Universitat Autònoma de Barcelona

Study of the regulatory response in the control of the development of active tuberculosis

María Paula Cardona

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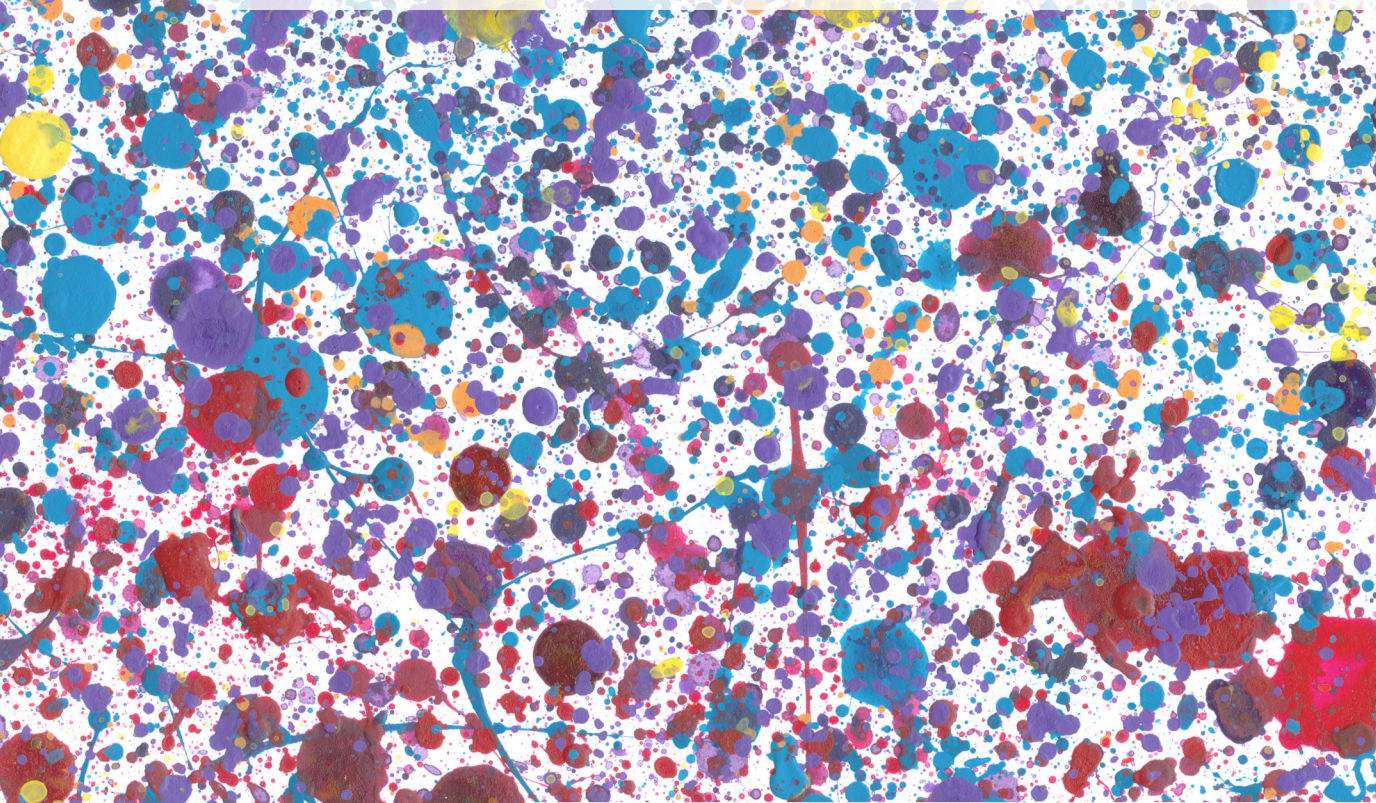

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Study of the regulatory response in the control of the development of active Tuberculosis

María Paula Cardona

Fundació Institut d'Investigació en Ciències de la Salut
Germans Trias i Pujol

Unitat de Tuberculosi Experimental

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Prof. Pere-Joan Cardona
Thesis Director

A la más buena

Abstract

It is estimated that a third of the world population is infected with *Mycobacterium tuberculosis* (Mtb). However, just a minor percentage develops the disease. It has been shown that inflammation is a key factor in the progress from infection to active tuberculosis (TB). This work addresses this issue, with a special attention on the immune regulatory response. By administering inactivated mycobacteria orally, we looked for the induction of tolerance to Mtb as a way of delaying the infection progression.

The oral administration of heat-killed *Mycobacterium manresensis* (hkMm) showed to be protective in the C3HeB/FeJ mice model of active TB. Treated animals had an increased survival, a reduction in the bacillary load and less lung infiltration. This was linked to an increase in specific memory regulatory T cells (Tregs) and IL-10 production in spleen. Moreover, there was a reduction in pro-inflammatory cytokines in lungs. Further studies on the same murine model showed that IL-17 is essential early in the infection, but detrimental later on. The neutralization of TNF-α or IFNAR-1 also delayed the progression of the infection. Mtb infection leads to a decrease in the microbial diversity in gut microbiota. The hkMm reverted this to comparable levels to non-infected animals, which appears to be a result of its protective and anti-inflammatory effect. Furthermore, the adoptive transfer of memory Tregs from hkMm treated mice into infected animals had a protective effect in terms of bacillary load and lung histopathology.

The safety and immunogenicity of hkMm was studied in healthy adults, both with or without latent TB infection (LTBI). Two doses were evaluated and showed an excellent tolerance profile. Treatment was associated to an increase in specific memory Tregs.

All in all, these results support that the immune modulation with hkMm to balance the response to Mtb might be a useful tool to prevent the progression from LTBI to TB.

Resumen

Se estima que un tercio de la población mundial está infectada por *Mycobacterium tuberculosis* (Mtb). Sin embargo, solo un porcentaje menor desarrolla la enfermedad. Se ha demostrado que la inflamación es un factor clave en el progreso de la infección a la tuberculosis activa (TB). Este trabajo aborda este problema, con una atención especial en la respuesta inmune regulatoria. Al administrar micobacterias inactivadas por vía oral, buscamos la inducción de la tolerancia a Mtb como una forma de retrasar la progresión de la infección.

La administración oral de *Mycobacterium manresensis* inactivada por calor (hkMm) mostró ser protectora en el modelo murino de TB activa. Los animales tratados tuvieron una mayor supervivencia, una reducción en la carga bacilar y una menor infiltración pulmonar. Esto se relacionó con un aumento en la producción de células Tregs específicas de memoria e IL-10 en el bazo. Además, hubo una reducción de citocinas proinflamatorias en los pulmones. Otros estudios en el mismo modelo demostraron que la IL-17 es esencial al principio de la infección, pero perjudicial más adelante. El bloqueo de TNF-α o IFNAR-1 también retrasó la progresión de la infección. La infección por Mtb lleva a una disminución de la diversidad microbiana en la microbiota intestinal. El hkMm revirtió esto a niveles comparables a los animales no infectados, lo que parece ser el resultado de su efecto protector y antiinflamatorio. Además, la transferencia adoptiva de Tregs de memoria de ratones tratados con hkMm a animales infectados tuvo un efecto protector en términos de carga bacilar e histopatología pulmonar.

La seguridad e inmunogenicidad de hkMm se estudió en adultos sanos, con o sin infección latente de TB (LTBI). Dos dosis fueron evaluadas y mostraron un excelente perfil de tolerancia. El tratamiento se asoció a un aumento en la memoria específica de Tregs.

En general, estos resultados respaldan que la modulación inmune con hkMm para equilibrar la respuesta a Mtb podría ser una herramienta útil para prevenir la progresión de LTBI a TB.

Abbreviations

AE	Adverse events	A
AM	Alveolar macrophage	
APCs	Antigen presenting cells	
BCG	Bacillus Calmette–Guérin	B
BL	Bacillary load	
CFU	Colony formation units	C
CRF	Case report form	
CRO	Contract research organization	
CT	Clinical trial	
DCs	Dendritic cells	D
DOT	Directly observed therapy	
DS	Drug susceptible	
FAO	The Food and Agriculture Organization of the United Nations	F
FoxP3	Forkhead box P3	
GALT	Gut associated lymph tissue	G
HACCP	Hazard analysis critical control points	H
HE	Haematoxylin-eosin	
HIV	Human Immunodeficiency Virus	
hk	Heat-killed	
hkBCG	Heat-killed *Mycobacterium bovis* BCG	
hkMm	Heat-killed *Mycobacterium manresensis*	
hkMtb	Heat-killed *Mycobacterium tuberculosis*	

i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenous
IFN	Interferon
IFNAR-1	Type I IFN receptor
IGRA	Interferon gamma release assay
IL	Interleukin
IQR	Interquartile range
KO	Knock Out
LN	Lymph node
LTBI	Latent Tuberculosis Infection
mAb	Monoclonal antibody
MDR	Multi-drug-resistant
Mm	*Mycobacterium manresensis*
Mtb	*Mycobacterium tuberculosis*
MTBC	*M. tuberculosis* complex
MTC	Masson trichromic
NETs	Neutrophil extracellular traps
NHP	Non-human primate
NR	Nyaditum resae
NTM	Nontuberculous mycobacteria
p.i.	Post-infection
PBMCs	Peripheral blood mononuclear cells
PMN	Polymorphonuclear cells
PPD	Purified protein derivative of Mtb
RHEZ	Rifampicin, Isoniazid, Ethambutol and Pyrazinamide

I

K

L

M

N

P

R

s.c.	Subcutaneous
SOPs	Standard operating procedures
TB	Tuberculosis
TGF	Transforming growth factor
TNF	Tumour necrosis factor
Tregs	Regulatory T cells
TST	Tuberculin skin test
UTE	Unitat de Tuberculosi Experimental
WHO	World Health Organization
ZN	Ziehl-Neelsen

S

T

U

W

Z

1| Introduction

1.1| Tuberculosis

Tuberculosis (TB) is an infectious disease, caused by the *Mycobacterium tuberculosis* (Mtb) bacillus. Even though many people think of it as a problem of the past, it is the leading cause of death from a single infectious agent (World Health Organization, 2017a). Figure 1 summarizes Tuberculosis' basic facts, which are addressed in this section.

1.1.1| Global health problem

The World Health Organization (WHO) has estimated that, in 2016 alone, TB caused 1.3 million deaths in the HIV-negative population and another 374,000 amongst the HIV-positive population. In the same year, approximately 10.4 million people fell ill with TB. Even though these numbers have been dropping over the last 16 years, it still isn't enough to reach the *End TB Strategy* goals for 2035 (World Health Organization, 2017a).

This scene is very heterogeneous over the globe; for example, 56% of the estimated cases in 2016 were present in only 5 countries: India, Indonesia, China, the Philippines and Pakistan. However, that does not leave us out of the picture: in Spain, there is an estimated incidence of 10/100,000 inhabitants (World Health Organization, 2017a). This number increases to 13.3/100,000 in Catalonia and 16.2/100,000 in Barcelona (Agència de Salut Pública de Barcelona, 2017; Generalitat de Catalunya - Departament de Salut, 2018). Similar dynamics are found in other big cities of Europe, presenting higher rates than the rest of the country (de Vries et al., 2014).

1.1.2| Tuberculosis & Latent Tuberculosis Infection

The infection with Mtb can affect almost every organ in the human's body, but pulmonary TB is the most common condition and the contagious form of the disease. The little droplets of aerosol, containing bacilli, that an

individual affected by pulmonary TB generates when coughing or speaking, are the responsible of spreading TB.

When infected with Mtb, there is a 5-15% chance of developing the disease. The risk is higher in adults and during the first 2 years after infection (Vynnycky and Fine, 1997). The vast majority of people remains infected, i.e. they have Latent Tuberculosis Infection (LTBI). Risk factors for developing TB include HIV infection, malnutrition, diabetes, smoking or alcoholism (Bates et al., 2015). However, it is still unknown why some apparently healthy people get TB and others control the infection.

People with LTBI do not present any symptoms. On the other hand, the symptoms associated with Active TB are often vague and nonspecific, so they can be present in other diseases. Among the most frequent clinical signs for pulmonary TB we find prolonged cough, fever, weight loss and night sweats (Loddenkemper et al., 2016).

1.1.3| Diagnosis & treatment

The steps to diagnose TB or LTBI vary according to the country's context: TB burden and available resources. However, there are some general recommendations by the WHO that will be exposed in this section.

1.1.3.1|LTBI

The first screenings to diagnose LTBI in population at high risk of having tuberculosis that don't present any symptoms are a tuberculin skin test (TST) or an interferon gamma (IFN-γ) release assay (IGRA). The first is based on the size of the induration generated by the cellular response to the intradermal injection of PPD (Purified protein derivative of Mtb). Its main drawback is that it may give false positives (e.g. in BCG-vaccinated subjects or other mycobacterial infections). IGRAs, in vitro assays that measure the IFN-γ produced by T cells in presence of Mtb antigens, are more specific than TST but more expensive and require special laboratory infrastructures. The problem with is that neither of the tests can differentiate between LTBI and TB (Rangaka et al., 2012). So, when

obtaining a positive result, TB should be excluded with further tests.

Figure 1| Basic facts of Tuberculosis.

The population considered "at risk", to be tested and treated for LTBI, is different in countries with low and high burden of TB and, unfortunately, it's also limited by the available resources. For example, in a low burden country we would consider people with HIV, contacts of pulmonary TB,

patients initiating anti-tumour necrosis factor (TNF) treatment, patients receiving dialysis, patients preparing for organ or hematologic transplantation and patients with silicosis. However, in countries with high TB burden, and generally limited resources, LTBI testing is recommended in pulmonary TB close contacts, but only if infected with HIV or under 5 years old (World Health Organization, 2018).

There are different treatment regimens for LTBI; some of them are: 6-month isoniazid, 9-month isoniazid, or 3-month regimen of weekly rifapentine plus isoniazid (World Health Organization, 2018).

1.1.3.2|TB

People presenting suggesting symptoms, such as unexplained cough for 2 weeks or abnormalities in a chest X-ray, should be tested for TB. As mentioned before, subjects with TST or IGRA positive results should also be tested. According to the WHO's guidelines (World Health Organization, 2015), the diagnosis of TB can be done by one of the following methods:

- Rapid molecular tests: The Xpert® MTB/RIF assay can confirm the presence of Mtb and susceptibility to rifampicin within 2 hours. However, this requires expensive equipment and laboratory facilities that are not available everywhere.
- Sputum smear microscopy: This traditional technique, which can detect most pulmonary TB cases, is based basically on the detection of Mtb in sputum samples. The required material and equipment for performing this rapid test is simpler and cheaper. On the downside, it cannot distinguish viable from nonviable Mtb or nontuberculous mycobacteria. It cannot be used to test drugs susceptibility either.
- Culture based methods: They detect more cases than the microscopy-based methods and provide the necessary material for the drug susceptibility testing. However, they are not rapid techniques and require more resources, especially those related with biosafety conditions.

Detection of extrapulmonary TB might require organ-oriented explorations, including biopsy and/or radiological methods such as X-ray, TAC or RMN.

The recommended treatment for drug susceptible TB (DS-TB) is 6-months long: 2 months of isoniazid, rifampicin, pyrazinamide and ethambutol, followed by 4 months of isoniazid and rifampicin (World Health Organization, 2017b). However, this tough picture gets even more complicated with the different antibiotic resistances, in which case treatment can last up to 2 years.

The antibiotics used in TB therapy have several side effects (Gülbay et al., 2006). This fact, together with the long periods of treatment, brings along cases of poor adherence. The WHO recommends patient care and support, including directly observed therapy (DOT) (World Health Organization, 2017b).

1.1.4| Vaccines against tuberculosis

The only available vaccine for TB at the moment is the Bacillus Calmette–Guérin (BCG). This vaccine, which has been used for almost 100 years, is based on a live attenuated form of *Mycobacterium bovis* (Calmette, 1931). Its protection against pulmonary TB is limited and variable, but it is still being used in more than 150 countries (World Health Organization, 2017a). This is because it does have a robust protective role against severe forms of the disease: meningeal or miliary TB (Mangtani et al., 2014).

There are currently 17 vaccines for TB in phases I, II or III of Clinical Trials (Treatment Action Group, 2018). Briefly, there are 6 candidates that are based on recombinant proteins and adjuvants (H56:IC31, H4:IC31, ID93+GLA-SE, M72/AS01E, GamTBvac, AEC/BCO2), 4 are based on viral vectors (Ad5Ag85A, ChAdOx185A+MVA85A, TB/FLU-04L, MVA85A), 4 are mycobacterial whole cell or extract (RUTI, DAR-901, Vaccae, MIP) and 2 are live mycobacteria (MTBVAC, VPM1002). The revaccination with BCG is also undergoing clinical trials.

In spite of these vaccine candidates, there is a tough outlook on the subject, since the promising vaccine candidate MVA85A (designed as a BCG boost) could not prove efficacy in its clinical trial phase IIb (Tameris et al., 2013). This was a bombshell on the field of TB vaccines, since MVA85A had shown encouraging results in mice, guinea pigs, cattle and rhesus macaques (Goonetilleke et al., 2003; Verreck et al., 2009; Vordermeier et al., 2009; Williams et al., 2005). These studies were done with MVA85A as an intradermal vaccine; it is now being tested as an aerosol vaccination (ClinicalTrials.gov Identifier: NCT02532036).

A difficult step on the development of TB vaccines is how to evaluate their potential protective effect. Traditionally, the TB vaccine research field has focused mainly on the Th1 response, mostly the production of IFN-γ (Tameris et al., 2013). Nevertheless, the Th1 immune response is also produced as a response to Mtb infection. Lately, an important role has been given to Th17 response as a correlate of vaccine protection (Da Costa et al., 2014; Monin et al., 2015). However, Th17 is likewise related to inflammatory onsets, such as TB. A relatively new player in TB, which has been typically associated to response against virus, is type I IFN. It has been detected in a gene signature for active TB (Berry et al., 2010). There is clearly an unfulfilled need of finding a protection biomarker, for which more studies on the mechanisms involved in the Mtb infection have to be carried out.

1.2| Pathogenesis of TB

1.2.1| Dynamic hypothesis of Mtb infection

People with active TB generate aerosol with Mtb bacilli when speaking or coughing (Figure 2-1). Once in the new host, these little droplets have to make their way through the respiratory tract, which entails several barriers to overcome, such as the bronchial epithelium and mucus (Torrelles and Schlesinger, 2017).

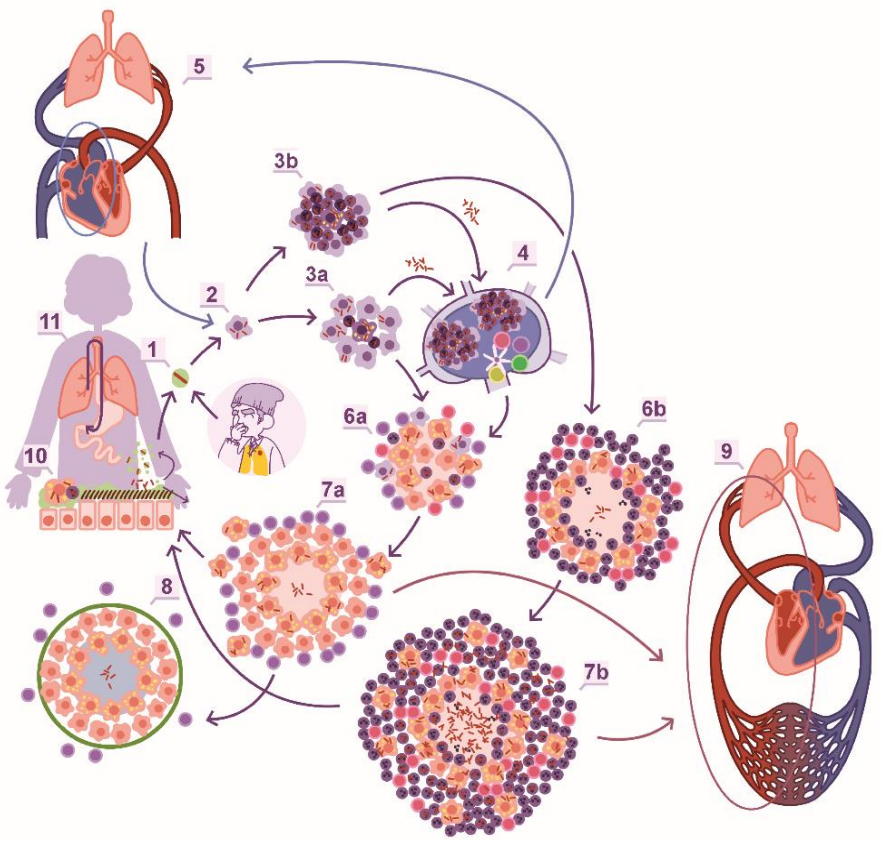

Figure 2| Dynamic Hypothesis of Mtb infection. This figure summarizes the stages involved in the development of TB. Bacilli arrive to the alveolus in aerosol droplets (1). The alveolar macrophage phagocytes Mtb (2), inducing two kinds of granuloma (3a or 3b). The bacilli are drained to the lymph node, where the T response is generated (4), allowing for the haematogenous dissemination (5). If the response is mainly Th1 (3b), there is a control of the granuloma (6a), most of the bacilli are destroyed (7a) and it is encapsulated (8). If the initial granuloma formation is dominated by neutrophils' infiltration (3a), the response is mostly Th17, attracting more neutrophils (6b) and growing until the development of TB (7b). The extrapulmonary haematogenous dissemination leads to systemic infection (9). Foamy macrophages are drained towards the bronchial tree (10) where they reach the gastrointestinal system and are expelled (11) or return to the lung through new aerosols (1). Adapted from Cardona (2017).

After reaching the alveoli, Mtb enters the alveolar macrophages (AM) (Figure 2-2), either by non-specific pinocytosis or through phagocytosis mediated by a variety of receptors (FcγRs, complement receptors, C-type lectin, TLR, scavenger receptors, among others). Once inside the AM, Mtb subverts its killing mechanisms, mainly by avoiding the acidification of the

phagosomes and their fusion with lysosomes. Mtb also attenuates the antigen presentation to T helper cells (Hmama et al., 2015). ESAT-6, a protein secreted by the bacillus, permits the rupture of the phagosome and the bacteria's translocation to the cytoplasm (Peng and Sun, 2016). Another strategy from the bacillus is to induce necrosis and inhibit apoptosis of the infected cell (Divangahi et al., 2013). This facilitates its way back to the extracellular media and thus the spread into neighbouring cells, which will eventually result in the onset of the inflammatory focus. In the meantime, however, the growth of Mtb is practically unnoticed.

The review of pathology studies from necropsies revealed there are two kinds of lesions: proliferative or exudative (Cardona, 2015). The development of one or the other is apparently linked to the bacillary load and the site of infection. The first are controlled lesions, with low bacillary load, formed mainly by lymphocytes and epithelioid cells (Figure 2-3a). Exudative lesions, on the other hand, have neutrophils as a main protagonist, are typical of the upper lobes and present high bacillary load (Figure 2-3b).

The dissemination of Mtb occurs before the development of the acquired immune response (Chackerian et al., 2002). There is a circulation of bacilli to the pulmonary lymph nodes (LN), thanks to the constant drainage of the alveoli (Figure 2-4). They do so freely at the beginning of the infection and later through antigen presenting cells (APCs), namely dendritic cells (DCs) (Wolf et al., 2007). There is a constant feedback run by infected DCs from the lesion to the LN in order to re-stimulate the lymphocytic proliferation. The activation of T cells depends on the bacillary load in LN (Wolf et al., 2008).

The haematogenous dissemination allows the bacilli's way from the LN towards the right atrium and ventricle, thus returning to the lungs (Figure 2-5) (Vilaplana et al., 2014). This may result in new infection foci and lesion, or the bacilli may get to a previously generated one, since granulomas have higher vascularisation and permeability (Datta et al., 2015; Oehlers et al., 2015). This may also allow the bacilli to get to the left

atrium and ventricle, which would permit the systemic dissemination (Figure 2-9) (Vilaplana et al., 2014).

In the LN, naive T cells are primed by infected DCs. These newly active T cells then migrate to the site of infection, thanks to the chemokines and cytokines released in the inflammatory focus. In the proliferative lesions, the immune response is mainly Th1. This permits the control of the granuloma (Figure 2-6a) and the destruction of most of the bacilli (Figure 2-7a), leading to the granuloma encapsulation process (Figure 2-8) which involves the production of fibrotic, collagen rich, connective tissues around the granuloma. There are arguments saying the granuloma is beneficial to the host, since it contains the mycobacteria in a "latent" state, and others saying it is detrimental, because it is a niche for Mtb growth. On the other hand, if the lesion is exudative, the response is mostly Th17 and the granuloma is infiltrated by increasing numbers of neutrophils (Figure 2-6b) and grows until the development of active TB (Figure 2-7b).

There is a small number of bacilli that enter a dormancy state, with minimal metabolic activity. The macrophages that contain these bacilli, also phagocyte necrotic tissue, up to a point in which they cannot metabolize the big amount of fatty acids this represents, and start accumulating lipid bodies in their cytoplasm becoming foamy macrophages. These cells are drained towards the bronchial tree (Figure 2-10) where they will be mainly drained by the gastrointestinal system (Figure 2-11). They can also return to the lung through new aerosols, infecting the host once again (Figure 2-1).

1.2.2| Of mice & pigs: TB and animal models

Many animal models have been used over the years to study TB (Cardona and Williams, 2017; Williams and Orme, 2016). They have helped understand the disease and test drugs or vaccines against it, despite their obvious limitations.

Mice models are the most widely used and characterised in research. One

of the main contributions from TB mice models was the confirmation of the key role of IFN-γ in the infection. This was accomplished thanks to the development of a knock-out (KO) mice: those lacking IFN-γ had a worse outcome (Cooper et al., 1993; Flynn et al., 1993).

Not all mouse strains have the same susceptibility to Mtb infection. In this sense, the most widely used laboratory mouse strains, C57BL/6 and BALB/c, have been traditionally considered as "resistant" although we should consider them as "tolerant" (Cardona, 2006, 2010). One of the first works addressing this issue was done by Medina & North, who classified mouse strains into "resistant" or "susceptible" according to their survival after intravenous (i.v.) or aerosol Mtb infection (Medina and North, 1998). Special efforts have been made into mimicking human pulmonary TB, where necrotizing granulomas are characteristic. The pulmonary lesions are also different among resistant and susceptible strains; while the first consist mainly of lymphocytes, the latter are mainly macrophage dominant (Beamer and Turner, 2005). In C57BL/6 and BALB/c mice, lesions are non-necrotic, with few neutrophils and intracellular bacilli (Harper et al., 2012; Kramnik and Beamer, 2016). Necrotizing lesions have been described in Mtb infected DBA/2, CBA/J, SCID or C3HeB/FeJ mouse strains (Cardona et al., 2003; Guirado et al., 2006; Major et al., 2013; Marzo et al., 2014).

The use of pigs for biomedical research has the advantages related to the similarities with humans: they share anatomical and physiological characteristics (in the cardiovascular, urinary, integumentary, and digestive systems). In fact, pigs have been increasingly used for pre-clinical toxicology studies (Swindle et al., 2012). An interesting aspect of using this animal as a model for studying TB is the lung's anatomy. Minipigs, as humans and larger mammals, have interlobular septa, which divide the lung into approximately 1 cm^3 pieces. These structures are not present in smaller mammal species, like mice or macaques, for which the bacteria can easily grow with no space limitations. By studying the lung histopathology of infected pigs, it was shown that lesions with a diameter as small as 0.5 mm could be encapsulated by the fibroblasts in the septa. This is a key factor

for controlling the progression of LTBI into TB (Gil et al., 2010).

1.2.2.1|C3HeB/FeJ mice

C3HeB/FeJ is a mouse strain highly susceptible to Mtb infection (Kramnik et al., 2000). Studies with this strain use either the intravenous or aerosol infection; however, the first induces TB human-like lesions in a faster and more robust way (Pan et al., 2005; Yan et al., 2006).

The particular interest in the use of this strain to study TB, lies in the fact that the lesions developed are quite similar to those found in the lung pathology in human TB, in spite of not presenting interlobular septa. It has been shown that after Mtb infection, these mice develop necrotic and hypoxic lung lesions (Harper et al., 2012; Pan et al., 2005). The C3HeB/FeJ strain has also been studied to mimic bovine TB, by intranasal infection with *M. bovis* (Bouté et al., 2017).

We have characterized the model, finding that the infected animals develop necrotic lesions, even reaching liquefaction in the centre. There is a massive infiltration of inflammatory cells, mainly neutrophils. These provide an excellent environment for the extracellular bacterial growth, thanks to the neutrophils extracellular traps (NETs) (Marzo et al., 2014). From now on, this animal model will be mentioned as the "Active TB" model, which could be considered as homologous to the human pulmonary TB.

The Active TB model's lesions were further studied using formalin-fixed, paraffin-embedded, lung sections stained with Masson trichromic (MTC). At day 21 post-infection (p.i.), the mean lesion area was 0.266 mm^2. Lesions grew exponentially with infection's progression and 7 days later the mean lesion area was 3.36 mm^2. The number of lesions increased with infection, but not in a constant manner, since those that were close enough would coalesce into one (Figure 3-A). All of these led to the development of a mathematical model that describes the process: the Bubble Model, because of the resemblance to the mechanics of soap bubbles (Prats et al., 2016).

Figure 3-B illustrates that, in the absence of neutrophils infiltration, the lesions' growth is controlled and they do not merge. This way, there is

enough time for them to be encapsulated, thus avoiding the development of active TB.

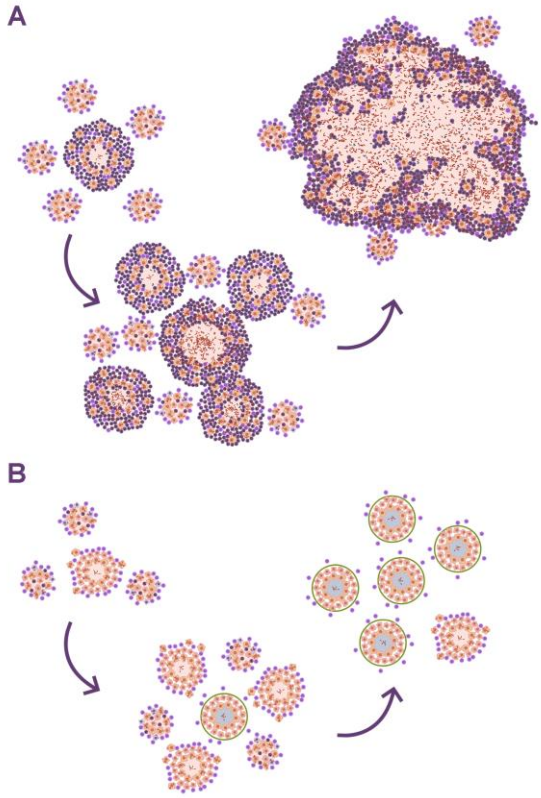

Figure 3| Evolution toward active tuberculosis (TB). (A) The process toward a neutrophilic infiltrated lesion favours the induction of new lesions and their rapid growth; this leads to lesion coalescence and the induction of an active TB lesion. On the contrary, (B) shows a well-controlled lesion, without neutrophilic infiltration, which hardly induces new lesions and soon becomes encapsulated, blocking the induction of active TB. Extracted from Cardona (2017).

1.2.3| "Damage-response" framework

The results from the studies carried out in the Active TB model have made us focus on the inflammatory response in tuberculosis. Is the lack of control of inflammation the reason why some people develop TB while others control the infection? Although the immune response is necessary for controlling the infection at early stages, an exacerbated response may be

detrimental later on. In this sense, we should take into consideration the "Damage-response" framework of microbial pathogenesis (Casadevall and Pirofski, 2003). This approach is based on three principles: the pathogenesis is the result from the interaction between the host and the microorganism, the pathological outcome is determined by the damage done to the host and this damage can be caused by the own host or/and the microorganism. Figure 4 illustrates this concept applied to the Mtb infection. While a weak response would result in a disseminated TB -like immunosuppressed individuals-, a strong response would be the responsible for pulmonary TB. In the middle there is a wide range of situations, where we'd find LTBI and most of the cases of Mtb infection (Cardona, 2010).

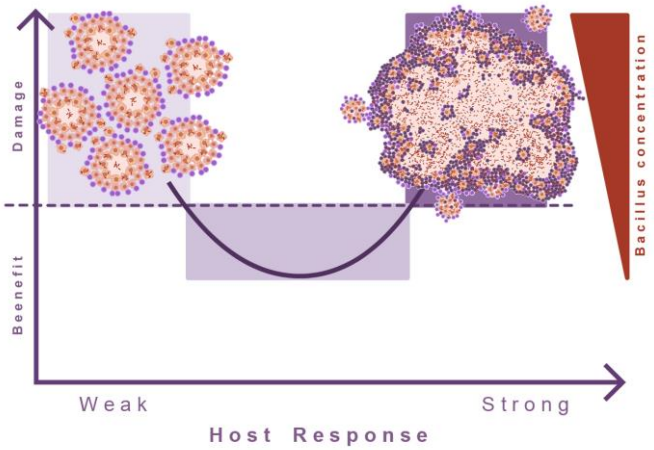

Figure 4| Damage-response framework in Tuberculosis. This picture illustrates Arturo Casadevall's "damage theory". In the case of TB, a weak immune response leads to the proliferation of lesions and dissemination of the bacilli. However, the induction of an excessive inflammatory response leads to exudative lesions characterized by massive tissue destruction. In this regard, we can consider that TB develops two kinds of diseases, and in between there is a wide range of situations where the immune response is balanced and allows the host to live with the bacilli without hampering its health status. Extracted from Cardona (2017).

As a first proof of concept, the effect of common anti-inflammatories in the Active TB model was studied at the Unitat de Tuberculosi Experimental (UTE). Excellent results were accomplished by administering ibuprofen or

aspirin (Kroesen et al., 2018; Vilaplana et al., 2013), which has resulted in a new line of research at the unit led by Dr. Vilaplana.

The idea of controlling the inflammation in an antigen specific way, made us search for other possible strategies. In this sense, we sought the development of regulatory T cells (Tregs) specific of mycobacteria, in order to balance the immune response against Mtb.

1.3| Immunotherapies

An immunotherapy treats or prevents a disease by stimulating, enhancing, suppressing or desensitizing the immune system. It is said that William B. Coley is the father of immunotherapy. In 1891, he injected streptococcal organisms into a cancer patient to cause erysipelas. This stimulation of the immune system is presumed to be the responsible for making the tumour disappear. In order to avoid the dangers of streptococcal infection, he began treating cancer patients with a combination of heat killed *Streptococcus pyogenes* and *Serratia marcescens*, which was known as Coley's Toxin (McCarthy, 2006). A widely used immunotherapy, based in this concept, is the treatment of some kinds of bladder cancer with *M. bovis* BCG, recently review by Pettenati and Ingersoll (2018).

1.3.1| Probiotics

An example of a common immunotherapy are probiotics, a term that is known pretty much by everyone nowadays. The Food and Agriculture Organization of the United Nations (FAO) together with the WHO reported a set of guidelines to evaluate probiotics, in which they defined them as "'live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Joint FAO/WHO Working Group, 2002).

The first example of probiotics application that comes to mind is their effect on gastrointestinal health. Nonetheless, it's been found that they also have an effect on the respiratory system. The ingest of probiotics reduces hay

fever symptoms (Dennis-Wall et al., 2017) and the incidence and duration of upper respiratory tract infections (Shida et al., 2017). The consumption of *Lactobacillus casei* was even associated with a better quality of sleep during stress periods (Takada et al., 2017).

However, it has been shown that the beneficial effects of probiotics are not accomplished only with live microorganisms. This "probiotics paradox" is nicely reviewed by Adams (2010). There are many studies with dead probiotics in animal models or human clinical trials. Mice treated orally with heat-killed (hk) *Lactobacillus pentosus* were protected against infection with *Streptococcus pneumonia*: they presented prolonged survival and less body weight loss compared with control mice (Tanaka et al., 2011). Administration of hk *Enterococcus faecalis* decreased the morbidity and lung inflammation in mice with influenza infection (Chen et al., 2017). The administration of hk *Lactobacillus* reduced the common cold's incidence (Shinkai et al., 2013) and improved symptoms and duration among susceptible subjects (Murata et al., 2018).

It's been shown that the microorganisms in our food may have an influence on the microbiota composition (Sánchez et al., 2017), i.e. the microorganisms that normally reside in our organism.

1.3.2| Microbiota

The study of microbiota is being used more and more as an indicator of human health, even if it's a whole new research field.

The microbiome can have big effects on the development of immune responses in the gut (Yoon et al., 2014). More specifically, there are several studies suggesting that bacteria from the Clostridia class may promote the development of tolerance in the gut (Atarashi et al., 2013). Different *Lactobacilli* bacteria reduce the inflammatory response through Tregs induction (Ding et al., 2017). Furthermore the colonization by *Bacteroides fragilis* has been related to the expansion of Tregs (Round and Mazmanian, 2010).

Nonetheless, the gut microbiota has effects not only in the tract, but also in organs distal to the intestine. Changes in the gut microbiota have been associated with allergy, asthma, COPD, cystic fibrosis and TB (Belkaid and Harrison, 2017; Gupta et al., 2018).

