PhD Thesis
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Exploring correlates of protection in a tuberculosis vaccine context

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Submitted February 9th 2018

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PREFACE

This PhD thesis covers four studies performed in the period February 2015 – February 2018.

The thesis was submitted to the Faculty of Health and Medical Sciences, University of Copenhagen, Denmark on February 9th 2018.

The work was performed at Department of Infectious Disease Immunology and the International Reference Laboratory of Mycobacteriology, Statens Serum Institut, Denmark, in collaboration with Department of Infectious Disease Immunology, Hvidovre Hospital, Denmark under the supervision of Thomas Benfield, MD, Prof.; Morten Ruhwald, MD, PhD and Erik Svensson, MD, PhD. The project was partly funded by EC HORIZON2020 TBVAC2020 (Contract no. 643381)

The thesis is based on the following four original manuscripts including one video demonstration:

I. A characterization of the human immune response to MPT70 peptides
   Holm LL., Blauenfeldt T., Aagaard C., Mortensen R., Graff S., Seersholm N., Andersen P. & Ruhwald M. (Manuscript ready for submission)

II. A suction blister protocol to study human T-cell recall responses in vivo

   Video available at:
   https://www.dropbox.com/sh/jrub2va7b6yjlua/AABBrRAVgBBJl0ouueYhE2eBa?dl=0

III. Optimisation of a murine splenocyte mycobacterial growth inhibition assay using virulent Mycobacterium tuberculosis

IV. Development and qualification of a human Mycobacterial growth inhibition assay using Mycobacterium tuberculosis
    Holm LL., Jensen C., Blauenfeldt T., Graff S., Seersholm N., Andersen P, Svensson E., Benfield T. & Ruhwald M. (Manuscript ready for submission)
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I would like to express my gratitude to EC Horizon2020 for funding parts of the project; to the staff at INFIMM and members of the human immunology group through the years for showing me how badass scientists can be; to Janne, Paula, Solveig and Lone for generously sharing happy vibes; to the staff at IRLM for providing such a lovely atmosphere and to the doctors and nurses at Gentofte Hospital, especially Nete Wrona, for hospitality and help with inclusion of patients.

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Thanks to my beautiful friends and family for love and support.

Thank you Oskar for simply being awesome.

Finally and most of all, I thank you Kaare, for providing me with love and shelter through this crazy time. I am forever grateful for your honesty, compassion and ongoing efforts to keep me balanced and close to you.

Line
February 8th, 2018
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<td>Ag</td>
<td>Antigen</td>
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<td>Alveolar macrophages</td>
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<td>Antigen presenting cell</td>
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<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
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<td>CAF01</td>
<td>Cationic Adjuvant Formulation 01</td>
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<td>CCR7</td>
<td>C-C chemokine receptor</td>
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<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
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<td>CXCR</td>
<td>C-X-C chemokine receptor</td>
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<td>CoP</td>
<td>Correlate of protection</td>
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<td>Dendritic cell</td>
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<td>Dimethyl dioctadecylammonium</td>
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<td>ICS</td>
<td>Intracellular stain</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>IGRA</td>
<td>Interferon gamma release assay</td>
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<td>iNOS</td>
<td>Inducible nictric oxide synthase</td>
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<td>LTBI</td>
<td>latent tuberculosis infection</td>
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<td>MDR-TB</td>
<td>Multidrug resistant tuberculosis</td>
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<td>MGIA</td>
<td>Mycobacterial growth inhibition assay</td>
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<td>Mycobacterial growth indicator tubes</td>
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<td>MHC</td>
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<td>NHP</td>
<td>Non-human primate</td>
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<td>Major secreted immunogenic protein 70</td>
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<td>Mtb</td>
<td>Mycobacterium tuberculosis</td>
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<td>NK</td>
<td>Natural Killer cell</td>
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<td>NTM</td>
<td>non-tuberculous mycobacteria</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<td>PET</td>
<td>Positron emission tomography</td>
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<td>PPD</td>
<td>Purified protein derivative</td>
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Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). More than 10 million people fall ill with TB, and over 1.6 million die from the disease every year. The current vaccine against *Mtb*, the Bacille Calmette-Guérin (BCG) vaccine, has variable efficacy in adults and development of new TB vaccines is a global priority. A major roadblock in TB vaccine development is the lack of a validated correlate of TB vaccine protection. The mycobacterial growth inhibition assay (MGIA) measures the summative capacity of host cells to restrict mycobacterial growth *in vitro*, and this assay has been suggested as a candidate for providing future correlates of protection.

The major objective of this PhD project was to explore human and murine MGIA in a TB vaccine context. Secondary objectives were to characterize the immune recognition of MPT70, a candidate *Mtb* antigen for TB vaccines, and describe practical aspects and applications of a cutaneous skin recall model in the context of vaccine evaluation.

In study I, we compared INF-γ responses and cytokine expression profiles in human MPT70 stimulated PBMCs with those of other differentially expressed mycobacterial antigens. In study II, we provided visual and written protocol for cutaneous purified protein derivative (PPD) recall and suction blister induction, and described and discussed characteristic features of T cells harvested by this method. In study III, we optimized a murine MGIA and demonstrated growth inhibition in splenocytes from mice vaccinated with BCG and the TB vaccine candidate H56-CAF01. In MGIA study IV, we described and optimized a human MGIA, but we never established proof of concept for growth inhibition induced by BCG vaccination in humans. Addition of cells isolated from PPD-inflamed skin to MGIA cultures did not result in increased Mtb control. In both study III and VI, we investigated T cell responses in Mtb infected MGIA cultures, but found no link between adaptive T cell immunity and growth inhibition.

Together the four studies exemplify the challenges and applications of single immune parameter analyses and functional assays in TB vaccine research. In study I, we concluded that MPT70 show promising features as a candidate latency antigen for TB vaccines. In study II, we demonstrated how high fractions of polyfunctional antigen-specific T cell are easily accessible via the skin. This method
have the potential to be applied in evaluations of novel TB vaccines or other T cell targeting agents. In study III, we found superior growth control by splenocytes from vaccinated mice corresponding to *in vivo* protection, but were never able to establish proof of concept for growth inhibition induced by BCG or latent Mtb infection in the human MGIA using a similar assay setup (study IV). These negative results might be explained by unrecognized technical error or limitations of the study population. However, as we were never able associate growth inhibition with adaptive T cell responses in either MGIA, the negative findings could also reflect inability of the assay to detect long-term immunity induced by vaccination. The underlying mechanism of growth inhibition in MGIA is not fully understood, and both innate and adaptive mechanisms have been suggested. In conclusion, more MGIA studies in are needed to clarify the relevance of this assay in TB vaccine evaluations.
SAMMENFATNING PÅ DANSK

Tuberkulose (TB) er en infektionssygdom forårsaget af Mycobacterium tuberculosis (Mtb). Sygdommen rammer mere end 10 millioner og dræber mere end 1,6 millioner mennesker årligt. Den eksisterende vaccine mod TB, Bacille Calmette-Guérin (BCG) vaccinen, beskytter ikke optimalt hos voksne, og der er behov for udvikling af nye vacciner mod TB. Udvikling af nye TB vacciner er vanskelig, fordi der ikke findes et brugbart korrelat til at estimere vaccinernes evne til at beskytte. En laboratorie metode, der måler immunforsvarets evne til at hæmme vækst af mykobakterier (MGIA metoden), er blevet foreslået som en potentiel kandidat til at kunne estimere vaccine beskyttelse i kliniske afprøvninger.

Hovedformålet i dette PhD projekt var at undersøge værdien af MGIA metoden i TB vaccinerede mus og mennesker. Sekundære formål var at karakterisere immungenkendelsen af et nyt potentielt vaccine antigen, MPT70, samt at beskrive praktiske aspekter og anvendelsesmuligheder for en model, hvor antigen specifikke T celler tilkaldes og høstes fra huden ved hjælp af vakuum vabler.


Tilsammen illustrerer de fire studier udfordringer og brug af immunparameter analyser og funktionelle metoder i TB vaccine forskning. I første studiet fandt vi ud af, at MPT70 har nogle lovende egenskaber som potentielt TB vaccine antigen. I andet studie viste vi, hvordan man nemt
kan isolere antigenspecifikke multifunktionelle T celler fra huden. Denne metode har potentiale til at indgå i evalueringer af nye TB vacciner. I tredje studie fandt vi ud af, at celler fra vaccinerede mus kan hæmme mykobakterier i MGIA og at denne hæmning svarer til vaccinebeskyttelse i levende mus. Det lykkedes os dog aldrig at påvise at celler fra BCG vaccinerede eller Mtb smittede mennesker kunne hæmme mykobakterie vækst ved MGIA metoden i det fjerde studie. De negative resultater kan skyldes tekniske fejl eller begrænsninger relateret til studiepopulationen. Vi var dog aldrig i stand til at bevise at væksthæmning var forårsaget af T celle genkendelse, og derfor er det også muligt, at MGIA ikke formår at detektere beskyttende immunologisk vaccine hukommelse. Den underliggende mekanisme for MGIA er endnu ikke afklaret, og andre studier har rapporteret mulige mekanismer inden for både tidlige og sene immunresponsor. Der er derfor brug for flere studier for at afgøre, om MGIA metoden er brugbar i en vaccine kontekst.
INTRODUCTION

Tuberculosis

Disease burden, transmission and pathogenesis

Tuberculosis (TB) is an infectious disease caused by the bacterium *Mycobacterium tuberculosis* (Mtb). TB has been epidemic for centuries and eradication of the disease has long been a global priority. Yet, TB remains the leading cause of death caused by a single infectious agent.\(^1\) In 2016, approximately 1.3 million people died from TB disease and an additional 374,000 died from TB/HIV co-infection.\(^1\) According to the World Health Organization (WHO), there were 10.4 million incident TB cases in 2016 with the majority of cases occurring in South East Asia, Africa and the Western Pacific region (figure 1).

