, and a dramatic increase of OVA-specific IFNγ response (Figure 1G).

Previous studies indicated that newly produced eosinophils in the bone marrow substantially contribute to lung eosinophilia in OVA-induced mouse asthma[6]. In addition, another study demonstrated that T cell–derived IFNγ abrogated eosinophil production in the bone marrow[7]. Based on these results, we next evaluated whether eosinophilopoiesis was affected by Mtb infection under our experimental settings (Figure S4). Our results indicated an increase of eosinophil precursors in the bone marrow following OVA challenge, as it had been previously described[6]. Remarkably, eosinophil progenitors were dramatically reduced in the H37Rv-infected mice, suggesting that inflammatory response induced by TB infection profoundly affects eosinophilopoiesis (Figure 2).

Our data suggest that T cell–driven IFNγ production in Mtb-infected OVA-challenged mice is not only generated by expanded lymphocytes that recognize Mtb antigens, but also by allergen-specific T cells, that seem to be re-educated from a Th2 to a Th1 profile due

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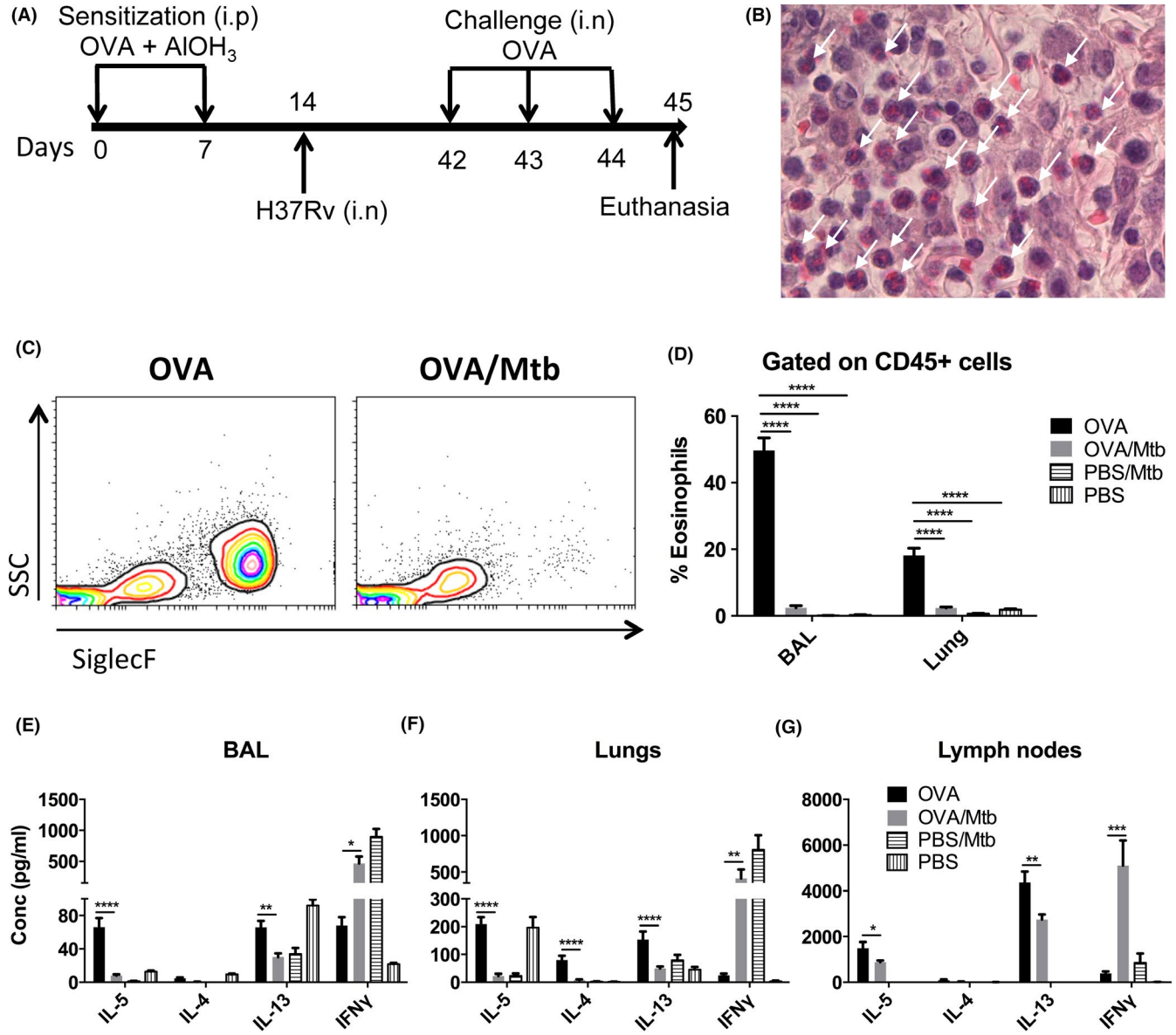