There is an increasing interest in microbiota on the field of TB. BALB/c mice showed a decrease in the microbiota diversity with Mtb aerosol infection, which went back up with infection's progression. However, with infection there was also a switch in the composition of the microbiota. The authors highlight the decrease in microorganisms from the Clostridiales and Bacteroidales orders (Winglee et al., 2014). A similar trend was observed in a microbiota study in Mtb infected C57BL/6J-CD45a(Ly5a) mice, but not as definite. In this work, the effect of TB antibiotics treatment on microbiota is addressed, which causes a rapid dysbiosis. This is described as a reduction in diversity at first, but also a change in the microbial composition that lasts long after the treatment is completed. The frequencies of different genera belonging to Clostridia class were reduced with chemotherapy. This was accompanied by an increase in the abundance of bacteria from the *Erysipelatoclostridium* genus (Namasivayam et al., 2017). Similar results were obtained in DS-TB patients, where antibiotics treatment caused a dysbiosis that lasted at least 1.2 years after finalizing treatment (Wipperman et al., 2017). The influence of antibiotics on microbiota, however, is not specific of TB chemotherapy (Langdon et al., 2016).

1.3.3| Oral tolerance

The human adult gut mucosa area is around 32 m^2 (Helander and Fändriks, 2014), the same size as many flats in Barcelona. It is in constant interaction with the outside world, through the food intake. There is an extensive lymphoid tissue associated to the gastrointestinal tract: the gut associated lymphatic tissue (GALT). It patrols over the gut, preventing the damaging role of pathogens, but does not react to food antigens. This is why it is referred to as a tolerogenic environment (it tolerates the presence of certain

antigens). The mechanism for which the GALT is taught no to react to certain antigens is referred to as “oral tolerance”.

1.4| Tuberculosis & Tregs

There is a recurring discussion on whether Tregs are beneficial or detrimental for TB. There are many studies, done mainly in blood samples, which show there are higher numbers of Tregs in TB patients. Most of them compare TB patients to LTBI and/or healthy controls. Some do a follow-up of TB patients under treatment. However, it is not clear whether Tregs are a consequence of inflammation or a risk factor for TB development. This topic will be addressed in this section after a brief introduction on Tregs, reviewing the available data on animal models or TB patients.

1.4.1| Regulatory T cells

Tregs have a suppressive role, counterbalancing the inflammatory response. Different mechanisms of action have been described, as reviewed by Vignali and colleagues (Vignali et al., 2009):

- Inhibitory cytokines, such as IL-10 and TGF-β
- Cytolysis
- Metabolic disruption
- Targeting dendritic cells (DCs)

The identification of cellular markers for characterizing Tregs has been a challenging field. Some of the markers used, aside from CD3 & CD4, are CD25, FoxP3, CD127 or CD39. Although $CD8^+$ Tregs have also been described, they are less characterized and haven’t been taken into account in this work.

It is widely accepted that CD25 is highly expressed in Tregs. However, this marker, which is the receptor for IL-2, is also upregulated in activated T cells (Zola et al., 2007). This is why it is generally used in combination with other markers.

FoxP3 is a transcription factor: Forkhead box P3. It has been long used as a marker for Tregs. Nevertheless, it has been identified in other cell types (Devaud et al., 2014). Besides, working with an intracellular marker is less appealing and many times it is not practical, since they interfere with functional assays.

Low levels or no expression at all of CD127, the α chain of the IL-7 receptor, among $CD4^+CD25^+$ cells has also been used for identifying Tregs (Seddiki et al., 2006).

CD39 is an ectonucleotidase that turns ATP into AMP and is expressed by $CD4^+CD25^+$ and $CD4^+FoxP3^+$ cells (Borsellino et al., 2007). It is used together with CD25 as a phenotype of CD4 Tregs.

1.4.1.1 | CD39 Tregs

The characterization of $CD4^+$ T cells according to the expression of CD25 and CD39 in humans makes the distinction of 4 populations (Dwyer et al., 2010):

- $CD25^+CD39^+$ (Tregs): 80-100% of these cells express high levels of FoxP3. Also, around an 80% express CD45RO, consistent with a memory regulatory phenotype.
- $CD25^-CD39^+$ (Tmeff): almost all of these cells express CD45RO, which denotes a memory effector profile. They produce IFN-γ and IL-17.
- $CD25^+CD39^-$ (Teff): this population represents the effector T cells, since after stimulation, proliferate and secrete cytokines from Th1, Th2, Th17 or even Tregs profile. Approximately 50% of them are positive for FoxP3, but in a lower intensity than $CD25^+CD39^+$.
- $CD25^-CD39^-$: naive T cells.

This classification of $CD4^+$ T cells was described in the field of organ transplant, where a low Tregs/Tmeff ratio in blood was observed in cases of renal allograft rejection (Dwyer et al., 2010). However, CD39 Tregs have also been studied in cancer, rheumatoid arthritis, multiple sclerosis and allergies (Herrath et al., 2014; Li et al., 2015b; Muls et al., 2015; Schuler et

al., 2012) as well as in TB (Chiacchio et al., 2009; Ye et al., 2011).

Gu et al. cultured human peripheral blood mononuclear cells (PBMCs) and characterized $CD4^+CD25^+CD127^-$ Tregs according to the expression of CD39. They showed that $CD39^{hi}$ Tregs secret more IL-10 and have stronger suppression activity. Furthermore, in the presence of IL-1β and IL-6, $CD39^{low}$ Tregs differentiated into Th1 or Th17 and downregulated the expression of FoxP3 in comparison to $CD39^{hi}$ Tregs. They also tested their protective effect on a xenograft versus host mice model, and found that only the $CD39^{hi}$ Tregs had a positive effect (Gu et al., 2017).

1.4.1.2| Th17 counterbalance

Regulatory T cells and pro-inflammatory or pathogenic Th17 are tightly bound. TGF-β induces both FoxP3 and RORγt. However, in order to induce Th17, TGF-β also needs the presence of IL-6 (Veldhoen et al., 2006). In that case, the balance is tipped towards Th17 instead of Tregs. Otherwise, FoxP3 inhibits the function of RORγt (Zhou et al., 2008). It has been shown that $CD4^+CD25^{hi}CD39^+$ Tregs have a role in constraining the pathogenic Th17 cells (Fletcher et al., 2009).

Once again, taking into account the "Damage-response" framework, it has been widely discussed that the balance between Tregs and Th17 is a key point in autoimmune and chronic inflammatory diseases, such as multiple sclerosis and rheumatoid arthritis (Abdolahi et al., 2015; Kim and Moudgil, 2017).

1.4.2| Tregs in TB animal models

In the guinea pig model, while studying the pathogenicity of different Beijing sublineages of Mtb, it has been shown that highly virulent strains have increasing levels of lungs' FoxP3, IL-10 and TGF-β mRNA with infection's progression (Shang et al., 2011). In another study, with a similar focus, it was also found that the expression of FoxP3 and TGF-β in lungs increases as the infection progresses, but also pro-inflammatory IL-17. This happens also in less virulent strains, but the increase is not as steep (Kato-

Maeda et al., 2012). Results from a nonhuman primate (NHP) Mtb infection model suggest that Tregs are augmented as a response to the inflammation rather than the cause of disease. In fact, these authors saw that NHP that didn't develop the disease (LTBI) had higher levels of Tregs prior to infection than those that developed active TB. Although all animals had an initial decrease in the frequencies of Tregs, LTBI NHP were higher levels. As infection progressed, Tregs returned to pre-infection levels in LTBI animals and continued to increase in TB NHP (Green et al., 2010).

When depleting $CD25^+$ cells in DBA/2 mice prior to Mtb aerosol infection, there is an improvement on the infection's outcome: a reduction in the bacillary load of lungs and spleen, together with less histopathology in lungs. Nevertheless, this effect is seen only at week 2 p.i., afterwards there are no differences between groups. There is also no significant effect if the $CD25^+$ cells depletion is done at the chronic stages of infection (Ozeki et al., 2010). Similar results were observed using a mixed bone chimera system, where $FoxP3^+$ cells are depleted with anti-Thy1.1 (Scott-Browne et al., 2007). However, the depletion of $CD25^+$ cells in *M. bovis* BCG or Mtb infected C57BL/6, 3 days prior to infection, did not result in a worse outcome, even though it was proved that there was a reduction in $CD4^+CD25^+$ cells for at least 23 days (Quinn et al., 2006).

Kapina et al. studied two mice strains, one hyper-susceptible to TB (I/StSnEgYCit) and the other relatively resistant (C57BL/6JCit). They observed that the first group had lower levels of $CD4^+CD25^+FoxP3^+$ cells in mediastinal lymph nodes, and they were practically constant during the follow up period (from baseline until week 17 p.i.) while on the resistant C57 mice this population increased over time. They conclude this is a host protective strategy (Kapina et al., 2013). Scott-Browne et al. failed to see this increase in percentages, but found an increase in total numbers of $CD4^+FoxP3^+$ cells in lungs and pulmonary LNs of Mtb infected C57BL/6 mice. In spite of this, they could not detect the *in* vitro production of the characteristic regulatory cytokine IL-10 by this population (Scott-Browne et al., 2007). Contrary to these observations, others have seen a reduction

of $CD4^+FoxP3^+$ on the lungs of TLR2 KO C57BL/6 as the infection progresses. Mice lacking TLR2 had increased bacterial burden and signs of exaggerated inflammatory response in lungs, accompanied by lower levels of $CD4^+FoxP3^+$ than the WT counterpart (McBride et al., 2013). Resistant strains (C57BL/6 and BALB/c) have higher numbers of $CD103^+$ DCs, important cells in the Tregs induction, and Tregs in lungs than a susceptible strain such as DBA/2 (Leepiyasakulchai et al., 2012).

It has been addressed that Tregs interfere with the protective effect of BCG vaccination. C57BL/6 mice were administered an adenoviral vector that expresses murine IL-28B, which is thought to down-regulate Tregs. When given together with BCG, although diminishing $CD4^+CD25^+FoxP3^+$ cells in spleen, it had no influence on vaccination's effect (Luo et al., 2016). These results support what was previously observed by Quinn et al., who inactivated Tregs with anti-CD25 prior to vaccination and found it didn't affect BCG's performance (Quinn et al., 2008).

The study of BCG boost vaccine candidates in Mtb infected BALB/c mice has shown an association between a lower bacillary load and a higher $CD4^+/CD4^+FoxP3^+$ ratio in lungs (Fedatto et al., 2012). The localization of Tregs in lungs appears to be the same as other T cells, within the lymphoid aggregates of granulomas (Scott-Browne et al., 2007).

1.4.3| Tregs in human TB

The importance of the phenotypic markers used to define the Treg cells, discussed in the beginning of the section, is exposed in the work carried out by Zewdie et al., who found that while $CD4^+CD25^{+/hi}$ T cells were higher in patients with active TB than LTBI, there were no differences in the frequency of $CD4^+CD25^+CD127^{lo}$, $CD4^+CD25^+FoxP3^+$ or $CD4^+CD25^+FoxP3^+CD127^{lo}$ T cells (Zewdie et al., 2016). A similar remark had been made before by Wergeland et al., who found lower levels of Tregs in healthy controls than in active TB or LTBI when using the Treg phenotype $CD4^+CD25^+CD127^-$ but not $CD4^+CD25^+FoxP3^+$ (Wergeland et al., 2011). It is also pertinent to know exactly to which parent population

we are referring. For example, there is a study where the percentage of $CD4^+CD25^{hi}$ cells expressing FoxP3 wasn't relevant, but the percentage of $CD4^+CD25^{hi}FoxP3^+$ out of the total $CD4^+$ cells was significantly different between healthy donors and PTB (Chen et al., 2007).

Burl et al. performed a study of FoxP3 gene expression in TB contacts. They found that non-infected contacts showed higher levels of FoxP3 mRNA than infected TB contacts, but lower than active TB patients. They state that this may be due to the presence of Tregs in lungs at early stages of the infection, which later on would become present in the periphery (Burl et al., 2007). It has also been shown that in patients with active TB the level of Tregs ($CD4^+CD25^+FoxP3^+$) is higher in bronchoalveolar lavage than in blood (Semple et al., 2013). Equally, after immunohistochemical analysis, it was shown that FoxP3 is expressed in Mtb affected lymph nodes (Chen et al., 2007).

The characterization of activated $CD4^+$ T cells, in PBMCs stimulated with the mycobacterial antigens PPD, ESAT-6 or CFP-10, showed higher percentage of $CD4^+CD25^+CD134^+$ expressing CD39 in active TB than LTBI. In accordance with this, the ratio of IL-10/ IFN-γ production culture was increased in active TB compared to LTBI (Kim et al., 2014). An interesting study on PBMCs of recently diagnosed TB patients, people with LTBI and healthy controls revealed differences in Tregs and Th17 levels, accompanied by differences in IL-10 and IL-17 levels. The percentages of Tregs but also Th17 cells (and their corresponding cytokines) were higher in active TB patients than in LTBI or controls. Furthermore, LTBI presented higher levels of Tregs and IL-10 than healthy controls (Luo et al., 2017). Contrary to this, it was found that TB patients had lower levels of IL-17 producing $CD4^+$ cells but higher levels of $FoxP3^+$ $CD4^+$ cells in blood, when comparing with healthy donors or LTBI. Nevertheless, they also presented higher levels of IL-6 and TGF-β in plasma, which are related to the induction of the Th17 population. The authors state that this might be explained by a reduction in the expression of IL-6R $CD4^+$ cells, which would be caused by Mtb (Chen et al., 2010).

TB patients, before starting treatment, have increased frequencies of $CD4^+CD25^{hi}$ Tregs and a higher expression of FoxP3 in PBMCs than healthy controls (Guyot-Revol et al., 2006). Chiacchio and colleagues found no differences in Tregs levels unless they did an Mtb specific *in vitro* stimulation. After 1 or 6 day of stimulus with culture filtrate protein-10 (RD1), TB patients had higher levels of Tregs than healthy controls, using both $CD4^+CD25^{hi}FoxP3^+$ or $CD4^+CD25^{hi}CD39^+$ phenotypes (Chiacchio et al., 2009). The characterization of BCG stimulated PBMCs in paediatric TB population agrees with these results: Tregs ($CD4^+CD25^+CD39^+FoxP3^+$) are higher in TB than in healthy controls. When stratifying pulmonary or extrapulmonary TB, the authors find that Tregs cannot be used to distinguish both populations. Interestingly, however, when they look at the percentages of Tregs after 6 months of treatment, they found that PTB children have significantly decreased the levels of Tregs but EPTB children have not (Whittaker et al., 2017).

A study carried out in a BCG vaccinated cohort found that patients with active TB presented higher levels of Tregs ($CD4^+CD25^+FoxP3^+$) than healthy controls. A follow-up performed during 9 months, including the 6 months of treatment, showed that these levels continued to increase until month 4, but later decreased until reaching the levels of healthy control population (Diaz et al., 2015). Xu et al. studied pulmonary TB patients at the time of diagnosis (sputum smear positive) and these same subjects after 3 weeks of treatment (sputum smear negative). They found that with the course of treatment, the frequency of Tregs ($CD4^+CD25^+CD127^-$) in PBMCs and the levels of IL-10 in plasma augmented, which also correlated negatively with the levels of IL-17 (Xu et al., 2016). Similar results were obtained in a study where they did the follow-up during the 6 months of treatment: Tregs ($CD4^+CD25^+FoxP3^+$ or $CD4^+CD25^+CD39^+$) levels were higher in pulmonary TB patients than healthy controls and they decreased with the chemotherapy (Agrawal et al., 2018).

Pang et al. also found a higher frequency of Tregs in blood in active TB than in LTBI or healthy controls. When they looked deeper into the group of

study, Tregs levels were higher in patients with sputum smear positive or cavitary TB (Pang et al., 2013). However, after stimulating PBMCs with PHA, PPD or ESAT-6, no significant differences were found in the levels of IFN-γ or IL-17 between cavitary and non-cavitary TB (Fan et al., 2015).

A different approach was done by Lim and colleagues, who looked into the differences between DS and MDR-TB. They did not find differences among groups, but once again Tregs ($CD4^+CD25^+FoxP3^+$) levels were higher in TB patients than in healthy controls (Lim et al., 2013). A more recent work in this target population shows, once more, that Tregs ($CD4^+CD25^+FoxP3^+$) are higher in TB patients than in healthy controls, but also that MDR-TB have higher frequencies of Tregs than DS-TB. This was supported by the blood levels of regulatory cytokines IL-10 and TGF-β. The same differences were found when looking at $CD4^+CD25^+$ cells that expressed PD-1 or CTLA-4 (Li et al., 2015a). Similarly, it was shown that MDR-TB cases had a higher percentage of Tregs ($CD4^+CD25^+$) in blood (although not significant) and higher IL-10 levels in serum than DS-TB (Fan et al., 2016).

1.5| Nontuberculous mycobacteria

Nontuberculous mycobacteria (NTM) is a term used to group mycobacteria that are not part of the *Mycobacterium tuberculosis* complex (MTBC) or *M. leprae*. NTM are also referred to as "environmental mycobacteria", since they're naturally present in water or soil (Falkinham, 2015).

Generally, NTM are not pathogenic, unless it is an immunocompromised host. The disease by NTM infection is associated with HIV infection, the intake of corticosteroids or biological therapies (such as anti-TNF) and organ transplant, among others (Henkle and Winthrop, 2015).

One of the believed reasons for which BCG's efficacy has a great variability over the world is the exposure to NTM (Fine, 1995). This is why there have been many studies focused on NTM and BCG.

It has been shown, in C57BL/6 mice, that oral exposure to live *M. avium* didn't protect against Mtb aerosol infection and interfered with BCG's

efficacy (Flaherty et al., 2006). Nevertheless, in the same infection model, when using hk *M. avium* intraperitoneally (i.p.), the protective effect of BCG was improved (Poyntz et al., 2014). On the other hand, it has been found that prior aerosol infection with NTM, such as *M. kansasii*, *M. simiae* or *M. avium*, not only didn't interfere with BCG but also protected animals upon further infection with Mtb (Orme and Collins, 1984).

The administration of live *M. bovis* BCG or *M. abscessus* in drinking water was proven to be protective in an i.n. Mtb infection model in BALB/c (Beverley et al., 2013).

Even though it is not a NTM, it is also interesting to highlight that oral administration of hk *M. bovis* to wild boar resulted in fewer TB lesions and lower bacillary load upon infection with *M. bovis* (Beltrán-Beck et al., 2014).

For the development of this thesis project, a newly discovered NTM was used: *Mycobacterium manresensis*. It belongs to the *fortuitum* complex and was isolated from the Cardener River in Manresa (Rech et al., 2015).

2| Hypothesis & Objectives

Our hypothesis was that by administering heat-killed mycobacteria orally, we could induce oral tolerance to *Mycobacterium tuberculosis*, stimulating a specific anti-inflammatory protective effect.

In order to demonstrate the hypothesis, 3 objectives were defined:

1. To evaluate the effect given by oral therapy with different heat-killed mycobacteria in the active TB model.
2. To test the role of different inflammatory players in the active TB model.
3. To evaluate the global tolerability of heat-killed *Mycobacterium manresensis* in healthy adults and its effect on specific memory regulatory T cells population.

3| Study I – Evaluation of oral therapy with heat-killed mycobacteria in active TB model

This work addresses the usefulness of a therapy based in inactivated mycobacteria to delay the progression from LTBI to TB. It was carried out in the active TB model in C3HeB/FeJ mice. The results have been published in Frontiers in Microbiology in 2016 (Annex I: Cardona et al., 2016).

3.1| Materials & Methods

3.1.1| Animals

Female C3HeB/FeJ specific-pathogen-free mice (6–8 weeks old) were obtained from Jackson Laboratories. All procedures were conducted in a BL3 security facility. Mice were infected with 2×10^4 CFU of Mtb H37Rv Pasteur strain via the caudal vein.

All procedures were performed according to a protocol reviewed by the Animal Experimentation Ethics Committee of the Hospital Universitari Germans Trias i Pujol (registered as B9900005) and approved by the Dept d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural of the Catalan Government, according to current national and European Union legislation regarding the protection of experimental animals.

Mice were supervised daily and euthanized, if required, with isoflurane (inhalation excess), following a strict protocol, in order to ensure animal welfare.

3.1.2| Treatment preparation

Different mycobacteria from the UTE strain collection, namely *M. tuberculosis, M. kansasii, M. avium*, and *M. manresensis* were used to prepare the treatments. *M. bovis* BCG Danish (Pfizer Inc.) was also used.

Bacteria were grown in 7H11 plates (BCG and *M. manresensis*) or in

Proskauer-Beck broth (*M. tuberculosis*) and subcultured in Proskauer-Beck broth (or in 7H10 broth, for *M. manresensis)* in aeration and agitation at 37ºC. The bacillary load (BL) of each culture was determined by serial dilution and culture on 7H11 plates, and a Blood Agar plate was also seeded to rule out contamination. Cultures were then inactivated by heating at 80ºC for 60 min. Sterilization was confirmed by negative culture in 7H11, Blood Agar, McConkey Agar, and Saboureaud Agar. The inactivated cultures were diluted 1:1 in sterile sucrose (10% sucrose in water) and aliquoted in 1 ml vials for storage at -80ºC (BCG and *M. tuberculosis*). In the case of *M. manresensis*, the inactivated culture was lyophilized, dry-diluted with mannitol and stored in aluminium bags at 4ºC.

3.1.3| Assessment of treatments

In order to characterize the effect of treatments with hk mycobacteria, we evaluated several parameters at week 3 p.i. For this purpose, a total of 20 C3HeB/FeJ mice were used (5 per group of treatment). Animals received seven oral doses of heat-killed *M. tuberculosis* (hkMtb, 10^5 bacilli/animal), heat-killed BCG (hkBCG, 10^6 bacilli/animal) or heat-killed *M. manresensis* (hkMm, 10^5 bacilli/animal) every other day from the day of infection and were sacrificed at day 21 p.i. At the final time point, pathology and bacillary load in lungs, and effect on T cell populations were studied (described in sections 3.1.4 to 3.1.6). A control group was included and treated with the corresponding dilution of the excipient (mannitol). The effect of treatment on the survival of infected mice was also studied (10 animals per group, thus a total of 40 mice). A second survival study was done to compare the effect of live versus hk *M. manresensis* on C3HeB/FeJ infected mice (a total of 34 mice were used, 10-12 animals per group).

The oral administration of hk mycobacteria as a coadjuvant therapy to the human standard treatment against active TB (a combination of rifampicin, isoniazid, ethambutol and pyrazinamide -RHEZ-) was assessed by studying mice survival. A total of 21 C3HeB/FeJ mice were used. They started to be treated at week 4 post-infection, when their weight started to decrease. All

animals received a commercial combination of the four drugs (RIMSTAR®, Sandoz Farmaceutica) adjusted to their weight, for a total of 4 weeks. Half of the animals also received hkMm (10^5 bacilli/animal) orally, 5 days a week for a total of 6 weeks (in addition to the 4-week-treatment with RHEZ, plus 2 more weeks). At the final time point, pathology in lungs was studied (described in section 3.1.5).

3.1.4| Bacillary load

Samples of lung lobes from each animal were collected, homogenized and several dilutions plated on nutrient Middlebrook 7H11 agar (BD Diagnostics). The number of CFU was counted after incubation for 28 days at 37ºC and the results expressed as CFU/mL.

3.1.5| Lung pathology

Lungs were fixed in 10% buffered formalin, embedded in paraffin and 5-µm sections stained with haematoxylin-eosin (HE), Masson trichromic (MTC) or Ziehl-Neelsen (ZN) stain for microscopic observation and histometric analysis using the NIS-Elements D version 3.0x software package (Nikon Instruments Inc.). Eight recuts of a block containing 1 lung lobe of each mice were stained with HE and used to determine the damaged area as a percentage of total lung area. In the case of animals treated with RHEZ, whole lung samples were used and measured individually.

3.1.6| Immuno-characterization

3.1.6.1|Cell isolation and cell cultures

Spleens were mechanically disrupted and filtered through a 40-µm cell strainer (BD Diagnostics), with erythrocytes being incubated for 8 min in lysis buffer (Tris 17 mM, NH_4Cl 0.14 M). 10^6 cells were cultured in supplemented RPMI 1640 (10% Fetal Calf Serum, streptomycin 100 µg/mL, penicillin 100 U/mL, 2-mercaptoethanol 0.025 mM, sodium pyruvate 1 mM) in 24-well plates at 37ºC and 5% CO_2, with or without PPD

stimuli (final concentration of 10 μg/mL; Statens Serum Institute). After 24 h, 4 days, or 7 days of culture, the content of each well was harvested and stained for flow cytometric analysis. Culture supernatants were stored at -80ºC for further study of the cytokine profile.

Lungs were snap-frozen when collected and kept stored at -80ºC until processed for cytokine profile analysis. They were then subjected to mechanical disruption and homogenized with lysis buffer (sodium azide 0.05%, Triton X-100 0.5%, protease inhibitor cocktail from Sigma-Aldrich at 1:500, in PBS).

3.1.6.2|PPD-specific T cell population analysis by flow cytometry

PPD-specific T cell populations, obtained from spleen cultures at 24 h, 4 days, or 7 days, were studied by flow cytometry. Cells were incubated for 30 min at 4ºC with the antibodies in PBS supplemented with 1% of FBS, followed by fixation with 4% formaldehyde in PBS for 10 min at room temperature. The four $CD4^+$ T cell types defined by the markers CD25 and CD39 according to Dwyer's characterization (Dwyer et al., 2010) were studied. The gating strategy and hierarchy applied for the flow cytometry analysis are shown in Figure 5.

The antibodies used were anti-mouse CD4 FITC (eBioscience Inc.), anti-mouse CD3e BV™421, anti-mouse CD25-PerCP-Cy™5.5 (BD biosciences) and anti-mouse CD39 PE (BioLegend Inc.).

Once stained, samples were read in a flow cytometer (BD LSRFortessa™, BD Biosciences). Data were analyzed using FACSdiva™ software (BD Biosciences).

3.1.6.3|Cytokine profile analysis

A cytokine profile study was performed in splenocyte culture supernatant and lung homogenates. The following cytokines were measured by Luminex xMAP® technology: IFN-γ, TNF-α, IL-5, IL-6, IL-10, IL-13, and IL-17. Results were expressed as pg per ml of supernatant or homogenate. The assay was performed with the MILLIPLEX® MAP kit (EMD Millipore Corporation) following the manufacturer's instructions and analysed with

xPONENT Software (Luminex Corporation).

3.1.7| Data analysis

GraphPad Prism version 7.00 for Windows (GraphPad Software) was used for graphics and statistics, with differences of $p<0.05$ being considered to be statistically significant.

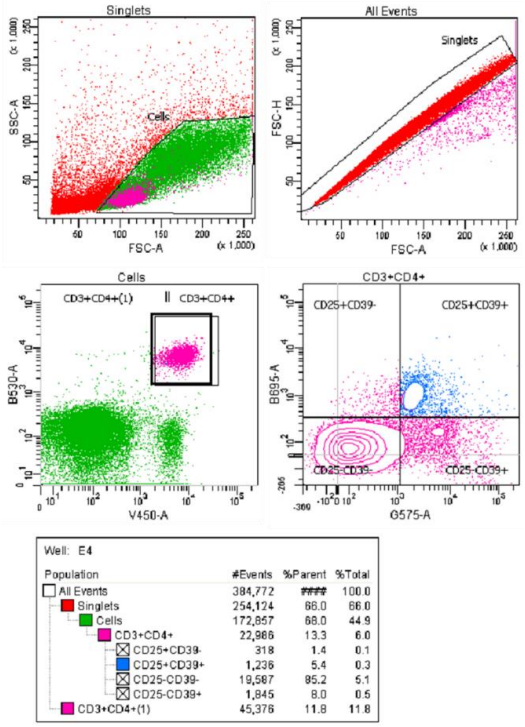

Figure 5| Flow cytometry analysis carried out. Singlets and lymphocytes were first gated. $CD3^{+}CD4^{+}$ T cells were classified into 4 groups: $CD25^{+}CD39^{+}$ Regulatory Memory T cells; $CD25^{-}CD39^{+}$ Effector Memory T cells, ex-Tregs IL-17 producers; $CD25^{+}CD39^{-}$ T Effector cells and $CD25^{-}CD39^{-}$ Naive T cells.

3.2| Results

3.2.1| Oral administration of heat-killed mycobacteria increased mice survival

The hkMtb bacilli were administered orally to C3HeB/FeJ infected mice to

test their influence on disease progression. Treatment was found to increase survival of the mice in a statistically significant manner (Figure 6).

Other mycobacterial species were also tested. Animals treated with hkBCG and hkMm showed an increased survival compared to untreated controls (Figure 6). This protection was also achieved when giving the same treatment with the same concentration of living *M. manresensis* (Figure 7). There were no statistically significant differences between hk and live Mm.

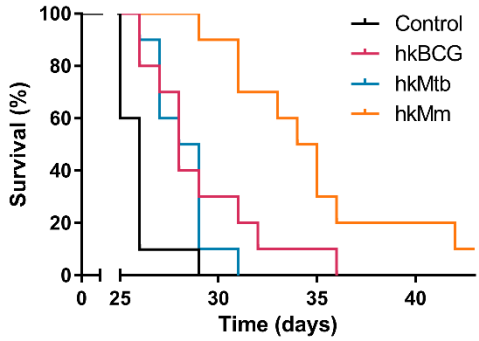

Figure 6| Effect of treatment on survival. Infected C3HeB/FeJ mice were treated every other day for 2 weeks from infection day with 10^5-10^6 heat-killed bacilli/animal. Statistically significant differences were observed between the survival curves of mannitol-treated control mice and mice treated with hk BCG (p=0.0038), Mtb (p=0.0029), or *M. manresensis* (p<0.0001) (Log-rank Test).

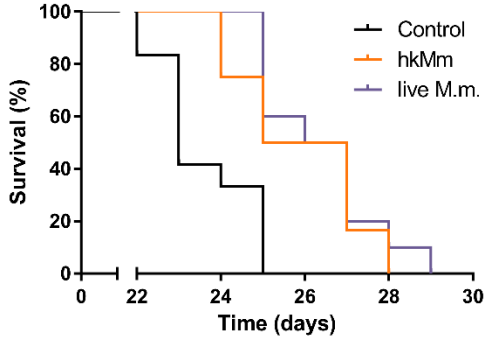

Figure 7| Effect of treatment on survival. Infected C3HeB/FeJ mice were treated every other day for 2 weeks from infection day with 10^5 heat-killed or live bacilli/animal. Statistically significant differences were observed between untreated mice and mice treated with both forms of *M. manresensis* (hkMm p=0.0015, live M.m. p=0.0002; Log-rank Test).

3.2.2| Oral administration of heat-killed mycobacteria reduced lung pathology

The effect on lung pathology was evaluated for oral treatments with three different hk mycobacteria: Mtb, BCG or *M. manresensis*.

Figure 8 shows how all treatments significantly reduced the infiltration in the parenchyma (statistically significant differences: p=0.0002; Mann Whitney test).

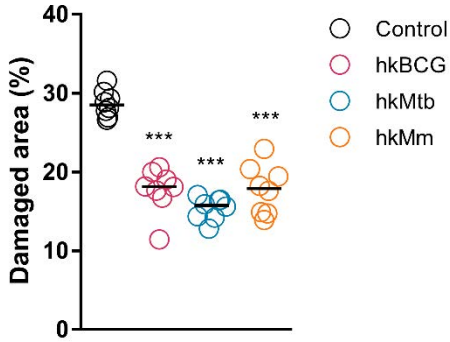

Figure 8| Effect of treatment on lung pathology: histometric analysis. Infected C3HeB/FeJ mice were treated every other day for 2 weeks from infection day with 10^5–10^6 hk bacilli/animal. The percentage of damaged area out of total lung area was studied in each treatment group on samples obtained on day 21 p.i. Each circle represents a recut and the lines are medians. Statistically significant differences were observed between mannitol-treated control mice and mice treated with heat-killed mycobacteria (***p=0.0002; Mann Whitney test).

Figure 9 shows one of the HE recuts, which was used to measure the percentage damaged area, for the untreated control group and the group treated with *M. manresensis*.

3.2.3| Oral administration of heat-killed mycobacteria decreases lung bacillary load

The effect on bacillary load in lungs was evaluated for oral treatments with three different hk mycobacteria, namely Mtb, BCG and *M. manresensis*. The three treatments decreased the BL in lungs at day 21 p.i. in a

statistically significant manner (hkBCG p=0.0317, hkMtb p=0.0079, hkMm p=0.0079; Mann Whitney test) as shown in Figure 10.

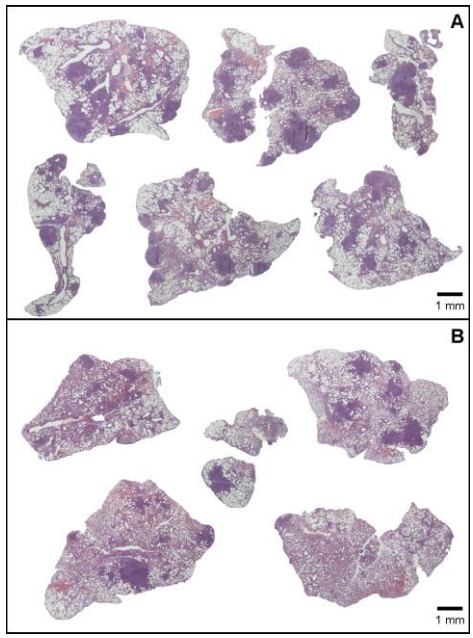

Figure 9| Effect of treatment on lung pathology: macroscopic view of lesions. HE stained lung recuts of untreated control mice (A) or mice treated with hkMm (B) of samples obtained on day 21 p.i.

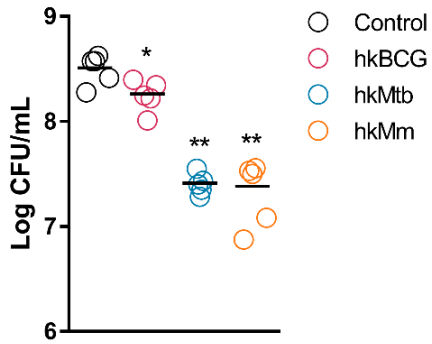

Figure 10| Effect of treatment on bacillary load in lungs. Infected C3HeB/FeJ mice were treated every other day for 2 weeks from infection day with 10^5-10^6 hk bacilli/animal. BL in lungs obtained on day 21 p.i. is expressed as log CFU/mL. Each circle represents an animal and the lines are medians. Statistically significant differences were observed between treated and control mice (hkBCG: *p=0.0317, hkMtb: **p=0.0079, hkMm: **p<0.0079; Mann Whitney test).

3.2.4| Immuno-characterization of heat-killed mycobacteria treatment

3.2.4.1|Splenocytes phenotype

The immunomodulation achieved by the oral treatment with hk Mtb, BCG or *M. manresensis* was also characterized. Flow cytometric analysis of splenocyte cultures was used to assess different $CD4^+$ T cell populations. The results are shown in Figure 11.

The naive $CD25^-CD39^-$ population, which represents the higher percentage of $CD3^+CD4^+$ cells, tended to be higher under unstimulated conditions than under PPD stimulation. In contrast, the $CD25^+CD39^-$ population, which represented approximately 2% of $CD3^+CD4^+$ cells, did not show any major differences between stimulated and non-stimulated culture conditions. A mild increase in this non-stimulated population was observed when cells were cultured for 7 days.

We also observed this trend to increase after 7 days of culture in $CD25^+CD39^+$ Tregs, which represent around 2–9% of all $CD3^+CD4^+$ cells. In contrast to naïve T cells, this population tends to be higher under PPD stimulation. Furthermore, a higher PPD-specific response of $CD25^+CD39^+$ was observed in splenocytes from mice treated with hkMtb and hkMm in comparison to untreated mice, but only when cells were cultured for 7 days (statistically significant differences: hkMtb p=0.0159, hkMm p=0.0079; Mann Whitney test).

The $CD25^-CD39^+$ population accounts for about 20% of all $CD4^+$ T cells. A higher percentage of PPD-stimulated cells (with statistically significant differences) is observed when cultured for 4 days.

3.2.4.2|Cytokine profile

We studied the presence of different cytokines in the splenocyte supernatant and lung homogenates of mannitol-treated control mice or mice treated with hkMm. Mice treated with hkMm showed higher levels of pro-inflammatory cytokines in cell culture, but a reduced inflammatory

milieu in lungs when compared to control mice. Detailed results are shown in Figure 12.

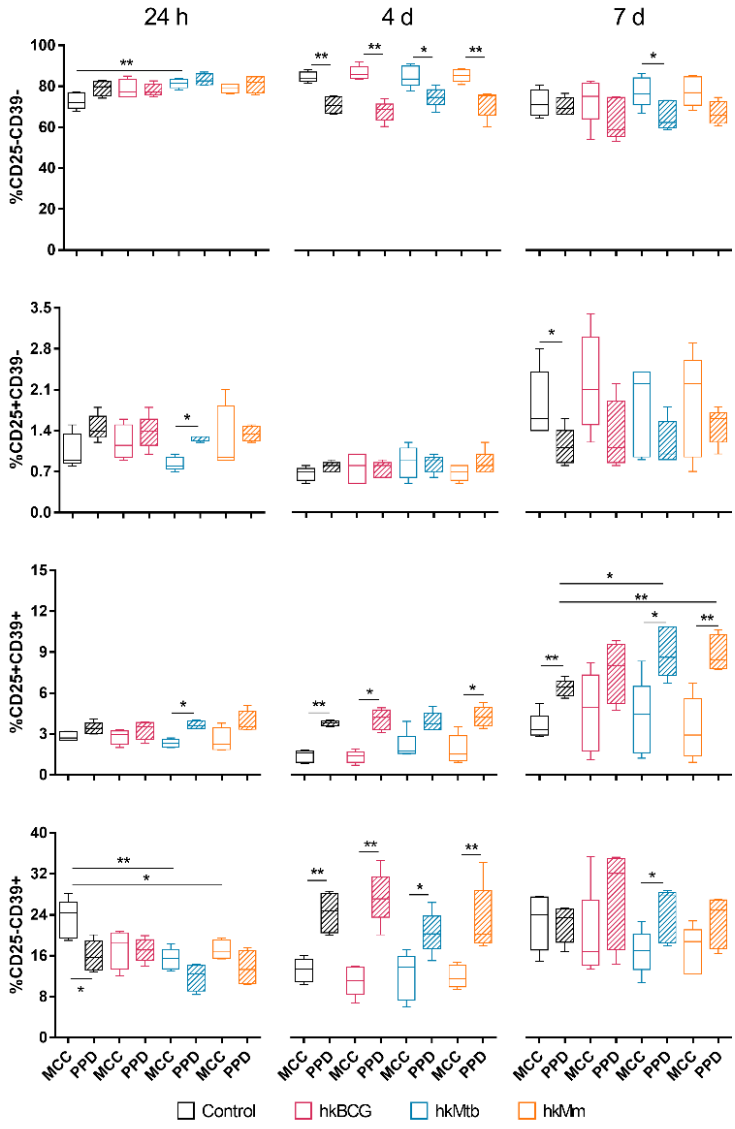

Figure 11| Effect of treatment with heat-killed mycobacteria on T cell populations in the spleen. Percentage of the four populations defined by CD25 and CD39 markers (out of the total of $CD3^+CD4^+$ cells). Splenocytes obtained on day 21 p.i. were cultured for 24 h, 4 days, or 7 days with PPD stimulus (PPD) or without stimulation (MCC). The boxplot shows the median, quartiles and minimum and maximum values, with a different colour for each treatment. Statistically significant differences are marked with asterisks (*p<0.05, **p<0.01; Mann Whitney test).