TB is predominantly an airborne disease and transmission requires inhalation and deposition of mycobacteria in the airways and alveoli. People with pulmonary TB transmit disease by coughing and exhalation of mycobacteria in aerosolized droplet nuclei. Infectiousness depends on a range of factors including bacillary load, degree and character of exposure, clinical and behavioral characteristics of the person with TB as well as the susceptibility of the contact person.\(^2\)–\(^4\) Generally, people who share a close physical environment with a person with symptomatic pulmonary TB have a high risk of becoming infected, and the overall prevalence of *Mtbc* infection is around 50% among household contacts.\(^3\)

Figure 1. Estimated TB incidence rates, 2016. WHO global tuberculosis report, 2017.\(^1\)
Clinical manifestation and spectrum of disease

TB is known as a great imitator since the disease can affect almost all organs and present with a range of symptoms. Pulmonary TB accounts for around 70% of all TB cases and classical symptoms include fever, cough, hemoptysis, respiratory insufficiency, wasting and night sweats.\textsuperscript{1,5,6} However, Mtb can also spread to other locations and cause extra-pulmonary disease such as TB meningitis, TB lymphadenitis and abdominal TB.\textsuperscript{6,7} Active TB can be verified by imaging (chest X-ray or CT), sputum smear microscopy, Mtb culture or molecular tests.\textsuperscript{8,9} Diagnosis and monitoring of pediatric TB is especially challenging, because children more often present with extra-pulmonary or unspecific symptoms and are more likely to have paucibacillary disease.\textsuperscript{8,10,11}

The recommended treatment for drug susceptible pulmonary TB in adults without comorbidity is a multidrug regimen based on rifampicin administered over minimum six months.\textsuperscript{12} Multidrug resistant TB (MDR-TB) is an emerging problem in some parts of the world with roughly half a million people developing MDR-TB in 2016.\textsuperscript{1}

Traditionally, TB infection has been classified as either symptomatic disease (active TB) or asymptomatic latent TB infection (LTBI). LTBI is recognized as evidence of immune sensitization by Tuberculin skin test (TST) or Interferon gamma release assays (IGRAs).\textsuperscript{13,14} People with LTBI may receive preventive treatment with anti-TB drugs, most commonly isoniazid, depending on national guidelines and individual risk factors.\textsuperscript{15,16}

However, although a roughly estimated quarter of the world’s population is infected with Mtb not all infected people will develop TB disease.\textsuperscript{17,18} In fact, Mtb can persist for decades in healthy individuals without ever causing symptomatic disease.\textsuperscript{19,20} The life-time risk of progression from infection to symptomatic disease is estimated to be around 10% in low exposure settings with the highest risk occurring within the first year of infection.\textsuperscript{5,18}

The classification of active TB and LTBI is widely used in clinical decision making and in TB research, however Mtb infection is probably more complex and dynamic than this binary conception. Positron emission tomography/computed tomography (PET/CT) studies have revealed subclinical lung lesions in asymptomatic individuals, and transcriptomic studies have clustered signatures from people with active TB and LTBI, suggesting that certain individuals classified as having LTBI actually
have subclinical disease.\textsuperscript{21-23} The dynamic spectrum of Mtb infection and disease, including predisposing factors for developing active disease following primary disease and precipitating factors for LTBI progression are presented in figure 2.\textsuperscript{18} Other factors include age, sex and tobacco smoking.\textsuperscript{8,24,25}

\begin{center}
\begin{figure} 
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Demonstration of the spectrum of Mtb infection with possible predisposing (Prd) and precipitating factors (Prc) that may influence progression to disease. Adapted from Esmail et al, 2014.\textsuperscript{18}}
\end{figure}
\end{center}
The immune response during Mtb infection

The immunology of Mtb infection is complex, dynamic and involves both innate and adaptive mechanisms. Most of our knowledge comes from animal models, and containment of Mtb requires an immunological equilibrium that is still poorly understood. Immunological mechanisms thought to be involved in human and animal immunity in natural Mtb infection are described in the following sections.

Primary infection

Mtb is essentially an intracellular pathogen that survives and replicates in modified phagosomes within antigen presenting cells (APCs). During the primary infection, inhaled droplets containing Mtb deposit in the airway and alveoli. As not all individuals develop evidence of immune sensitization following Mtb exposure, mechanical as well as mucosa associated humoral and cellular components may play a preventive role before Mtb even reaches the host cells. Once deposited in the lungs, Mtb is phagocytosed by APCs, such as alveolar macrophages, dendritic cells (DCs), and neutrophils. Digestion of Mtb activates the APCs and induce secretion of antimicrobial peptides, chemokines and cytokines such as IL-12, IL-6 and TNF-α. This attracts more neutrophils, Natural Killer (NK) cells, γδ cells and monocytes to the site of infection. In symphony, these cells and cytokines cause the first local inflammatory response against Mtb and serve as reservoirs for Mtb replication.5,28

The adaptive response is initiated when Mtb infected activated DC’s migrate to draining lymph nodes and present mycobacterial antigen (ag) to naive T and B cells. Together with co-stimulatory and polarizing signals, such as IL-12 and IL-18, this leads to priming and expansion of ag specific effector T cell populations, primarily type 1 T helper (Th1) cells, which become the key mediators of in the response against Mtb (figure 3).29–33 The adaptive response is characterized by a long delay in onset, approximately 3 – 8 weeks following exposure in humans, and 11 – 14 days in mice following both primary and secondary infection.5,29,34,35 The slow onset is believed to be caused by inhibition of APC and T cell function and chemotaxis mediated by IL-10 and Foxp3+ T cell regulatory pathways, and Mtb itself is known cause further delay by inhibiting APC ag presentation and apoptosis, and DC migration to the lymph nodes.5
Once established, Th1 cells migrate back to the site of infection and initiate a response dominated by IFN-γ secretion, which promotes macrophage activation, and enhance microbicidal activity and granuloma formation.\textsuperscript{5,36} From here, the course of infection can take many directions. In most cases, primary Mtb infection is self-limiting as the immune response manage to contain or even sterilize the infection. Contained Mtb may persist in the lungs for many years in a dormant state.\textsuperscript{37} In some cases, the bacterium reactivates and cause disease at a later time point.\textsuperscript{37} Emerging disease is characterized by replicating mycobacteria, necrotic host cell death and destabilized granulomas allowing for dissemination of mycobacteria (figure 3).\textsuperscript{28,38}

While effector T cell populations undergo apoptosis following primary infection (or vaccine antigenic challenge), effector memory and central memory T cells persist, and upon reinfection these memory T cells can mount an accelerated secondary response.\textsuperscript{32} Memory T cell differentiation is described in detail in the Correlates of protection section.

\textbf{Figure 3.} Overview of the immune response in tuberculosis. Kaufmann et al, 2010.\textsuperscript{33}
The granuloma

The granuloma is often referred to as the hallmark of pulmonary TB. The classical granuloma is characterized by a circular solid structure with a central caseous necrosis. However, post-primary granulomata in humans and non-human primates are much more diverse, dynamic and display a range of structures, even within the individual lungs.\textsuperscript{28,39} The structure forms when recruited macrophages differentiate into epithelioid cells, lipid-rich foamy macrophages and merged multinucleated giant cells and join around Mtb infected cells. Together with recruited neutrophils, DCs, NK cells and fibroblasts, these specialized cells form a compact structure around the infected core. The granuloma periphery consist of a lymphoid cuff comprising T and B cells. Little is known about the cell turnover in granulomas during latent infection, however the balance between pro- and anti-inflammatory responses seem crucial for Mtb containment.\textsuperscript{38,40,41} During disease progression, more and more infected cells undergo necrosis, classically resulting in accumulation of caseum, cavitation and eventually collapse causing dissemination of Mtb.\textsuperscript{28,38}

Innate immunity

Innate immune cells such as macrophages, DCs, neutrophils and NK cells constitute the first line of defense in Mtb infection. Mycobacteria are recognized by pattern recognition receptors, such as Toll-like, Nod-like and C-type lectin receptors, that via multiple signaling pathways activate phagocytosis, inflammasome activation and autophagy or apoptosis.\textsuperscript{42,43} Traditionally, innate mechanisms are not associated with immunological memory, however recent studies have shown that epigenetic reprogramming in response to exposure may enhance the response by innate cells upon a second exposure. This concept is called trained immunity and has been associated with Mtb infection/re-infection as well as Bacillus Calmette-Guérin (BCG) vaccination.\textsuperscript{44–52}

Antigen presenting cells

Alveolar macrophages (AMs) are important throughout Mtb infection. They serve as reservoirs for Mtb and act in the first line of defense against Mtb infection by secretion of antimicrobial peptides and phagocytosis of mycobacteria. Mycobacteria are engulfed via receptor-mediated uptake, such as scavenger, complement and the mannose receptors.\textsuperscript{5} In humans, AM phagocytosis is primarily mediated by the complement receptor 4 and both Mtb uptake and the following cytokine response
are highly variable during the course of infection. Following uptake, the mycobacteria reside inside the phagosome. If autophagy is activated e.g. by IFN-γ and TNF-α stimulation, the phagosome matures and acidifies resulting in intracellular killing. However, viable Mtb may prevent this process by various evasion mechanisms (not covered here) and survive inside the phagosome.