FIGURE 1 *Mycobacterium tuberculosis* (Mtb) infection prevents airway eosinophilia in an OVA-driven asthma mouse model. A, Model of allergic asthma. ip: intraperitoneal; in: intranasal. B, Representative hematoxylin-eosin image (amplified 40×) of a lung from an OVA-treated mouse. White arrows indicate eosinophils. C, Eosinophils detection in bronchoalveolar lavage (BAL) samples by flow cytometry. D, Percentage of eosinophils in BAL and lungs at day 45. E-G, Cytokine concentration in BAL (E), lungs (F) or in supernatants from mediastinal lymph node cells incubated with ovalbumin OVA (data are shown after subtracting baseline values obtained from unstimulated controls) (E). Graphs represent mean ± SEM from pooled data of two experiments (n = 12 mice for OVA-challenged and 6 for PBS groups). D, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$, by two-way ANOVA and Bonferroni post-test. E-G, For each cytokine, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$, by one-way ANOVA and Bonferroni post-test

to the inflammatory environment associated with Mtb infection. In this regard, switch from Th2 to Th1 profile has been previously described, and it is mediated by IL-12 and interferons[8], cytokines classically associated with TB infection.

To our knowledge, our results provide the first evidence about the capacity of Mtb infection to impair eosinophil production at a bone marrow level. Studies in the literature indicate that Mtb can colonize bone marrow stem cells during LTBI[9]. Thus, we could speculate that Mtb could alter local inflammatory response in the host bone marrow, enhancing IFNγ production, and impairing IL-5, which would have an impact on generation of eosinophil progenitors and therefore in restricting production of mature eosinophils that could be recruited to peripheral tissues upon allergen exposure in asthmatic individuals.

The global niche of LTBI individuals is currently estimated in around 1.7 billion people[1], with only a small proportion (5%-10%) expected to develop active TB over their lifetime. In this sense, Mtb could be considered both as a pathogen in active TB, or as a symbiont in 90% of individuals with LTBI. We could speculate that selection of tolerance to latent TB throughout human evolution could have been favored due

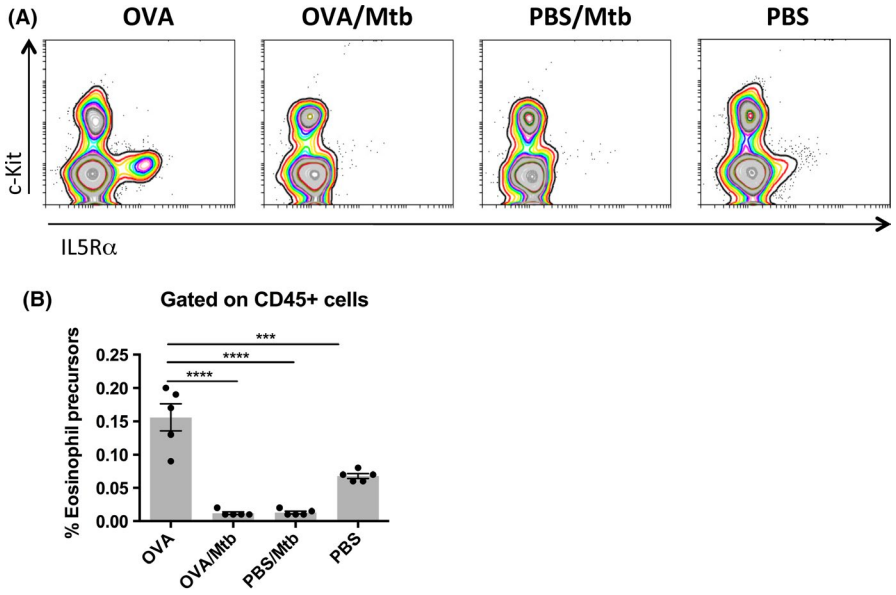

FIGURE 2 *Mycobacterium tuberculosis* (Mtb) infection abrogates bone marrow eosinophil precursors. A, Eosinophil precursor detection in bone marrow samples by flow cytometry, defined as $Lin^{-}Sca1^{-}CD34^{+}IL5R\alpha^{+}cKit^{low}$ cells. B, Percentage of eosinophil precursors in bone marrow at day 45. Data are mean ± SEM from one experiment (n = 5 mice). ***$P < 0.01$; ****$P < 0.0001$, by one-way ANOVA and Bonferroni post-test

to unspecific protection conferred by Mtb-mediated Th1 response against certain Th2 cytokine-driven allergies, including asthma. Mtb and humans have coevolved for more than 70 000 years, long before human migrations out of Africa, when societies were rudimentarily composed of small groups of people dedicated to hunt and gathering[10]. In this regard, considering lifestyle of these ancient societies, we can speculate that asthma protection conferred by asymptomatic LTBI could have resulted advantageous to humans, particularly if we consider asthma-associated symptoms, which should be especially detrimental in individuals whose survival crucially depended on physical activity.

At present, multiple clinical trials in TB endemic countries are on course to develop novel tools against this disease. Based on our results and the observational studies available, we consider that the systematic inclusion of asthma detection as an additional exploratory endpoint in TB clinical trials could provide highly valuable information. These trials represent an excellent opportunity to study in depth the relationship between asthma and LTBI and could help to elucidate the immunological bases of asthma protection induced by Mtb infection.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.