Treated mice showed a statistically significant increase in IFN-γ, TNF-α, IL-6, and IL-10 levels in splenocytes' culture, whereas they showed a statistically significant decrease in IFN-γ, TNF-α, IL-6, and IL-17 levels in lung homogenates. IL-5 levels could not be detected in any sample, thus suggesting a poor Th2 response.

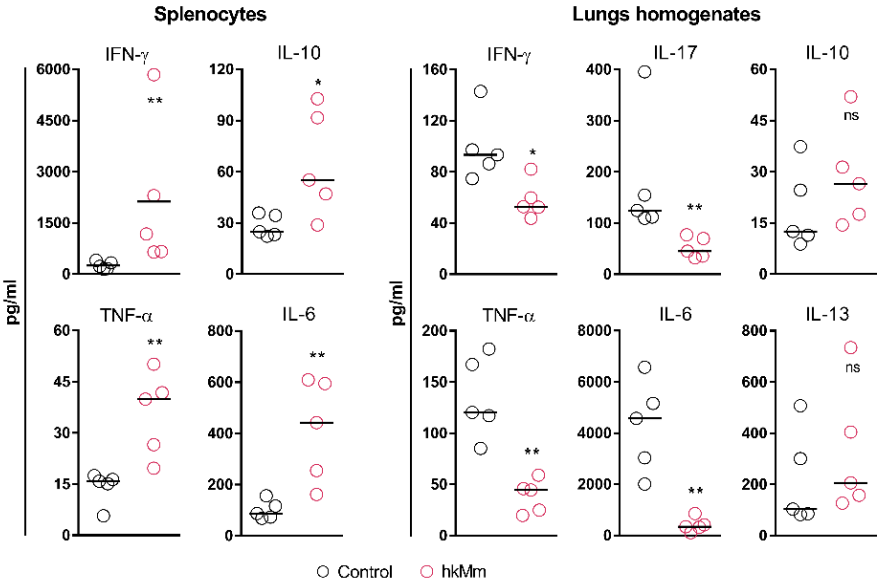

Figure 12| Cytokine profile in spleen and lungs. Effect of treatment of infected C3HeB/FeJ mice every other day for 2 weeks with hkMm. Samples were obtained on day 21 p.i. Each circle represents an animal and lines are medians. Cytokine levels are expressed as pg/mL. Statistically significant differences are marked with asterisks (*p<0.05, **p<0.01; Mann Whitney test).

3.2.5| hkMm as a coadjuvant therapy

The effect of hkMm on mice survival when administered as coadjuvant treatment to RHEZ was evaluated, with hkMm treated mice showing a statistically significant increase in survival (p<0.0001, Log-rank test; Figure 13).

Comparison of the histopathology between the animals from the control group that had to be euthanized according to the welfare monitoring control (between weeks 15 and 22 post-infection) with the survivors from the *M.*

manresensis-treated group that were euthanized to terminate the experiment (week 24) showed a clear difference in terms of intrapulmonary infiltration (51 vs 68%; p=0.0059, Mann Whitney test; Figure 14).

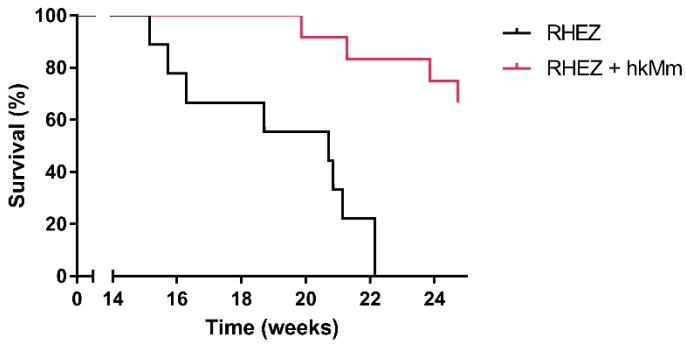

Figure 13| Heat-killed *M. manresensis* as a coadjuvant therapy. Survival curves for infected C3HeB/FeJ mice treated with RHEZ or RHEZ in combination with hkMm. Treatment started at week 4 post-infection, RHEZ therapy was administered for 4 weeks and hkMm therapy lasted 6 weeks. Statistically significant differences were observed between groups (p<0.0001; Log-rank Test).

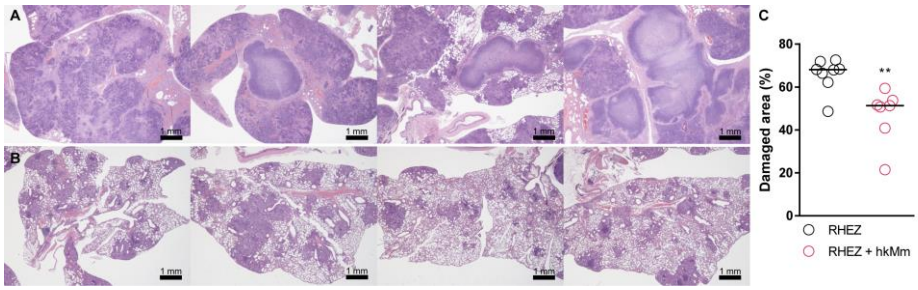

Figure 14| Heat-killed *M. manresensis* as a coadjuvant therapy: histometric analysis. Macroscopic view of HE-stained lung recuts from mice treated with RHEZ (A) or treated with RHEZ and hkMm (B). The percentage of damaged area out of total lung area was studied in both groups (C). Each circle represents an animal and the lines are medians. Statistically significant differences were observed between antibiotics' treated mice and mice with coadjuvant therapy of hkMm (**p=0.059; Mann Whitney test).

Looking at the quality of the lesions (Figure 15), all the samples of the control group showed a massive necrosis with liquefaction in the centre and big patches of massive accumulation of nuclear debris plenty of bacilli. In the case of the group treated with *M. manresensis* lesions resembled granulomas of chronic tuberculosis infection seen in resistant mice

(C57BL/6) where the bacilli are accumulated in foamy macrophages (FM), and where big cholesterol crystals can be seen as described before (Cáceres et al., 2009; Cardona et al., 2003). There is also a progressive fibrosis of the parenchyma caused by the proliferation of the fibroblasts of the alveolar wall, in a honeycomb pattern as described by Dunn and North (Dunn and North, 1996). The difference is that there are little infiltrations of PMN, especially around the infected FM. Different animals have lesions with different degrees of evolution, and it can be seen how this PMN infiltration become bigger and is infected with bacilli, that multiplies over them, as described before (Marzo et al., 2014).

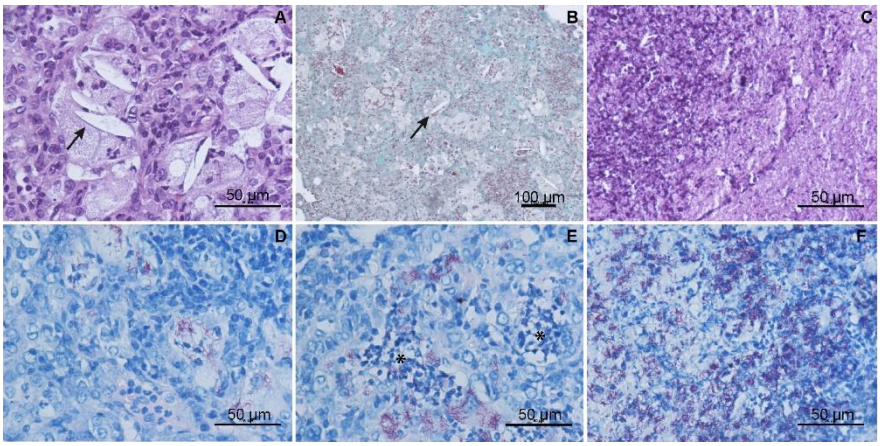

Figure 15| Heat-killed *M. manresensis* as a coadjuvant therapy: qualitative histological analysis. Pulmonary infiltration in mice treated with RHEZ and hkMm at week 24 p.i. (A,B,D,E) is compared with mice only treated with RHEZ that had to be euthanized according to the welfare monitoring control (C,F). Pictures (A) and (B) show the HE and MTC stains respectively showing the structure of murine chronic TB infection with a lot of foamy macrophages and where cholesterol crystals (marked with arrows) can be seen. MTC stain showing the honeycomb patron caused by the progressive fibrosis originated at the alveolar wall. Pictures (D) and (E) show the localization of bacilli with the ZN stain, initially only in foamy macrophages (D) to growth on PMN rafts (marked with asterisks) afterwards (E). Picture C shows the accumulation of cellular debris, which is the main property of the infiltration in terminal mice, after HE stain. Massive bacillary presence is shown with ZN stain in picture (F).

3.3| Discussion

The data provided herein suggest, for the first time, that a low dose tolerance regimen (i.e., administration of 10^5 hkMm daily for 14 days)

could be used to control progression towards active TB. This treatment regimen seems to interfere with the excessive inflammatory response, a fact that appears to be key to the development of human-like lesions, and thus is also able to reduce the bacillary concentration in lungs (Marzo et al., 2014). In our opinion, this could be related to the induction of Tregs.

It is well established that dose and route of administration determine the immune response to an administered antigen (Faria and Weiner, 2005), therefore we have good reason to think that our regimen induces tolerance to the mycobacteria bacilli. Continuous oral administration modifies the response triggered by the infection through Tregs, as has also been seen in other infectious or autoimmune diseases (Harats et al., 2002; Levy and Ilan, 2007; Ochi et al., 2006; Weiner et al., 2011).

We have shown that treatment with hk bacilli is effective for reducing both the infiltration area and the bacillary load in lungs, in addition to increasing survival. As such, we then decided to further characterize the effect of treatment on the immune system to prove that tolerance is induced.

In this regard we first investigated the role of Tregs in our model as it was feasible that oral tolerance could be articulated through this type of cell. The role of Tregs in Mtb infection is controversial, with some authors considering the induction of Tregs to be detrimental (Chiacchio et al., 2009; Ribeiro-Rodrigues et al., 2006) but others pointing to a protective role for Tregs (Leepiyasakulchai et al., 2012). To investigate the role of Tregs in our experimental system we had previously compared the Treg population in C3HeB/FeJ mice with their positive controls (C3H/HeN mice), which share the MHC haplotype but have a different susceptibility to Mtb (Marzo et al., 2014; Marzo Escartín, 2014). We found that the better outcome was associated with a stronger regulatory immune response in the C3H/HeN strain, as the Treg percentage was higher (also PPD-specific response), and when we depleted C3H/HeN mice of Tregs by administration of anti-CD25 the susceptibility to Mtb increased. Two out of five mice developed necrotic lesions, whereas in several experiments conducted by our group with this mouse strain, no animals have either died or developed such necrotic

lesions. Although, T $CD4^+CD25^+$ cells include Tregs as well as potential T $CD4^+CD25^+CD39^-$ Th subsets (Dwyer et al., 2010), the latter account for about 1% of all T $CD4^+$, therefore Tregs depletion is far more relevant. Taken together, these results strongly support the hypothesis that Tregs are protective in TB, therefore we continued our research by studying the effect of treatment with hk mycobacteria on Tregs.

As we were interested in the induction of memory Tregs, we designed the concept of 7 days incubation, together with a study of the CD39 marker. We believe this marker to be very relevant as it has been related to both the memory phenotype and induction of tolerance (Chiacchio et al., 2009; Dwyer et al., 2010; Roberts et al., 2014). Furthermore, unlike Foxp3, it is a surface marker, which translates into less aggressive cell processing and easier and faster protocols. Other relevant T cell subtypes, such as T memory effector cells $CD25^-CD39^+$, which have shown to play a detrimental role in organ transplantation in humans, or T effector cells, both of which are capable of secreting IL-17, and thus having a pro-inflammatory profile (Dwyer et al., 2010), were also studied with the marker combination used. Apart from being used in the study of autoimmune diseases, this CD25 CD39 marker combination has been validated as a Tregs marker in the study of TB, exhibiting a negative correlation with IL-17 T cells in peripheral blood (Chiacchio et al., 2009; de Cassan et al., 2010).

The use of these markers showed an increase in Tregs in splenocyte cultures from treated mice, together with a slight global stimulation. The levels of IFN-γ, TNF-α and IL-6 were increased in cultured spleen samples from mice treated with hkMm. This suggest a global stimulation that also includes an increase in the immunosuppressive cytokine IL-10, which can be related to the increase in Treg, as seen by other authors who used 10^6 hk *M. chelonae* intraperitoneally once a week for 3 weeks (Ho et al., 2010). In that study, the authors also demonstrated a parallel increase in IFN-γ, although they did not check for IL-6 or TNF-α. The most important aspect, however, is that the increase in IL-6 was not linked to the presence of IL-17 (Gao et al., 2009), which was undetectable, and this is logical as an

exaggerated inflammatory response with neutrophilic attraction is not seen in the spleen in the C3HeB/FeJ model, where there is also effective control of bacillary load (data not shown). This could be due to the fact of the presence of Tregs counterbalancing IFN type I production (Aida et al., 2014; Srivastava et al., 2014), thus favouring the increased IFN-γ response (Manca et al., 2001); or because of the lack of PMN infiltration in the spleen prevents the production of type I IFN (Berry et al., 2010), thus favouring the presence of both Tregs and IFN-γ in this organ in the context of Mtb infection. However, further experiments would be needed to investigate this issue.

A lower inflammatory milieu was found in the lungs of mice treated with hkMm when compared to control animals, with lower levels of IFN-γ, TNF-α, IL-17, and IL-6. In our opinion, the population of PPD-specific memory Tregs is attracted to the lungs and must be crucial for reducing the inflammatory response in situ, especially by counterbalancing the Th17 response (Zheng, 2013; Zhou et al., 2008).

Lung histopathology of mice treated with RHEZ shows how the addition of hkMm is able to stop the progression of the lesions, favouring the fibrosis of the tissue which might contribute to create an anti-inflammatory milieu able to abrogate the growth of the bacilli by curtailing the infiltration of the lesion with monocytes or PMN. This phenomenon is amplified in big-mammals thanks to the stimulation of the fibroblasts of the intralobar septae, that encapsulate the lesions at very early stages, promoting this anti-inflammatory milieu and stopping the bacillary growth (Cardona, 2015; Gil et al., 2010).

The modulatory effect of NTM was demonstrated years ago after intravenous inoculation was shown to induce a non-specific cellular immune response (Collins, 1971) and protect against subsequent aerosol Mtb infection to a similar degree as in BCG vaccinated mice (Orme and Collins, 1984). On the other hand, subcutaneous sensitization with *M. avium* interfered with BCG vaccination by stopping its growth, although this was not seen after sensitization with *M. chelonae* or *M. fortuitum*

(Brandt et al., 2002). The study by Poyntz et al. (2014) is interesting as it shows how important the route of administration for NTM is (in this case, *M. avium*). An increase in Th1 response can be detected when inoculating hk *M. avium* i.p. several times, thereby increasing the efficacy of BCG, whereas oral administration of living bacilli tended to increase Th2 response in the lung during Mtb infection and reduced BCG's protective effect. Treatment with hk *Mycobacterium vaccae*, which has been used for the treatment of active TB (von Reyn et al., 2010) and has recently been reviewed by Gröschel et al. (2014), deserves particular attention due to its ability to reduce Th2 responses and increase Th1 ones.

Finally, it is relevant to recall that studies in BCG-vaccinated infants in the United Kingdom or in Malawi showed no evidence that the initial response to NTM affected the vaccine-induced change in IFN-γ response (Weir et al., 2006). In this regard, it is interesting to note that, to date, no surrogates of protection have been found in TB. Th1 responses, including poly-functional cells, have received attention for a number of years, although an evaluation of their protective value in a large BCG trial in South Africa showed that protected and unprotected newborns exhibited an equivalent immunological profile (Kagina et al., 2010). In this sense, our work may also help to shed some light on other biomarkers that are more closely related to the induction of a balanced immune response and are able to avoid an excessive inflammatory response than can lead to active TB, instead of focussing on an immune response that exclusively targets destruction of the bacilli.

In conclusion, induction of a balanced immune response triggered by PPD-specific memory Tregs through the administration of heat-killed *M. manresensis* has demonstrated an ability to protect against the progression of Mtb infection to active disease and relapse after TB treatment. This has been demonstrated in the experimental model induced in C3HeB/FeJ mice, which reproduce "human-like" lesions. This finding might help to focus on a new kind of "host-directed" prophylactic and therapeutic approach as well as the development of new predictive biomarkers.

4| Study II – Modulation of the inflammatory response in experimental active TB

4.1| Materials & Methods

4.1.1| Experimental Design

We did different approaches on the modulation of the inflammatory response in the active TB model, using C3HeB/FeJ mice. This study consists of four experiments, outlined in Figure 16, which could divided in two parts.

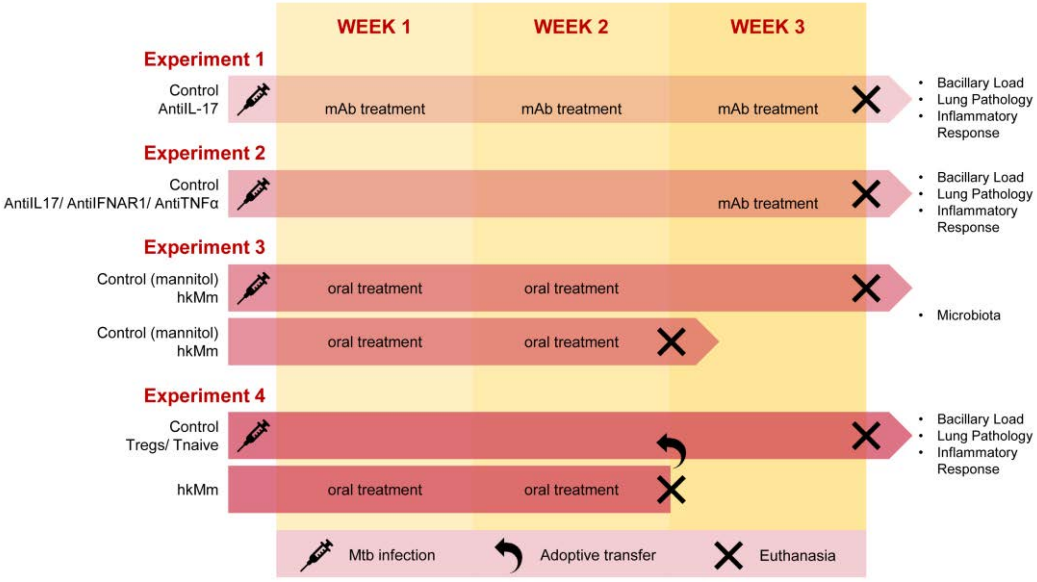

Figure 16| Experimental plan for Study II.

First of all, we studied the neutralization of IL-17 from the beginning of infection (6 mice per group, n=12). Afterwards, we decided to neutralize this pro-inflammatory cytokine when the infection was already set, and tested in parallel the blockage of type I IFN receptor (IFNAR-1) or neutralization of TNF-α (6 mice per group, n=24).

On the other hand, we studied the effect of hkMm on gut microbiota of

infected and non-infected C3HeB/FeJ mice (between 4 and 10 mice per group, n=29). Finally, we tested the influence of adoptive transfer of sorted T cells from hkMm treated mice into Mtb infected animals (6 animals per group, n=18).

4.1.2| Animals

Female or male C3HeB/FeJ specific-pathogen-free mice (6–8 weeks old) were obtained from Jackson Laboratories or from our facility's colony. All procedures were conducted in a BL3 security facility. Mice were infected with 2×10^4 CFU of *M. tuberculosis* H37Rv Pasteur strain via the caudal vein.

All procedures were performed according to a protocol reviewed by the Animal Experimentation Ethics Committee of the Hospital Universitari Germans Trias i Pujol (registered as B9900005) and approved by the Dept d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural of the Catalan Government, according to current national and European Union legislation regarding the protection of experimental animals.

Mice were supervised daily and euthanized, if required, with isoflurane (inhalation excess), following a strict protocol, in order to ensure animal welfare.

4.1.3| Treatments

4.1.3.1|Monoclonal antibodies

Each group of mice received intraperitoneally the assigned monoclonal antibody (mAb). Treatment details are shown in Table 1. The dosages were based on previous works (Pinto et al., 2011; Plessner et al., 2007; Redford et al., 2010). The days of treatment of Anti IL-17 groups vary according to the experiment.

4.1.3.2|Heat-killed *Mycobacterium manresensis*

Heat-killed *M. manresensis* was administered orally, every other day, from day 1 p.i., for 14 days. Each dose consisted of 10^5 hk bacilli. The control

group was treated with the same concentration of excipient (mannitol).

4.1.3.3|T cells adoptive transfer

Cell obtaining is described in sections 4.1.6 and 4.1.8. Mice received 150,000 Tregs ($CD4^+CD3^+CD25^+CD39^+$) or Tnaive ($CD4^+CD3^+CD25^-CD39^-$) cells via the caudal vein on day 14 p.i. These were obtained from mice treated with hkMm, as described before.

Table 1| Monoclonal antibodies treatment for experiments 1 & 2.

Group	Antibody	Dosage	
Anti IL-17	Anti-Mouse IL-17A (mouse IgG1, kappa) - Affymetrix	100 ug, twice a week	Exp 1: days 2, 6, 9, 13, 16 & 20 p.i. Exp 2: days 14 & 17 p.i.
Anti IFNAR-1	Anti-Mouse IFN alpha/beta receptor I (IFNAR-1) (mouse IgG1) - Affymetrix	500 ug, once	Day 14 p.i.
Anti TNF-α	Infliximab (chimeric mAb) (Remicade)	500 ug, once	Day 14 p.i.

4.1.4| Bacillary load

Samples of lung lobes or spleen from each animal were collected, homogenized and several dilutions were plated on nutrient Middlebrook 7H11 agar (BD Diagnostics). The number of CFU was counted after incubation for 28 days at 37ºC and the results were expressed as CFU/mL.

4.1.5| Lung pathology

Lungs were fixed in 10% buffered formalin, embedded in paraffin and 5-µm sections stained with haematoxylin-eosin (HE) or Ziehl-Neelsen (ZN) stain for microscopic observation and histometric analysis using the NIS-Elements D version 3.0x software package (Nikon Instruments Inc.). Between 4 and 6 recuts of a block containing 1 lung lobe of each mice were stained with HE and used to determine the damaged area as a percentage of total lung area.

4.1.6| Cell isolation and cell cultures

Spleens were mechanically disrupted and filtered through a 40 μm cell strainer (BD Diagnostics), with erythrocytes being incubated for 8 min in lysis buffer (Tris 17 mM, NH_4Cl 0.14 M).

Lungs were snap-frozen when collected and kept stored at -80ºC until processed for cytokine profile analysis. They were then subjected to mechanical disruption and homogenized with lysis buffer (sodium azide 0.05%, Triton X-100 0.5%, protease inhibitor cocktail from Sigma at 1:500, in PBS).

Cell culture was conducted in supplemented RPMI 1640 (10% Fetal Calf Serum, streptomycin 100 μg/mL, penicillin 100 U/mL, 2-mercaptoethanol 0.025 mM, sodium pyruvate 1 mM) in 96-well plates at 37ºC and 5% CO_2. 20,000 cells were plated per well, previously covered with 0.5 μg of anti-CD3 (BioLegend). Media was also supplemented with anti-CD28 (2 μg/mL, BioLegend) and PPD stimuli (10 μg/mL, Statens Serum Institute). After 24 h, the supernatant of each well was harvested and stored at -80ºC until the study of the cytokine profile.

4.1.6.1|Inflammatory response analysis

A cytokine profile study was performed in splenocyte culture supernatant and lung homogenates. The following cytokines and chemokines were measured by Luminex xMAP® technology: IFN-γ, TNF-α, TGF-β, IL-1β IL-2, IL-6, IL-10, IL-12(p40), IL-13, IL-17, CXCL-1 and CXCL-5. Results were expressed as pg per ml of supernatant or homogenate. The assay was performed with the MILLIPLEX® MAP kit (EMD Millipore Corporation) following the manufacturer's instructions and analysed with xPONENT Software (Luminex Corporation).

4.1.7| Microbiota analysis

4.1.7.1|Sample processing and DNA extraction

Cecum from animals was isolated and stored at -80ºC until DNA extraction.

Cecum was thawed on ice and stool samples were separated from mice epithelia. DNA was extracted using a QIAamp Fast DNA Stool Mini Kit (QIAGEN GmbH) according to manufacturers' instructions. Three 4 mm glass beads were added in the first step before vortexing to enhance the cell lysis. Total volume of eluate was stored at -20ºC until DNA concentration measurement and16s rRNA gene library preparation.

4.1.7.2| 16S rRNA Gene Sequencing

The microbial DNA was analysed with the Illumina 16S Metagenomic Sequencing Library Preparation guide with some modifications. The protocol targets the V3-V4 regions of the 16S rRNA gene and was amplified using KAPA HiFi HotStart PCR Kit (KAPA Byosystems). The expected product size was ~550 bp. The PCR products were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter) on the Magnetic Stand-96 (Thermo Fisher Scientific) and quality controlled with a 1.4% Agarose Gel. The Index PCR was done using Nextera XT DNA Library Preparation Kit (Illumina) and KAPA HiFi HotStart PCR Kit. The procedure was followed as in 16S Metagenomic Sequencing Library Preparation guide. Library products were purified as PCR products. The final DNA concentrations of the purified products were measured with a Qubit 3.0 fluorometer (Thermo Fisher Scientific) and validated with the Agilent 2100 Byoanalizer (Agilent Technologies). The purified products were diluted to a final concentration of 4 nM and pooled. The pool was denatured and sample loaded as in 16S Metagenomic Sequencing Library Preparation guide in a final concentration of 7 pM. The 16S rRNA gene libraries were sequenced with 2 x 300 paired-end reads on the Illumina MySeq system (Illumina).

4.1.7.3| Bacterial diversity analysis

The resulting read files were analysed using mothur pipeline (vs 1.39.5). The first step was to join paired-end reads into contigs and joined reads were quality controlled: reads were removed if they were less than 420 base pairs or more than 490 base pairs or contained ambiguous nucleotides or contained more than eight homopolymer nucleotides. Once the sequences were quality controlled, they were aligned using SILVA reference database.

The alignments that did not fit exactly with our region of interest were removed. Sequences were pre-clustered if they contained as much as one difference for every 100 base pairs. At this point, chimeric sequences were identified and removed. Sequences were taxonomically classified using RDP (ribosomal database project) and eliminated those sequences classified as chloroplasts, mitochondria, archaea and eukaryote. Distances between sequences were calculated with a distance cut-off of 0.03. After creating the distance matrix, sequences with >97% of similarity were clustered into OTUs (operational taxonomic units). OTUs were filtered and only those that had more than 0.1% of relative abundance and were in at least two samples were conserved. Each OTU was assigned to a taxonomic classification.

4.1.8| Flow cytometry

Cells were obtained as described in section 4.1.6 and incubated for 30 min at 4ºC with the antibodies in PBS supplemented with 1% of FBS. The antibodies used were anti-mouse CD4 FITC, anti-mouse CD3e BV™421, anti-mouse CD25-PerCP-Cy™5.5 (BD Biosciences) and anti-mouse CD39 PE (BioLegend Inc.).

The four $CD4^+$ T populations defined by the markers CD25 and CD39 (Dwyer et al., 2010) were isolated using the flow cytometer FACSAria II (BD Biosciences). Data were analysed using FACSdiva™ software (BD Biosciences).

4.1.9| Data analysis

GraphPad Prism version 7.00 for Windows (GraphPad Software) was used for most of the graphics and statistics, with differences of $p<0.05$ considered to be statistically significant.

Rarefaction curves, diversity and richness indexes and distances between samples were calculated and plotted using RStudio (vs 1.0.143). Diversity was quantified using Bray-Curtis dissimilarity test and plotted using non-

metric multidimensional scaling (NMDS). Beta diversity (Shannon's, Simpson's and Inverse Simpson's index) was calculated to analyse differences in diversity between samples.

4.2| Results

4.2.1| IL-17 neutralization from beginning of infection

Anti IL-17 monoclonal antibody was administered from 1 day before infection, in order to study this pro-inflammatory cytokine's role in the active TB model. Bacillary load in spleen and lung at day 21 post-infection increased with treatment (Figure 17).

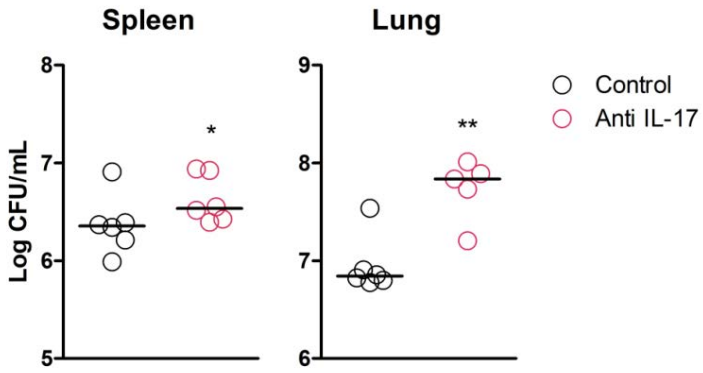

Figure 17| Effect of anti IL-17 on bacillary load. Infected C3HeB/FeJ mice were treated twice a week from 1 day prior to infection with anti IL-17 mAb. BL in spleen and lungs obtained on day 21 p.i. is expressed as log CFU/mL. Each circle represents an animal and the lines are medians. Statistically significant differences were observed (spleen: *p=0.0260, lung: **p=0.0087; Mann Whitney test).

We also assessed the effect of anti IL-17 mAb on lungs pathology. Panels A and B from Figure 18 show the macroscopic view of HE stained lung sections from control and treated animals, respectively. The total damaged area in lungs was 5 times greater in treated mice (Figure 18-C).

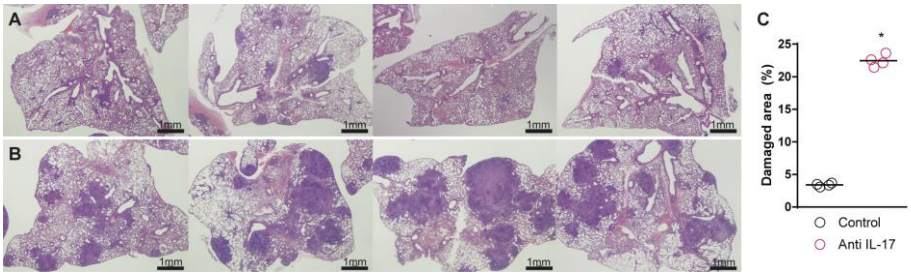

Figure 18| Effect of anti IL-17 on lung's pathology. Macroscopic view of HE stained lung sections of infected C3HeB/FeJ mice untreated (A) or treated twice a week from 1 day prior to infection with anti IL-17 mAb (B). The percentage of damaged area out of total lung area was studied in both groups on day 21 post infection (C). Each circle represents a recut of the whole group and the lines are medians. Statistically, significant differences were observed between treated and untreated control mice (*p=0.0286; Mann Whitney test).

At the same time point, we studied different cytokines and chemokines in lungs homogenates (Figure 19).

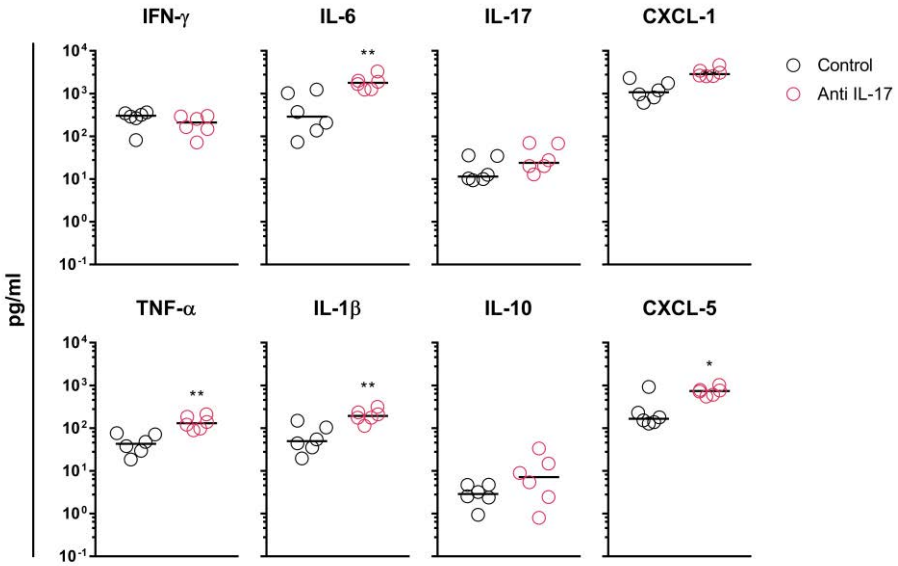

Figure 19| Inflammatory response in lungs. Infected C3HeB/FeJ mice were treated twice a week from 1 day prior to infection with anti IL-17 mAb. Samples were obtained on week 3 p.i. Each circle represents an animal and lines are medians. Cytokines and chemokines levels are expressed as pg/mL. Statistically significant differences between treated and control animals are marked with asterisks (*p<0.05, **p<0.01; Mann Whitney test).

Animals treated with anti IL-17 mAb had higher levels of pro-inflammatory

cytokines: IL-6, TNF-α and IL-1β. However, the levels of IFN-γ were equivalent in both groups. There were also no significant differences in the levels of IL-17 or IL-10. The chemokines CXCL-1 and CXCL-5 were present in higher concentrations in treated animals.

4.2.2| IL-17, type I IFN or TNF-α neutralization after infection's onset

Due to the detrimental effect seen after initiating the treatment together with the Mtb challenge, we concluded that this may be reducing too much the initial protective effect of IL-17, causing an exacerbated inflammatory response. For this reason, we decided to wait until the immune response was well set, but before infiltration took place. That's why we administered the anti-IL-17 mAb from day 14 after infection.

In this case, bacillary load in spleen and lung at day 21 post-infection decreased with treatment (Figure 20; p=0.0152 in spleen & ns in lung, Mann Whitney test). The total damaged area in lungs was also reduced with treatment (Figure 21; p=0.0286, Mann Whitney test).

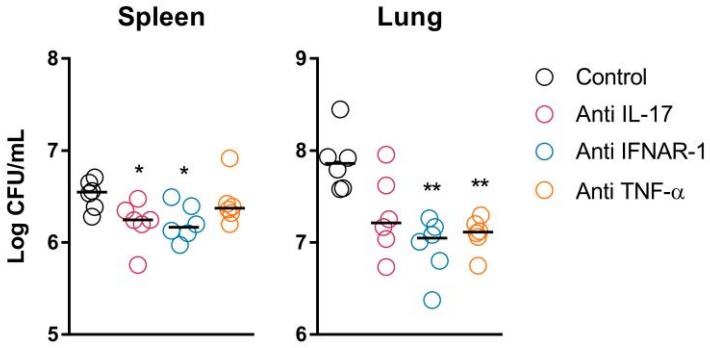

Figure 20| Effect of Anti IL-17, anti IFNAR-1 or anti TNF-α on bacillary load. Infected C3HeB/FeJ mice were treated from day 14 post-infection with mAb. BL in spleen and lungs obtained on day 21 post infection is expressed as log CFU/mL. Each circle represents an animal and the lines are medians. Statistically significant differences are marked with asterisks (*p<0.05, **p<0.01; Mann Whitney test).

Together with this, the blockage of IFNAR-1 and neutralization of TNF-α from day 14 after infection was tested, as we had seen an increase of these cytokines with infection's progression in the active TB model (Marzo et al., 2014). Bacillary load in spleen and lung at day 21 post-infection decreased with treatment (Figure 20; Anti IFNAR-1 p=0.0260 & p=0.0022, Anti TNF-α ns & p=0.0022, for spleen and lung respectively, Mann Whitney test). The total damaged area in lungs was also reduced with treatment (Figure 21; p=0.0286 for both treatments, Mann Whitney test).