TNF-α is induced by both APCs, innate and adaptive T cells and has a broad range of functions such as macrophage activation, chemokine production and autophagy. In murine neutralization and deficiency models, lack of TNF is associated with increased susceptibility, lethality and bacillary load, as it is believed that the cytokine has a key role in boosting Mtb intracellular killing via macrophage activation (via iNOS and autophagy) and in maintaining granulomata. In humans, anti-TNF therapy is known to increase the risk of TB reactivation, supporting a central need for TNF-α for Mtb containment. However, AMs from TB patients with high bacillary loads seem to be hyper reactive and AM in vitro studies show that high levels of TNF-α induced by virulent Mtb infection actually increases bacillary load and apoptotic cell death.

**Th1**

CD4+ T cells primed via the MHC class II pathway are believed be important in the immune response against Mtb. They promote inflammation and orchestrate the immune response during natural infection in animals and humans. The Th1 response is triggered by IL-12 secretion by activated macrophages in response to Mtb infection and Th1 CD4+ effector cells secrete the pro-inflammatory cytokines IFN-γ and TNF-α.

In mice, INF-γ producing CD4+ T cells are believed to be essential in the adaptive immune response against Mtb. This dogma is based on initial observations that CD4+ T cell, MHC-II and IFN-γ deficient mice are highly susceptible to Mtb disease. IFN-γ stimulates macrophages to kill Mtb via activation of various downstream pathways including iNOS, IFN-γ inducible ATPase and Vitamin D receptor signaling, phagosome acidification and apoptosis. Furthermore, IFN-γ is of special importance in the early infection, as it drives naïve T cell differentiation towards Th1 and stimulates the upregulation of MHC-I and II. NK cells, innate T cells and CD8+ T cells also produce IFN-γ, but secretion from these other cellular sources is not sufficient to control (early) infection.
In humans, the role of functional CD4+ T cell responses for Mtb control seems less clear and more ambiguous. For example, people with inherent deficiencies in IFN-γ and IL-12 signaling pathways are highly susceptible to disease caused by non-tuberculous mycobacteria (NTM) and BCG, but these individuals rarely suffer from actual Mtb disease.73–78 Likewise, people with HIV infection, characterized by progressive loss of functional CD4+ T cells, have increased risk of developing TB; however, this association is not directly related to CD4+ T cell counts during progression.79–82 In people with active disease, frequencies of IFN-γ (and TNF-α) producing cells are high at the local pulmonary site of infection and correlate with bacterial load, and high production of IFN-γ in cells from peripheral blood has been associated with progression to active disease, again probably reflecting bacterial load.36,64,83–85

In summary, Th1 cells and cytokines have been widely studied in human and animal TB models, and Th1 responses are commonly evaluated in TB immunogenicity studies (covered in more detail later). Most studies agree that the Th1 response is very important, but not sufficient, for Mtb control.

Th17

IL-17 producing CD4+ T cells (Th17) have gained increased interest in recent years as several studies have emphasized the importance of (a balanced) Th17/IL-17 response for protection against Mtb disease.34,86 The Th17 subset is polarized from CD4+ T cells in presence of various cytokines such as IL-6 and TGFβ (varies between species) and produce cytokines, mainly IL-17a, which promote the inflammatory response, recruit and activate neutrophils, and promote granulopoietic differentiation (figure 3).86 In addition, the Th17 response seems necessary for priming and maintaining an effective Th1 response and counteracts regulatory T cell inhibition.40,87–89

CD8+ T cells

The role of CD8+ T cells in TB is not as dominant as the CD4+ response and less studied, however the contribution of CD8+ T cells in TB immunity is not insignificant, as demonstrated by the impaired CD8+ T cell function associated with active TB disease in humans.90,91 Classically restricted CD8+ T cells recognize Mtb antigens (peptides) by MHC Ia and act by contact-dependent cytolysis of Mtb infected cells via FAS/FAS-ligand receptor activation or exocytosis of granules containing cell death mediators such as granulysin (in humans), granzymes and perforin (figure 3).5,32,92 Like CD4+ T cells,
CD8+ T cells also have the capacity to produce IFN-γ, TNF-α and IL-2 and differentiate into memory subsets.\textsuperscript{32,93} Furthermore, CD8+ T cells may play a regulatory role during Mtb infection.\textsuperscript{92} Finally, CD8+ T cells also include a range of non-classically restricted subsets that can recognize Mtb peptides in the context of HLA-E, CD1 and MR1 and have roles in both innate and adaptive immunity.\textsuperscript{32,92}

Counter regulation of the inflammatory response

Importantly, the Th1 inflammatory response is counter-regulated via inhibitory cytokines, in particular IL-10, secreted by Th2 and FoxP3+ regulatory T cells, and by inhibitory molecules (PD1) expressed by the Th1 effector cells themselves (figure 3).\textsuperscript{34,94–96} Excess regulatory activity is associated with impaired TB immunity in both humans and animals. However, some degree of regulatory response is required to counteract lung damage and granuloma disruption induced by overly active Th1 inflammatory responses.\textsuperscript{34,95}

B cells

B cells are present in high numbers in the granuloma, but traditionally they have not been considered important in TB immunology. However, there is increasing evidence that B cells play a role in modulation of protective responses against intracellular mycobacteria.\textsuperscript{56,97–100} B cells have the ability to process and present antigen to T cells, modulate inflammation by IL-10 secretion and secrete antibodies. Antibodies can bind directly to mycobacteria followed by complement binding (by classical lection and alternative pathways) and mediate lysis or macrophage uptake (by opsonophagocytosis or Fc receptor binding).\textsuperscript{53,101} Furthermore, antibodies may contribute functionally to Mtb control by enhancing macrophage responses.\textsuperscript{100}
The murine TB model

Animal models are widely used in Mtb immunology and TB vaccine discovery research, and comprise a range of species such as non-human primates (NHPs), cows, guinea pigs, mice and zebrafish, which all differ in regard to pathology and immunity. This section focuses on the murine model, in particular the in-bred strain C57BL/6, which is the model used in manuscript III. The use of in-bred mice allows for setting up various experimental models and increase standardization between experiments and laboratories. However, the C57BL/6 model is limited by its poor resemblance to human TB disease. As mentioned, human Mtb infection is a dynamic spectrum ranging from cleared or latent infection to symptomatic disease classically characterized by heavy inflammation, lung tissue necrosis and cavitation. Conversely, C57BL/6 mice have a balanced immune response against Mtb and a rather limited pathology profile. Following low dose aerosol challenge, mice develop chronic pulmonary infection and gradually, but inevitably, fail to eliminate infection. Lung pathology consist of diffuse lesions with poorly organized granulomas that lack fibrosis and hypoxia, and Mtb readily disseminate to other organs such as the spleen and liver. BCG vaccination induce good protection in mice, however, vaccination only restrict bacterial growth and delay the course of disease, but do not prevent progression and death. Consequently, reduction of lung bacillary load following acute infection is the most common indicator of vaccine potency in mice.
TB vaccine development

TB incidence and mortality rates have slowly declined over the last decades, largely due to increased focus on TB case detection and management. However, additional strategies are needed to reach the WHO and United Nations vision to reduce the TB incidence rate by 90% by 2035 (from 2015).\textsuperscript{108} The development of a new vaccine to supplement or replace the current BCG vaccine is considered a cost-effective strategy for achieving this goal (figure 4).\textsuperscript{108-113} Moreover, development of a novel TB vaccine would reduce the financial and individual costs of drug resistant TB.\textsuperscript{1} Only one TB vaccine candidate, the MVA85A, has so far been evaluated for efficacy of in humans. Disappointingly, the vaccine did not prevent disease in BCG vaccinated South African infants.\textsuperscript{114} This chapter describes current rationales and strategies for TB vaccine development.

**Figure 4.** Desired decline in global TB incidence rates. Lines illustrate the projected acceleration of decline in global TB incidence rates by optimization of current and new tools.