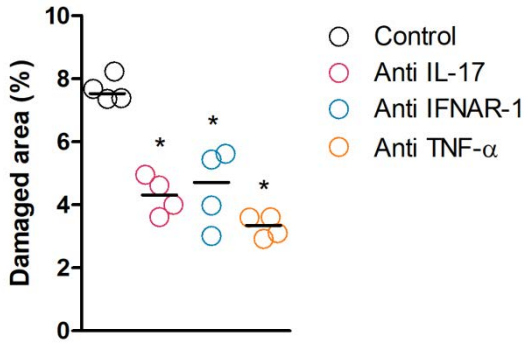

Figure 21| Effect of Anti IL-17, anti IFNAR-1 or anti TNF-α on lung's pathology. Infected C3HeB/FeJ mice were treated from day 14 p.i. with mAb. The percentage of damaged area out of total lung area was studied in the four experimental groups on day 21 p.i. Each circle represents a recut and the lines are medians. Statistically significant differences are marked with asterisks (*p<0.05, **p<0.01; Mann Whitney test).

Figure 22 shows the results of the cytokines and chemokines analysed in lungs' homogenates of mice treated with mAb.

The three treatments studied reduced the concentration of IL-6 and CXCL-1. Anti IL-17 mAb treatment reduced the concentration of IL-17 in lungs. The administration of anti IFNAR-1 mAb resulted in significantly less TNF-α and CXCL-5 than control animals. Finally, the neutralization of TNF-α reduced the levels of IFN-γ, TNF-α, IL-10 and IL-12(p40) in lungs. There were no statistically significant differences in TGF-β levels among groups. IL-1β was not analysed in these set of mice.

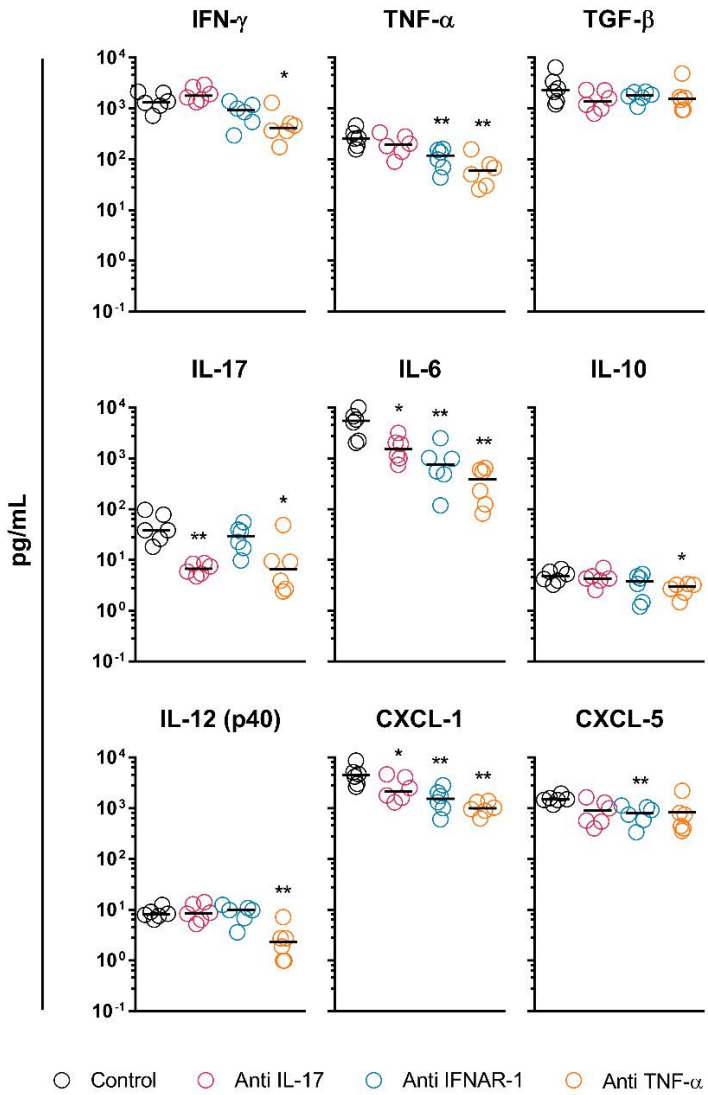

Figure 22| Levels of cytokines and chemokines in lungs. Effect of mAb treatment on infected C3HeB/FeJ mice. Samples were obtained on day 21 post-infection. Each circle represents an animal and lines are medians. Cytokines levels are expressed as pg/mL. Statistically significant differences between treated and control animals are marked with asterisks (*p<0.05, **p<0.01; Mann Whitney test).

4.2.3| Effect of hkMm on microbiota

The immunomodulation induced by hkMm treatment at an intestinal level was assessed through the study of gut microbiota, by sequencing the 16s

rRNA gene. This was done using the active TB model and non-infected C3HeB/FeJ mice. To facilitate the expression of these results, the groups will be mentioned as follows:

- Mtb: C3HeB/FeJ mice, infected, treated with mannitol
- Mtb-hkMm: C3HeB/FeJ mice, infected, treated with hkMm
- Ct: C3HeB/FeJ mice, non-infected, treated with mannitol
- hkMm: C3HeB/FeJ mice, non-infected, treated with hkMm

Figure 23, Figure 26 and Figure 25 show the results of microbiota composition analysis.

According to the rarefaction curves, all the samples reached a plateau in the number of OTUs (Figure 23-A), meaning that the number of reads included in the analysis were sufficient and we are not missing information. In this same figure, we can see that the samples from Mtb group had the lowest number of OTUs.

Secondly, the NMDS plot -that separates samples according to how similar they are- shows that we can distinguish the four experimental groups, and the biggest differences are found between Mtb and Mtb-hkMm groups (Figure 23-B).

The plot on Figure 23-C represents three diversity indexes: Shannon, Simpson and Inversed Simpson. As expected from the low number of OTUs, Mtb samples presented the lowest diversity of the four groups. The treatment of infected animals produced a recovery in the microbiota diversity, similar to that of non-infected animals. Differences between Mtb and Mtb-hkMm or Ct were statistically significant ($p=0.0200$ and $p=0.0058$, respectively; Mann Whitney test). Interestingly, the diversity indexes of Mtb-hkMm were higher than those of hkMm mice ($p=0.0120$, Mann Whitney test). There were no differences between Ct and hkMm groups.

Figure 24 shows the relative abundance of bacterial genera for each of the samples analysed. Although this data representation already suggests there are differences between experimental groups, we have subdivided the

information in order to further understand the results. Figure 25 and Figure 26 represent the relative abundance of the found OTUs depending on the phylum, family or genus to which they belong.

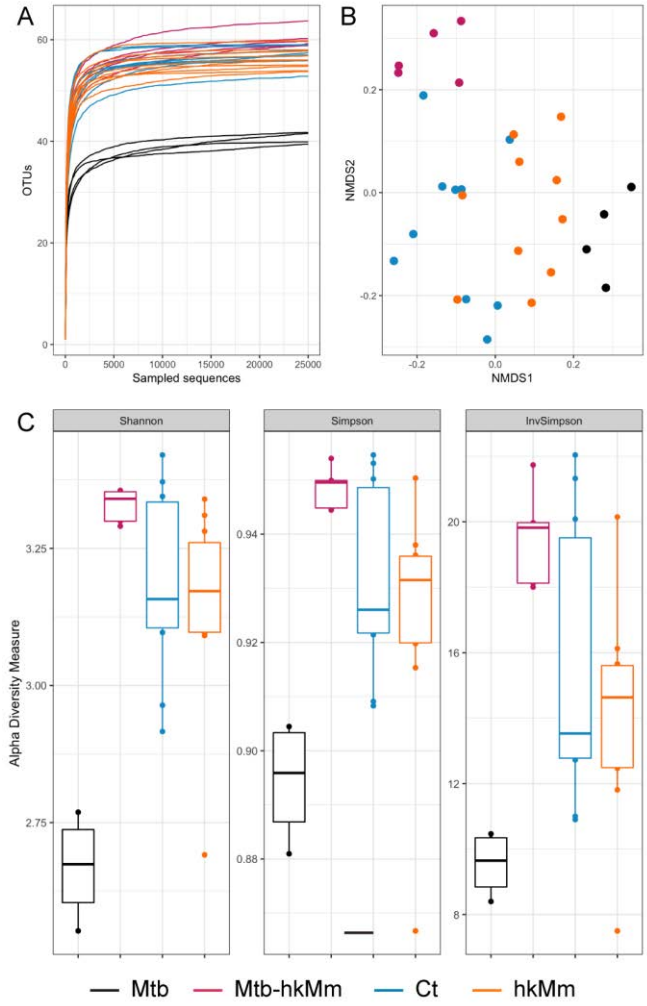

Figure 23| Analysis of the microbiota diversity based on 16S rRNA gene sequencing. Rarefaction curves (A), NMDS ordination (B) and Shannon, Simpson and Inverse Simpson diversity indexes (C).

The most represented phyla where Bacteroidetes and Firmicutes. As shown in Figure 25-A, samples from the four experimental groups have a higher relative abundance of Bacteroidetes than Firmicutes. Non-infected mice tend to have more Bacteroidetes and less Firmicutes than infected animals,

but this difference is statistically significant only between hkMm treatment groups. The ratio between these phyla is lower in non-infected animals, but once again this difference is not statistically significant in non-treated animals.

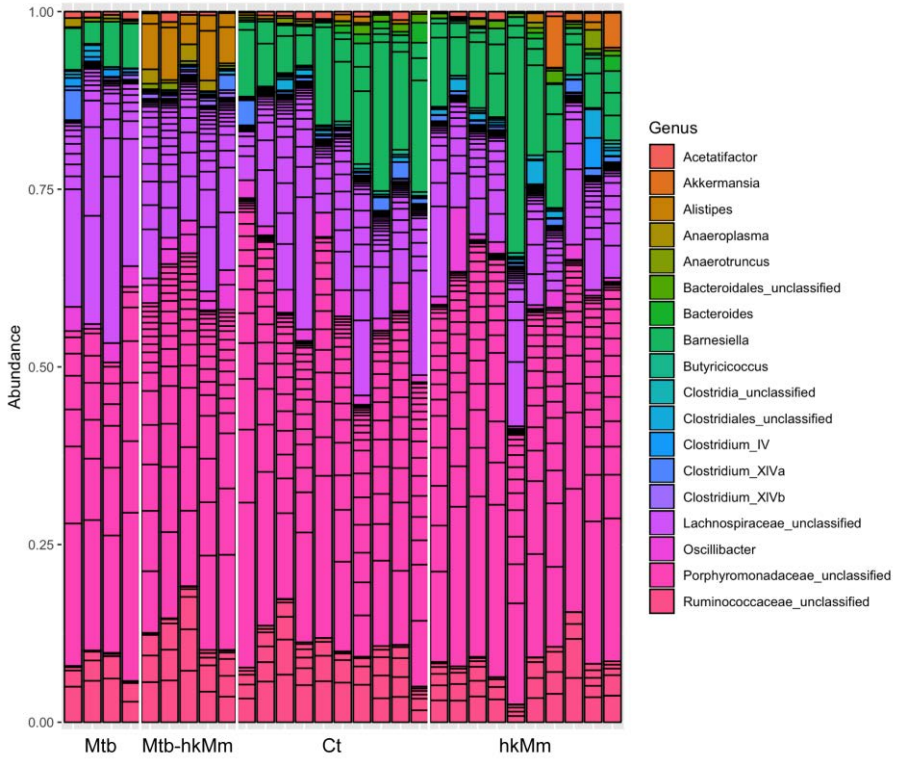

Figure 24| Taxonomic composition of the intestinal microbiota based on 16S rRNA gene sequencing. Each bar represents an animal and experimental groups are separated with white lines. Black horizontal lines represent different OTUs.

Regarding the families, Porphyromonadaceae, Ruminococcaceae and Lachnospiraceae were the most abundant ones. Their relative abundance in each of the groups is shown in Figure 25-B. There is a loss of Porphyromonadaceae with infection, but an increase in the presence of Lachnospiraceae. Mtb-hkMm animals have a higher percentage of Ruminococcaceae than the rest of the mice.

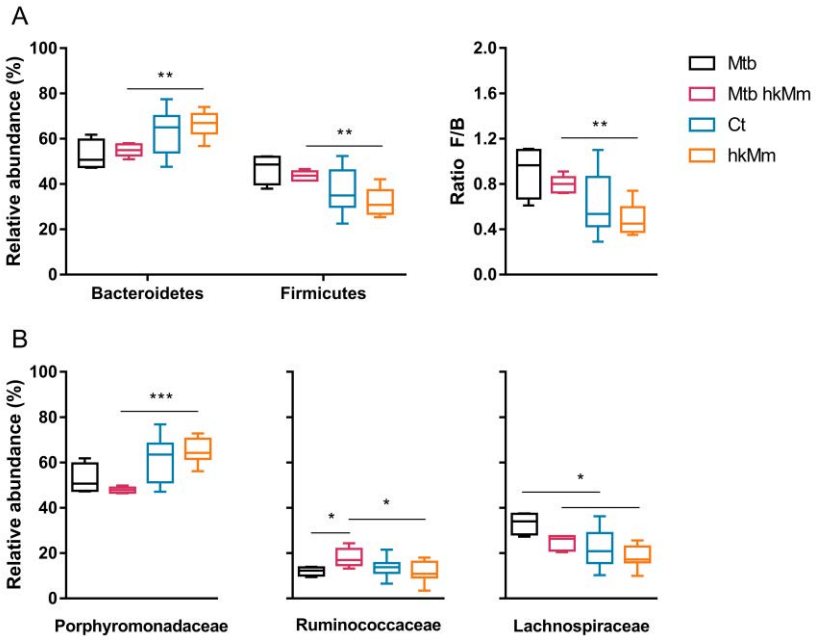

Figure 25| Taxonomic composition of the intestinal microbiota. OTUs obtained with 16S rRNA gene sequencing were taxonomically classified. (A) Relative abundance of the principal phylum and analysis of the Firmicutes/Bacteroidetes ratio. (B) Relative abundance of the principal families. Plots are shown with median, IQR and minimum/ maximum values. Statistically significant differences are marked with asterisks (*p<0.05, **p<0.01, ***p<0.001; Mann Whitney test).

We then analysed the relative abundance of each of the genus into which the obtained OTUs were grouped (Figure 26). Mtb infection resulted in lower percentages of *Bacteroides*, *Barnesiella* and *Butyricicoccus* in both treated and non-treated animals; while *Clostridium* IV and *Anaeroplasma* were augmented in infected groups. The OTUs classified as *Alistipes* were less abundant in Mtb mice than in Ct, but higher in Mtb-hkMm than in non-infected hkMm. The hkMm treatment on infected mice resulted in higher abundance of *Alistipes* and *Clostridium* XIVb; while lower numbers of *Barnesiella* and *Clostridium* IV when compared to non-treated infected animals. The only differences between treatment groups in non-infected mice were in the abundance of *Butyricicoccus* and *Akkermansia*. Finally, if we look at the differences between hkMm and Mtb-hkMm, that weren't found in infected mice, we see that *Clostridium* XIVb and *Oscillibacter* were

overrepresented.

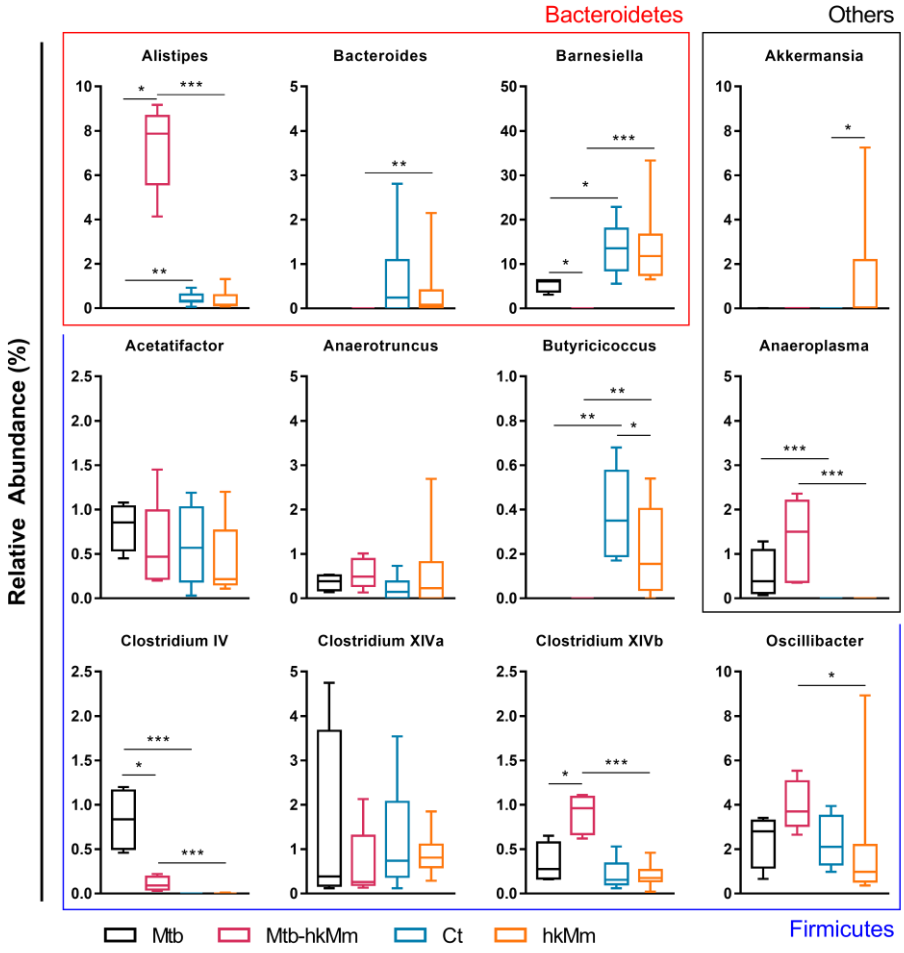

Figure 26| Taxonomic composition of the intestinal microbiota according to their genus. OTUs obtained with 16S rRNA gene sequencing were taxonomically classified. Plots are shown with median, IQR and minimum/ maximum values. Statistically significant differences are marked with asterisks (*p<0.05, **p<0.01, ***p<0.001; Mann Whitney test).

4.2.4| T cells adoptive transfer

Tregs cells ($CD25^{+}CD39^{+}$) were isolated from splenocytes coming from non-infected C3HeB/FeJ mice treated with hkMm. The same was done with Tnaive ($CD25^{-}CD39^{-}$) cells, as a control of the adoptive transfer. These cells were transferred into infected animals at day 14 post-infection. In this

experiment, an infection control group was also included.

The bacillary load in spleen and lung was studied (Figure 27). The transfer of Tregs reduced the BL on lungs (p=0.0238, Mann Whitney test), while the transfer of Tnaive increased the BL both in spleen and lungs (p=0.0087 & p=0.0260 respectively, Mann Whitney test)

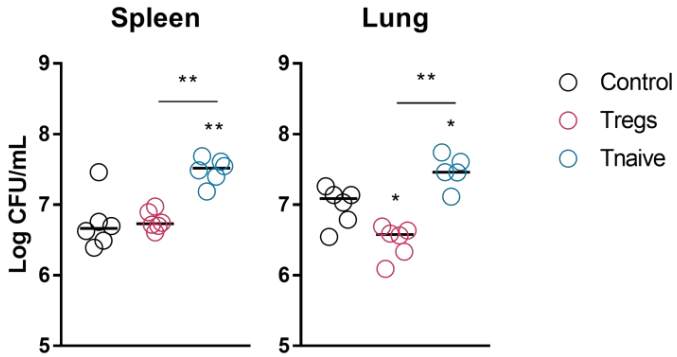

Figure 27| Effect of adoptive transfer on bacillary load. Infected C3HeB/FeJ mice received Tregs or Tnaive on day 14 p.i. The Control group was infected but not treated. BL in spleen and lungs obtained on day 21 p.i. is expressed as log CFU/mL. Each circle represents an animal and the lines are medians. Statistically significant differences between treated and control animals are marked with asterisks, while differences between both cell treatments are marked with lines and asterisks (*p<0.05, **p<0.01; Mann Whitney test).

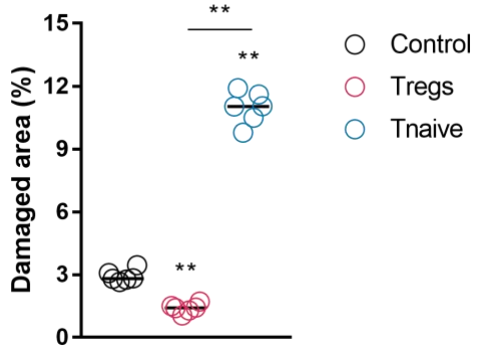

Figure 28| Effect of adoptive transfer on lung's pathology. Infected C3HeB/FeJ mice received Tregs or Tnaive on day 14 p.i. The Control group was infected but not treated. The percentage of damaged area out of total lung area was studied on day 21 p.i. Each circle represents a recut and the lines are medians. Statistically significant differences between treated and control animals are marked with asterisks, while differences between both cell treatments are marked with lines and asterisks (**p<0.01; Mann Whitney test).

We also looked into the lung pathology at day 21 post-infection (Figure 28). There was a reduction in the lung's total damaged area of animals that had received Tregs compared with untreated controls, while those that had received Tnaive had increased lung infiltration.

Furthermore, the cytokines' profile in lungs homogenates was studied (Figure 29).

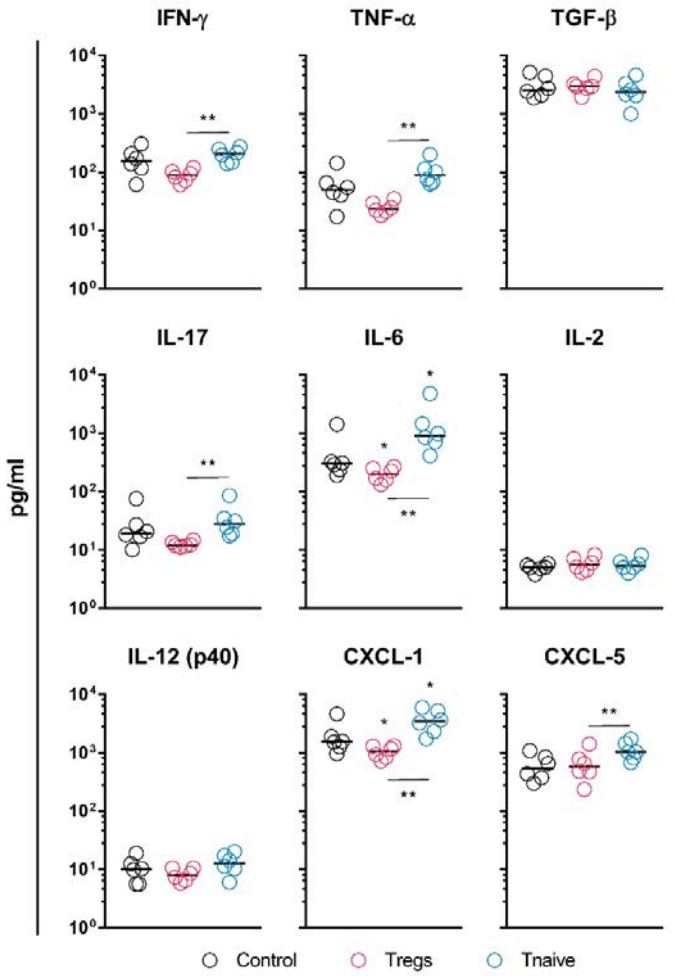

Figure 29| Levels of cytokines and chemokines in lungs. Effect of Tregs and Tnaive adoptive transfer on infected C3HeB/FeJ mice. Samples were obtained on day 21 p.i. Each circle represents an animal and lines are medians. Cytokines levels are expressed as pg/mL. Statistically significant differences between groups are marked with asterisks (*p<0.05, **p<0.01; Mann Whitney test).

When comparing treated mice with control animals, we found that IL-6 and CXCL-1 were lower in Tregs but higher in Tnaive treated mice. There were also differences between Tregs and Tnaive treated mice: IFN-γ, TNF-α, IL-17, IL-6, CXCL-1 and CXCL-5 levels were higher in Tnaive than in Tregs groups. There were no statistically significant differences among groups in the levels of TGF-β, IL-2 or IL-12(p40).

4.2.4.1| *In vitro* characterization of T cells

The four $CD3^{+}CD4^{+}$ populations defined by the markers CD25 and CD39 were isolated from splenocytes of hkMm treated mice and cultured for 24 h in presence of PPD in order to characterize the cytokine production. Results are shown in Figure 30.

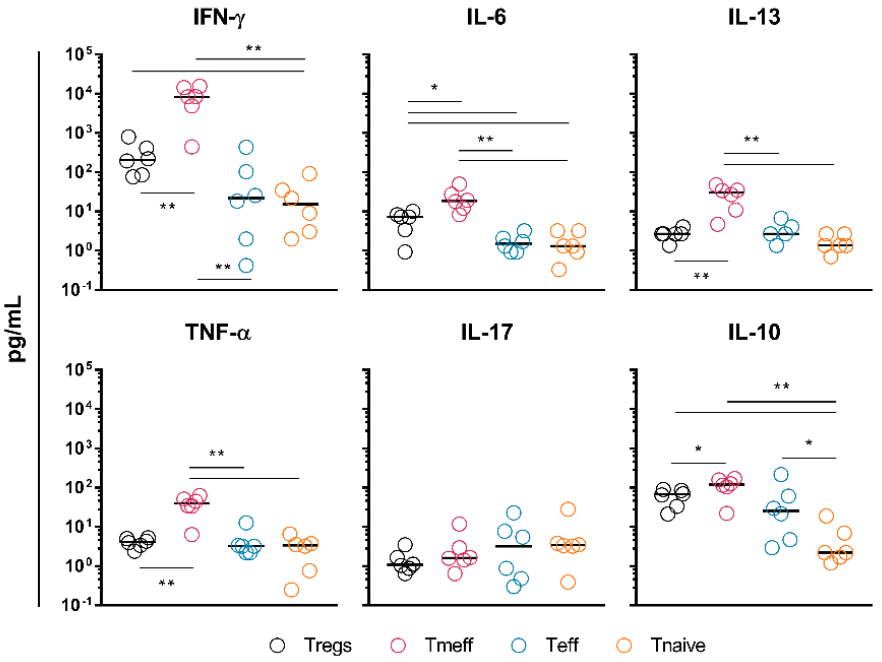

Figure 30| Levels of cytokines and chemokines in cell culture supernatant. Tregs, Tmeff, Teff and Tnaive from hkMm treated non-infected C3HeB/FeJ mice were isolated and cultured with PPD. Supernatants were obtained at 24 h. Each circle represents a replicate and lines are medians. Cytokines levels are expressed as pg/mL. Statistically significant differences between cell types are marked with lines and asterisks (*p<0.05, **p<0.01; Mann Whitney test).

The first interesting finding was that Tmeff cells produced higher levels of

most of the cytokines studied compared to the other populations: IFN-γ, IL-6, IL-13, TNF-α and IL-10. The Tnaive population was, in general, a poor cytokine producer. Tregs cells produced higher levels of IFN-γ, and IL-10 than Tnaive cells. IL-6 was also detected in a higher concentration in Tregs population than Teff or Tnaive cells. There were no differences among cell types in the IL-17 production

We also analysed the IFN-γ/ IL-10 production ratio (Figure 31). This index was higher for the Tmeff population, supporting their pro-inflammatory role. There were no differences between Tregs and Tnaive cells. The Teff population presented the lower values of this ratio.

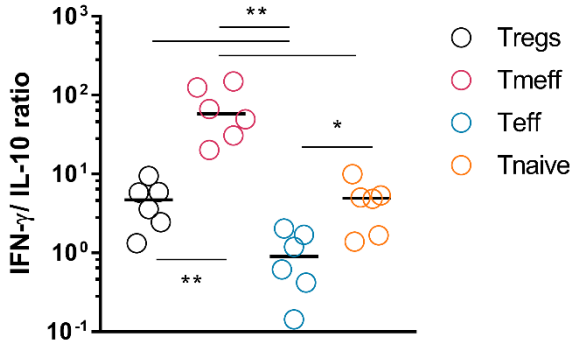

Figure 31| Ratio of IFN-γ and IL-10 concentrations in cell culture supernatant. Tregs, Tmeff, Teff and Tnaive from hkMm treated non-infected C3HeB/FeJ mice were isolated and cultured with PPD. Supernatants were obtained at 24 h. Each circle represents a replicate and lines are medians. Cytokines levels are expressed as pg/mL. Statistically significant differences between cell types are marked with lines and asterisks (*p<0.05, **p<0.01; Mann Whitney test).

4.3| Discussion

This study has helped further understand the importance of the inflammatory factor in the active TB model. It is a complex scenario where each player may have a beneficial or detrimental role in the course of infection.

IL-17 promotes granulopoiesis, by regulating G-CSF and its receptor, and neutrophils recruitment to the site of infection, through chemokines such as CXCL-1 or CXCL-5 (Gaffen, 2008). Since we had shown that the massive

neutrophilic infiltration was key in the development of lung lesions in the C3HeB/FeJ active TB model (Marzo et al., 2014), we decided to test the neutralization of IL-17. Interestingly, this cytokine appears to have a dual role in active TB: it is essential at early stages of infection, but detrimental later on.

When neutralizing IL-17 from beginning of infection, we found an increase in Mtb load, both in spleen and lungs, and a worse pathogenesis in lungs at day 21 p.i. There are previous studies on Mtb infection in C57BL/6 mice KO for the IL-17 receptor A subunit (IL17RA$^{-/-}$), which could be considered equivalent to this first experiment. Intratracheally Mtb infected IL17RA$^{-/-}$ mice have accelerated mortality compared to the WT (Freches et al., 2013). However, another study, with i.n. BCG or Mtb infection, showed no differences in the bacterial load in lungs of IL17RA KO mice compared to WT (Lombard et al., 2016). IL17RA$^{-/-}$ mice showed lower levels of CXCL-1 and a trend in CXCL-5 in lungs, both in Mtb-infected and non-infected mice (Freches et al., 2013; Lombard et al., 2016). Interestingly, we found both CXCL-1 and CXCL-5 augmented in lungs of C3HeB/FeJ infected mice treated with anti IL-17 mAb from the beginning of infection. This might be explained by the levels of TNF-α, which is also involved in recruiting neutrophils through CXCL-1 and CXCL-5 (Vieira et al., 2009). While we found increased levels of TNF-α, Freches and colleagues reported a decrease in IL17RA$^{-/-}$ mice. The lack of IL-17 has also been associated with a disorganized granuloma (Okamoto Yoshida et al., 2010). In this study, the IL-17A KO presented higher bacillary load in lungs, but fewer numbers of neutrophils than the C57BL/6 WT. In this context, it is important to recall that neutrophils don not have such an important role in the Mtb lung lesions of C57BL/6 mice (Kramnik and Beamer, 2016).

On the other hand, when this mAb was administered after day 14, CXCL-1 and CXCL-5 were diminished in comparison to the control. There are no studies, to our knowledge, that neutralize IL-17 when the infection is already set. By limiting the inflammation from day 14 p.i. onward, through anti IL-17 mAb administration, we found a decrease in the bacterial load

and better lung pathology, together with a reduction of IL-17 and IL-6. These results go along with the Damage-response framework (Casadevall and Pirofski, 2003) and the concept that the exacerbated inflammatory response to this pathogen ends up being detrimental for the host, but we cannot completely delete IL-17, since it's a key player in the defence against Mtb. In fact, in the TB vaccine field, an early Th17 immune response has been related with better protection against infection (Gopal et al., 2013).

Since we wanted to reduce the inflammatory response caused by Mtb infection, we also decided to neutralize type I IFN or TNF-α on day 14 post-infection. We had seen that both cytokines were expressed in higher levels in lungs with the infection's progression in the active TB model (Marzo et al., 2014).

The first approach was based on blocking the alpha chain of the type I IFN receptor (IFNAR-1). Type I IFN has been typically associated to immunity against virus. Nevertheless, after being associated to a TB blood signature (Berry et al., 2010), IFN-α/β got a lot of attention in the study of TB. The effects of anti IFNAR-1 mAb on Mtb infection in the active TB model were positive: lower bacillary load and less lung infiltration. This matched the results found on a susceptible mice strain (129S) IFNAR-1 KO model, where mice lacking IFNAR-1 survived Mtb infection and had lower bacillary load. These authors show that the absence of IFN I signalling did not affect the T cell response to TB, but reduced the migration of neutrophils to lungs (Dorhoi et al., 2014). In accordance with this, we found a decrease in the concentration of CXCL-1 and CXCL-5 in lungs. To complement these results, Dorhoi et al. did a similar study with resistant C57BL/6 mice and their IFNAR-1 KO. There were no differences in the survival rates, but there was a reduction in the bacillary load when comparing to the WT. In the context of Mtb virulence studies, using B6D2/F_1 mice strain, the administration of an IFN-α/β neutralizing antibody increased animals' survival, but had no impact on bacillary load (Manca et al., 2005). Once again, this highlights the relevance of the animal model employed. There is still some controversy on whether type I IFN is

beneficial or detrimental for TB, although most results point towards the later (Moreira-Teixeira et al., 2018). Type I IFN appears to interfere with the responsiveness of macrophages to IFN-γ (McNab et al., 2014).

In the active TB model, we also accomplished a protective effect by neutralizing TNF-α. Before this, there have been only few positive experiences in the usage of anti-TNF, while treating active TB in HIV-positive patients undergoing chemotherapy together with anti-TNF therapy (Wallis et al., 2004). In C57BL/6, starting the treatment before infection, Segueni and colleagues found a detrimental effect of TNF-α neutralization (Segueni et al., 2016). Previous results had also related the neutralization of TNF-α with a worse outcome of infection, more specifically with interference in the granuloma formation showing a higher number of non-organized lesions, an effect compatible with a constant dissemination of the Mtb (Flynn et al., 1995). In our case, anti TNF-α treatment was related with a better outcome, stopping the inflammatory response and the induction of the typical human-like TB lesion with liquefaction and progression to cavitation. We believe the key is in the time from infection of administration of the antibody. This does not mean it would not generate a disseminated TB with time.

In view of the results presented in Study I, where oral administration of hkMm modulated the immune response to Mtb infection, we studied deeper its influence on C3HeB/FeJ mice. This experiment was actually carried out in 2 phases. Firstly, we analysed the microbiota of infected animals that had received hkMm. We found that mice that had not been treated (and thus had higher BL and lung pathology) had a loss in microbiota diversity and number of OTUs. These results were in accordance to those published before, where Mtb's infection progression was related with a loss of microbiota diversity (Namasivayam et al., 2017; Winglee et al., 2014). Nevertheless, we wondered whether the changes induced by hkMm were actually related to the control of Mtb infection or to its direct influence on microbiota. For this reason we then included samples of non-infected C3HeB/FeJ mice. We found there were little changes in microbiota

diversity associated to hkMm in healthy mice, which suggests that the abovementioned differences were a consequence of infection's control. Surprisingly, Mtb-hkMm animals had higher diversity than non-infected animals. This may be influenced by environmental differences, even though both experiments were hold in the same housing facility (Hufeldt et al., 2010; Thoene-Reineke et al., 2014).

Although Mtb-hkMm recovered the microbiota diversity, there were several differences when comparing to the relative abundance of the different OTUs found in hkMm mice, i.e. there is a switch in the gut microbiome. Lachnospiraceae was found augmented in murine models of stress (Li et al., 2017) and collagen induced arthritis (Liu et al., 2016). This was associated to higher levels of Th17, which might explain why we found higher abundance of this family in infected mice. Surprisingly, we did not detect OTUs from the Lactobacillaceae family, which had previously been associated to the induction of Tregs (Ding et al., 2017).

Given that the results obtained so far supported the protective effect of an anti-inflammatory or regulatory milieu in the active TB model, we decided to test the adoptive transfer of Tregs generated after the hkMm treatment. These cells were isolated based on the $CD25^{+}CD39^{+}$ phenotype, which denotes a memory profile (Dwyer et al., 2010). Based on the results obtained with anti IL-17 mAb, we decided to do the transfer on day 14 post-infection. We found a reduction on bacillary load and lung's pathology. Tnaive cells ($CD25^{-}CD39^{-}$) were used as a control. We did not expect to see any influence on the infection with this population, since they are not activated $CD4^{+}$ T cells. However, animals that had been treated with Tnaive had a worse outcome. We believe this could be due to a sort of physical competence/interference with the specific lymphocytes, taking into account that it also increases the bacillary load in the spleen. Most works on Tregs & TB associate high numbers of this population with the disease. It is natural, since their role is to counterbalance the inflammatory response, which is likewise augmented (Luo et al., 2017). The results obtained with the transfer of Tregs, however, reveal that this "extra" population is

beneficial.

When characterizing the cytokines induced *in vitro* by each one of the 4 populations, the Tmeff population was the one that produced the highest concentration of total cytokines. It was shown that these cells, under Th17 promoting conditions, secrete both IFN-γ and IL-17 (Dwyer et al., 2010). Although we did not add conditioning cytokines to the media, only anti-CD3/anti-CD28 and the mix of antigens PPD, we found these cells produced 100 times more IFN-γ than the rest. We expected Tregs to produce higher levels of IL-10 than Tmeff, given that it is one of the main regulatory cytokines, but it was the contrary. Other authors have failed to detect this cytokine *in vitro* when culturing FoxP3$^+$ Tregs (Scott-Browne et al., 2007). Nonetheless, when looking at the IFN/IL-10 ratio, as an indicator of pro/anti-inflammatory ratio, we saw that the ratio was higher in Tmeff. In fact, in TB patients the IFN/IL-10 ratio was found to be lower than in LTBI (Kim et al., 2014). This is coherent with all the data that shows an increase on Tregs frequency related to active TB. We interpret this as a logical reaction to prevent more damage once active TB is developing, while being protective to avoid the progression from LTBI to active TB. It would be interesting to test in further studies the adoptive transfer of Tmeff cells and evaluate their pro-inflammatory effect on the active TB model.

5| Study III – Nyadatreg clinical trial: safety and immunogenicity

The study presented here is a pilot, double-blind, randomised, masked and placebo controlled clinical trial (CT) conducted in adult volunteers to evaluate the tolerability and the immunogenicity of 2 oral doses of hkMm administered daily for 14 days to the general population, both LTBI positive or negative. The obtained results have been published in PLOS One (Annex II: Montané et al., 2017).

5.1| Materials & Methods

5.1.1| Ethics

The protocol of the study was reviewed and approved by the Ethics Committee at the investigational centre (Hospital Universitari Germans Trias i Pujol). All investigators and collaborators agreed to rigorously observe the Helsinki declaration with all its amendments and to follow the Good Clinical Practice guidelines of the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use). The objectives and methodology, as well as possible drawbacks and risks due to the study, were explained to each subject orally and in writing (Subject Information Sheet) before their inclusion. They were also informed of the different treatments to be tested, the way they would be assigned to the groups, the option to withdrawal from the study at any time and of the existence of an insurance contract. Informed consent was obtained of all the participating volunteers by consent form signature before starting any study procedure. Participants were also informed and signed their consent to be included in a local register from the Health Department of the Catalan Government to control the participation of healthy volunteers in Phase I clinical trials. The trial has been registered in ClinicalTrials.gov: NCT02076139.

5.1.2| Participants

Subjects were interviewed and screened for enrolment at the Phase I Unit of the Hospital Germans Trias i Pujol (Badalona, Catalonia, Spain) by the clinical pharmacologists. The target population of the study was healthy adults, with or without LTBI. A screening visit was made for each volunteer where a complete anamnesis and physical exam, laboratory parameters tests (hemato-biochemical and immunogenicity parameters), TST, serology against HIV and a pregnancy test were performed. Previous TST was acceptable and was not repeated when positive in the last 5 years or negative in the last 6 months. Radiological chest X-ray exam was performed in TST-positive volunteers. Subjects were not included in the study if any of the following criteria were present: TB, immunodeficiencies, chronic immunosuppressant therapy, reception of blood products or derivatives six months prior randomization, pregnancy or lactation.

Furthermore, the investigator's team certifies that to their knowledge all subjects were considered able to be included in the CT, in terms of fulfilling all the study's requirements.

5.1.3| Interventions

After the inclusion period, the volunteers were randomized in a proportion 1:1:1 to receive either placebo or hkMm in low (10^4) or high (10^5) doses, stratified by TST status (Figure 32).

The trial was originally designed to allocate 10 subjects per treatment group and TST status. Once the groups with TST-negative were fulfilled, the screening process was focused in looking for TST-positive subjects.

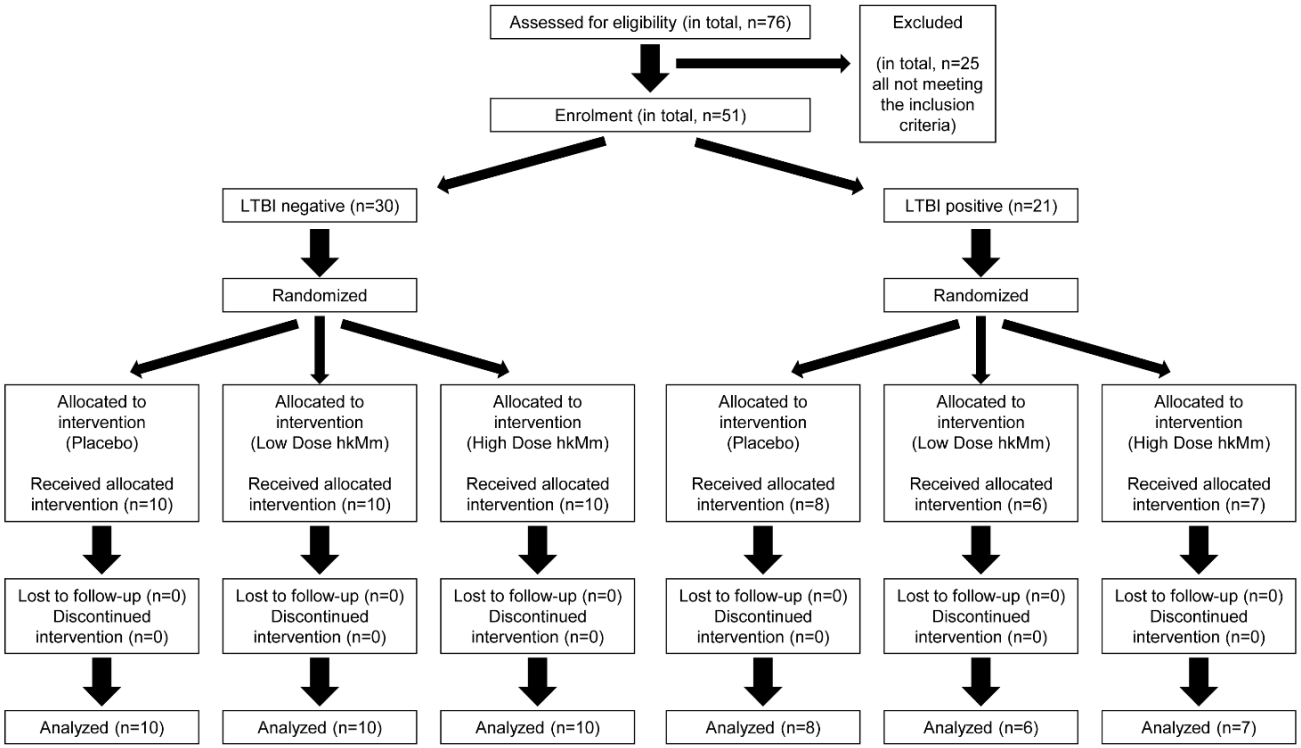

Figure 32| Chart representing the participant flow of the CT. From 76 people interviewed for eligibility, 51 could be included. All the 51 were randomized and allocated to treatment, receiving it, being fully followed-up, and ending the trial and being analysed.

The treatment was supplied by the sponsor (Manremyc S.L.) as a preparation of drinkable vials containing hkMm dissolved in distilled water. It was produced under Good Manufacturing Practice by the Laboratory Reig Jofre. Placebo was identically supplied and formulated except that it only contained distilled water. Only a single batch of both hkMm and placebo were used in the whole study. The distribution of the treatment vials for the CT was carried out by the Contract Research Organisation (CRO) of the study, Fundació per la Lluita contra la Sida -FLS Research Support-, where the entire study treatment received was inventoried and accounted for throughout the study.

Once randomized, volunteers received a box with 14 drinkable vials with one of the 2 doses of hkMm or Placebo at the Phase I Unit of the Clinical Pharmacology Department, supervised by the investigators and following the random plan specified in the study protocol. They drank one vial every day at breakfast during 2 weeks.

Every subject included in the trial had the right to withdraw at any moment by immediately contacting the investigators to inform them, without any obligation to provide reasons. This participant was supposed to be replaced by another, except in the case the trial had to be ended because of safety issues, and the new participant would have received the corresponding treatment of the subject he or she replaced. If a participant was withdrawn because of an adverse event, he or she would have been followed-up by the investigators until resolution or reaching a clinically stable endpoint. The investigators had the right to decide to terminate or suspend the trial without prior agreement of the sponsor, by promptly informing the trial subjects (assuring them appropriate therapy and follow-up), the sponsor and the regulatory authority.

5.1.4| Outcomes

After the administration of the first drinkable vial, each volunteer was followed-up for 6 weeks. The monitoring plan (Table 2) shows every procedure of the follow-up planned during the study.

Table 2| Chronogram and monitoring plan

	Selection		Pre-administration	Administration	Follow-up		
Visit	Selection		Baseline		w1	w2	w6
Day	-28	-25	0		7	15	42
Week	-4	-4	0		1	2	6
Window (days)	±14		0		±3	±3	±3
Subject Information, Informed consent	X						
Inclusion/Exclusion criteria	X		X				
Medical History	X						
HIV test	X						
Tuberculin Skin Test (TST)	X[A]	X[B]					
Chest X ray		X[C]					
Pregnancy test in serum	X		X				X
Randomization			X				
Treatment administration				X			
Physical exam and vital signs	X		X		X	X	X
Biochemistry & Hematology	X		X[D]		X	X	X
Immunogenicity sample			X		X	X	X
Volunteer diary*				X	X	X	X[E]
Concomitant medication	X		X		X	X	X
Adverse events	X			X	X	X	X

[A] Previous TST was acceptable and not repeated when positive in the last 5 years or negative in the last 6 months.
[B] TST readout.
[C] In the case of Positive TST.
[D] Not repeated if selection's analysis performed in the previous 15 days.
[E] Collection of Volunteer's Diary.
*Volunteer's Diary records every day: day and hour of the treatment intake; stool deposition (number/day) and aspect (soft/hard); nausea (degree 1 to 3); vomits (degree 1 to 3); abdominal pain (degree 1 to 3); other adverse events (degree 1 to 3). Degree 1: mild; degree 2: moderate; degree 3: severe.

Adverse events (AE) detected by the investigator through interrogation or

reported by the subject during the defined period of collection were recorded. AE was considered as any unwanted medical event in a subject in a clinical trial, regardless of its relationship with the intervention under evaluation. A serious AE was defined as cause of death, life threatening, requiring inpatient hospitalization, producing disability or incapacity persistent or significant or threatening the patient. The investigators determined the relationship between the study treatment and the AE as 'not related', 'unlikely', 'possibly', 'probably', and 'definite' according to a predefined algorithm based on the modified Karch and Lasagna algorithm used by the Spanish Pharmacovigilance System (Karch and Lasagna, 1977; Meyboom and Royer, 1992) and after a consensus reached between the clinical pharmacologists study investigators. Laboratory abnormalities were graded following the Toxicity Grading Scale Guidance provided by the Food and Drug Administration (FDA) (Norquist et al., 2012). The main safety analyses have been focused on 'possibly', 'probably', and 'definite' classified AE.

Blood was extracted from the volunteers by the nurse of the Phase I Unit, under fasting conditions and before any other procedure was done, at the time-points indicated in the chronogram (Table 2). The samples were properly labelled and sent to the hospital laboratories for laboratory safety testing (15 mL) and to the UTE (8 mL) for the immunogenicity testing as soon as possible. The Departments involved in the safety laboratory results (Haematology, Clinical Analysis and Microbiology) as well as the UTE, are accredited by ISO 9001 procedures. The CRO of the study monitored all the study in order to ensure the use of standard terminology and the collection of accurate, consistent, complete and reliable data.

Blood samples were tested for circulating cell counts (erythrocytes, leucocytes and platelets), Hb, haematocrit, fasting glucose, aspartate aminotransferase, alanine aminotransferase, g-glutamyl transpeptidase, alkaline phosphatase, total bilirubin, direct and indirect bilirubin, urea N, creatinine, glomerular filtration rate, Na and K.

The reliability of the Immunogenicity data was ensured by the performance

of all assays always made by the same technician (previously trained during a 6 month period) and under supervision. Accuracy of the performance of the techniques as well as the values obtained were ensured by following strict specific procedures (standard operating procedures: SOPs) that were previously designed and set up specifically for this CT and always under blinding premises. The sponsor also used an external auditing to review the following issues: data management and statistics, both the UTE and the Phase I Unit, source data / source documents and the Investigator File and the Trial Master File.

Measure of specific Tregs was done according to previous studies (Chiacchio et al., 2009; de Cassan et al., 2010; Dwyer et al., 2010; Feruglio et al., 2015). PBMCs were isolated in cell preparation tubes (CPT Becton Dickinson, BD) with sodium citrate according to the manufacturer proceedings, and processed immediately. PPD batch 49 (Statens Serum Institute) was used at a final concentration of 10 μg/mL to measure antigen specific responses. $1x10^6$ cells/well were non-stimulated or PPD-stimulated in a complete medium (RPMI-L-Gln, 10% heat-inactivated FCS, 10 U/mL penicillin, 10 U/mL streptomycin, 1 mM sodium pyruvate, 0.025 mM 2-ME) for 7 days at 37°C and 5% of CO_2. The phenotypic analysis was performed by flow cytometry, using the following antibodies for membrane staining: PerCP-Cy 5.5 mouse anti-human CD3, APC-H7 mouse anti-human CD4, PE mouse anti-human CD25 (BD Biosciences) and Brilliant Violet 421 mouse anti-human CD39 (BioLegend). Data acquisition was performed using an LSRFortessa flow cytometer (BD Biosciences) and analysed with FACSDiva software (BD Biosciences).

None of the Immunogenicity assessments performed in the present CT were standard procedures. As no cut-off exists for those non-validated and research-only techniques, a statistical difference ($p<0.05$) between the time-points among the same treatment group has been considered enough to prove the reactivity of the treatment.

5.1.5| Sample size

Given that this CT was an exploratory study of the first administration of hkMm in humans, basically intending to demonstrate the safety of the probiotic, a formal predetermination of sample size based on numeric or statistic criteria was not made. In any case, the numbers shuffled in the trial (51 subjects, 18 placebo and 33 of treatment) were within the standard for this type of studies.

5.1.6| Randomization and blinding

A random allocation scheme in which each participant has equal likelihood of being assigned to treatment versus placebo groups (ratio 1:1:1) was used. The CRO generated an allocation list by blocks using numbers drawn from the uniform distribution. Allocation was performed considering the stratification for TST-positive and TST-negative.

Treatment is masked, meaning that neither the investigator nor the volunteer knows if the vial contains hkMm or Placebo. Both treatments were physically identical with the same excipients. Labelling was also identical, indicating the study code, the numerical identification of the subject and the data referred to the promoter, administration route and posology.

In an emergency case that would require to open the double blind, the researcher had to contact to the CRO which would inform the composition of the treatment of the subject. The investigator had to write a justification explaining the reasons for opening the double blind.

Investigational treatment kits were conserved in UTE and transferred to the Phase I Unit until their use. Both places performed temperature log on a daily basis.

5.1.7| Statistical methods

Results were expressed as median with the interquartile range (IQR), or as

otherwise specified. All the analyses corresponding to the demographic variables, basal characteristics, as well as the variables to evaluate the Immunogenicity were made with the population per protocol (all randomized subjects who met the selection criteria, received the study treatment, and did not present major protocol deviations). All the analyses corresponding to the security variables were carried out in the safety population (all participants who received at least ten doses of the treatment).

Continuous variables were compared using non parametrical tests, Mann Whitney and Kruskal Wallis for non-paired and Wilcoxon test for paired data. The Chi-square or Fisher's exact test, as appropriate according to the variables distribution, was used to compare categorical variables. Given the exploratory nature of this study of phase I, no multiplicity adjustments were considered. The analysis was performed using SPSS(®) v.15 and the level of significance was established at the 0.05 level (two-sided).

5.2| Results

5.2.1| Participant flow and recruitment

The CT lasted 5 months (from 31th of March to 29th of August 2014) from the data the first participant enrolled to the time the last participant finished. After screening a total of 76 volunteers during the whole CT, 51 were included and randomized. A total of 25 volunteers were excluded for not fulfilling the study's requirements: presenting hematologic abnormalities such as anaemia (3 participants) or thrombocytopenia (2), vaccinated 3 months before the CT (2), and having an autoimmune disease (1). Recent vaccination and autoimmune disease were excluded as considered able to interfere with the immunological results. After groups with negative TST were completed, 14 screened participants had a TST negative result, and thus were not included. And finally, 3 participants were excluded because the recruitment period was promptly ended (Figure 32).

The other 51 volunteers were included and randomized. Eighteen

participants were in the placebo group, 17 in the hkMm high dose and 16 in the hkMm low dose group.

Each volunteer was treated with the allocated intervention (hkMm or placebo) and followed-up for a total of 6 weeks. No loss of follow-up or discontinuation of the treatment occurred, with the all 51/51 volunteers being analysed at the end of the CT. None of the subjects included in the present CT decided to withdraw from the study, nor was removed by the investigators. All volunteers declared to take the whole treatment (14 vials on a daily basis).

All the results and data obtained from the volunteers during the whole study are considered confidential. Furthermore, the investigator's team certifies that to their knowledge all subjects were healthy and thus considered able to be included in the CT, in terms of fulfilling all the study's requirements.

The only protocol deviation from the study protocol occurred was the recruitment promptly stopped because the TST reagent (PPD RT-23) was out of stock (Agencia Española de Medicamentos y Productos Sanitarios, 2014), resulting in a smaller sample size than expected (n=60) in the TST-positive groups.

5.2.2| Baseline data

All demographic and clinical baseline characteristics for the 51 volunteers finally included in the CT, with the corresponding ratio or median with the IQR, are summarized in Table 3.

The anamnesis and physical exams performed to the volunteers during screening did not show any clinical significant abnormality. The results of the laboratory analysis of all the volunteers included in the study performed during the screening were in the normal range; otherwise, the investigators considered them without clinical significance.

Table 3| Demographic characteristics of the clinical trial.

	Placebo (n=18)	Low dose hkMm (n=16)	High dose hkMm (n=17)
Age (years)*	25.5 (22 – 36.3)	31 (23 – 44.3)	29 (21.5 – 42.5)
Gender (% men)	28%	43.80%	52.90%
Underlying disease (% yes)	88.90%	93.80%	94.10%
Height (cm)*	165.5 (160.5 – 170.3)	169.0 (162.5 – 175.3)	171.0 (163.5 – 179.5)
Weight (kg)*	65.7 (51.8 – 79.9)	70.1 (58.8 – 80.8)	67.4 (61.5 – 81.5)
Systolic blood pressure (mm Hg)*	111 (102 – 122)	116 (103 – 141)	113 (106 – 123)
Diastolic blood pressure (mm Hg)*	64 (58 – 67)	65 (58 – 77)	63 (59 – 69)
Cardiac frequency*	69 (60 – 79)	64 (57 – 72)	68 (60 – 75)
Respiratory frequency*	18 (16 – 20)	20 (17 – 20)	18 (13 – 21)

*median (IQR)

5.2.3| Outcomes and estimation

5.2.3.1|Results of laboratory tests

The abnormal laboratory tests possibly or probably related to the investigational treatment recorded during the CT include kidney function, hepatic enzymes, haematology and glycemia. No significant statistical differences were found when compared between groups (Table 4).

Seven participants (13.7%) presented an increase of bilirubin or transaminase values. All of the elevations were asymptomatic, mild and resolved in the following weeks or at the end of the trial.

Five participants (9.8%) presented a decrease of haemoglobin or an abnormal value of leucocytes, all of them were also asymptomatic, mild and resolved in the following weeks or at the end of the trial (excepting one volunteer in the placebo group with a probably myeloproliferative syndrome currently in study). A participant presented a moderate thrombocytopenia that spontaneously resolved within 5 weeks.

Hyperglycemia was recorded in 2 participants and hypoglycemia in one.

These were also asymptomatic, mild and resolved in the following weeks or at the end of the trial.

5.2.3.2| Physical examination and vital signs

No anomalies regarding to vital signs were detected during the trial visits.

Table 4| Number of subjects presenting possible or probable related adverse events.

Adverse Events			Participants		Placebo (n=16)	Low dose hkMm (n=16)	High dose hkMm (n=17)	P-value
			n	%				
Gastrointestinal	Abdominal pain		18	35.3	8	3	7	0.221
	Stool frequency	Increased	18	35.3	8	6	4	0.412
	Stool consistency	Decreased	14	27.5	5	5	4	0.883
	Nausea		9	17.6	6	2	1	0.084
	Diarrhoea		6	11.8	3	2	1	0.588
	Dyspepsia		4	7.8	1	3	0	0.087
	Stool frequency	Decreased	3	5.9	3	0	0	0.054
	Stool consistency	Increased	3	5.9	3	0	0	0.054
	Vomits		2	3.9	1	0	1	0.462
	Flatulence		2	3.9	1	1	0	0.434
	Constipation		1	2	1	0	0	0.346
	Epigastralgia		1	2	1	0	0	0.346
	Rectal Tenesmus		1	2	0	1	0	0.307
Non-Gastrointestinal	Hepatic enzymes alterations		7	13.7	1	4	2	0.206
	Hematologic alterations		5	9.8	3	1	1	0.378
	Cephalea, migraine		4	7.9	1	3	0	0.346
	Respiratory Infection		3	5.9	1	1	1	0.996
	Hyperglycemia		2	3.9	0	1	1	0.221
	Hypoglycemia		1	2	0	0	1	0.346
	Thrombocytopenia		1	2	0	1	0	0.307
	Others		5	9.8	1	3	1	0.378

5.2.3.3| Adverse events

The 51 participants reported a total of 322 AE. The 92.5% (298) of the AE were mild, and only one was classified as severe but was considered improbably to be related to the investigational treatment (radial bone fracture after slipping and falling). No statistical differences were found when comparing the number of AE between the placebo group and both treatment groups (Table 5).

Table 5| Comparison of the median (IQR) number of AE by groups.

	Gastrointestinal	Non-Gastrointestinal	Total
Placebo	4 (2 – 6)	2 (1 – 3)	6 (4 – 9)
Low dose hkMm	2.5 (1.3 – 4)	2 (1.3 – 4.5)	4.5 (4 – 9)
High dose hkMm	2 (1.0 – 3.5)	2 (1 – 4.5)	5 (2.5 – 7.5)
P-value	0.057	0.444	0.467

The 46.3% (149/322) of the overall reported AE were considered possibly or probably related to the investigational treatment. No AE were causally related as definite. The 47% of the AE probably or possibly related to the treatment (70/149) occurred in the placebo group, 29.5% (44/149) in the hkMm low dose group, and 23.5% (35/149) in the hkMm high dose group. None of them were severe (94% were mild and 6% moderate). The most common reported AE were gastrointestinal events (82%, 122/149).

The 78.4% (40/51) of the participants presented at least one gastrointestinal event (no significant differences were found between treatment groups, p=0.078), reporting most frequently mild abdominal pain and/or increase of the stool frequency (in 35.3% of the participants each one [18/51]) (Table 5).

5.2.3.4| Immunogenicity assays

The phenotyping described in the work of Dwyer (Dwyer et al., 2010) according to the differential expression of CD25 and CD39 on circulating $CD4^+$ T cells was used. As mentioned before, the FACS analysis strategy used allowed us to identify 4 different types of cells.

Figure 33 shows the evolution of the PPD stimulated cells in all of the

participants, while Figure 34 shows the same results stratified by the TST status. Essentially both TST-negative and TST-positive volunteers treated with hkMm experimented a global increase on Treg response, showed in both populations of $CD25^{+}CD39^{-}$, mainly effector T cells, or $CD25^{+}CD39^{+}$ memory Treg cells, according to different authors (Dwyer et al., 2010; Guyot-Revol et al., 2006).

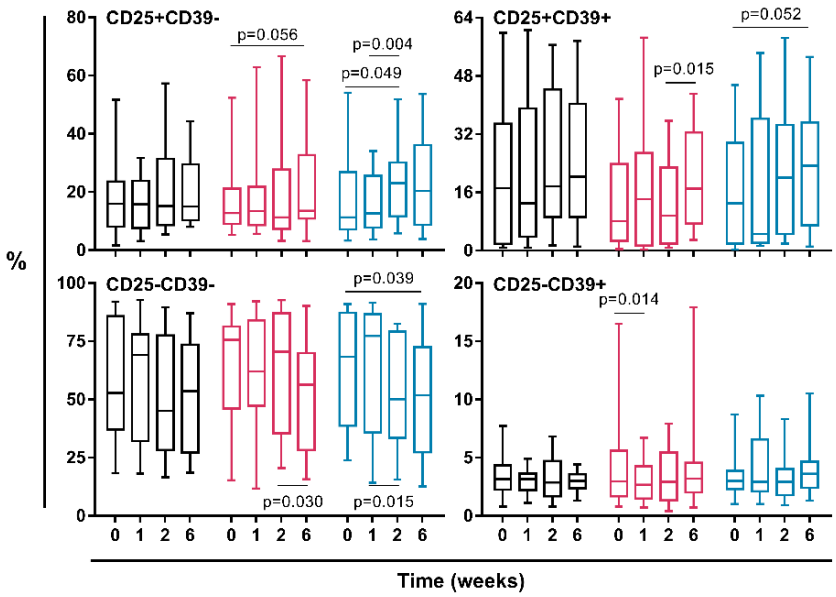

Figure 33| Evolution of the PPD-stimulated T cells in all volunteers regardless their TST status. Treatment groups are represented in black, red and blue, corresponding to Placebo, low dose and high dose hkMm respectively. P-values calculated by Wilcoxon matched pairs test. Plots are shown with median, IQR and minimum/maximum values.

Furthermore, the levels of Tregs were in general higher in TST-positive subjects during and after the treatment, being the group treated with the high dose hkMm the one that experienced more significant increases with time (Figure 35). Indeed, in the group of TST-positive subjects treated with placebo the baseline levels were really high and stable, probably reflecting an already protected population. In some cases a significant decrease in the $CD25^{-}CD39^{+}$ population has also been noted.

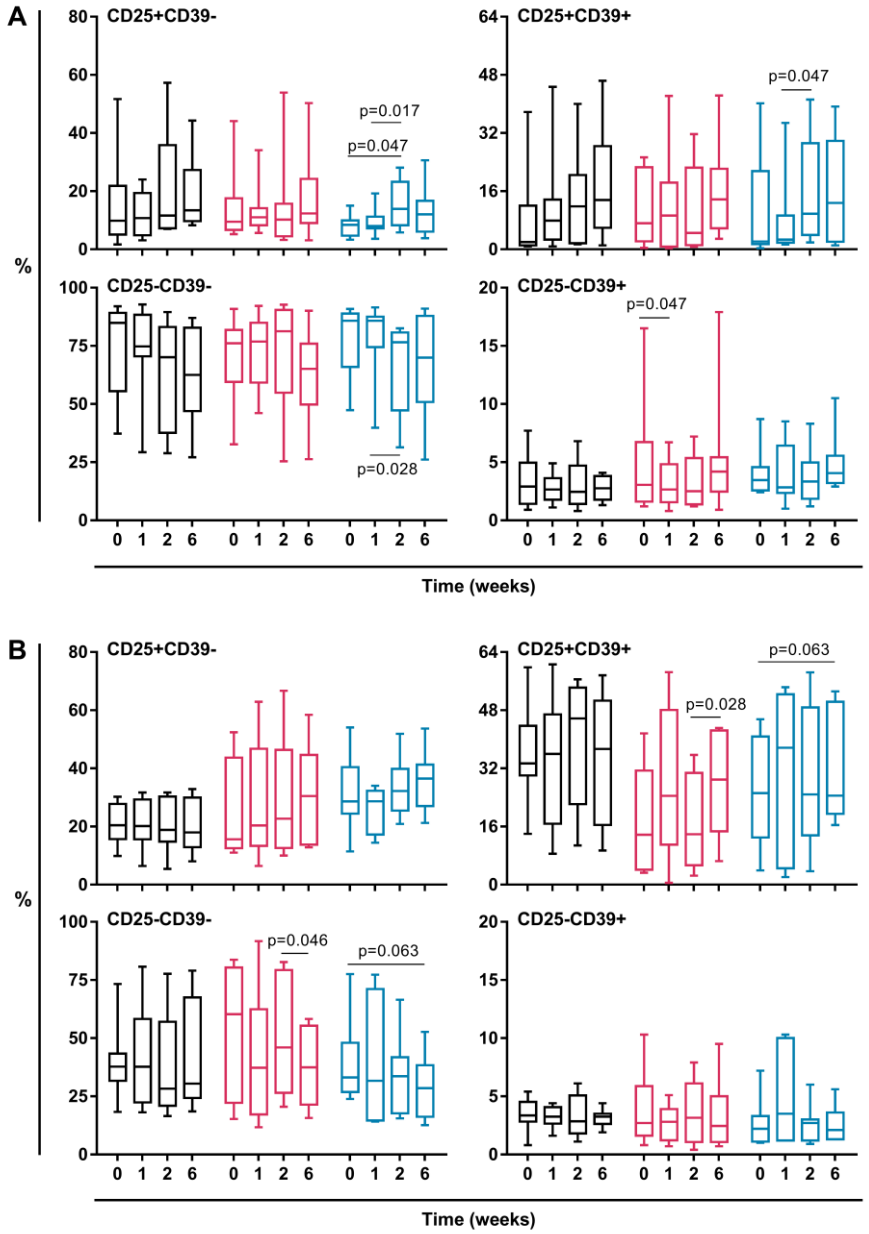

Figure 34| Evolution of the PPD-stimulated T cells in TST-negative (A) and TST-positive (B) volunteers. Treatment groups are represented in black, red and blue, corresponding to Placebo, low dose and high dose hkMm respectively. P- values calculated by Wilcoxon matched pairs test. Plots are shown with median, IQR and minimum/maximum values.

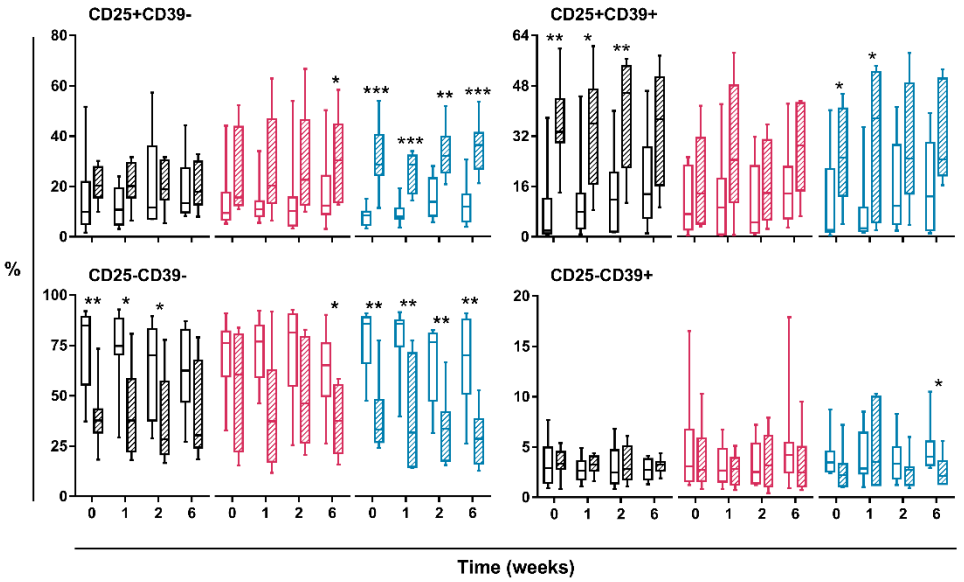

Figure 35| Evolution of the PPD-stimulated T cells according to TST status. Comparison between TST-negative and TST-positive volunteers. Treatment groups are represented in black, red and blue, corresponding to Placebo, low dose and high dose hkMm respectively. P- values calculated by Mann Whitney test, expressed in intervals: * from 0.05 to 0.01; ** from <0.01 to 0.001 and *** <0.001. Plots are shown with median, IQR and minimum/maximum values.

Protection index as assayed by Dwyer (2010) was also determined as a ratio between the data obtained by the ratio of stimulated and non-stimulated $CD25^{+}CD39^{+}$ and $CD25^{-}CD39^{+}$ cells. In this case, even when naturally Placebo groups can have increased levels of Tregs, only hkMm treated groups experienced an increase in this index, maybe relating to a "real" protection, or at least a better cellular distribution able to avoid a pro-inflammatory milieu. The levels were higher in TST-positive volunteers and after being treated with the highest dose of hkMm (Figure 36).

5.3| Discussion

The objectives of this CT were to demonstrate the safety of the oral therapy with hkMm and evaluate its effect on specific Tregs population.

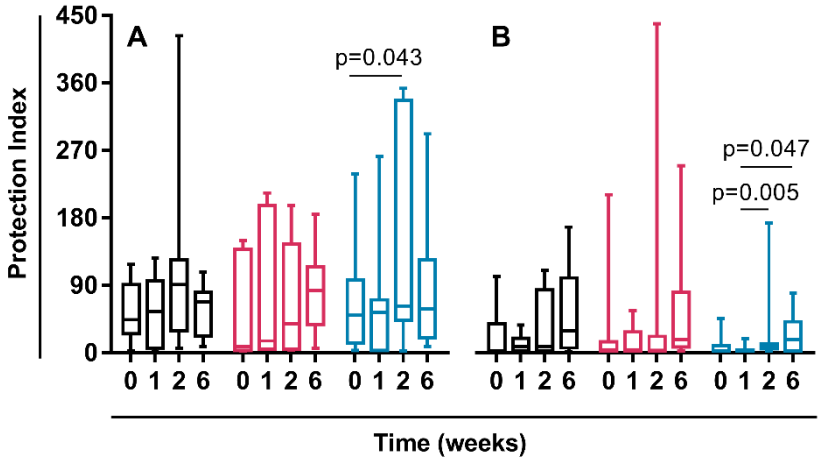

Figure 36| Protection index as assayed after Dwyer et al. (2010) determined as a ratio between the data obtained by the ratio of stimulated and non-stimulated $CD25^{+}CD39^{+}$ and the obtained by the ratio of stimulated and non-stimulated $CD25^{-}CD39^{+}$ cells. Results divided according TST status: (A) TST-positive, (B) TST-negative. Treatment groups are represented in black, red and blue, corresponding to Placebo, hkMm low dose and hkMm high dose respectively. P-values calculated by Wilcoxon matched pairs test. Plots are shown with median, IQR and minimum/maximum values.

Surprisingly, the median number of AE in placebo treated participants was higher than in groups treated with the investigational treatment, but no significant differences were found when comparing between groups. More than two-thirds of participants presented a gastrointestinal event with a similar distribution between groups. Gastrointestinal adverse events are very unspecific and usually frequent when the investigational treatment is administered orally. Moreover, gastrointestinal events were those we could mainly expect because of administration route (oral) as well as the bacterial origin of the probiotic. As all the participants were informed about this potential effect, this could explain the high rate of AE considered probably and possibly. On the other hand, it is well known that the administration of placebo can induce AE, related to the informed potential AE of the investigational product. It is logical then the fact that placebo side effect profile is mostly similar to the side effect profile of the investigational treatment (Rosenzweig et al., 1993). Variability on the adverse events

profile in healthy volunteers has been related to personality and lifestyle of volunteers. Although randomisation, women were more frequent in the placebo group than in the investigational treatment groups. Somatization and hypocondriacal features, and adverse reaction in general, are predominantly related to women (Rief et al.), which could explain the highest incidence in this group.

Laboratory abnormalities such as liver or glycemia alterations were more frequent in the probiotic groups than placebo, without significant differences found. Contrary to these alterations, leucocytes abnormalities were more frequent in the placebo groups, also without significant differences. The small sample size of this study is a limitation and could difficult the possibility to find statistical significant differences between groups. A bigger sample size could be necessary to elucidate the incidence and relation to treatment of those rare AE.

In the present clinical trial we have demonstrated the safety of the hkMm, because, although the AE were frequent, they were mild and spontaneously resolved within the following weeks, and no significant differences were found when compared between groups.

Data provided show for the first time that the administration of low doses of hk non tuberculous mycobacteria is able to induce both effector and memory specific Tregs. The induction of Tregs by probiotics is well known (West et al., 2009) even when using hk probiotics (Adams, 2010). It has been recently demonstrated that in some cases the effect induced by probiotics is not affected by its viability (Lahtinen, 2012), emerging the concept of "paraprobiotic" or "ghost probiotics" (Taverniti and Guglielmetti, 2011) which have clear advantages in terms of production, stability and security of the product. Shinkai et al. (2013) have recently evaluated the usefulness of the oral intake of hk *Lactobacillus pentosus* strain b240 as immunoprotective by reducing the incidence rate of the common cold in elderly adults. Previously, Zhang et al (Zhang et al., 2012) demonstrated that the administration of hk *Enterococcus faecalis* FK-23 was able to attenuate the Th17 response in the lung, thus suppressing the

allergic response in a murine model of asthma induced by ovalbumin.

We have demonstrated that IL-17 plays a paramount role in the evolution from infection to disease in a TB model in mice, by increasing the inflammatory response in the granuloma through a neutrophilic infiltration, followed by the coalescence of different lesions (Cardona et al., 2016; Marzo et al., 2014; Vilaplana et al., 2013). Furthermore, we have demonstrated that mice from a strain that never develops TB had higher levels of Tregs and when these mice were treated with mAbs anti CD25 for the Tregs depletion, TB appeared (Cardona et al., 2016).

After the induction of a low dose tolerance with a daily oral administration of hk mycobacteria, progression towards TB was abrogated as demonstrated by increasing from 30 to 50% the survival time of the mice. When translating this phenomenon to humans, another local defence mechanism not present in mice must be taken into account: the septae that surrounds secondary lung lobules able to encapsulate lesions and abrogate its growth (Gil et al., 2010).

So far, the induction of Tregs after Mtb infection has been understood as deleterious. Tregs have been claimed to be responsible for a weaker cellular immune response, stopping Th1 cellular proliferation (Guyot-Revol et al., 2006) that would fuel the progression from LTBI to TB (Chen et al., 2007; Hougardy et al., 2007), and inducing a limited protection after BCG vaccination (Li et al., 2007). Further investigations have given them a neutral role (Jaron et al., 2008; Quinn et al., 2006; Shafiani et al., 2010) mostly in models where protection was measured through the reduction of the bacillary load. Some authors have seen a protective role of the presence of Tregs linked to a control of the inflammatory response (Chen et al., 2012).

The presence of *M. fortuitum* complex bacilli in the tap water has been demonstrated by different authors in different regions of the planet (Fernandez-Rendon et al., 2012; Imwidthaya et al., 1989; Kubalek and Mysak, 1996; Moghim et al., 2012; Nasr-Esfahani et al., 2012; Scarlata et al., 1985) including the region where the clinical trial was run (Martín

Casabona and Rosselló Urgell, 2000). This was the reason for developing hkMm as a supplement food once an equivalent protective effect than the induced by the hkMtb was demonstrated (Cardona et al., 2016). On the other hand this fact might had played a role in our trial as there was a risk that subjects from the placebo group had drank tap water with *M. fortuitum* bacilli, or even that treatment with the investigation product boosted the immune response of subjects that has been previously in contact with this bacillus. When designing the trial, we assumed to include this risk, as it is part of real life.