Adapted from the END TB Strategy, WHO, 2015.\textsuperscript{108}
The Bacille Calmette-Guérin Vaccine

The only licensed vaccine against TB is the Bacille Calmette-Guérin (BCG) vaccine - an attenuated strain of *Mycobacterium bovis* (M. bovis).\textsuperscript{115} The BCG vaccine has been administered for over 90 years; it is safe to use in immuno-competent individuals and it is currently one of the most widely used vaccines.\textsuperscript{116,117} BCG vaccination effectively prevents disseminated and pulmonary TB disease in young children and the WHO recommends neonate BCG vaccination in high burden TB countries.\textsuperscript{117–119} However, BCG protection wane over time and has variable immunogenicity and efficacy in adults, depending on the population and geographical setting.\textsuperscript{116,120,121} While BCG may provide long-term protection in some low TB incidence settings, it only offers limited efficacy against pulmonary TB in adults living in high burden settings.\textsuperscript{122–125} In addition, sensitization with environmental mycobacteria or non-tuberculous mycobacteria (NTM) seems to interfere with BCG performance, or at least the detection of efficacy.\textsuperscript{119,126–128} Attenuation of BCG is caused by the loss of several gene segments, i.e. the region of difference 1 (RD1), that encode for virulence factors and T cell antigens such as the secretory antigenic target 6kD (ESAT-6) and culture filtrate protein 10 (CFP-10).\textsuperscript{126,129,130} Consequently, immune responses induced by BCG vaccination may differ from those occurring in natural infection and this potentially explains the limited induction of protective immunity.\textsuperscript{131,132} BCG vaccination induce both innate and Th1 responses in humans.\textsuperscript{46,51,121,133–135} Yet, despite the long history of BCG, knowledge on the mechanisms behind BCG immunity remains limited and there are no confirmed T cell correlates of BCG protection.\textsuperscript{116} Nevertheless, being the only TB vaccine available, the performance of BCG is often used for comparison in experimental TB vaccine studies and in models exploring correlates of protection. Furthermore, most novel TB vaccines are developed for boosting preexisting BCG-induced immunity, as described in the following sections.
Clinical TB vaccine development

Currently, there are at least 12 novel TB vaccine candidates in clinical development, of which 9 are in phase I or phase II (figure 5).\textsuperscript{1,136} The vaccines in clinical development can be classified as either mycobacterial vaccines or subunit vaccines. The mycobacterial vaccines comprise Vaccae and DAR01, which are both based on whole cell NTMs; MTBVAC is a whole cell Mtb with gene deletions to ensure safety; VPM 1002 is a recombinant M. bovis BCG; and RUTI\textsuperscript{R} is a detoxified fragmented Mtb contained in liposomes. Since the mycobacterial vaccines include a range of mycobacterial lipids and proteins and they induce diverse immune responses and may have various applications such as BCG replacement (i.e. VPM1002 and MTBVAC), prevention of TB disease or recurrence (DAR09, VPM1009, Vaccae) or immunotherapy in people with TB disease (RUTI, Vaccae).\textsuperscript{137,138} Strategies for specific vaccine strategies are summarized in table 1.

\textbf{Figure 5.} The global development pipeline for new TB vaccines, August 2017. WHO global tuberculosis report, 2017.\textsuperscript{1}
Subunit vaccines, on the other hand, are prime-boost vaccines designed to supplement or boost pre-existing immunity induced by neonatal BCG vaccination.\textsuperscript{26,138–140} These vaccines are composed of one or several recombinant Mtb antigens and formulated with adjuvant or expressed in viral vectors, a design which allows for directing and boosting specific immune responses as well as more controlled and safe manufacturing.\textsuperscript{141} Adjuvant formulation is needed to enhance the immunogenicity of protein vaccines. In example, the cationic liposome adjuvant CAF01, which is used in the immunization experiments in manuscript III, combines a delivery system (dimethyl dioctadecylammonium, DDA) with an immunostimulator (trehalose dibehenate, TDB) to induce strong Th1 and Th17 responses and create a long depot effect at the site of injection.\textsuperscript{141–143}

### Table 1. TB vaccine strategies. Adapted from the WHO Tuberculosis vaccine Development webpage.\textsuperscript{144}

<table>
<thead>
<tr>
<th>Prevention of infection</th>
<th>vaccines that can be given prior to Mtb exposure, in order to prevent initial infection and therefore disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevention of disease</td>
<td>vaccines that can be administered after exposure to Mtb, to people who are infected but may be asymptomatic and at risk of developing disease in the future. This type of vaccine would protect against manifestation of active disease and therefore reduce transmission</td>
</tr>
<tr>
<td>Prevention of recurrence</td>
<td>vaccines that can be administered after infection and treatment of Mtb disease, to prevent reactivation and subsequent transmission</td>
</tr>
</tbody>
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Subunit vaccines - the multistage approach

Historically, subunit Mtb vaccine antigens have been selected based on their ability induce strong immune responses and their differential expression between LTBI and active TB, with emphasis on Th1 CD4+ responses and to some degree CD8+ and Th17.\textsuperscript{136,137,145,146} Consequently, the subunit vaccines in clinical development today are characterized by a lack of antigen diversity. Five vaccines express the antigen 85 (Ag85 A/B) and two express ESAT-6. However, during natural infection, Mtb can enter a state of dormancy where replicative and metabolic activity is shut down and the bacterium becomes highly immune resistant.\textsuperscript{41,145} The alterations in Mtb gene expression result in a change in antigenic repertoire. For example, Ag85B is mainly expressed during early infection while ESAT-6 seems to be constitutively secreted (figure 6).\textsuperscript{147–152} This differential expression and
variation in antigen availability have great consequences for specific T cell differentiation and functional exhaustion (discussed in the next chapter).\textsuperscript{147,153}

Accordingly, recent subunit vaccines aim to target antigens represented at different stages of infection, including actively replicating and dormant forms.\textsuperscript{140,145,154} An example of such a multi-stage vaccine is the purified recombinant fusion protein H56 (Ag85B-ESAT-6-Rv2660c), which was used in combination with CAF01 for immunization experiments in manuscript III.\textsuperscript{143,149}

\textbf{Figure 6.} Antigen expression and induction of ESAT-6 and Ag85B specific CD4+ T cells during Mtb infection. Adapted from Moguche et al, 2017.\textsuperscript{147}
Correlates of protection

Development of TB vaccines from discovery to large-sized clinical trials is challenging, expensive and time consuming. A major roadblock in this process is the lack of a reliable biomarker for early vaccine triage and selection.\textsuperscript{155} As described in chapter I, immunity against TB is primarily cell mediated and IFN-\(\gamma\) producing Th1 cells are important, but not sufficient, for protection against TB. Though several biomarkers and biosignatures have been suggested, our knowledge on vaccine induced immunity remains incomplete and there are currently no validated correlates of TB vaccine protection.\textsuperscript{156} The scope of this chapter is not to review all proposed biomarkers for TB vaccine immunity and protection. The focus of the following sections is to provide some background information on the signatures and assays applied in manuscript I-IV: mainly Th1 responses, CD4+ T cell memory and the Mycobacterial growth inhibition assay (MGIA).

Definitions

WHO defines a biomarker as “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease”.\textsuperscript{157,158} In vaccinology, the term correlate of protection (CoP) is used for biomarkers that are statistically associated with vaccine induced protection against infection and/or disease.\textsuperscript{159,160} CoPs can be biologically responsible for vaccine induced protection (mechanistic CoPs) or merely predictive of protection without direct causality (non-mechanistic CoP).\textsuperscript{159,160} Potential markers of protective immunity during TB infection and disease (correlates of disease and risk) can be used to inspire discovery of candidate CoPs.

T cell memory, homing and differentiation markers

Ideally, TB booster vaccines aim to induce long-lived immunological memory capable of mounting boosted and accelerated adaptive response at any stage of Mtb infection. In recent years, induction of specific T cell subsets or profiles have been associated with long-lived protection in animal models and investigated as potential CoPs in human vaccine trials.\textsuperscript{59,161–166} These subsets can be described by their phenotype, homing potential and functional cytokine profile by intracellular stain (ICS) flow cytometry and proposed linear differentiation models (illustrated in figure 7).\textsuperscript{59,93}
Central memory T cells (T\textsubscript{CM}) have the ability to continuously proliferate, produce abundant IL-2 and pass through lymphoid tissue (high expression of CCR7 and CD62L).\textsuperscript{93,167,168} Conversely effector memory T cells (T\textsubscript{EM}) are found in peripheral lymphoid tissues and have low expression of CCR7 and CD62L.\textsuperscript{145} At their final stage of differentiation there are called terminal effector T cells and loose their ability to produce IL-2.\textsuperscript{93,147}

Several murine studies have shown, that polyfunctional CD4+ T cells (defined by simultaneous co-expression of two or more pro-inflammatory cytokines) are induced by both BCG and subunit vaccination, and that the presence or magnitudes of this polyfunctional response correlate with control of bacterial replication (reviewed in Lewinsohn et al, 2017).\textsuperscript{34,59,93,149,161,162,169,170} However, there is yet no evidence of polyfunctional T cells as a mechanistic correlate of protection in the murine model.\textsuperscript{59} In mice, protective immunity has also been associated with the ability of Mtb specific CD4 T cell to migrate into the lung parenchyma and interact with infected APCs.\textsuperscript{171–174} Compared with cells restricted to the vasculature, lung homing CD4+ T cell populations show
characteristic features, such as lower differentiation status, high IL-2 and IL-17 production and CXCR3 expression.\textsuperscript{171,172,174} Together these murine data suggest a protective capacity of less differentiated T cells reflected by superior proliferative, cytokine producing and homing abilities. Of note, these findings may be specific for the murine model, as defect lung homing ability was recently found not to be the limiting factor for T cell mediated immunity in granulomas of Mtb infected rhesus macaques.\textsuperscript{175}

Human studies aiming to examine associations between T cell subsets and TB protection often rely on peripheral blood samples and \textit{in vitro} stimulation assays. The study subjects are typically recently vaccinated people or persons with Mtb infection. Such studies have associated IL-2 producing T cells with Mtb containment in LTBI, whereas IFN-γ/TNF-α producing T cells has been associated with increased antigen pressure as seen in active TB.\textsuperscript{93,147,164,176–180} This is in line with the linear T cell differentiation model (figure 7), where loss of IL-2 expression reflects CD4+ T cells driven towards advanced differentiation.\textsuperscript{93} This was also recently illustrated by \textit{Moguche et al} who associated continuous ESAT-6 exposure during Mtb infection with higher levels of T cell differentiation (figure 6).\textsuperscript{93,147} However, human studies are limited by the obvious lack of challenge models and low frequencies of circulating memory T cells. The method of cutaneous antigenic recall and sampling from suction blisters, described in study II, represent an alternative way for sampling and study of antigen specific T cells, which have proven the capacity to home to the skin and undergone expansion and differentiation \textit{in vivo}.\textsuperscript{181}