The hkMm has also demonstrated its capacity to induce specific Tregs and also memory Tregs ($CD39^+$). Overall, the highest dose was more clearly related to an increase of this cell population. TST status has also shown to play a major role. TST positive participants had in general more Tregs than TST-negative, both in Placebo or hkMm treated subjects. This fact was expected because Mtb infection itself also induces the presence of Tregs. Interestingly, the Placebo group of TST-positive subjects had a very high number of Tregs from the baseline, although it did not experiment any increase during the clinical trial. In this regard we could theorize that those subjects have already been protected, and it can be hypothesized that this is either because they have had contact with non-tuberculosis mycobacteria or because the Mtb infection has naturally triggered a higher proportion of Tregs in those volunteers. This latter hypothesis would be in concordance with the concept of genetic resistance (Möller and Hoal, 2010; Stein, 2011). In fact, in our experience, we have been able to demonstrate higher Treg % in those mice strains that better resist the Mtb infection (Cardona et al., 2016).

Regarding the data presented to monitor regulatory response, we mainly presented raw data of PPD stimulated PBMCs, following the methodology of other authors (Feruglio et al., 2015) that have observed a better response-window. In fact, non-stimulated PBMCs have shown very low levels of Tregs through time in all treatment groups. When we analysed the protection ratio as a measure of protection firstly described by Dwyer et al.

(2010), we have been able to show a significant increase through time in the group of the highest dose of hkMm (10^5 CFU) in both TST-positive and negative participants.

In conclusion, data supports that the administration of heat-killed bacilli of *Mycobacterium manresensis*, is able to induce a specific increase of the regulatory response including memory cells, with an excellent safety profile, thus being a new tool to reduce the risk of the progression from latent infection to TB in humans.

6| Internship in Manremyc

After the promising results with heat-killed mycobacteria, a patent for the oral use of inactivated mycobacteria against tuberculosis was gotten. Also, a spin-off to take this concept to the market was founded: Manresana de Micobacteriologia s.l. (Manremyc). Nyaditum resae® (NR) is a supplement food composed of heat-killed bacilli of *Mycobacterium manresensis*.

In the context of a collaboration contract between the UTE and Manremyc, and thanks to the iPFIS contract from Instituto de Salud Carlos III (ISCIII), I have also done an internship in Manremyc.

During these years I have collaborated in the experiments required for the products' dossier. I was also involved in the planning of an efficacy clinical trial: Nyadageorg. And last, during 2017, I worked with the quality department in order to obtain the HACCP certification.

6.1| Experimental work

6.1.1| Stability assays

We evaluated the stability of NR stored in bags or capsules. Kymos Pharma oversaw the storage of the product in controlled atmosphere: 75% of humidity and 30ºC (for up to 24 months) or 40ºC (for up to 6 months). At different time points, the product that had been exposed to high temperatures was used to perform survival studies in the active TB model, as explained in Study I. Mice were treated orally, every other day, with NR or mannitol (Control group).

Figure 37 shows the survival curves for the first year of stability at 30ºC, comparing control with NR treated animals. The environmental conditions tested did not affect the product's efficacy, evidenced in the statistically significant increase in mice survival.

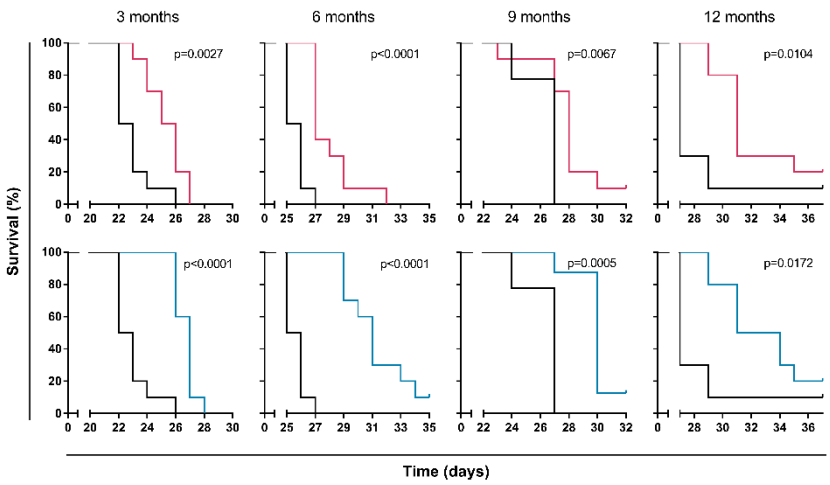

Figure 37| Effect of NR exposed to 30ºC/75%RH on survival. Infected C3HeB/FeJ mice were treated every other day for 2 weeks from infection with NR stored in bags (red lines) or capsules (blue lines) that had been exposed to 30ºC for different periods of time. Statistically significant differences were observed between treated animals and control (mannitol treated, black lines) (Log-rank test).

6.1.2| Virulence assay in SCID mice

We studied the potential virulence of *M. manresensis* in SCID mice (severe combined immunodeficiency). Animals were infected intravenously with *M. bovis* BCG (10^5 or 10^6 CFU) or *M. manresensis* (10^5, 10^6 or 10^7 CFU). A survival study was done, as explained in Study I. The survival curves are shown in Figure 38.

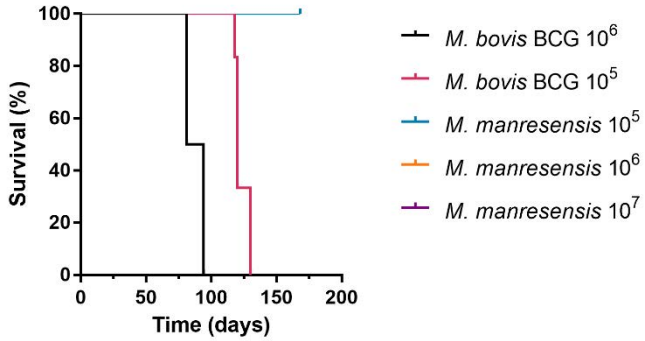

Figure 38| Survival in SCID mice infected with high doses of *M. bovis* or *M. manresensis*.

Results showed that SCID mice infected with *M. bovis* BCG had a median survival of 87.5 days (10^6 CFU) or 120 days (10^5 CFU). On the other hand, no animal infected with *M. manresensis* had to be sacrificed since there was no loss of weight or signs of suffering. For this reason, we decided to establish the experiment endpoint at six months after infection.

There were statistically significant differences between the survival rates of mice infected with one strain or the other (Table 6).

Table 6| Statistical differences between infection groups in SCID survival rates.

Experimental groups compared		p-value
M. bovis BCG 10^5 CFU	*M. manresensis* 10^5 CFU	0.0008
	M. manresensis 10^6 CFU	0.0008
	M. manresensis 10^7 CFU	0.0008
M. bovis BCG 10^6 CFU	*M. manresensis* 10^5 CFU	0.0009
	M. manresensis 10^6 CFU	0.0009
	M. manresensis 10^7 CFU	0.0009

These results show that Mm is significantly less virulent than *M. bovis* BCG in the SCID mice, even when the dose is 10-100 times higher.

6.1.3| NR and BCG vaccination

Given that BCG is widely used in countries with high incidence of TB, we wanted to test whether there were some kind of interference when administering NR.

12 weeks before infection, 2 groups of C57BL/6 mice were s.c. vaccinated with BCG. After 6 weeks, one of these groups started to be treated orally with NR. Mice received a total of 10 doses in a 2-weeks-period. Animals were aerosol infected with Mtb and euthanized on week 4 p.i. The bacillary load in lungs and spleen was assessed, as explained in Study I.

We found a reduction in the Mtb load, both in spleen and lungs, of BCG vaccinated animals. This protective effect was not altered by the NR

treatment Figure 39.

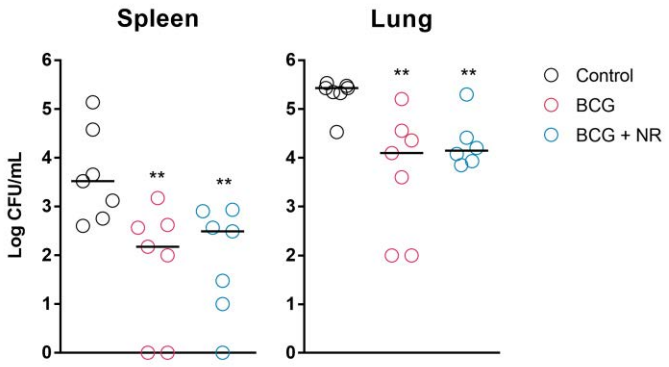

Figure 39| Effect of NR and BCG vaccination on bacillary load. C57BL/6 mice received BCG alone or in combination with NR. Their effect on BL was studied 4 weeks p.i., expressed as log CFU/mL. Each circle represents an animal and the lines are medians. Statistically significant differences were observed between control animals and those vaccinated, both receiving NR treatment or not (**p<0.01; Mann Whitney test).

6.2| Nyadageorg: efficacy clinical trial

A clinical trial for testing the efficacy of NR is currently being carried out in Georgia (NYADAGEORG, ClinicalTrials.gov Identifier: NCT02897180). It is a double-blind, randomized, masked, placebo-controlled trial, where 3,300 volunteers are expected to be included. The target population is contacts from active TB not eligible for chemoprophylaxis, i.e. children of <5 years old and HIV-positive adults.

Participants receive 14 daily doses of 10^5 hkMm or placebo (composed of the excipient, mannitol). The follow-up is done over the phone at weeks 1 & 2 and months 6, 12, 18 & 24. The primary endpoint of the trial is incidence of active TB.

In this CT, I participated in the elaboration of the SOPs and CRFs of the trial. Currently I am monitoring the evolution of the trial through the electronic CRFs. Up until early September 2018, a total number of 585 participants have been included in the study, with less than 10% of lost to follow up or withdrawal from the study. So far, only 23 participants have

presented some kind of medical event during the treatment weeks. There were a total of 28 events, of which 25% were of gastrointestinal origin. The most frequent event was dizziness (n=6). None of the events was severe and most of them had recovered at the time of the follow-up phone call (82%).

6.3| HACCP

Judit Amposta introduced me into the field of quality assurance. To obtain the HACCP certification, we updated the working documents of Manremyc and worked together with the personnel from the sub-contracted producing plants to improve the manufacturing process.

HACCP is a quality system applied in the food industry, which stands for Hazard Analysis Critical Control Point. It is based on the detection of possible hazards during the whole production process and the establishment of critical control points in order to prevent the risks. It is a way to guarantee a safe product for the consumer (Herrera, 2004).

7| Final Remarks

Despite many efforts done on fighting tuberculosis, this disease is still one of the leading causes of death worldwide. The development of antibiotics during the 20th century revolutionized the management of TB. However, the appearance of drug-resistant Mtb strains stands out the need of looking for new approaches. In this context, therapies directed to the modulation of the host response, as hkMm, are an emerging strategy to fight TB.

The starting point of the work included in this thesis were the findings done in the active TB model (Marzo et al., 2014) which set the focus of the group on the inflammatory factor of the disease. It has been a long road since the first experiments with ibuprofen and aspirin, which delayed the progression of TB (Kroesen et al., 2018; Vilaplana et al., 2013). The observations from the Mtb lung lesions in minipigs were also paramount for conceiving and understanding this work (Gil et al., 2010).

We have shown that the oral therapy with heat-killed mycobacteria is a useful strategy for delaying the progression towards active TB. Using an active TB model, we have proved that the oral administration of hkMm increases survival time, reduces bacillary load and improves lung pathology in Mtb infected mice. These studies were carried out using the C3HeB/FeJ mice strain, which is a particularly valuable model, since the characteristics of the lung lesions developed after Mtb infection resemble the ones in human TB (Cardona and Williams, 2017; Marzo et al., 2014). It would be interesting, however, to try this therapy in other animal models, especially larger mammals with the ability of encapsulating granulomas, to further characterize its effect.

We have also demonstrated in mice, that the standard chemotherapy for DS-TB (RHEZ) is improved with hkMm. This reveals the possibility of using hkMm as a co-adjuvant to standard anti-TB treatment. Bearing in mind the problems related to toxicity and low adherence of the conventional therapy, there is a need for reducing the time of treatment. The administration of hkMm together with antibiotics might be a good approach to solve this

problem. Of course this should be further addressed after specific clinical trials.

In countries where there is a high incidence of TB, there is generally no chemoprophylaxis in LTBI individuals. In fact, LTBI is not tested, since probably most people are infected. This population is at risk of developing the disease and could benefit from the use of oral hkMm. In this context, it will be exciting to obtain the results of the Nyadageorg study. This trial could prove that hkMm might be the solution to a medical need currently unattended, which is to treat the contacts of TB patients in areas with high incidence.

When considering the possibility of hkMm as a coadjuvant therapy, both for TB or LTBI, we should also contemplate its protective effects on the microbiota population. The hkMm restored the microbiota diversity lost with Mtb infection. In this direction, we are planning to test if hkMm can reverse the dysbiosis associated to RHEZ.

Moreover, by using a non-pathogenic environmental mycobacterium, the possible safety issues in the production and implementation of the therapy are close to none. In fact, the safety profile of the product has been deeply evaluated. In SCID mice, *Mycobacterium manresensis* is even less virulent than *M. bovis* BCG, the most extensively used vaccine in history. There were no signs of toxicity in rats or pigs (data not shown). Furthermore, the clinical trial Nyadatreg showed an excellent safety profile in adults treated orally with hkMm for 14 days. Another factor on the plus side of this product is its stability: it may be stored at room temperature.

Besides from being an environmental microorganism, it is found in drinking water, even after chlorination. This has permitted the development of the product as a food supplement, which is not trivial at all. The drug development process takes at least 20 years, while hkMm is expected to be on the market after only 5 years of its conception. Looking at the world picture of tuberculosis over time, the possibility of contributing to the control of the disease as soon as possible should be a priority to all.

On the other hand, and to further support our hypothesis, we studied the modulation of the inflammatory response during Mtb infection with mAb. Our results show that a reduction in the pro-inflammatory cytokines produced during infection may ameliorate its outcome. However, as evidenced with the neutralization of IL-17, the timings are extremely delicate. This experimental work might also hold the clue to explain why it is so hard to find correlates of infection and protection in TB: it is not just a matter of which cell or molecule is induced, but when in the infection's course.

Supporting the benefits of reducing inflammation, the adoptive transfer of Tregs also controlled the progression of the disease. In this sense, it is important to consider that the hkMm therapy by increasing the production of Tregs -which we proved in treated C3HeB/FeJ mice and healthy adults-, reduces the inflammatory response but doesn't completely eliminate it.

To sum up, with the hkMm therapy we do not expect that Mtb infection will be avoided. The rationale behind this is that by modulating the inflammatory response, we will allow the lung parenchyma to encapsulate the lesions and thus control the development of the disease.

8| Conclusions

1) Oral administration of heat-killed mycobacteria delayed infection's progression in active TB model: increased survival, improved lung pathology and reduced bacillary load.
2) Oral administration of heat-killed mycobacteria increased specific memory Tregs in active TB model.
3) Standard TB chemotherapy was improved by concomitant hkMm treatment in active TB model: increased survival and reduced lung pathology.
4) Neutralization of IL-17 from the beginning of infection increased spleen's and lung's bacillary load in active TB model.
5) Neutralization of IL-17, type I IFN receptor or TNF-α when the infection has already set, reduced spleen's and lung's bacillary load, which was accompanied by a reduction in pro-inflammatory cytokines and neutrophil's recruitment chemokines.
6) Mtb infection reduced gut microbiota diversity in C3HeB/FeJ mice.
7) The administration of hkMm in infected mice promoted a higher diversity in microbiota and a change in the OTUs relative abundance.
8) Adoptive transfer of Tregs from hkMm treated mice reduced bacillary load in infected animals, which was accompanied by a reduction in pro-inflammatory cytokines and neutrophil's recruitment chemokines.
9) The hkMm was safe and well tolerated in healthy adults, with or without LTBI, treated with 14 doses.
10) The hkMm increased specific memory Tregs in PBMCs of healthy adults.

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Annex I

frontiers in Microbiology

ORIGINAL RESEARCH
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Oral Administration of Heat-Killed *Mycobacterium manresensis* Delays Progression toward Active Tuberculosis in C3HeB/FeJ Mice

*Paula Cardona[†], Elena Marzo-Escartín[†], Gustavo Tapia[2], Jorge Díaz[1], Vanessa García[1], Ismael Varela[1], Cristina Vilaplana[1] and Pere-Joan Cardona[1]**

[1] Unitat de Tuberculosi Experimental, Fundació Institut Germans Trias i Pujol, CIBER Enfermedades Respiratorias, Universitat Autònoma de Barcelona, Badalona, Spain, [2] Pathology Department, Universitat Autònoma de Barcelona, Hospital Germans Trias I Pujol, Badalona, Spain

Low-dose tolerance using heat-killed mycobacteria has been tested as a means of stopping progression toward active tuberculosis (TB) lesions in a human-like murine model using C3HeB/FeJ mice. In the present study, we studied the effect of different treatment schedules with heat-killed non-tuberculous-mycobacteria (NTM) species when given orally, based on the hypothesis of generating oral tolerance. This study included *M. manresensis*, a new species belonging to the *fortuitum* group, present in drinking water. Oral treatment with *M. manresensis* for 2 weeks was able to induce a PPD-specific Tregs population, which has been related to a decrease in the neutrophilic infiltration found in TB lesions. Further mechanistic analysis using PPD-stimulated splenocytes links this 2-week treatment with heat-killed *M. manresensis* to IL-10 production and memory PPD-specific Tregs, and also to a weak PPD-specific global immune response stimulation, increasing IL-6, TNF, and IFN-γ production. In lungs, this treatment decreased the bacillary load, granulomatous infiltration and pro-inflammatory cytokines (TNF, IFN-γ, IL-6, and IL-17). Oral administration of *M. manresensis* during standard treatment for TB also significantly reduced the relapse of active TB after ending the treatment. Overall the data suggest that the use of heat-killed *M. manresensis* could be a new and promising tool for avoiding active TB induction and as adjunctive to TB treatment. This supports the usefulness of generating a new kind of protection based on a complex balanced immune response focused on both destroying the bacilli and including control of an excessive inflammatory response.

Keywords: tuberculosis, *Mycobacterium manresensis*, tolerance, Tregs, neutrophils, C3HeB/FeJ, mice

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Jordi Torrelles,
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Oklahoma State University, USA

****Correspondence:***
Pere-Joan Cardona
pjcardona@igtp.cat

[†] These authors have contributed equally to this work.

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INTRODUCTION

Tuberculosis (TB) is a major global health problem, with 9 million new TB cases and 1.5 million deaths reported in 2013 (WHO, 2014). It is estimated that one third of the world's population is infected with latent tuberculosis infection (LTBI). Fortunately, only a small proportion of infected subjects go on to develop the disease. Although, some host factors, mainly immunosuppression conditions such as human immunodeficiency virus (HIV) coinfection, drug abuse, renal failure or

diabetes mellitus, are known to be related to generation of the disease, it still remains unclear why almost half of healthy adult cases develop TB.

As reviewed recently (Vilaplana and Cardona, 2014), research efforts to develop new treatment or preventive strategies have focused on destruction of the bacilli by identifying metabolic pathway targets (development of antimicrobial agents) or studying how the host's immune system identifies it (new vaccine development), whereas little effort has been invested in trying to modulate the host's response against the bacilli, which is paramount for understanding the development of infectious diseases (Casadevall and Pirofski, 2003), with TB not being an exception (Cardona, 2010).

Understanding the infection's progression to active TB is essential for finding new strategies to fight TB. In this regard, induction of liquefaction in TB lesions has been identified as a key factor that allows extracellular growth of the bacilli and the development of cavities, thereby favoring spread of the infection (Grosset, 2003; Cardona, 2011). For this reason, we developed a murine model of active TB using a C3HeB/FeJ mice strain in which lesions liquefact, thus mimicking human-like progression (Marzo et al., 2014). Characterization of this model showed us that extracellular bacillary growth starts before the liquefaction process and is fuelled by the constant attraction of neutrophils and the induction of Neutrophilic Extracellular Traps (NETs), thereby starting a local and uncontrollable bacillary spread. This, in turn, allows fast growth of the granulomas, the induction of new lesions and the coalescence of neighboring lesions, which are responsible for the logarithmic growth of the granulomas. Once a certain size is exceeded, the center of the granulomas first develops caseous necrosis and then the caseum softens, thus leading to liquefaction (Marzo et al., 2014; Vilaplana and Cardona, 2014). In this regard, a recent review of the pathology of human lesions based on necropsies from the pre-antibiotic era also highlights the key role of neutrophils as inducers of exudative lesions, which are clearly responsible for the progression toward active TB (Cardona, 2015). In summary, we can conclude that the onset of active TB is a consequence of an excessive inflammatory response and the coalescence of different lesions (Vilaplana and Cardona, 2014).

Evidence for the key role of inflammation in progression to TB disease has also been reported by others. Thus, a detrimental role of excessive accumulation of neutrophils has been described in mice (Eruslanov et al., 2005; Keller et al., 2006) and, in humans, genetic studies performed with TB and leprosy patients indicated that insufficient but also excessive inflammation are key to TB pathogenesis (Tobin et al., 2010). Similarly, Berry et al. found a signature of active TB that measures interferon IFN type I-inducible genes in whole blood (Berry et al., 2010). Unexpectedly, the genes induced in susceptible population were expressed in neutrophils and not in T-cells, thus suggesting that over-activation of neutrophils by IFNs may contribute to TB pathogenesis.

Host-Directed Therapies (HDT) are currently becoming increasingly popular, as reviewed by Hawn et al. (2013), Zumla et al. (2015), and Zumla and Maeurer (2015). In this regard, we previously tested ibuprofen (Vilaplana et al., 2013) and other non-steroidal anti-inflammatory drugs (NSAID) (Marzo et al., 2014) in the C3HeB/FeJ model, with very promising results. We are now exploring their potential therapeutic use in humans, but as NSAIDs can produce adverse effects related to dose and length of treatment, these should be taken into account when considering long-term therapy (Michels et al., 2012; Bjarnason, 2013).

After the success obtained with the aforementioned anti-inflammatory treatment, and for safety reasons, we decided to look for a strategy that avoids the potential toxicity of these drugs and the perspective of constant administration to obtain an effective prophylactic effect. The prophylactic/therapeutic use of low dose tolerance to avoid an excessive inflammatory response has been widely used by different authors in the field of experimental autoimmune encephalitis (Ochi et al., 2006), diabetes mellitus and other autoimmune diseases (Weiner et al., 2011), as well as infectious diseases (Levy and Ilan, 2007) and atherosclerosis (Harats et al., 2002). The role of regulatory T cells (Tregs) in TB has been controversial as they were initially thought to fuel progression toward active TB. However, recent reports seem not to support this idea (Green et al., 2010; Leepiyasakulchai et al., 2012), thus leading to a more neutral role in their interference against the Th1 response.

METHODOLOGY

Experimental Design

We first conducted a proof-of-concept study to assess the role of Tregs in protection against the development of active TB by comparing the susceptible strain (C3HeB/FeJ) with the resistant one (C3H/HeN). We then tested the usefulness of low-dose heat-killed *Mycobacterium tuberculosis* (Mtb) cells and their protective role. Finally, we tested different environmental NTM species in order to ensure any possible transfer of this strategy to the market and further characterized the effect of the species that provided the best results.

Animals

Female C3HeB/FeJ and C3H/HeN specific-pathogen-free mice (6–8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and Harlan Labs (Castellar del Vallès, Catalonia, Spain). All procedures were conducted in a BL3 security facility. Mice were infected with 2×10^4 CFU of *M. tuberculosis* H37Rv Pasteur strain via the caudal vein.

All procedures were performed according to protocol DMAH6119, which was reviewed by the Animal Experimentation Ethics Committee of the Hospital Universitari Germans Trias i Pujol (registered as B9900005) and approved by the Dept d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural of the Catalan Government, according to current national and European Union legislation regarding the protection of experimental animals (Law 1997 of the Catalan Government; Spanish Royal Decree 1201/2005; and European legislation 86/609/EEC; 91/628/EEC; 92/65/EEC and 90/425/EEC).

Mice were supervised daily and euthanized, if required, with isoflurane (inhalation excess), following a strict protocol, in order to ensure animal welfare.

Study of the Role of Tregs in Protection Against the Development of Active TB

To study the role of Tregs in TB, three experiments were performed with C3HeB/FeJ and C3H/HeN mice. First, 6 mice from each strain were infected and sacrificed at 3 weeks post-infection to evaluate Treg population in spleen. This experiment was repeated with a final time point of week 2 post-infection, the splenocytes collected being cultured for 7 days. In the third experiment, a total of 12 C3H/HeN mice were infected, and one group was Treg-depleted *in vivo* by administration of anti-mouse CD25 antibody (Clone PC61.5, eBioscience Inc. San Diego, CA) the day before the infection. Blood was collected at different time points to evaluate Treg depletion by flow cytometry. Animals were sacrificed at day 46 post-infection to evaluate lung histopathology.

Treatment Preparation

Different mycobacteria from the Experimental Tuberculosis Unit strain collection, namely *M. tuberculosis*, *M. kansasii*, *M. avium,* and *M. manresensis* (new species, Rech et al., 2015 CECT 8638) were used to prepare the treatments. *M. bovis* BCG Danish (Pfizer Inc., NY, USA) was also used. Bacteria were grown in 7H11 plates (BCG, *M. kansasii,* and *M. manresensis*) or in Proskauer-Beck broth (*M. tuberculosis* and *M. avium*) and subcultured in Proskauer-Beck broth (or in 7H11 plates, for *M. manresensis)* in aeration and agitation at 37°C. The bacillary load (BL) of each culture was determined by serial dilution and culture on 7H11 plates, and a Blood Agar plate was also seeded to rule out contamination. Cultures were then inactivated by heating at 80°C for 60 min. Sterilization was confirmed by negative culture in 7H11 (10 plates), Blood Agar, McConkey Agar, and Saboureaud Agar. The inactivated cultures were diluted 1:1 in sterile sucrose (10% sucrose in water) and aliquoted in 1 ml vials for storage at −80°C.

Assessment of Treatments

The effect of treatment on the survival of infected mice was studied. This assessment was done for each treatment when given prophylactically and therapeutically at different doses (10^3–10^6 heat-killed bacilli/animal) and administration schedules (every day, every other day, three times-a-week). The results presented here were repeated 3 times, using 10 C3HeB/FeJ mice per group (a total of 120 animals).

In order to further characterize the effect of treatment, we evaluated several parameters at week 3 post-infection. For this purpose a total of 20 C3HeB/FeJ mice were used (5 per group of treatment). Animals received seven oral doses of *M. tuberculosis* (10^5 heat-killed bacilli/animal), BCG (10^6 heat-killed bacilli/animal), and *M. manresensis* (10^5 heat-killed bacilli/animal) every other day from the day of infection and were sacrificed at day 21 post-infection. At the final time point, pathology and BL in lungs, and effect on T cell populations were studied (see Sections 2.6 to 2.8). A control group was included and treated with the corresponding dilution of the excipient (mannitol).

The oral administration of heat-killed mycobacteria as a coadjuvant therapy to the human standard treatment against active TB (a combination of rifampicin, isoniazid, ethambutol, and pyrazinamide (RHEZ)) was also studied. A total of 21 C3HeB/FeJ mice were used. They started to be treated at week 4 post-infection, when their weight started to decrease. All animals received a commercial combination of the four drugs (RIMSTAR®, Sandoz Farmaceutica, Barcelona) adjusted to their weight, for a total of 4 weeks. Half of the animals also received heat-killed *M. manresensis* (10^5 heat-killed bacilli/animal) orally, 5 days a week for a total of 6 weeks (in addition to the 4-week-treatment with RHEZ, plus 2 more weeks). At the final time point, pathology in lungs was studied (see Section Lung Pathology).

Bacillary Load

Samples of lung lobes from each animal were collected, homogenized and several dilutions plated on nutrient Middlebrook 7H11 agar (BD Diagnostics, Spark, USA). The number of CFU was counted after incubation for 28 days at 37°C and the results expressed as CFU/mL.

Lung Pathology

Lungs were fixed in 10% buffered formalin, embedded in paraffin and 5-μm sections stained with haematoxylin-eosin (HE), Masson trichromic (MTC) or Ziehl-Neelsen (ZN) stain for microscopic observation and histometric analysis using the NIS-Elements D version 3.0x software package (Nikon Instruments Inc., Tokyo, Japan). Eight recuts of half-lung of each mice stained with HE were used to determine the damaged area as a percentage of total lung area.

Immuno-Characterization

Cell Isolation and Cell Cultures

Spleens were mechanically disrupted and filtered through a 40-μm cell strainer (BD Diagnostics, Spark, USA), with erythrocytes being incubated for 8 min in lysis buffer (Tris 17 mM, NH_4Cl 0.14 M). 10^6 cells were either directly stained for flow cytometric analysis or cultured, depending on the experiment. Cell culture was conducted in supplemented RPMI 1640 (10% Fetal Calf Serum, streptomycin 100 μg/ml, penicillin 100 U/ml, 2-mercaptoethanol 0.025 mM, sodium pyruvate 1 mM) in 24-well plates at 37°C and 5% CO_2, with or without PPD stimuli (final concentration of 10 μg/ml; Statens Serum Institute, Kobenhavn, Denmark). After 24 h, 4 days, or 7 days of culture, the content of each well was harvested and stained for flow cytometric analysis. Culture supernatants were stored at −80°C for further study of the cytokine profile. Lungs were snap-frozen when collected and kept stored at −80°C until processed for cytokine profile analysis. They were then subjected to mechanical disruption and homogenized with lysis buffer (sodium azide 0.05%, Triton X-100 0.5%, protease inhibitor cocktail from Sigma at 1:500, in PBS; Sigma-Aldrich Co. LLC, St. Louis, MO, USA).

PPD-Specific T Cell Population Analysis by Flow Cytometry

Mouse Regulatory T Cell Staining Kit #2 (eBioscience Inc., SD, USA) was used for intracellular staining (Foxp3) according to the manufacturer's indications. For membrane staining, cells were incubated for 30 min at 4°C with the antibodies, followed by fixation with 4% formaldehyde in PBS for 10 min at room temperature.

PPD-specific T cell populations, obtained from spleen cultures at 24 h, 4 days, or 7 days, were studied by flow cytometry. Different CD4+ T cell types, namely CD25+CD39+ Regulatory Memory T cells; CD25-CD39+ Effector Memory T cells, ex-Tregs IL-17 producers; CD25+CD39− T Effector cells and CD25-CD39− Naïve T cells, according to Dwyer et al.'s characterization (Dwyer et al., 2010), were studied.

The antibodies used were anti-mouse CD4 FITC, anti-mouse CD25 PE and anti-mouse Foxp3 APC (eBioscience Inc., SD, USA), anti-mouse CD3e BV™421, anti-mouse CD25-PerCP-Cy™5.5 (BD biosciences, CA, USA) and anti-mouse CD39 PE (BioLegend Inc., CA, USA).

Once stained, samples were read in a flow cytometer (BD LSRFortessa™, BD Biosciences, CA, USA). Data were analyzed using FACSdiva™ software (BD Biosciences, CA, USA; **Figure 1**).

Cytokine Profile Analysis

A cytokine profile study was performed in splenocyte culture supernatant and lung homogenates. The following cytokines were measured by Luminex xMAP® technology: IFNγ, TNFα, IL-5, IL-6, IL-10, IL-13, and IL-17. Results were expressed as pg per ml of supernatant. The assay was performed with the MILLIPLEX® MAP kit (EMD Millipore Corporation, Billerica, MA, USA) following the manufacturer's instructions and analyzed with xPONENT Software (Luminex Corporation, Austin, TX, USA).

Data Analysis

GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego California USA) was used for graphics and statistics, with differences of $p < 0.05$ being considered to be statistically significant.

RESULTS

Protective Role of Regulatory T Cells (Treg)

In order to study the mechanisms that modulate the inflammation in active TB, the Treg population was studied in C3HeB/FeJ mice and compared with the TB-resistant mouse strain C3H/HeN. Mice from both strains were infected IV and the Treg population in spleen studied by flow cytometry 3 weeks post-infection. As shown in **Figure 2A**, the median percentage of Treg (CD4+CD25+Foxp3+ cells) out of total CD4+ in spleen was higher in C3H/HeN than in C3HeB/FeJ mice (C3H/HeN: 10.28%, C3HeB/FeJ: 8.09%; *t*-test, $p = 0.0022$). The same experiment was repeated with the animals being sacrificed at day 14 and the resulting splenocytes cultured and PPD-stimulated. The C3H/HeN resistant mice had higher basal and PPD-specific Treg percentage in culture compared to C3HeB/FeJ mice, as shown in **Figure 2B**. Intergroup differences were statistically significant ($p < 0.05$, Mann Whitney test).

In the third experiment, C3H/HeN mice were depleted of Treg cells by administration of anti-CD25 prior to Mtb infection. All animals treated were effectively depleted of Treg cells, as shown in **Figure 2C**, except for one animal, which was excluded from the analysis. One animal from the Treg-depleted group was sacrificed at day 32 for ethical reasons as it exhibited a marked TB infection in the lungs. The remaining animals were sacrificed at day 46 as scheduled and their lung pathology assessed by qualitative and quantitative (histometry) histological analysis. The histometry showed differences in the percentage of damaged lung area between both groups (not statistically significant) (**Figure 2D**). The results for the Treg-depleted group were highly scattered: three animals had a similar percentage of damaged lung area to the controls (20–40%), whereas the other two animals had a higher percentage of damaged area (around 80%). The qualitative analysis matched these findings: the animals with mild TB infection (less than 40% of damaged lung area) presented medium size lesions with no signs of necrosis or other forms of cell death, with mainly macrophage and lymphocyte infiltration, and scattered neutrophils around foamy macrophages, while those with severe TB infection (more than 75% of damaged lung area) presented very big lesions with central caseous necrosis and liquefaction (**Figure 2F**). When classifying the animals according to the degree of lung affection it was found that the frequency of severe affection (>75% of damaged lung) was 0 in the control group and 2/5 in the depleted group (**Figure 2E**; $p < 0.1$, z-test of proportions, one-tailed).

Taken together, these results suggest a protective role of regulatory T cells in our model.

Oral Administration of Inactivated Mycobacteria spp. Increased Mice Survival

Mycobacterium tuberculosis (Mtb) heat-killed bacilli were administered orally to C3HeB/FeJ infected mice to test their influence on disease progression. Treatment was found to increase survival of the mice in a statistically significant manner. Different doses and administration patterns were tested and positive results were obtained both when administered pre-infection or post-infection (data not shown). The survival curve of a representative experiment is shown in **Figure 3**.

Other mycobacterial species, including *M. bovis* Bacille-Calmette-Guérin (BCG) and environmental species, were also tested. Animals treated with heat-killed BCG, *M. kansasii* (data not shown) and *M. manresensis*, a newly discovered environmental mycobacteria species belonging to the *M. fortuitum* complex (CECT 8638) (Rech et al., 2015) showed an increased survival compared to untreated controls (**Figure 3**). This protection was also achieved when giving the same treatment with the same concentration of living *M. manresensis* (data not shown). As was the case for Mtb, different doses and administration schedules were tested for all of these strains and positive results were obtained both when administered pre-infection or post-infection (data not shown). Oral treatment with *M. avium* was also tested but found not to improve mice survival (data not shown).

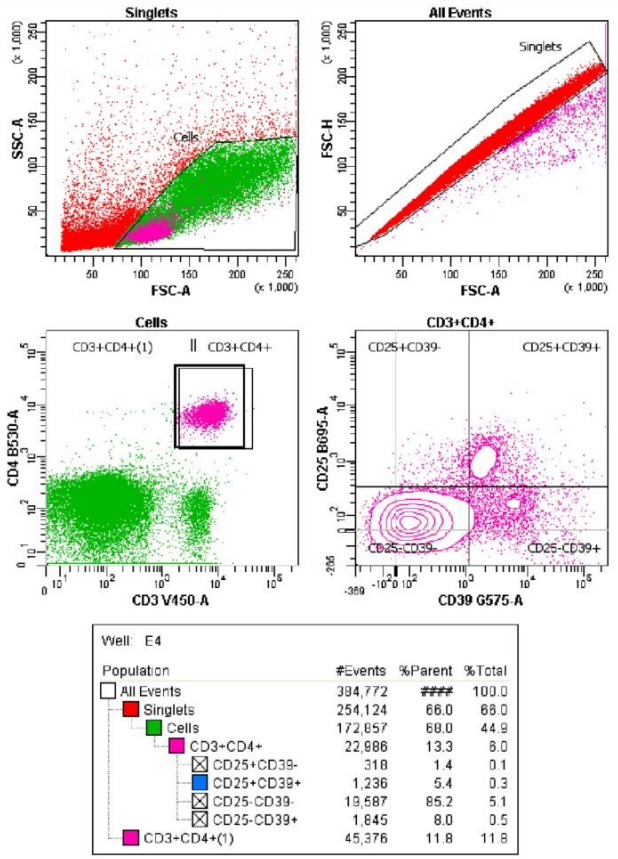

FIGURE 1 | Flow cytometry analysis carried out. CD3+CD4+ T cells were classified into 4 groups: CD25+CD39+ Regulatory Memory T cells; CD25-CD39+ Effector Memory T cells, ex-Tregs IL-17 producers; CD25+CD39− T Effector cells and CD25-CD39− Naïve T cells.

Oral Administration of Heat-Killed Mycobacteria Reduced Lung Pathology

The effect on lung pathology was evaluated for oral treatments with three different heat-killed mycobacteria, namely Mtb, BCG and *M. manresensis*. **Figure 4** shows how all treatments significantly reduced the infiltration in the parenchyma (statistically significant differences: $p = 0.0002$; Mann Whitney test). **Figure 5** shows one of the H/E recuts, which was used to measure the percentage damaged area, for the untreated control group and the group treated with *M. manresensis*. In agreement with the histometry analysis, the picture enclosed in **Figure 5** shows the presence of fewer and smaller lesions in the lungs of treated mice than in the untreated control group.

Oral Administration of Heat-Killed Mycobacteria Decreases Lung Bacillary Load

The effect on bacillary load in lungs was evaluated for oral treatments with three different heat-killed mycobacteria, namely Mtb, BCG and *M. manresensis*. The three treatments decreased

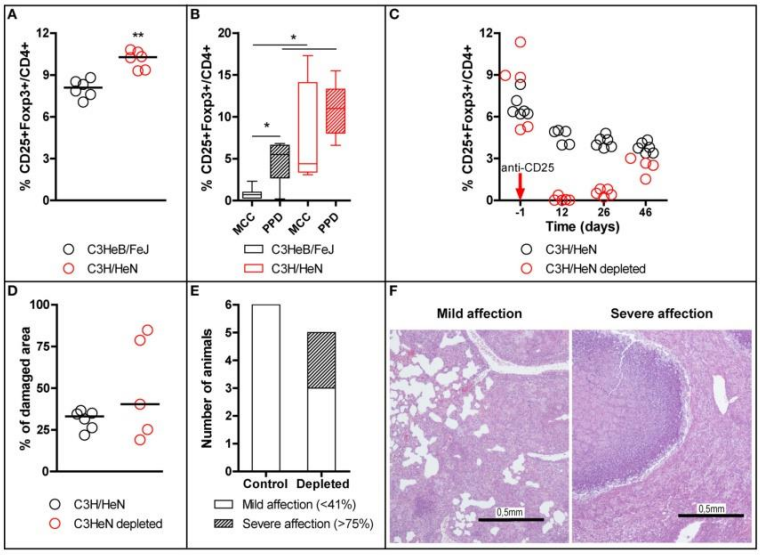

FIGURE 2 | Role of Treg in TB-resistant C3H/HeN mice strain. Treg Foxp3+ population was studied in C3HeB/FeJ and C3H/HeN mice in splenocytes **(A,B)**. Splenocytes obtained on day 21 post-infection **(A)**, statistically significant differences (**$p = 0.0022$, Mann Whitney test); each circle represents an animal and the median is represented with a line. Splenocytes obtained on day 14 post-infection were cultured for 7 days with PPD stimulus (PPD) or with no stimulation (MCC) **(B)**, statistically significant differences between groups (*$p < 0.05$; Mann Whitney test); the boxplot shows the median, quartiles and minimum and maximum values. A Treg-depletion study was performed and lung pathology was studied in depleted and undepleted animals **(C–F)**. Treg depletion was confirmed by measuring the percentage of Treg Foxp3+ cells at different time points **(C)**. Percentage of damaged area out of total lung area **(D)**; each circle represents an animal and the lines are medians. Proportion of animals with mild or severe affection in lungs in control undepleted group and Treg depleted group **(E)**; bars indicate number of animals. H/E stained lung recuts showing mild and severe infection **(F)**.

the BL in lungs at day 21 post-infection in a statistically significant manner (BCG $p = 0.0317$, Mtb $p = 0.0079$, *M. manresensis* $p = 0.0079$; Mann Whitney test) as shown in **Figure 6**.

Immuno-Characterization of Heat-Killed Mycobacteria Treatment

The immunomodulation achieved by the oral treatment with heat-killed Mtb, BCG and *M. manresensis* was also characterized. Flow cytometric analysis of splenocyte cultures was used to assess different CD4+ T cell populations (M&M, Section PPD-specific T cell population analysis by flow cytometry). The results are shown in **Figure 7** (*M. manresensis* treatment) and **Supplementary Image 1** (Mtb, BCG, and *M. manresensis* treatments). The naïve CD25-CD39− population, which represents the higher percentage of CD3+CD4+ cells, tended to be higher under unstimulated conditions than under PPD stimulation. In contrast, the CD25+CD39− population, which represented approximately 2% of CD3+CD4+ cells, did not show any major differences between stimulated and non-stimulated culture conditions. A mild increase in this population was observed when cells were cultured for 7 days.

We also observed this trend in CD25+CD39+ Regulatory T cells, which represent around 2–9% of all CD3+CD4+ cells. In contrast to naïve T cells, this population tends to be higher under PPD stimulation. Furthermore, a higher PPD-specific response of CD25+CD39+ was observed in splenocytes from mice treated with *M. tuberculosis* and *M. manresensis* in comparison to untreated mice, but only when cells were cultured for 7 days (statistically significant differences: Mtb $p = 0.0159$, *M. manresensis* $p = 0.0079$; Mann Whitney test).

The CD25-CD39+ population accounts for about 20% of all CD4+ T cells. A higher percentage of PPD-stimulated cells (with statistically significant differences) is observed when cultured for 4 days, with levels of both stimulated and unstimulated cells decreasing after culture for 7 days.

Cytokine Profile

We studied the presence of different cytokines in the splenocyte supernatant and lung homogenates of control untreated mice

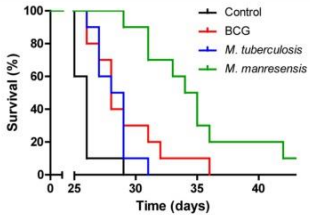

FIGURE 3 | Effect of treatment on survival. The survival curves for three different oral treatments are represented together with the survival curve for the untreated control group. Infected C3HeB/FeJ mice were treated every other day for 2 weeks from infection day with 10^5–10^6 heat-killed bacilli/animal. Statistically significant differences were observed between untreated mice and mice treated with heat-killed BCG ($p = 0.0038$), Mtb ($p = 0.0029$), or *M. manresensis* ($p < 0.0001$) (Log-rank Test).

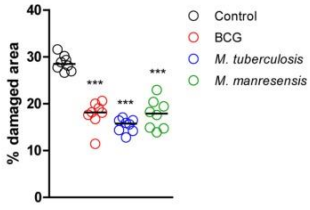

FIGURE 4 | Effect of treatment on lung pathology: histometric analysis. Infected C3HeB/FeJ mice were treated every other day for 2 weeks from infection day with 10^5–10^6 heat-killed bacilli/animal. The percentage of damaged area out of total lung area was studied in each treatment group on samples obtained on day 21 post-infection. Each circle represents an animal and the lines are medians. Statistically, significant differences were observed between untreated mice and mice treated with heat-killed mycobacteria (***$p = 0.0002$; Mann Whitney test).

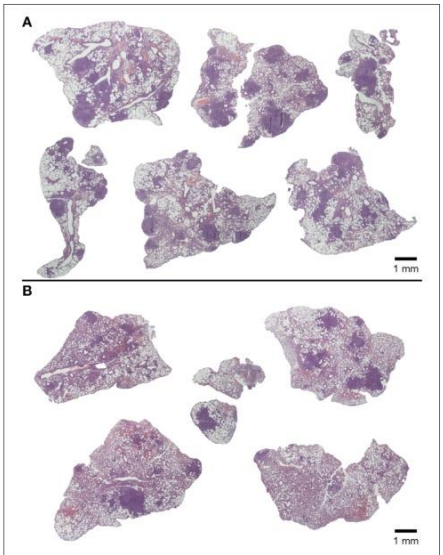

FIGURE 5 | Effect of treatment on lung pathology: macroscopic view of lesions. HE-stained lung recuts of untreated control mice **(A)** or mice treated with heat-killed *M. manresensis* **(B)** of samples obtained on day 21 post-infection.

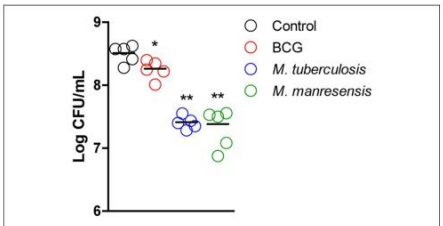

FIGURE 6 | Effect of treatment on bacillary load in lungs. Infected C3HeB/FeJ mice were treated every other day for 2 weeks from infection day with 10^5–10^6 heat-killed bacilli/animal. BL in lungs obtained on day 21 post infection is expressed as log CFU/ml. The boxplot shows the median, quartiles and minimum and maximum values, with a different pattern for each treatment group. Statistically, significant differences were observed between treated and untreated control mice (BCG: *$p = 0.0317$, Mtb: **$p = 0.0079$, *M. manresensis*: **$p < 0.0079$; Mann Whitney test).

or mice treated with heat-killed *M. manresensis*. Mice treated with *M. manresensis* showed higher levels of pro-inflammatory cytokines but a reduced inflammatory milieu in lungs when compared to control mice. Detailed results are shown in **Figure 8**. In splenocyte culture, treated mice showed a statistically significant increase in IFNγ, TNFα, IL-6, and IL-10 levels in spleens, whereas they showed a statistically significant decrease in IFNγ, TNFα, IL-6, and IL-17 levels in lung homogenates.

IL-5 levels could not be detected in any sample, thus suggesting a poor Th2 response.

Heat-Killed Mycobacteria as Coadjuvant Therapy

The effect of heat-killed *M. manresensis* on mice survival when administered as coadjuvant treatment to RHEZ was evaluated, with *M. manresensis*-treated mice showing a statistically significant increase in survival ($p < 0.0001$, Log-rank test; **Figure 9**). Comparison of the histopathology between the animals from the control group that had to be euthanized according to the welfare monitoring control (between weeks 15 and 22 post-infection) with the survivors from the

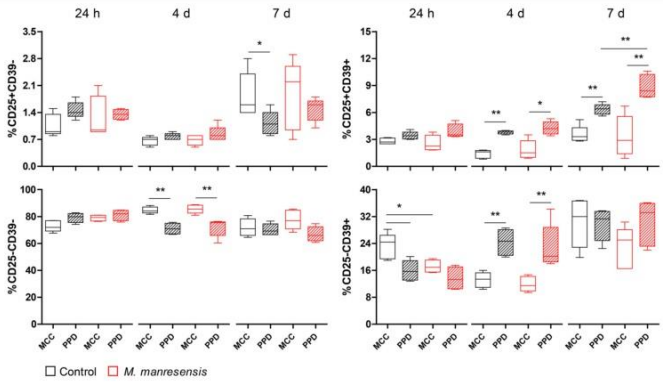

FIGURE 7 | Effect of treatment with heat-killed *M. manresensis* on T cell populations in the spleen. Percentage of the four populations defined by CD25 and CD39 markers (out of the total of CD3+CD4+ cells). Splenocytes *obtained on day 21 post-infection were* cultured for 24 h, 4 days, or 7 days with PPD stimulus (PPD) or without stimulation (MCC). The boxplot shows the median, quartiles and minimum and maximum values, with a different color for each treatment. Statistically, significant differences are marked with asterisks (*$p < 0.05$, **$p < 0.01$; Mann Whitney test).

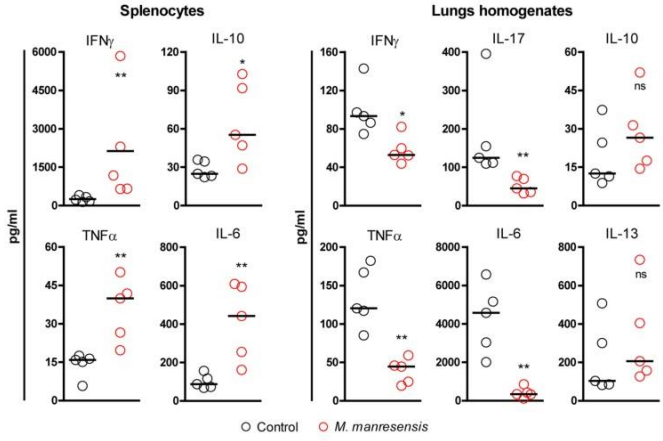

FIGURE 8 | Cytokine profile in spleen and lungs. Effect of treatment of infected C3HeB/FeJ mice every other day for 2 weeks with heat-killed *M. manresensis*. Samples were obtained on day 21 post-infection. Each circle represents an animal and the median is represented with a line. Cytokine levels are expressed as pg/ml. Statistically, significant differences are marked with asterisks (*$p < 0.05$, **$p < 0.01$; Mann Whitney test).

M. manresensis-treated group that were euthanized to terminate the experiment (week 24) showed a clear difference in terms of intrapulmonary infiltration (51 vs. 68%; $p = 0.0059$, Mann Whitney test; **Figure 10**). Looking at the quality of the lesions (**Figure 11**), all the samples of the control group showed a massive necrosis with liquefaction in the center and big patches of massive accumulation of nuclear debris plenty of bacilli. In the case of the group treated with *M manresensis* lesions resembled

granulomas of chronic tuberculosis infection seen in resistant mice (C57BL/6) where the bacilli are accumulated in foamy macrophages (FM), and where big cholesterol crystals can be seen as described before (Cardona et al., 2003; Cáceres et al., 2009). There is also a progressive fibrosis of the parenchyma caused by the proliferation of the fibroblasts of the alveolar wall, in a honeycomb pattern as described by Dunn and North (1996). The difference is that there are little infiltrations of PMN, especially around the infected FM. Different animals have lesions with different degrees of evolution, and it can be seen how this PMN infiltration become bigger and is infected with bacilli, that multiplies over them, as described before (Marzo et al., 2014).

DISCUSSION

The data provided herein suggest, for the first time, that a low dose tolerance regimen (i.e., administration of 10^5 heat-killed *M. manresensis* daily for 14 days) could be used to control progression toward active TB. This treatment regimen seems to interfere with the excessive inflammatory response, a fact that appears to be key to the development of human-like lesions (Cardona, 2015), and thus is also able to reduce the bacillary concentration in lungs (Marzo et al., 2014). In our opinion, this could be related to the induction of Tregs.

It is well established that dose and route of administration determine the immune response to an administered antigen (Faria and Weiner, 2005), therefore we have good reason to think that our regimen induces tolerance to the mycobacteria bacilli. Continuous oral administration modifies the response triggered by the infection through Tregs, as has also been seen in other infectious or autoimmune diseases (Harats et al., 2002; Ochi et al., 2006; Levy and Ilan, 2007; Weiner et al., 2011).

We have shown that treatment with heat-killed bacilli is effective for reducing both the infiltration area and the bacillary load in lungs, in addition to increasing survival. As such, we then decided to further characterize the effect of treatment on the immune system to prove that tolerance is induced.

In this regard we first investigated the role of Tregs in our model as it was feasible that oral tolerance could be articulated through this type of cell. The role of Tregs in Mtb infection is controversial, with some authors considering the induction of Tregs to be detrimental (Ribeiro-Rodrigues et al., 2006; Chiacchio et al., 2009) but others pointing to a protective role for Tregs (Leepiyasakulchai et al., 2012). To investigate the role of Tregs in our experimental system we compared the Treg population in C3HeB/FeJ mice with their positive controls (C3H/HeN mice), which share the MHC haplotype but have a different susceptibility to Mtb (Marzo et al., 2014). We found that the better outcome was associated with a stronger regulatory immune response in the C3H/HeN strain, as the Treg percentage was higher (also PPD-specific response), and when we depleted C3H/HeN mice of Treg by administration of anti-CD25 the susceptibility to Mtb increased. Two out of five mice developed necrotic lesions, whereas in several experiments conducted by our group with this mouse strain, no animals have either died

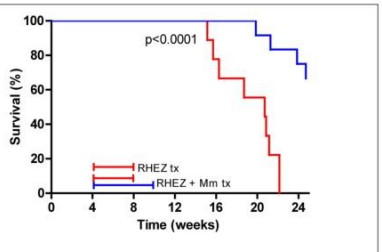

FIGURE 9 | Heat-killed *M. manresensis* as a coadjuvant therapy. Survival curves for infected C3HeB/FeJ mice treated with RHEZ (red line) or RHEZ in combination with heat-killed *M. manresensis* (Mm) (blue line). Treatment started at week 4 post-infection, RHEZ therapy was administered for 4 weeks and Mm therapy lasted 6 weeks.

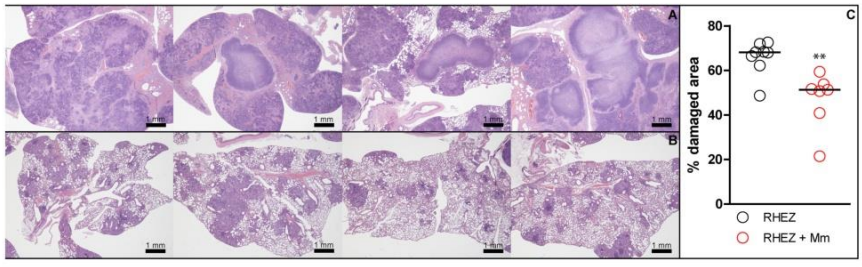

FIGURE 10 | Heat-killed *M. manresensis* as a coadjuvant therapy: histometric analysis. Macroscopic view of HE-stained lung recuts from mice treated with RHEZ **(A)** or treated with RHEZ and heat-killed *M. manresensis* **(B)**. The percentage of damaged area out of total lung area was studied in both groups **(C)**. Each circle represents an animal and the lines are medians. Statistically, significant differences were observed between untreated mice and mice treated with heat-killed mycobacteria (**$p = 0.059$; Mann Whitney test).

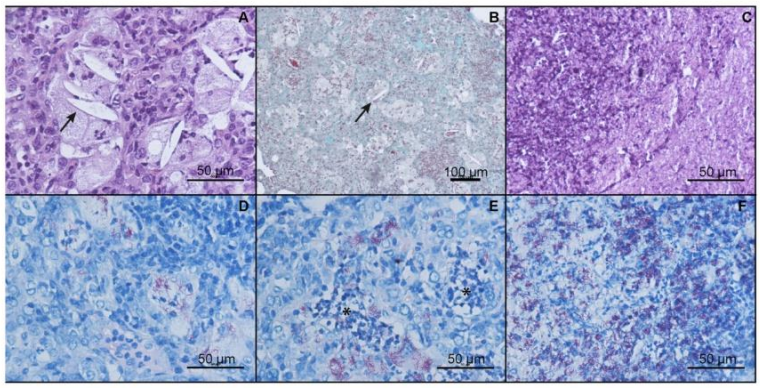

FIGURE 11 | Heat-killed *M. manresensis* as a coadjuvant therapy: qualitative histological analysis. Pulmonary infiltration in mice treated with RHEZ and heat-killed *M. manresensis* at week 24 post-infection **(A,B,D,E)** is compared with mice only treated with RHEZ that had to be euthanized according to the welfare monitoring control **(C,F)**. Pictures **(A)** and **(B)** show the haematoxylin-eosin (HE) and Masson trichromic (MTC) stains respectively showing the structure of murine chronic TB infection with a lot of foamy macrophages and where cholesterol crystals (marked with arrows) can be seen. MTC stain showing the honeycomb patron caused by the progressive fibrosis originated at the alveolar wall. Pictures **(D)** and **(E)** show the localization of bacilli with the Ziehl-Neelsen (ZN) stain, initially only in foamy macrophages **(D)** to growth on PMN rafts (marked with asterisks) afterwards **(E)**. Picture **C** shows the accumulation of cellular debris, which is the main property of the infiltration in terminal mice, after HE stain. Massive bacillary presence is shown with ZN stain in picture **(F)**.

or developed such necrotic lesions. Although, T CD4+CD25+ cells include Tregs as well as potential T CD4+CD25+CD39− Th subsets (Dwyer et al., 2010), the latter account for about 1% of all T CD4+, therefore Treg depletion is far more relevant. Taken together, these results strongly support the hypothesis that Tregs are protective in TB, therefore we continued our research by studying the effect of treatment with heat-killed mycobacteria on Tregs.

As we were interested in the induction of memory Tregs, we designed the concept of 7 days incubation, together with a study of the CD39 marker. We believe this marker to be very relevant as it has been related to both the memory phenotype and induction of tolerance (Borsellino et al., 2007; Dwyer et al., 2010; Roberts et al., 2014). Furthermore, unlike Foxp3, it is a surface marker, which translates into less aggressive cell processing and easier and faster protocols. Other relevant T cell subtypes, such as T memory effector cells CD25-CD39+, which have shown to play a detrimental role in organ transplantation in humans, or T effector cells, both of which are capable of secreting IL-17, and thus having a pro-inflammatory profile (Dwyer et al., 2010), were also studied with the marker combination used. Apart from being used in the study of autoimmune diseases, this CD25 CD39 marker combination has been validated as a Treg marker in the study of TB, exhibiting a negative correlation with IL-17 T cells in peripheral blood (Chiacchio et al., 2009; de Cassan et al., 2010).

Use of these markers showed an increase in Treg cells in splenocyte cultures from treated mice, together with a slight global stimulation. The levels of IFN-γ, TNF, and IL-6 were increased in cultured spleen samples from mice treated with *M. manresensis*. This suggest a global stimulation that also includes an increase in the immunosuppressive cytokine IL-10, which can be related to the increase in Treg, as seen by other authors who used 10^6 heat-killed *M. chelonae* intraperitoneally once a week for 3 weeks (Ho et al., 2010). In that study, the authors also demonstrated a parallel increase in IFN-γ, although they did not check for IL-6 or TNF. The most important aspect, however, is that the increase in IL-6 was not linked to the presence of IL-17 (Gao et al., 2009), which was undetectable, and this is logical as an exaggerated inflammatory response with neutrophilic attraction is not seen in the spleen in the C3HeB/FeJ model, where there is also effective control of bacillary load (data not shown). This could be due to the fact of the presence of Treg counterbalancing IFN type I production (Srivastava et al., 2014; Aida et al., 2014), thus favoring the increased IFNγ response (Manca et al., 2001); or because of the lack of PMN infiltration in the spleen prevents the production of type I IFN (Berry et al., 2010), thus favoring the presence of both Tregs and IFN-gamma in this organ in the context of Mtb infection. However, further experiments would be needed to investigate this issue.

A lower inflammatory milieu was found in the lungs of mice treated with *M. manresensis* when compared to control animals, with lower levels of IFNγ, TNFα, IL-17, and IL-6. In our opinion, the population of PPD-specific memory Tregs is attracted to the lungs and must be crucial for reducing the inflammatory response in situ, specially by counterbalancing the Th17 response (Zhou et al., 2008; Zheng et al., 2013).

Lung histopathology of mice treated with RHEZ shows how the addition of heat-killed *M. manresensis* is able to stop the progression of the lesions, favoring the fibrosis of the tissue which might contribute to create an anti-inflammatory milieu able to abrogate the growth of the bacilli by curtailing the infiltration of the lesion with monocytes or PMN. This phenomenon is amplified in big-mammals thanks to the stimulation of the fibroblasts of the intralobar septae, that encapsulate the lesions at very early stages, promoting this anti-inflammatory milieu and stopping the bacillary growth (Gil et al., 2010; Cardona, 2015).

The modulatory effect of NTM was demonstrated years ago after intravenous inoculation was shown to induce a non-specific cellular immune response (Collins, 1971) and protect against subsequent aerosol Mtb infection to a similar degree as in BCG vaccinated mice (Orme and Collins, 1984). On the other hand, subcutaneous sensitization with *M. avium* interfered with BCG vaccination by stopping its growth, although this was not seen after sensitization with *M. chelonae* or *M. fortuitum* (Brandt et al., 2002). The recent study by Poyntz et al. (2014) is interesting as it shows how important the route of administration for NTM is (in this case, *M. avium*). An increase in Th1 response can be detected when inoculating heat-killed IP several times, thereby increasing the efficacy of BCG, whereas oral administration of living bacilli tended to increase Th2 response in the lung during Mtb infection and reduced the protection against Mtb infection. Treatment with heat-killed *Mycobacterium vaccae*, which has been used for the treatment of active TB (von Reyn et al., 2010) and has recently been reviewed by Gröschel et al. (2014), deserves particular attention due to its ability to reduce Th2 responses and increase Th1 ones.

Finally, it is relevant to recall that studies in BCG-vaccinated infants in the United Kingdom or in Malawi showed no evidence that the initial response to NTM affected the vaccine-induced change in IFN-γ response (Weir et al., 2006). In this regard, it is interesting to note that, to date, no surrogates of protection have been found in TB. Th1 responses, including poly-functional cells, have received attention for a number of years, although an evaluation of their protective value in a large BCG trial in South Africa showed that protected and unprotected newborns exhibited an equivalent immunological profile (Kagina et al., 2010). In this sense, our paper may also help to shed some light on other biomarkers that are more closely related to the induction of a balanced immune response and are able to avoid an excessive inflammatory response than can lead to active TB, instead of focussing on an immune response that exclusively targets destruction of the bacilli.

In conclusion, induction of a balanced immune response triggered by PPD-specific memory Tregs through the administration of heat-killed *M. manresensis* has demonstrated an ability to protect against the progression of Mtb infection to active disease and relapse after TB treatment. This has been demonstrated in the experimental model induced in C3HeB/FeJ mice, which reproduce "human-like" lesions. This finding might help to focus on a new kind of "host-directed" prophylactic and therapeutic approach as well as the development of new predictive biomarkers.

AUTHOR CONTRIBUTIONS

EM and PJC had substantially contributed to the conception and design of the work. PC, EM, GT, JD, VG, IV, CV, and PJC contributed to the acquisition, analysis, and interpretation of data for the work. All authors contributed to drafting the work and revising the work; and gave final approval of the version to be published. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01482

Supplementary Image 1 | Effect of treatment with different heat-killed mycobacteria treatments on T cell populations in the spleen. Percentage of the four populations defined by CD25 and CD39 markers (out of the total of CD3+CD4+ cells). Splenocytes obtained on day 21 post-infection were cultured for 24 h, 4 days, or 7 days with PPD stimulus (PPD) or without stimulation (MCC). The boxplot shows the median, quartiles and minimum and maximum values, with a different color for each treatment. Statistically significant differences are marked with asterisks (*$p < 0.05$, **$p < 0.01$; Mann Whitney test).

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Conflict of Interest Statement: "Inactivated mycobacteria for oral use in the prevention of tuberculosis" is a patent (PCT/ES2013/000145) owned by IGTP and CIBER Enfermedades Respiratorias and invented by PC, CV, and EM. Manremyc sl is a new spin-off from IGTP and CIBER Enfermedades Respiratorias that has been created exclusively for the development of this patent. PC is the CEO of this spin-off.

The reviewer JT and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

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Annex II

PLOS ONE

RESEARCH ARTICLE

Pilot, double-blind, randomized, placebo-controlled clinical trial of the supplement food Nyaditum resae® in adults with or without latent TB infection: Safety and immunogenicity

Eva Montané[1,2], Ana Maria Barriocanal[2,3], Ana Lucía Arellano[1,2], Angelica Valderrama[1], Yolanda Sanz[1], Nuria Perez-Alvarez[4,5], Paula Cardona[6], Cristina Vilaplana[6], Pere-Joan Cardona[6]*

1 Department of Clinical Pharmacology, Hospital Universitari Germans Trias i Pujol, Badalona, Catalonia, Spain, **2** Department of Pharmacology, Therapeutics and Toxicology, Universitat Autònoma de Barcelona, Catalonia, Spain, **3** Fundació Institut Germans Trias i Pujol, Badalona, Catalonia, Spain, **4** Lluita Contra la Sida Foundation, Badalona, Catalonia, Spain, **5** Statistics and Operations Research Department, Universitat Politècnica de Catalunya- BarcelonaTech, Barcelona, Catalonia, Spain, **6** Unitat de Tuberculosi Experimental, Universitat Autònoma de Barcelona, CIBERES, Fundació Institut Germans Trias i Pujol, Badalona, Catalonia, Spain

* pjcardona@igtp.cat

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Abstract

Background

Nyaditum resae® (NR) is a galenic preparation of heat-killed *Mycobacterium manresensis*, a new species of the *fortuitum* complex, that is found in drinkable water, and that has demonstrated to protect against the development of active TB in a murine experimental model that develop human-like lesions.

Methods

Double-blind, randomized, placebo-controlled Clinical Trial (51 volunteers included). Two different doses of NR and a placebo were tested, the randomization was stratified by Latent Tuberculosis Infection (LTBI)-positive (n = 21) and LTBI-negative subjects (n = 30). Each subject received 14 drinkable daily doses for 2 weeks.

Results

All patients completed the study. The 46.3% of the overall reported adverse events (AE) were considered related to the investigational treatment. None of them were severe (94% were mild and 6% moderate). No statistical differences were found when comparing the median number of AE between the placebo group and both treatment groups. The most common AE reported were gastrointestinal events, most frequently mild abdominal pain and increase in stool frequency. Regarding the immunogenic response, both LTBI-negative and LTBI-positive volunteers treated with NR experienced a global increase on the Treg

IFI14/00015 (PC); and Manremyc sl. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: "Inactivated mycobacteria for oral use in the prevention of tuberculosis" is a patent (PCT/ES2013/000145) owned by IGTP and invented by PJC and CV. Manremyc sl is a new spinoff from IGTP that has been created exclusively for the development of this patent. PJC is the CEO of this spin-off. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

response, showed both in the population of CD25+CD39-, mainly effector Treg cells, or CD25+CD39+ memory PPD-specific Treg cells.

Conclusion

This clinical trial demonstrates an excellent tolerability profile of NR linked to a significant increase in the population of specific effector and memory Tregs in the groups treated with NR in both LTBI-positive and negative subjects. NR shows a promising profile to be used to reduce the risk of active TB.

1 Introduction

Tuberculosis is one of the most frequent infectious diseases in the world, in spite of being a curable disease [1]. Even if a vaccine (Bacille Calmette Guérin -BCG-) has been available since 1927 and extensively used (3 billion doses used), there is a consensus that it only helps to stop the development of disseminated and meningeal TB [2]. In fact it has been experimentally demonstrated that BCG vaccination stops the bacillary growth in vaccinated subjects sooner than in the unvaccinated, but does not avoid the infection by *Mycobacterium tuberculosis* (Mtb) [3].

One of the characteristics of Mtb infection, is that it is transmitted through aerosol and that disease is not usually the immediate consequence of the infection [4], resulting in LTBI, were the bacilli is able to remain in the subjects for a long period, even years. It has been estimated that approximately a third of mankind already has LTBI [5] without hindering the host. Lesions of 1 mm in diameter at the lung parenchyma are irrelevant to challenge normal lung function [6], which is why most of them ignore the fact of being infected. The only way to know that someone has LTBI is by performing the tuberculin skin test (TST) or the T-cell interferon-gamma release assays (TIGRA) [7]. A low percentage (around 10%) will develop active TB (TB) [5], and besides severe immunodeficiencies (like AIDS) [1], the mechanism of other comorbid factors are poorly known.

A new theory supported by experimental data and published recently suggests that the development of active TB is caused by an exaggerated response against Mtb. This response is based on a progressive massive neutrophilic infiltration of the lesions (causing a sudden increase in size) and with the presence of nearby coincident lesions; a process favoured by the environment of the upper lobes, that leads to the coalescence of the lesions [8,9]. This mechanism could also explain why subjects with diabetes mellitus (DM) have 3 times more risk to develop TB [10,11]. DM is a consequence of an imbalance in the Treg/Th17 response [12], generating a global exaggerated inflammatory response.

An excessive inflammatory response in LTBI would be materialized through a neutrophilic infiltration fuelled by Th17 cells, stimulated by the Mtb infection itself in an intensity that depends on the host reactivity [13]. It has been demonstrated that in subjects with LTBI there is an inverse relation between the Th17 and Treg cells [14]; and that Th17 response can be counterbalanced by the presence of Tregs [15–17]. In this context, we decided to induce a Treg response through a low dose tolerance process using heat-killed Mtb (HKMtb) cells, and several environmental mycobacteria for their cross-immunity with Mtb [18], with successful results. Among the environmental mycobacteria tested, a species usually found in drinking water and thus able to be considered as a food supplement [19] was isolated. It was identified to be part of the *fortuitum* complex, according to a state-of-the-art multiplex probe assay [20]

which is the standard for mycobacterium identification in clinical laboratories. After its sequencing, it was considered a new species, called *M. manresensis* (Certificate of Deposit CECT 8638 at the Spanish Type Culture Collection) [21]. This strain has been chosen to be further developed as food supplement for its similar ability to induce the protection obtained with HKMtb [22].

Oral administration of alive or heat-killed *M. manresensis* was found to stop the inflammatory progression towards TB in an experimental murine model, a process linked to the increase of PPD-specific memory Tregs (CD25+CD39+) [22]. This new tool that decreases the risk of progression towards TB was patented by the Institut d'Investigació Germans Trias i Pujol (IGTP) (PCT/ES2013/000145) and transferred to a spin-off of the same Institute (Manremyc sl) for its development.

The study presented here is a pilot, double-blind, randomised, masked and placebo controlled clinical trial (CT), conducted in volunteers, to evaluate the tolerability and the immunogenicity of 2 oral doses of Nyaditum resae® (a preparation of heat-killed *M. manresensis*) administered daily for 14 days to the general population, both LTBI positive or negative.

2 Materials and methods

2.1 Ethics

The protocol of the study was reviewed and approved by the Ethics Committee at the investigational centre (Hospital Universitari Germans Trias i Pujol, Badalona, Catalonia, Spain). All investigators and collaborators agreed to rigorously observe the Helsinki declaration with all its amendments and to follow the Good Clinical Practice guidelines of the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use). The objectives and methodology, as well as possible drawbacks and risks due to the study, were explained to each subject orally and in writing (Subject Information Sheet) before their inclusion. They were also informed of the different treatments to be tested, the way they would be assigned to the groups, the option to withdrawal from the study at any time and of the existence of an insurance contract. Informed consent was obtained of all the participating volunteers by consent form signature before starting any study procedure. Participants were also informed and signed their consent to be included in a local register from the Health Department of the Catalan Government to control the participation of healthy volunteers in Phase I clinical trials. The trial has been registered in ClinicalTrials.gov: NCT02076139.

2.2 Participants

Subjects were interviewed and screened for enrolment at the Phase I Unit of the Hospital Germans Trias i Pujol (Badalona, Catalonia, Spain) by the clinical pharmacologists. The target population of the study was healthy adults, with or without LTBI. A screening visit was made for each volunteer where a complete anamnesis and physical exam, laboratory parameters tests (hemato-biochemical and immunogenicity parameters), Tuberculin Skin Test (TST), serology against HIV and a pregnancy test were performed. Previous TST was acceptable and was not repeated when positive in the last 5 years or negative in the last 6 months. Radiological chest X-ray exam was performed in TST-positive volunteers. Subjects were not included in the study if any of the following criteria were present: TB, immunodeficiencies, chronic immunosuppressant therapy, reception of blood products or derivatives six months prior randomization, pregnancy or lactation.

Furthermore, the investigator's team certifies that to their knowledge all subjects were considered able to be included in the CT, in terms of fulfilling all the study's requirements.

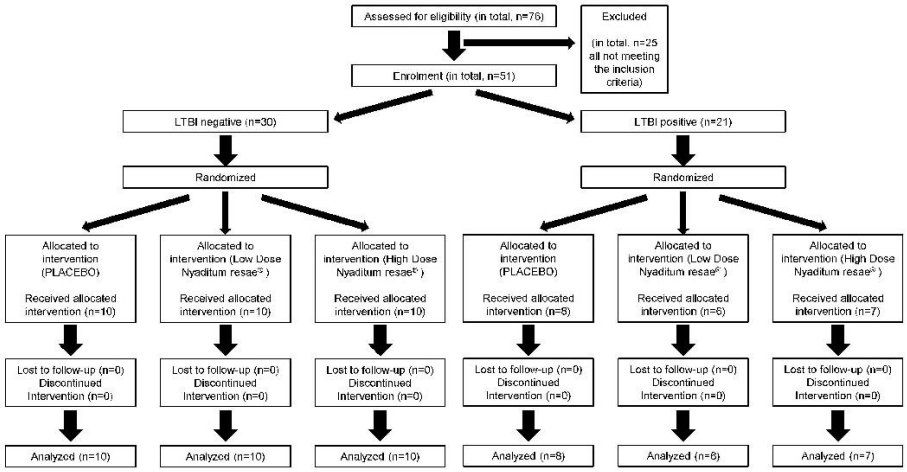

Fig 1. Chart representing the participant flow of the CT. From 76 people interviewed for eligibility, 51 could be included. All the 51 were randomized and allocated to treatment, receiving it, being fully followed-up, and ending the trial and being analysed.

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2.3 Interventions

After the inclusion period, the volunteers were randomized in a proportion 1:1:1 to receive either placebo or the Nyaditum resae® (NR) in low (10^4) or high (10^5) doses, stratified by TST status (Fig 1). The trial was originally designed to allocate 10 subjects per treatment group and TST status. Once the groups with TST-negative were fulfilled, the screening process was focused in looking for TST-positive subjects. NR was supplied by the sponsor (Manremyc S.L., Manresa, Catalonia, Spain) as a preparation of drinkable vials containing heat-killed *Mycobacterium manresensis* dissolved in distilled water. NR was produced under Good Manufacturing Practice by the Laboratory Reig Jofre (Sant Joan Despí, Catalonia, Spain). Placebo was identically supplied and formulated except that it only contained distilled water. Only a single batch of both NR and placebo were used in the whole study. The distribution of the treatment vials for the CT was carried out by the Clinical Research Organisation (CRO) of the study, Fundació per la Lluita contra la Sida -FLS Research Support- (Badalona, Catalonia, Spain), where the entire study treatment received was inventoried and accounted for throughout the study.

Once randomized, volunteers received a box with 14 drinkable vials with one of the 2 doses of NR or Placebo at the Phase I Unit of the Clinical Pharmacology Department, supervised by the Investigators and following the random plan specified in the study protocol. They drank one vial every day at breakfast during 2 weeks.