Possible Th1 CoPs in human vaccine trials

Based on the findings and models described above, T cell polyfunctionality and especially IL-2 producing T cells have been investigated as possible correlates of vaccine protection. Several studies have demonstrated that BCG and subunit vaccines induce polyfunctional T cells in children and adults (reviewed in \textit{Lewinsohn et al}, 2017).\textsuperscript{59} Some have argued for the importance of Th1 responses by emphasizing the contrast between increased BCG-specific IFN-γ responses in populations where BCG vaccination is known to induce high protection, and lower BCG responses in populations with less protection.\textsuperscript{85,120,156,182,183} Recently, \textit{Fletcher et al} reported that increased CD4+ cell numbers and
BCG-specific IFN-γ producing T cells associated with reduced risk of TB, using samples from BCG-vaccinated infants from the South African MVA85A study population. However, another recent prospective 2-year follow-up study comprising 5,726 BCG-vaccinated South African infants, found no correlation between risk of TB and the profile or phenotypes of BCG-specific CD4+ or CD8+ T-cells (including multifunctional T cells) following BCG vaccination. The MVA85A vaccine trials also exemplifies a discrepancy between T cell readouts believed to be associated with protection and actual protective efficacy in humans, as the vaccine induced prominent IFN-γ T cell responses and high frequencies of polyfunctional ag-specific T cells in initial immunogenicity studies, but subsequently failed to induce protection against Mtb disease in real life.

In conclusion, there is no solid evidence of correlation between Th1 correlates, including polyfunctionality, and vaccine induced protection in humans. Consequently, the TB research community have less faith in the classic Th1 single parameter readouts and look towards alternative or supplementary ways of evaluating vaccine efficacy.

Mycobacterial growth inhibition assays

Mycobacterial growth inhibition assays (MGIAs) have been proposed as promising candidates to predict protective efficacy of TB vaccines. MGIAs are functional in vitro assays that measure the summative effect of a range of immunological mechanisms and interactions. The assay readout is commonly reported as a reduction of mycobacterial growth in infected in vitro cultures. Comparison of samples obtained pre- and post-vaccination or samples from non- vs. immunized individuals, can be used to produce this measure of in vitro growth inhibitory capacity induced by vaccination, which possibly could reflect the protective capacity in vivo. As such, MGIAs may provide a more direct measure of vaccine induced anti-mycobacterial immunity and provide an alternative to the single immunological parameters commonly measured in preclinical and early clinical vaccine trials. In addition, the assay allows simultaneous evaluation of growth inhibition using different Mtb strains and facilitates bridging between human and animal research.

The basic principle of all MGIAs is to evaluate the ability of host cells to reduce mycobacterial replication in vitro. Several assay setups and readout methods have been suggested through the
years. Tanner et al recently proposed a classification of MGIAs based on choice of host cells, quantification method and the level of specimen manipulation (e.g. isolation or specific antigen stimulation of different cell phenotypes used in the assay) (figure 8).\textsuperscript{190}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Classification of MGIAs applied in TB vaccine studies. Tanner et al, 2016.\textsuperscript{190}}
\end{figure}

MGIAs have been applied in animal research using cells from mice, cattle and NHPs.\textsuperscript{143,191–199} Compared with \textit{in vivo} challenge models, MGIA endorse the principles of the 3Rs (replacement, reduction and refinement) in animal research.\textsuperscript{200,201} Firstly, MGIAs minimize animal suffering as Mtb infection occurs \textit{ex vivo}. Secondly, MGIAs allow for testing multiple samples from the same animal, i.e. infection of replicate samples with different Mtb strains, hereby reducing the animals required for experiments. In larger species, such as cattle and NHPs, MGIAs can be performed using peripheral blood obtained without sacrificing the animal.\textsuperscript{196,199}

The murine MGIA is the most widely used animal MGIA. The assay use splenocytes, either alone or in combination with Mtb infected bone marrow derived macrophages.\textsuperscript{190} Growth inhibition
following BCG- or experimental vaccination have been reported by several groups.\textsuperscript{143,192–194} Importantly, animal MGIAs provide an opportunity to link \textit{in vitro} readout with \textit{in vivo} protection. Several studies, including our study III, have found that growth inhibition using splenocytes from vaccinated mice was consistent with the level of protection \textit{in vivo} Mtb challenge experiments.\textsuperscript{143,193,194} These findings strengthen the relevance of MGIAs in both murine and human TB research.

Human MGIAs are commonly based on whole blood samples or peripheral blood mononuclear cells (PBMCs). MGIA has also been performed with bronchoalveolar lavage mononuclear cells from BCG vaccinated TB patients and contacts, however without any success.\textsuperscript{202} PBMC based MGIAs allow for use of cryopreserved samples and may have less variation caused by individual differences in cell numbers and hemoglobin.\textsuperscript{199} Conversely, whole blood assays include humoral components of immunity, which is not comprised in PBMC MGIAs unless cultures are supplemented with autologous serum or plasma.

One version of the whole blood assay, the BCG-\textit{lux} assay, uses recombinant reporter mycobacterial for bacterial enumeration.\textsuperscript{203,204} This assay have successfully discriminated differences in mycobacterial growth in purified protein derivative (PPD) -positive vs. PPD-negative adults, HIV-positive vs. HIV-negative children and following secondary BCG vaccination in adults and primary BCG-vaccination in South African children.\textsuperscript{203,205–207} Furthermore, the assay has been optimized for low-volume for pediatric studies.\textsuperscript{208} Another version of the whole blood assay use Mycobacterial growth indicator tubes (MGIT) to evaluate growth inhibition, a method that was originally designed for studies of anti-TB drugs and mycobacterial strains.\textsuperscript{209–211} The whole blood MGIT MGIAs have also been applied in BCG vaccination studies, where vaccination has been associated with growth inhibition in adults from low endemic settings.\textsuperscript{212,213}

The PBMC based MGIAs rely on infection of fixed numbers of PBMCs or co-cultures of pre-infected monocytes and lymphocytes. The PBMC assay design can also take more deconstructed forms, where infected monocytes are co-cultured with unstimulated or antigen expanded lymphocytes (the primary and secondary lymphocyte assay, figure 8).\textsuperscript{190} Growth inhibition has been detected in PPD-positive individuals by the primary lymphocyte assay, and following BCG vaccination in adults by all types of PBMC MGIAs, although the studies provide somewhat inconsistent reports on growth
inhibition after primary vs. secondary vaccination.\textsuperscript{206,213–216} In children, growth inhibition following BCG vaccination has been proved in PBMCs from UK infants, but not in South African infants.\textsuperscript{184,217} Only one study have found growth inhibition in adults where BCG vaccination was administered many years prior of sampling.\textsuperscript{213}

In recent years, there has been a movement towards standardization and harmonization of the non-human and human MGIAs.\textsuperscript{188,218} Recently, a range of human studies have been published using the MGIT incubator system, and a standardized murine MGIT MGIA protocol has been proposed.\textsuperscript{193,213,217,219} The methodology of the MGIT assay is described in detail in manuscript IV.

Manuscript III and IV describe the optimization and evaluation of murine and human MGIAs based on \textit{in vitro} infection with virulent Mtb and the MGIT methodology.
AIMS

I. To characterize human immune recognition of peptides from Mtb antigen MPT70

II. To explore practical aspects and applications of a PPD cutaneous recall method in a TB vaccine context

III. To describe and optimize a murine MGIA for novel TB vaccine evaluation

IV. To describe and evaluate a human MGIA using BCG vaccination for proof of concept
SUMMARY OF RESULTS

Manuscript I

In this short paper, we aimed to characterize immune recognition of predicted peptides from Mtb antigen MPT70 in people with LTBI (n = 11), TB (n = 16), BCG vaccinated (n = 15) and unvaccinated healthy controls (n = 8). We measured IFN-γ in fresh PBMCs cultures stimulated with pools of in silico predicted MPT70 peptides and characterized CD4+ T cell cytokine profiles by ICS flowcytometry. For comparison, PBMCs were also stimulated with peptide pools from an antigen expressed early during Mtb infection (Ag85B) as well as two latency antigens (EsxL and α-crystalline).

We found strong responses to MPT70 peptides in the LTBI group (median IFN-γ levels: 1181 pg/ml (IQR: 533 – 5595)) and IFN-γ release was comparable to median levels induced by Ag85B, EsxL and α-crystalline peptides (p = 0.056, levels not shown). The responder rate for MPT70 (IFN-γ >150 pg/mL) was 73 % (8/11), 50 % (8/16), 40% (6/15) and 13 % (1/8) in LTBI, TB, BCG vaccinated and unvaccinated control groups, respectively. In individuals with TB, Ag85B peptides induced higher IFN-γ responses compared with latency antigens EsxL, α-crystalline and MPT70 peptides (median levels: 416 pg/mL (IQR: 16 – 1186), 127 pg/mL (IQR: 4 – 519), 14 pg/mL (0.5 – 336) and 29 pg/ml (IQR: 4 – 422), respectively, p = 0.018). Median IFN-γ levels in controls were below 150 pg/ml for all peptide stimulations.

Intracellular cytokine production was low in all antigen peptide stimulations, measured in a subset of donors. MPT70 peptides induced a CD4+ cytokine profile dominated by IFN-γ, TNF-α and IL-2 single expressing cells. When calculating the ratio between IFN-γ expressing and IFN-γ non-expressing CD4+ T cells (functional differentiation score, FDS), the score tended to be highest for latency antigens and lowest for the Ag85B peptides, but these trends were not significant.

In conclusion, we found that predicted MPT70 peptides induced strong IFN-γ responses in people with LTBI and low responses in controls. Furthermore, the MPT70 presented an immune profile similar to other latency antigens and less similar to the early expressed Ag85B, indicating a potential of MPT70 a candidate latency antigen in multistage subunit vaccines.
Manuscript and video II

The objective of this report was to describe and discuss a human cutaneous recall model, and provide a detailed protocol and video demonstration of the technical procedure.

In the video demonstration, purified protein derivative was deposited into the dermal layer of the skin of a BCG vaccinated individual, followed by induction of fluid filled skin suction blisters seven days post challenge and harvest of PPD-specific cells by syringe aspiration.

Blister cells and PBMCs from the BCG vaccinated volunteer were compared by intracellular stain flow cytometry. The fraction of PPD specific cytokine producing CD3+CD4+CD8- skin blister cells was 33 % compared with 0.2 % in PBMCs. PPD stimulated CD3+CD4+CD8- skin blister cells showed high fractions of triple or double positive cytokine profiles (17 % IFN-γ+TNF-α+IL-2+ and 15 % IFN-γ+TNF-α+).

Eight BCG vaccinated volunteers were recruited in order to demonstrate representative cell yield and cytokine levels using a protocol with simultaneous challenge with 2 x 2 T.U. PPD depositions. Median time from BCG vaccination was 5.5 years, range 1 – 30 years. Median skin induration size was 10.25 mm (range 0 – 20 mm) with a median yield of 50,000 cells/blister (range: 15,000 – 210,000 cells, n blisters = 15). Cell yield correlated to some degree with skin test induration size (Spearman R = 0.643, p=0.094). Median IFN-γ, TNF-α and IL-2 levels were 339 pg/mL, 19 pg/mL and 1 pg/mL in blister fluid (n=6).

In conclusion, the report provides practical instructions on harvest of cells from the skin following antigenic recall – a relatively non-invasive method, which could be applied in vaccinology studies or studies of human adaptive immunity.
Manuscript III

In this paper, we aimed to optimize a murine splenocyte MGIA using virulent Mtb Erdman for in vitro infection and assess the growth inhibitory potential following vaccination with BCG and H56:CAF01. Secondly, we wanted to look for T cell responses in the MGIA system.

We set off using a state-of-art splenocyte MGIA protocol but discovered that the continuous rotation of MGIA cultures applied by this protocol compromised PBMC viability. Changing the protocol to still-culture with enriched medium increased viability (from 21% to 46% at the last day of culture). We found low inoculum variability (CV < 6%) and acceptable assay variability.

Immunization with BCG, H56:CAF01 or H56:CAF01 administered side-by-side with BCG induced significant growth reduction in splenocyte cultures compared to placebo (delta 0.3 log\(_{10}\) CFU (+/- 0.2, p = 0.049), delta 0.5 (+/- 0.2, p = 0.016) and delta 0.6 (+/- 0.1, p = 0.0007), respectively). MGIA growth inhibition corresponded to the levels of protection seen in in vivo challenge experiments. Interestingly, we also found growth inhibition in the CAF01 control group (delta 0.3 log\(_{10}\) CFU (+/- 0.2, p = 0.047) and the growth inhibition observed at 1 and 5 weeks following BCG vaccination waned to insignificant levels 29 weeks post-vaccination.

Finally, we looked for the cellular source of the vaccine induced growth inhibition. We found abundant vaccine specific T cells, and spontaneous IFN-γ release in MGIA cultures correlated with growth inhibition levels (p = 0.02). However, Mtb infection did not induce changes in CD4+ T cell frequency, cytokine expression profile or cytokine levels, indicating the lack of an adaptive response to explain the reduction in bacterial replication. Hence, we did not find a link between growth inhibition and vaccine induced CD4+ T cells.
In this study, we aimed to optimize a human MGIA using virulent Mtb H37Rv and investigate the growth inhibitory potential of PBMCs from BCG vaccinated individuals. Secondly, we wanted to assess the effect of adding autologous plasma and cells from PPD-primed skin to MGIA cultures.

In the optimization experiments, we found low PBMC viability in MGIA cultures (22 % viable cells) caused by continuous rotation and we increased cell survival over the four-day culture period by introducing still-culture (59 %, p = 0.012). Assay variability was low both within-run (CV 4 %, range 0 – 17 %) and between-run (CV 8 %, range 0 – 28 %). In the optimization phase, we tried to pool independent experiment data and by this approach, we found an indication of growth inhibition in BCG vaccinated individuals compared with controls in cultures containing autologous plasma (delta 0.3 log₁₀ CFU, p = 0.065). To explore this further, we conducted plasma titration and crossover experiments, but found no clear indication that the presence of plasma facilitated growth inhibition.

We next tried to establish proof of concept for vaccine induced growth inhibition by comparing MGIA results in 13 adults with pre-existing BCG vaccination with 13 healthy controls. Despite IFN-γ release in BCG group cultures, we found no growth reduction in the BCG group compared with controls (p = 0.990 and 0.870 for MGIA cultures in RPMI and RPMI + 10 % autologous plasma). We next proceed to investigate growth inhibitory potentials in individuals with LTBI, active TB and a new group of BCG vaccinated individuals, but found no indication of superior growth control by either group compared with unvaccinated controls (p= 0.351, 0.274 and 0.914 for cultures in RPMI +/- 10 % autologous plasma and pooled human serum, respectively).

Finally, we enriched MGIA cultures with high frequencies of PPD-specific T cells isolated from the skin. However, these cells did not induce growth inhibition in MGIA cultures when compared with autologous control cultures (p = 0.563, n = 8).

In conclusion, we established and described a reproducible human MGIA, but we did not prove a growth inhibitory capacity in groups of individuals with distant BCG vaccination, LTBI or active TB, and we found no superior growth control by enriching MGIA cultures with PPD-specific cells isolated from PPD-inflamed skin.
DISCUSSION

The overall scope of this PhD project was to explore possible correlates of protection for use in TB vaccine development. The majority of work and primary focus of the work involved characterization of the Mycobacterial growth inhibition assay, a functional assay highlighted as a lead candidate to provide future correlates of protection in TB vaccine trials.\textsuperscript{187,188,218} However, the four studies presented in this thesis also cover other aspects of the preclinical TB vaccine development process. Findings from study I and II will be discussed briefly in the two first sections, while the work involving the Mycobacterial growth inhibition assay (study III and IV) will be discussed in more detail in the following sections.

Antigen MPT70 (study I)

In study I, we described human immune recognition of peptides from mycobacterial antigen MPT70, and found that MPT70 show promising features as a candidate latency antigen for TB vaccines. Although it was not within the scope of the study to explore candidate correlates of TB vaccine protection, the study illustrates applications Th1 parameter readouts, such as IFN-\(\gamma\) secretion and T cell cytokine expression profiling, commonly used in early evaluations of candidate vaccine antigens. We found that MPT70 peptides were strongly immunogenic in people with LTBI, which is generally considered important for latency vaccine antigens.\textsuperscript{136} The study also illustrates the complexity in choosing antigens for TB multistage vaccines. Novel multi-stage vaccines aim to broaden the antigenic repertoire and target immunogenic antigens expressed at different stages during Mtb infection, and inclusion of latency antigens is believed to be important in preventing progression from latent to active disease.\textsuperscript{140} In our study, MPT70 peptides showed phenotypical characteristics that grouped the antigen with other latency antigens, thereby supporting the role of MPT70 in multistage vaccines in line with findings from recent transcription regulation studies.\textsuperscript{221,222}
Study I also illustrate some of the methodological considerations and challenges working with human TB vaccinology. As demonstrated, we found low to intermediate rates of MPT70 responders also among healthy BCG-vaccinated and unvaccinated controls, probably illustrating influential factors related to the study population, such as exposure to environmental mycobacteria, vaccination status and possibly genetic background (not discussed in the manuscript). Finally, study I exemplifies the scarcity of antigen specific T cells is available in peripheral blood, which is a challenge in studies of specific vaccine or TB antigen responses.\cite{164,217} Low cell frequencies further impairs the sensitivity and introduce variability in flow cytometric phenotypical characterizations, why such analyses in humans should be interpreted with a great deal of caution.

**Addressing the compartment (Study II)**

In study II, we demonstrated a method for harvest of antigen specific cells following recall to the skin. The kinetics of the PPD recall response and the characteristic of the antigen specific T cells isolated by suction blisters have been thoroughly described elsewhere, but in study II we exemplified how high fractions of polyfunctional PPD-specific memory T cells was easily obtained from a BCG vaccinated volunteer.\cite{181,223,224}

The presented method is interesting in the context of correlates of protection for several reasons. Firstly, the method bypasses some of the practical challenges in TB vaccine immunogenicity studies that rely on peripheral blood samples, which only contain limited numbers of antigen specific T cells.\cite{164,217} Furthermore, the immunology observed in peripheral blood is not necessarily representative of the immunology that occurs *in situ* in the Mtb infected lung.\cite{36,56,147,225,226} Cells and cytokines from human lungs can be studied via bronchoalveolar lavage, but this method is invasive and the samples cells probably reflect the lining of the airways rather than the parenchyma.\cite{227} Compared with bronchoscopy, induction of suction blisters is much less invasive and allows for the study of adaptive T cell responses generated *in vivo*, still in a tissue specific context. Naturally, the immune response in PPD-injected skin is not directly comparable to the immunology in Mtb infected lungs, but compared with peripheral blood, the immune transcriptome found in tuberculin skin reactions seem to have a closer resemblance with the transcripts found in human granulomata.\cite{228}
Furthermore, recent murine studies have emphasized the importance of homing and proliferative capacities in protective T cell responses induced by subunit TB vaccination.\textsuperscript{173,174} In this context, T cells isolated by the cutaneous recall method have at least shown the capacity to home to a peripheral tissue and proliferate locally in response to antigenic challenge, which increases their relevance as objects of interest in TB vaccine immunogenicity studies.