Every subject included in the trial had the right to withdraw at any moment by immediately contacting the investigators to inform them, without any obligation to provide reasons. This participant was supposed to be replaced by another, except in the case the trial had to be ended because of safety issues, and the new participant would have received the corresponding treatment of the subject he or she replaced. If a participant was withdrawn because of an adverse

Table 1. Chronogram and monitoring plan.

	Selection		Pre administration	Administration	Follow-up		
Visit	**Selection**		**Baseline**		**w1**	**w2**	**w6**
Day	-28	-25	0		7	15	42
Week	-4	-4	0		1	2	6
Window (days)	±14		0		±3	±3	±3
Subject Information, Informed consent	X						
Inclusion/Exclusion criteria	X		X				
Medical History	X						
HIV test	X						
Tuberculin Skin Test (TST)	X[A]	X[B]					
Chest X ray		X[C]					
Pregnancy test in serum	X		X				X
Randomization			X				
Treatment administration				X			
Physical exam and vital signs	X		X		X	X	X
Biochemistry & Hematology	X		X[D]		X	X	X
Immunogenicity sample			X		X	X	X
Volunteer diary*				X	X	X	X[E]
Concomitant medication	X		X		X	X	X
Adverse events	X			X	X	X	X

[A] Previous TST was acceptable and not repeated when positive in the last 5 years or negative in the last 6 months.

[B] TST readout.

[C] In the case of Positive TST.

[D] Not repeated if selection's analysis performed in the previous 15 days.

[E] Collection of Volunteer's Diary.

* Volunteer's Diary records every day: day and hour of the treatment intake; stool deposition (number/day) and aspect (soft/hard); nausea (degree 1 to 3); vomits (degree 1 to 3); abdominal pain (degree 1 to 3); other adverse events (degree 1 to 3). Degree 1: mild; degree 2: moderate; degree 3: severe.

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event, he or she would have been followed-up by the Investigators until resolution or reaching a clinically stable endpoint. The investigators had the right to decide to terminate or suspend the trial without prior agreement of the sponsor, by promptly informing the trial subjects (assuring them appropriate therapy and follow-up), the sponsor and the regulatory authority.

2.4 Objectives

This trial aimed to evaluate 1) the global tolerability of the probiotic Nyaditum resae® and 2) the effect on specific memory regulatory T cells (Treg) cells population.

2.5 Outcomes

After the administration of the first drinkable vial, each volunteer was followed-up during 6 weeks. The monitoring plan (Table 1) shows every procedure of the follow-up planned during the study.

The volunteers were monitored by the investigators team of the Phase I Unit in order to perform the following safety evaluations: 1) record of adverse events, both gastrointestinal and non-gastrointestinal, being reported by the subject either spontaneously or after questioning or looking at the Volunteer's Diary, during the first 4 weeks of the monitoring (Table 1); 2) vital constants; 3) physical examination; 4) laboratory safety and immunogenicity tests.

AE detected by the investigator through interrogation or reported by the subject during the defined period of collection were recorded. AE was considered as any unwanted medical event in a subject in a clinical trial, regardless of its relationship with the intervention under evaluation. A serious AE was defined as cause of death, life threatening, requiring inpatient hospitalization, producing disability or incapacity persistent or significant or threatening the patient. The investigators determined the relationship between the study treatment and the AE as 'not related', 'unlikely', 'possibly', 'probably', and 'definite' according to a predefined algorithm based on the modified Karch and Lasagna algorithm used by the Spanish Pharmacovigilance System [23,24] and after a consensus reached between the clinical pharmacologists study investigators. Laboratory abnormalities were graded following the Toxicity Grading Scale Guidance provided by the Food and Drug Administration (FDA) [25]. The main safety analyses have been focused on 'possibly', 'probably', and 'definite' classified AE.

Blood was extracted from the volunteers by the nurse of the Phase I Unit, under fasting conditions and before any other procedure was done, at the time-points indicated in the chronogram (Table 1). The samples were properly labelled and sent to the hospital laboratories for laboratory safety testing (15 mL) and to the Experimental Tuberculosis Unit (UTE) (8 mL) for the immunogenicity testing as soon as possible. The Departments involved in the safety laboratory results (Haematology, Clinical Analysis and Microbiology) as well as the UTE, are accredited by ISO 9001 procedures. The CRO of the study (FLS Research Support) monitored all the study in order to ensure the use of standard terminology and the collection of accurate, consistent, complete and reliable data.

Blood samples were tested for circulating cell counts (erythrocytes, leucocytes and platelets), Hb, haematocrit, fasting glucose, aspartate aminotransferase, alanine aminotransferase, g-glutamyl transpeptidase, alkaline phosphatase, total bilirubin, direct and indirect bilirubin, urea N, creatinine, glomerular filtration rate, Na and K.

The reliability of the Immunogenicity data was ensured by the performance of all assays always made by the same technician (previously trained during a 6 month period) and under supervision. Accuracy of the performance of the techniques as well as the values obtained were ensured by following strict specific procedures (Standard Operating Procedures SOPs) that were previously designed and set up specifically for this CT and always under blinding premises. The sponsor also used an external auditing to review the following issues: data management and statistics, both the UTE and the Phase I Unit, source data / source documents and the Investigator File and the Trial Master File.

Measure of specific Tregs was done according to previous studies [14,26–28]. Peripheral blood mononuclear cells (PBMC) were isolated in cell preparation tubes (CPT Becton Dickinson, BD) with sodium citrate according to the manufacturer proceedings, and processed immediately. Protein Purified Derivative (PPD) batch 49 (Statens Serum Institute, Copenhagen, Denmark) was used alone at a final concentration of 10 μg/mL to measure antigen specific responses. 1 x 10^6 cells/well were non-stimulated or PPD-stimulated in a complete medium (RPMI-L-Gln, 10% heat-inactivated FCS, 10 U/ml penicillin, 10 U/ml streptomycin, 1 mM sodium pyruvate, 0.025 mM 2-ME) for 7 days at 37˚C and 5% of CO_2. The phenotypic analysis was performed by flow cytometry, using the following antibodies for membrane staining: PerCP-Cy 5.5 mouse anti-human CD3, APC-H7 mouse anti-human CD4, PE mouse anti-human CD25 (BD Biosciences) and Brilliant Violet 421 mouse anti-human CD39 (BioLegend). Data acquisition was performed using an LSRFortessa flow cytometer (BD Biosciences) and analysed with FACSDiva software (BD Biosciences) (Fig 2).

None of the Immunogenicity assessments performed in the present CT were standard procedures. As no cut-off exists for those non-validated and research-only techniques, a statistical

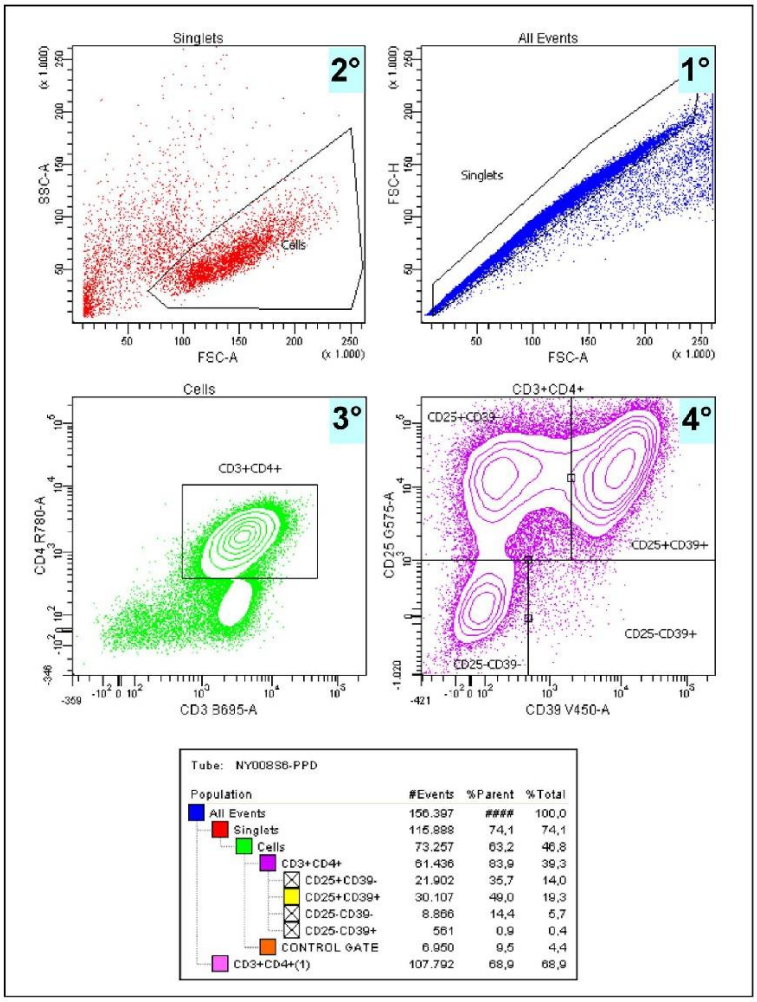

Fig 2. FACS analysis strategy used allowed us to find 4 different types of populations. CD25+CD39-: mainly effector Tregs (80–90%) [29]; CD25+CD39+: memory Tregs [26]; CD25-CD39-: Non Treg Effector cells; CD25-CD39+: ex-Treg cells mainly IL-17 producers [26].

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difference ($p<0.05$) between the time-points among the same treatment group has been considered enough to prove the reactivity of the treatment.

2.6 Sample size

Given that this clinical trial was an exploratory study of the first administration in humans, basically intending to demonstrate the safety of the probiotic, a formal predetermination of sample size based on numeric or statistic criteria was not made. In any case, the numbers shuffled in the trial (51 subjects, 18 placebo and 33 of treatment) were within the standard for this type of studies.

2.7 Randomization and blinding

A random allocation scheme in which each participant has equal likelihood of being assigned to treatment versus placebo groups (ratio 1:1:1) was used. The CRO (FLS Research Support) generated an allocation list by blocks using numbers drawn from the uniform distribution. Allocation was performed considering the stratification for TST-positive and TST-negative.

Treatment is masked, meaning that neither the investigator nor the volunteer knows if the vial contains NR or Placebo. Both treatments were physically identical with the same excipients. Labelling was also identical, indicating the study code, the numerical identification of the subject and the data referred to the promoter, administration route and posology.

In an emergency case that would require to open the double blind, the researcher had to contact to the CRO which would inform the composition of the treatment of the subject. The investigator had to write a justification explaining the reasons for opening the double blind.

Investigational treatment kits were conserved in UTE and transferred to the Phase I Unit until their use. Both places performed temperature log on a daily basis.

2.8 Statistical methods

Results were expressed as median with the interquartile range (IQR), or as otherwise specified. All the analyses corresponding to the demographic variables, basal characteristics, as well as the variables to evaluate the Immunogenicity were made with the population per protocol (all randomized subjects who met the selection criteria, received the study treatment, and did not present major protocol deviations). All the analyses corresponding to the security variables were carried out in the safety population (all participants who received at least ten doses of the treatment).

Continuous variables were compared using non parametrical tests, Mann-Whitney and Kruskal-Wallis for non-paired and Wilcoxon test for paired data. The Chi-square or Fisher's exact test, as appropriate according to the variables distribution, was used to compare categorical variables. Given the exploratory nature of this study of phase I, no multiplicity adjustments were considered. The analysis was performed using SPSS(®) v. 15 (SPSS Inc., Chicago, IL, USA) and the level of significance was established at the 0.05 level (two-sided).

3 Results

3.1 Participant flow and recruitment

The CT lasted 5 months (from 31th of March to 29th of August 2014) from the data the first participant enrolled to the time the last participant finished. After screening a total of 76 volunteers during the whole CT, 51 were included and randomized. A total of 25 volunteers were excluded for not fulfilling the study's requirements: presenting hematologic abnormalities such as anaemia (3 participants) or thrombocytopenia (2), vaccinated 3 months before the CT

(2), and having an autoimmune disease (1). Recent vaccination and autoimmune disease were excluded as considered able to interfere with the immunological results. After groups with negative TST were completed, 14 screened participants had a TST negative result, and thus were not included. And finally, 3 participants were excluded because the recruitment period was promptly ended (Fig 1).

The other 51 volunteers were included and randomized. Eighteen participants were in the placebo group, 17 in the high dose NR and 16 in the low dose NR group.

Each volunteer was treated with the allocated intervention (NR or placebo) and followed-up for a total of 6 weeks. No loss of follow-up or discontinuation of the treatment occurred, with the all 51/51 volunteers being analysed at the end of the CT. None of the subjects included in the present CT decided to withdraw from the study, nor was removed by the investigators. All volunteers declared to take the whole treatment (14 vials on a daily basis).

All the results and data obtained from the volunteers during the whole study are considered confidential. Furthermore, the investigator's team certifies that to their knowledge all subjects were healthy and thus considered able to be included in the CT, in terms of fulfilling all the study's requirements.

The only protocol deviation from the study protocol occurred was the recruitment promptly stopped because the TST reagent (PPD RT-23) was out of stock (http://www.aemps.gob.es/informa/notasInformativas/medicamentosUsoHumano/problemasSuministro/2014/NI-MUH_20-2014-tuberculina.htm), resulting in a smaller sample size than expected (n = 60) in the TST-positive groups.

3.2 Baseline data

All demographic and clinical baseline characteristics for the 51 volunteers finally included in the CT, with the corresponding ratio or median with the interquartile range (IQR), are summarized in Table 2.

The anamnesis and physical exams performed to the volunteers during screening did not show any clinical significant abnormality. The results of the laboratory analysis of all the volunteers included in the study performed during the screening were in the normal range; otherwise, the investigators considered them without clinical significance.

3.3 Outcomes and estimation

3.3.1 Results of laboratory tests. The abnormal laboratory tests possibly or probably related to the investigational treatment recorded during the CT include kidney function,

Table 2. Demographic characteristics of the clinical trial.

	Placebo (n = 18)	Nyaditum resae® low dose (n = 16)	Nyaditum resae® high dose (n = 17)
Age (years)*	25.5 (22–36.3)	31 (23–44.3)	29 (21.5–42.5)
Gender (% men)	28%	43.80%	52.90%
Underlying disease (% yes)	88.90%	93.80%	94.10%
Height (cm)*	165.5 (160.5–170.3)	169.0 (162.5–175.3)	171.0 (163.5–179.5)
Weight (kg)*	65.7 (51.8–79.9)	70.1 (58.8–80.8)	67.4 (61.5–81.5)
Systolic blood pressure (mm Hg)*	111 (102–122)	116 (103–141)	113 (106–123)
Diastolic blood pressure (mm Hg)*	64 (58–67)	65 (58–77)	63 (59–69)
Cardiac frequency*	69 (60–79)	64 (57–72)	68 (60–75)
Respiratory frequency*	18 (16–20)	20 (17–20)	18 (13–21)

* median (IQR)

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hepatic enzymes, haematology and glycemia. No significant statistical differences were found when compared between groups (Table 3).

Seven participants (13.7%) presented an increase of bilirubin or transaminase values. All of the elevations were asymptomatic, mild and resolved in the following weeks or at the end of the trial.

Five participants (9.8%) presented a decrease of haemoglobin or an abnormal value of leucocytes, all of them were also asymptomatic, mild and resolved in the following weeks or at the end of the trial (excepting one volunteer in the placebo group with a probably myeloproliferative syndrome currently in study). A participant presented a moderate thrombocytopenia that spontaneously resolved within 5 weeks.

Hyperglycemia was recorded in 2 participants and hypoglycemia in one. These were also asymptomatic, mild and resolved in the following weeks or at the end of the trial.

3.3.2 Physical examination and vital signs. No anomalies regarding to vital signs were detected during the trial visits.

3.3.3 Adverse events. The 51 participants reported a total of 322 AE. The 92.5% (298) of the AE were mild, and only one was classified as severe but was considered improbably to be related to the investigational treatment (radial bone fracture after slipping and falling). No statistical differences were found when comparing the median (IQR) number of AE between the placebo group and both treatment groups (Table 4).

The 46.3% (149/322) of the overall reported AE were considered possibly or probably related to the investigational treatment. No AE were causally related as definite. The 47% of the AE probably or possibly related to the treatment (70/149) occurred in the placebo group, 29.5% (44/149) in the NR low dose group, and 23.5% (35/149) in the NR high dose group. None of them were severe (94% were mild and 6% moderate). The most common reported AE were gastrointestinal events (82%, 122/149).

The 78.4% (40/51) of the participants presented at least one gastrointestinal event (no significant differences were found between treatment groups, $p = 0.078$), reporting most frequently mild abdominal pain and/or increase of the stool frequency (in 35.3% of the participants each one [18/51]) (Table 3).

3.3.4 Imunogenicity assays. According to the work of Dwyer [26] differential expression of CD25 and CD39 on circulating CD4+ T cells distinguishes between memory Treg (CD25+CD39+) and pathogenic cellular populations that secrete proinflammatory cytokines such as IL-17 (CD25-CD39+) and that an increase in the latter population is related with a decrease on the Treg population. Our data in the TB murine model of TB demonstrate that the evolution from LTBI to TB is also related to an increase of this CD25-CD39+ T cell population through the production of IL-17 [22]. FACS analysis strategy used allowed us to identify 4 different types of cells. **CD25+CD39-**: mainly effector Tregs (80–90%); **CD25+CD39+**: memory Tregs; **CD25-CD39-**: Non Treg Effector cells; **CD25-CD39+**: ex-Treg cells mainly IL-17 producers.

Figs 3, 4 and 5 show the evolution of the PPD stimulated cells. Essentially both TST-negative and TST-positive volunteers treated with NR experimented a global increase on Treg response, showed in both populations of CD25+CD39-, mainly effector Treg cells or CD25+CD39+ memory Treg cells, according to different authors [26,29].

Furthermore, the levels of Tregs were in general higher in TST-positive subjects during and after the treatment, being the group treated with the high dose NR the one that experienced more significant increases with time (Fig 6). Indeed, in the group of TST-positive subjects treated with placebo the baseline levels were really high and stable, probably reflecting an already protected population. In some cases a significant decrease in the CD25-CD39+ population has also been noted.

Table 3. Number of subjects presenting possible or probable related adverse events.

ADVERSE EVENTS			Participants		Placebo (n = 18)	Nyaditum resae® low dose (n = 16)	Nyaditum resae® high dose (n = 17)	P-value
			N	%				
Gastrointestinal	Abdominal pain		18	35.3	8	3	7	0.221
	Stool frequency	Increased	18	35.3	8	6	4	0.412
	Stool consistency	Decreased	14	27.5	5	5	4	0.883
	Nausea		9	17.6	6	2	1	0.084
	Diarrhoea		6	11.8	3	2	1	0.588
	Dyspepsia		4	7.8	1	3	0	0.087
	Stool frequency	Decreased	3	5.9	3	0	0	0.054
	Stool consistency	Increased	3	5.9	3	0	0	0.054
	Vomits		2	3.9	1	0	1	0.462
	Flatulence		2	3.9	1	1	0	0.434
	Constipation		1	2	1	0	0	0.346
	Epigastralgia		1	2	1	0	0	0.346
	Rectal Tenesmus		1	2	0	1	0	0.307
Non-Gastrointestinal	Hepatic enzymes alterations		7	13.7	1	4	2	0.206
	Hematologic alterations		5	9.8	3	1	1	0.378
	Cephalea, migraine		4	7.9	1	3	0	0.346
	Respiratory Infection		3	5.9	1	1	1	0.996
	Hyperglycemia		2	3.9	0	1	1	0.221
	Hypoglycemia		1	2	0	0	1	0.346
	Thrombocytopenia		1	2	0	1	0	0.307
	Others		5	9.8	1	3	1	0.378

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Table 4. Comparison of the median (IQR) number of AE by groups.

	Gastrointestinal	Non-Gastrointestinal	Total
Placebo	4 (2–6)	2 (1–3)	6 (4–9)
Nyaditum resae® low dose	2.5 (1.3–4)	2 (1.3–4.5)	4.5 (4–9)
Nyaditum resae® high dose	2 (1.0–3.5)	2 (1–4.5)	5 (2.5–7.5)
P-value	0.057	0.444	0.467

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Protection index as assayed by Dwyer [26] was also determined as a ratio between the data obtained by the ratio of stimulated and non-stimulated CD25+CD39+ and CD25-CD39+ cells. In this case, even when naturally Placebo groups can have increased levels of Tregs, only NR treated groups experienced an increase in this index, maybe relating to a "real" protection, or at least a better cellular distribution able to avoid a pro-inflammatory milieu. The levels were higher in TST-positive volunteers and after being treated with the highest dose of NR (Fig 7).

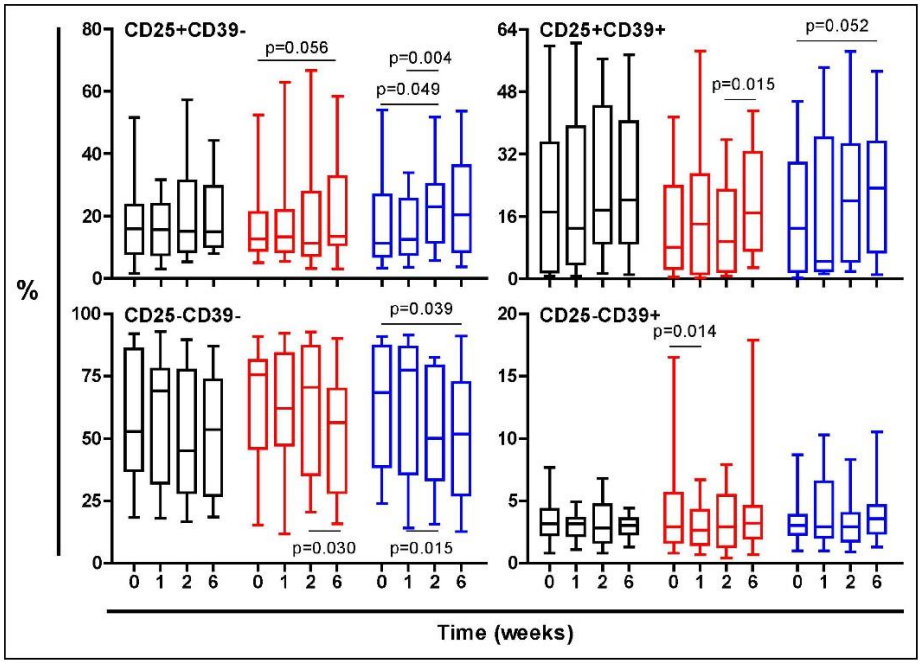

Fig 3. Evolution of the PPD-stimulated T cells in all volunteers regardless their TST status. Treatment groups are represented in black, red and blue, corresponding to Placebo, low dose and high dose Nyaditum resae® respectively. P- values calculated by Wilcoxon matched pairs test. Plots are shown with median, IQR and minimum/maximum values.

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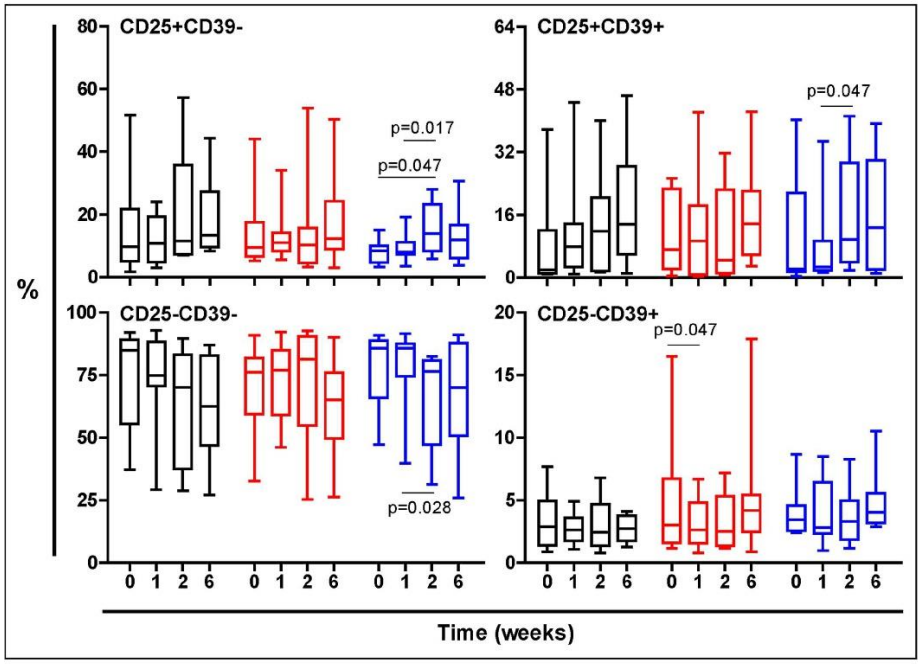

Fig 4. Evolution of the PPD-stimulated T cells in TST-negative volunteers. Treatment groups are represented in black, red and blue, corresponding to Placebo, low dose and high dose Nyaditum resae® respectively. P- values calculated by Wilcoxon matched pairs test. Plots are shown with median, IQR and minimum/maximum values.

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4 Discussion

The objectives of this CT were to demonstrate the safety of the new product Nyaditum resae® and evaluate its effect on specific Regulatory T cells (Treg) cells population.

Surprisingly, the median number of AE in placebo treated participants was higher than in groups treated with the investigational treatment, but no significant differences were found when comparing between groups. More than two-thirds of participants presented a gastrointestinal event with a similar distribution between groups. Gastrointestinal adverse events are very unspecific and usually frequent when the investigational treatment is administered orally. Moreover, gastrointestinal events were those we could mainly expect because of administration route (oral) as well as the bacterial origin of the probiotic. As all the participants were informed about this potential effect, this could explain the high rate of AE considered probably and possibly. On the other hand, it is well known that the administration of placebo can induce AE, related to the informed potential AE of the investigational product. It is logical then the fact that placebo side effect profile is mostly similar to the side effect profile of the

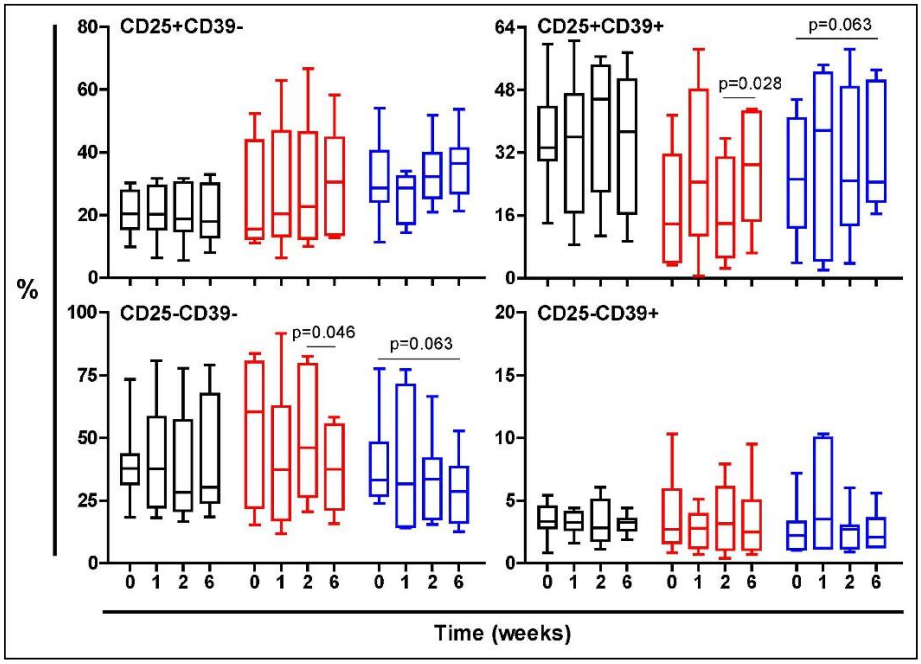

Fig 5. Evolution of the PPD-stimulated T cells in TST-positive volunteers. Treatment groups are represented in black, red and blue, corresponding to Placebo, low dose and high dose Nyaditum resae® respectively. P- values calculated by Wilcoxon matched pairs test. Plots are shown with median, IQR and minimum/maximum values.

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investigational treatment [30]. Variability on the adverse events profile in healthy volunteers has been related to personality and lifestyle of volunteers. Although randomisation, women were more frequent in the placebo group than in the investigational treatment groups. Somatization and hypocondriacal features, and adverse reaction in general, are predominantly related to women [31], which could explain the highest incidence in this group.

Laboratory abnormalities such as liver or glycemia alterations were more frequent in the probiotic groups than placebo, without significant differences found. Contrary to these alterations, leucocytes abnormalities were more frequent in the placebo groups, also without significant differences. The small sample size of this study is a limitation and could difficult the possibility to find statistical significant differences between groups. A bigger sample size could be necessary to elucidate the incidence and relation to treatment of those rare AE.

In the present clinical trial we have demonstrated the safety of the NR, because, although the AE were frequent, they were mild and spontaneously resolved within the following weeks, and no significant differences were found when compared between groups.

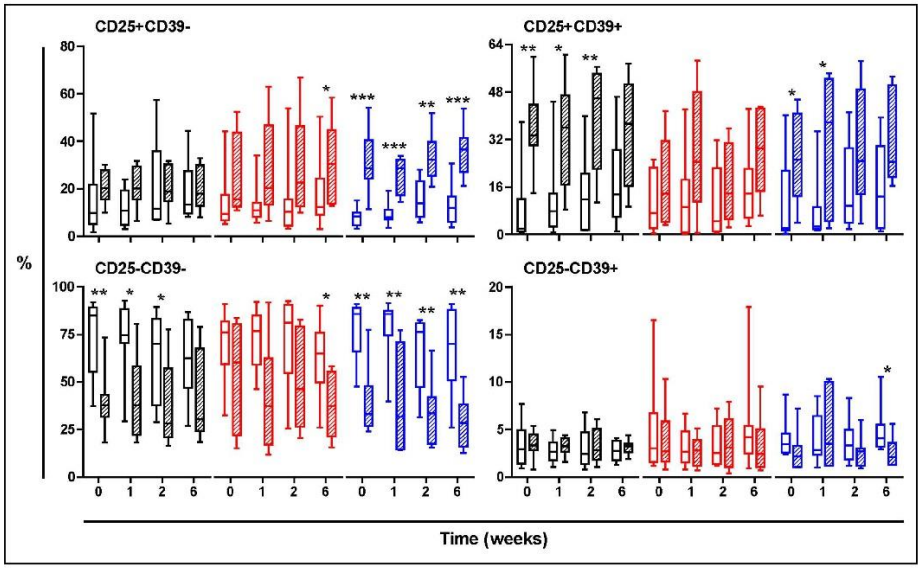

Fig 6. Evolution of the PPD-stimulated T cells according to TST status. Comparison between TST-negative and TST-positive volunteers. Treatment groups are represented in black, red and blue, corresponding to Placebo, low dose and high dose Nyaditum resae® respectively. P- values calculated by Mann Whitney test, expressed in intervals: * from 0.05 to 0.01; ** from <0.01 to 0.001 and *** <0.001. Plots are shown with median, IQR and minimum/maximum values.

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Data provided show for the first time that the administration of low doses of heat-killed non tuberculous mycobacteria is able to induce both effector and memory specific Tregs. The induction of Tregs by probiotics is well known [32] even when using heat-killed probiotics [33]. It has been recently demonstrated that in some cases the effect induced by probiotics is not affected by its viability [34], emerging the concept of "paraprobiotic" or "ghost probiotics" [35] which have clear advantages in terms of production, stability and security of the product. Shinkai *et al* [36] have recently evaluated the usefulness of the oral intake of heat-killed *Lactobacillus pentosus* strain b240 as immunoprotective by reducing the incidence rate of the common cold in elderly adults. Previously, Zhang *et al* [37] demonstrated that the administration of heat-killed *Enterococcus faecalis* FK-23 was able to attenuate the Th17 response in the lung, thus suppressing the allergic response in a murine model of asthma induced by ovalbumin.

Recently it has been demonstrated that IL-17 plays a paramount role in the evolution from infection to disease in a TB model in mice, by increasing the inflammatory response in the granuloma through a neutrophilic infiltration, followed by the coalescence of different lesions [8,9,22]. Furthermore, it has been demonstrated that mice from a strain that never develops TB had higher levels of Tregs and when these mice were treated with mAbs anti CD25 for the Tregs depletion, TB appeared [22].

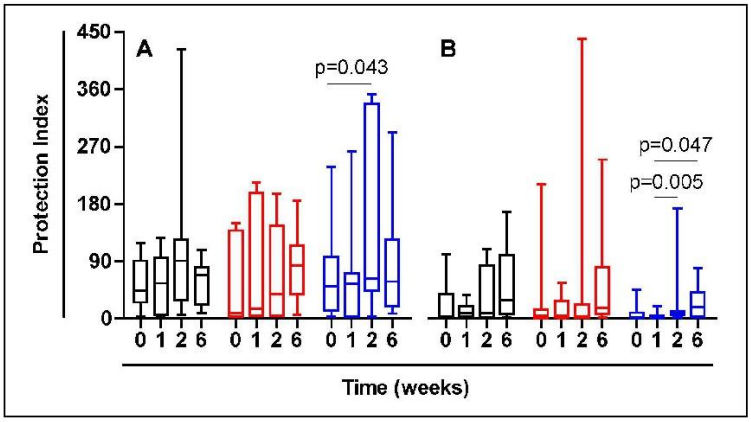

Fig 7. Protection index as assayed after Dwyer [26] determined as a ratio between the data obtained by the ratio of stimulated and non-stimulated CD25+CD39+ and the obtained by the ratio of stimulated and non-stimulated CD25-CD39+ cells. Results divided according TST status: (A) TST-positive, (B) TST-negative. Treatment groups are represented in black, red and blue, corresponding to Placebo, low dose and high dose Nyaditum resae® respectively. P-values calculated by Wilcoxon matched pairs test. Plots are shown with median, IQR and minimum/maximum values.

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After the induction of a low dose tolerance with a daily oral administration of heat killed mycobacteria, progression towards TB was abrogated as demonstrated by increasing from 30 to 50% the survival time of the mice. When translating this phenomenon to humans, another local defence mechanism not present in mice must be taken into account: the septae that surrounds secondary lung lobules able to encapsulate lesions and abrogate its growth [38].

So far, the induction of Tregs after Mtb infection has been understood as deleterious. Tregs have been claimed to be responsible for a weaker cellular immune response, stopping Th1 cellular proliferation [29] that would fuel the progression from LTBI to TB [39,40], and inducing a limited protection after BCG vaccination [41]. Further investigations have given them a neutral role [42–44] mostly in models where protection was measured through the reduction of the bacillary load. Recently, when also evaluating the role of the pathology, some authors have seen a protective role of the presence of Tregs linked to a control of the inflammatory response [45].

The presence of *M. fortuitum* complex bacilli in the tap water has been demonstrated by different authors in different regions of the planet [46–51] including the region where the clinical trial was run [52]. This was the reason for developing NR as a supplement food once an equivalent protective effect than the induced by the HKMtb was demonstrated [22]. On the other hand this fact might had played a role in our trial as there was a risk that subjects from the placebo group had drank tap water with *M. fortuitum* bacilli, or even that treatment with the investigation product boosted the immune response of subjects that has been previously in contact with this bacillus. When designing the trial, we assumed to include this risk, as it is part of real life.

NR has also demonstrated its capacity to induce specific Tregs and also memory Tregs (CD39+). Overall, the highest dose was more clearly related to an increase of this cell population.

TST status has also shown to play a major role. TST positive participants had in general more Tregs than TST-negative, both in Placebo or NR treated subjects. This fact was expected because Mtb infection itself also induces the presence of Tregs. Interestingly, the Placebo group of TST-positive subjects had a very high number of Tregs from the baseline, although it did not experiment any increase during the clinical trial. In this regard we could theorize that those subjects have already been protected, and it can be hypothesized that this is either because they have had contact with non-tuberculosis mycobacteria or because the Mtb infection has naturally triggered a higher proportion of Tregs in those volunteers. This latter hypothesis would be in concordance with the concept of genetic resistance [53,54]. In fact, in our experience, we have been able to demonstrate higher Treg % in those mice strains that better resist the Mtb infection (data not published).

Regarding the data presented to monitor Treg response, we mainly presented raw data of PPD stimulated PBMCs, following the methodology of other authors [28] that have observed a better response-window. In fact, non-stimulated PBMCs have shown very low levels of Tregs through time in all treatment groups. When we analysed the protection ratio as a measure of protection firstly described by Dwyer et al [26], we have been able to show a significant increase through time in the group of the highest dose of NR (10^5 CFU) in both TST-positive and negative participants.

In conclusion, data supports that the administration of Nyaditum resae®, a product based on heat killed bacilli of *Mycobacterium manresensis*, is able to induce a specific increase of the Treg response including memory cells, with an excellent safety profile, thus being a new tool to reduce the risk of the progression from latent infection to TB in humans.

Supporting information

S1 File. Consort checklist.
(PDF)

S2 File. Nyadatreg protocol (English version).
(PDF)

S3 File. Annex 1 & 2: Data collection notebook & SAE form.
(PDF)

S4 File. Annex 4: Technical file of the product.
(PDF)

S5 File. Annex 5: Participant information sheet.
(PDF)

S6 File. Annex 6: Informed consent for participants.
(PDF)

S7 File. Annex 7: Patient diary.
(PDF)

S8 File. Annex 8: Inclusion diagram.
(PDF)

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Author contributions

Conceived and designed the experiments: EM AMB ALA AV NP PC CV PJC.

Performed the experiments: EM AMB ALA AV YS NP PC CV PJC.

Analyzed the data: EM AMB ALA AV NP PC CV PJC.

Contributed reagents/materials/analysis tools: EM AMB ALA AV YS NP PC CV PJC.

Wrote the paper: EM AMB NP PC CV PJC.

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