**MGIAs – candidates to provide future correlates of protection? (study III and IV)**

The link to long-term protection

Currently, there is no validated correlate of TB protection and the MGIA has been highlighted as a candidate to predict TB vaccine efficacy by \textit{in vitro} evaluation. However, MGIAs are still under development and several questions need to be answered to evaluate the potential of this assay. The key question is of course to determine if MGIA readout correlate with protection against TB disease in real life, and for most vaccines in clinical development, the ultimate goal is long-term protective efficacy in adult populations. In study III, we showed that vaccine induced growth inhibition in mice correlated with \textit{in vivo} protection in experimentally matched animals.\textsuperscript{143} Answering the same question in humans would require long-term follow up, large trials and an effective vaccine. Consequently, most human studies have investigated MGIA in the context of recent BCG vaccination, primarily in children, with optimal time points for detection of growth inhibition within weeks or months following vaccination.\textsuperscript{206,207,212,213,217} We, however, aimed to study MGIA in the context of long-term BCG vaccination in the case-control experiments in study IV. This approach may have reduced our chances to detect growth inhibition, as BCG protection and immunogenicity wanes over time and is highly variable in adult populations.\textsuperscript{116,122,125,229}
Chasing T cell mechanisms

In lack of a validated human correlate of protection, other explanatory variables may indicate the potential of assays to provide a plausible correlate of protection. Proving a mechanistic link not a requirement for a future correlate of protection. Nonetheless, investigating and possibly providing a link to adaptive Th1 immunity seems desirable if MGIA is to be credible and unbiased endpoints in future vaccine triage.\textsuperscript{188,218}

Several studies have tried to investigate the role of Th1 immunity in MGIA. Determining immune mechanisms in MGIA was not the primary scope of this thesis, however both study III and IV provide some mechanistic insight. In the murine study III, we had hoped to link growth inhibition with adaptive T cell responses induced by BCG and H56-CAF01 vaccination, which have previously been linked with \textit{in vivo} protection.\textsuperscript{143,149,174} However, we did not find evidence of a CD4+ T cell dependent mechanism, despite of significant growth inhibition and induction of cytokine producing T cells in vaccinated groups.\textsuperscript{143} In line with others, we did find an association between IFN-\(\gamma\) levels and murine growth inhibition, but this release proved not to be driven by Mtb infection.\textsuperscript{143,190,193,194,197} In the human study IV, we found increased IFN-\(\gamma\) levels following \textit{in vitro} Mtb infection in the BCG vaccinated group, suggesting immune activation, but in the absence of growth inhibition. The majority of human MGIA studies do not support IFN-\(\gamma\) as a key mediator of growth inhibition in MGIA, but since we did not see a growth inhibitory signal at any time, we should be careful to derive mechanistic conclusions based on our data.\textsuperscript{203,206,207,213,214,216,230}

In study IV, we experimented with enriching MGIA cultures with CD4+ cells isolated from PBMCs, but found no change in mycobacterial numbers by this approach. We had hoped to augment an adaptive growth inhibitory signal, as previous studies have emphasized the T cellular component in human MGIA. For example, depletion of CD4+ and CD8+ T cells has been shown to reduce the growth inhibitory potential in whole blood and monocyte MGIA, and enrichment with lymphocytes and ag-expanded CD8+ and CD4+ T cells has been associated with reduced mycobacterial growth in monocyte cultures.\textsuperscript{212,214–216} The latter approach is somewhat similar to our experiments in study IV, where we added suction blister cells to MGIA cultures. As described in manuscript II, these cells comprise large fractions of PPD-specific polyfunctional T cells activated and expanded \textit{in vivo}.\textsuperscript{181} Although there is no evidence that polyfunctional T cells correlate with \textit{in vivo} protection in humans,
vaccine specific polyfunctional T cells have been associated with protection in murine challenge models, and with MGIA growth inhibition in recently BCG vaccinated UK infants. Nonetheless, we detected no significant effect on mycobacterial numbers in by addition of suction blister cells to MGIA cultures in study IV. This could indicate an inability of the MGIA to detect adaptive responses, but again, it is difficult to interpret our data in the absence of growth inhibition. However, two large studies have recently questioned the adaptive component in MGIA, as both studies found no association between growth inhibition and various T cell subsets, including polyfunctional T cells, in cohorts of PPD-positive and -negative South African children, and TB contacts and BCG-vaccinated Dutch adults, respectively (Joosten et al, JCI, in press).

In conclusion, several studies have underlined the importance of the presence of T cells for growth inhibition in MGIA and some have argued, that adaptive T cell responses may be present and cause growth inhibition in MGIA, but that such responses are just to discrete to be measured. However, altogether there is currently no strong evidence to support an adaptive T cell mechanism being the primary inducer of growth inhibition in MGIA.

Humoral immunity in MGIA

Very few studies have investigated the importance of humoral immunity in MGIA, and in study IV we aimed to be the first to perform head-to-head comparisons of PBMC MGIA cultures with and without autologous plasma. Heat inactivated serum/plasma potentially contain antibodies which may enhance mycobacterial killing, and there is some indication that the presence of serum enhance the growth inhibitory capacity in vitro. In study IV, we did observe some individual tendencies to strong growth control in samples containing plasma, but there was no significant effect of plasma on a group level, and the plasma approach lead to increased assay variability. More studies are needed to shed light on the humoral aspect in MGIA.

Innate immunity in MGIA

Interestingly, just a few days before submission of this thesis, we were kindly provided a copy of an accepted manuscript from a large Dutch MGIA study comprising both LTBI cohorts and samples from an adult BCG vaccination trial (Joosten et al, JCI, in press). The authors found that growth inhibition in PBMCs from recently exposed TB contacts was transient and strongly correlated with the
presence of CD14<sup>dim</sup> monocytes and CXCL10 production, linking growth inhibition with trained immunity. Growth inhibition also required the presence of T cells and CXCR3 signaling; hereby suggesting an interplay between T cells and trained monocytes. Interestingly, these findings contrast recent reports from a TB high endemic setting, where T cell and innate immune signatures were not associated with whole blood growth inhibition in PPD-positive and -negative children.\textsuperscript{219}

Other studies have also emphasized the role of innate cellular components in MGIA. Monocyte:lymphocyte ratio has been associated with risk of TB \textit{in vivo} as well as growth inhibitory capacity \textit{in vitro}, and Hoft \textit{et al} associated γδ cells with growth inhibition in the secondary lymphocyte MGIA and later proved a mechanism by which γδ subsets inhibits intracellular Mtb by soluble granzyme A.\textsuperscript{215,233–237} Additionally, the importance of neutrophils has been demonstrated in the \textit{lux} MGIA, where neutrophil depletion in whole blood from TB contacts resulted in impaired ability to restrict BCG and Mtb growth.\textsuperscript{203,238}

In our MGIA studies, we did not look for innate mechanisms. However, we were never able to couple vaccine induced growth inhibition with adaptive immunity in the murine assay (study III) and interestingly, we found that the adjuvant CAF01, induced growth inhibition at the same level as BCG as well as a loss of growth inhibitory signal 29 weeks after immunization.\textsuperscript{143} These observations could suggest vaccine unspecific immune activation of cells in the MGIA in line with the observations by other groups discussed in this section.

LTBI models

In lack of good vaccination models to investigate long-term protection in humans, researchers draw on knowledge from studies of natural Mtb infection and correlates of disease. LTBI is known to protect against Mtb reinfection, and several studies have explored \textit{in vitro} growth inhibition in the context of LTBI with varying results.\textsuperscript{203,214,219,239–241} In study IV, we also investigated the growth inhibitory capacity of individuals with LTBI, but found no growth reduction by this group. Interestingly, in the study by Joosten \textit{et al}, only very recent Mtb exposure correlated with strong Mtb control in MGIA, while the authors found no growth inhibitory capacity in other cohort with long-term LTBI. Our LTBI group, on the other hand, represented a heterogeneous population of both recently exposed and long-term Mtb infected individuals, and this may explain
why we did not see growth inhibition by this group. Of note, time from diagnosis did not correlate with mycobacterial numbers in this small group.

**MGIA in vaccine trials – a question of specificity?**

As discussed, the role of innate immunity in MGIA may be bigger than anticipated at the initiation of this PhD project. The majority of studies have focused on innate mechanisms in an LTBI context, and this mechanistic knowledge becomes especially important if MGIA s are used for exploring correlates of disease, treatment outcome etc. However, it could have great consequences for the interpretation and application of MGIA s in TB vaccine trials if the assays indeed are sensitive for detection of innate or trained immunity. This leads to a question that frequently puzzled me during this PhD: “What is actually the specificity of MGIA s in a vaccine context?”.

The need for novel TB vaccines is greatest in areas with high prevalence of LTBI, ongoing Mtb exposure and neonatal BCG vaccination programs. However, exposure to these factors may induce both unspecific and cross reactive immune responses upon re-exposure to mycobacterial antigens, i.e. in MGIA.\(^{44-46,126,242,243}\) Innate BCG responses may be detected in MGIA in the weeks, months or years following vaccination, as it was the case in BCG trial by Joosten et al.\(^ {243}\) Moreover, high levels of Mtb and NTM sensitization may cause universally raised growth inhibitory responses in MGIA, as speculated by Baguma et al, following a failure to detect growth inhibitory differences between PPD-positive and PPD-negative individuals in South Africa.\(^ {219}\) Altogether, such unspecific ‘noise’ may mask or reduce the likelihood of MGIA s to detect vaccine induced signals and provide useful correlates in target populations.

So far, the MGIA readout has not been applied in early phase human trials of candidate TB vaccines, apart from a retrospective analysis of MVA85A trial samples, where MGIA readout did not correlate with protection by either BCG or MVA85A.\(^ {184}\) Possible assay detection problems suggested here may prove to be specific for BCG vaccination, and MGIA s could potentially perform stronger in the context of human subunit vaccination. However, at present more studies are needed to evaluate the performance of MGIA s in relevant populations.
PERSPECTIVES AND APPLICATIONS

The Mtb antigen MPT70 could be an interesting candidate antigen for a novel TB multi-stage vaccine. However, further evaluation of MPT70 expression and immunogenicity in animals and humans is needed.

The cutaneous recall method provides an alternative to peripheral blood sampling and allows for studying adaptive T cell responses in a tissue specific context – an approach that definitely has a potential for application in early human trials testing novel TB vaccine candidates. In this context, the recall antigens should to be vaccine specific, which would of course require additional safety evaluations. In addition, it would be nice to include markers for other cellular sources possibly involved in the skin reaction, including innate cells and resident skin cells, to get a better understanding of the character of the skin response. For practical reasons, studies using this method would probably have to be restricted to adult populations of a limited size. Finally, the cutaneous recall method has a broad potential for application in adaptive immunology studies or evaluations of other T cell targeting agents, i.e. therapeutic cancer vaccines.

The MGIA definitely has some attractive qualities compared with single parameter immune correlates. It is important to remember, that the assays could also be of relevance in TB research areas other than vaccine evaluation. For example, MGIAs allow for bridging human and animal research, and with increased focus on mechanistic understanding and standardization, MGIAs could be good sources of insight for immunological translation across species. However, if MGIAs are to be used for prediction of vaccine efficacy, more studies are needed to reproduce or challenge our current understanding of the underlying mechanism in MGIA and describe the variability and specificity of the assay in target populations. For example, it would be interesting to test if immunization with a live vaccine such as MMR or yellow fever could induce a mycobacterial growth inhibition. In my opinion, the confidence in MGIAs to measure protective adaptive immune responses has been compromised by recent studies, including the original studies included in this thesis. Ultimately, uncovering the prospect of MGIAs for providing plausible correlates of protection
would require validation in samples from long-term follow-up vaccination studies in relevant populations.

Of final mention, the studies in this thesis covers only a small fraction of immune markers and assays proposed to correlate with TB protection. The studies objectives presented here is fairly CD4+ T cell centric, and it is important to underline that a broad range of cell subsets, signatures and assays are currently investigated as possible markers of TB risk and TB vaccine protection. The way forward is probably not to focus on single correlates and definitely not focusing on Th1 parameters only. In these years, there is an increased focus on “omics” and systems biology for identification of new biomarkers, alongside with studies exploring the quality of the TB immune response and functional assays such as the MGIA. Combining, evaluating and validating future correlates in relevant and diverse cohorts is a major task, emphasizing the importance of biobanks and research collaboration in TB vaccine development.
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APPENDIX I

Manuscript I

A characterization of the human immune response to MPT70 peptides

Ready for submission
Title

A characterization of the human immune response to MPT70 peptides

Authors

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Introduction

Tuberculosis is the cause of 1.7 million deaths annually and more than a third of the world’s population is estimated to be infected with \textit{Mycobacterium tuberculosis} (\textit{Mtb}).\textsuperscript{1,2} New initiatives are needed to meet the WHO goals of eradication of the TB epidemic by 2050.\textsuperscript{3} The only licensed vaccine against \textit{Mtb} infection, the Bacille-Calmette Guérin (BCG) vaccine, has limited protective efficacy in adults.\textsuperscript{4} Consequently, new vaccine initiatives are needed and development of BCG-booster vaccines, such as recombinant protein subunit vaccines, are considered cost-effective tools for prevention of TB disease and transmission.\textsuperscript{3,5,6}

CD4+ T cell responses are considered important for \textit{Mtb} control in both humans and animal models.\textsuperscript{7,8} Human \textit{Mtb} infection represent a dynamic spectrum with stages ranging from asymptomatic infection and \textit{Mtb} containment to active disease and bacterial dissemination.
During these stages, *Mtb* adapt and manipulate host immunity by shifting between metabolic states of active replication or non-replicating dormancy, characterized by alterations in bacterial gene expression and protein secretion.\(^9\text{-}^{12}\) Stage-specific bacterial proteins serve as antigenic targets and are considered critical for *Mtb* immune evasion and manipulation. During latency early secreted antigens, such as Antigen 85B, are downregulated whereas dormancy antigens are upregulated.\(^11\text{-}^{15}\) Novel TB vaccine constructs aim to prevent progression from latent to active TB by targeting *Mtb* antigens presented at multiple stages of infection, including those expressed during latency.\(^16,17\)

MPT70 (Rv2875) is a candidate antigen for multi-stage subunit vaccines. MPT70 and MPB70 are isoforms, but the proteins belong to *Mycobacterium tuberculosis* (Mtb) and *M. bovis* BCG, respectively.\(^18\text{-}^{21}\) MPT70/MPB70 share the same gene sequence and organization, but due to mutation in their gene transcriptional regulators, expression of the antigens varies considerably across mycobacteria. Virulent *M. bovis* and some BCG substrains (i.e. BCG Tokyo) express high amounts of MPB70, while the expression is very low in other BCG strains (i.e. BCG Glaxo, Copenhagen, Tice, and Pasteur).\(^19\text{-}^{23}\)

Virulent *Mtb* is known to produce very small amounts of MPT70 in *vitro* cultures.\(^21\) However, it was recently demonstrated that *Mtb* express high amounts of MPT70 in IFN-γ activated macrophages and that expression is induced by a redox sensitive mechanism, indicating that MPT70 is secreted late during natural *Mtb* infection.\(^24\) Strong cellular immune responses to this antigen have been detected in animals and humans, and vaccination with MPT70/MPB70 peptides has shown protection against *Mtb* in mice.\(^21,25\text{-}^{30}\) Altogether this suggest a relevance for this antigen in multistage subunit vaccines.

In this study, we aimed to investigate the human immune response to pools of *in silico* predicted MPT70 peptides in groups of individuals with active TB, latent TB infection (LTBI), BCG vaccinated and unvaccinated healthy controls. Secondly, assuming a late expression of MPT70 during *Mtb* infection, we aimed to compare phenotypical immune profiles induced by MPT70 to profiles induced by the early secreted Ag85B (Rv1886c), and EsxL (Rv1198) and α-crystalline (Rv2031c), which are considered latency antigens.\(^31\text{-}^{33}\)
Materials and Methods

Prediction of HLA Class II epitope-binding

We used The Immune Epitope Database (IEDB) Analysis Resources server for prediction of HLA class II binding core regions in Rv2875. Prediction was done for 27 HLA alleles representing seven superalleles and covering > 99% of the general population as proposed by IEDB. The prediction algorithms SMM and NN-align were used and binding core regions were defined as having a binding affinity (IC$_{50}$) < 500nM. Five 9aa binding core peptides identified by both algorithms and recognized by minimum 7 different HLA II alleles were chosen and 5 20-mer peptides each containing a binding core were generated. Using a similar approach, nine, three and three 20-mer peptides were selected for Rv1886c, Rv1198 and Rv2031c, respectively. Number of peptides varied according to size of antigen (Blauenfeldt et al, in prep). Predicted peptides were purchased from JPT, Berlin, Germany and synthesized using their Fast Track technology.

Peptide pools for PBMC stimulation

Peptides were dissolved in 100% DMSO and further diluted in PBS to a concentration of 0.1 mg per peptide/mL in PBS with maximum 10% DMSO and stored at -80°C. Individual peptide sequences are listed in online supplement 1.

Study population

Study volunteers were 1) adults recently diagnosed with pulmonary TB, 2) adults with LTBI confirmed by positive QuantiFERON or 3) healthy Danish adults without previous TB exposure or evidence of LTBI. Volunteers were recruited at the TB outpatient clinic at Gentofte Hospital, Copenhagen, Denmark and at Statens Serum Institut, Copenhagen Denmark. Information on age,
BCG vaccination status, TB exposure, TB/LTBI diagnosis, duration of anti-TB treatment (if any), comorbidities and medication was obtained at inclusion. All volunteers provided verbal and written informed consent. The study was approved by the Danish Committee on Health Research Ethics (H-17006335) and registered via the Danish Data Protection Agency (2015-54-0102).

**PBMC isolation**

Peripheral blood was collected using heparinized tubes. Within four hours from collection, PBMCs were isolated by density gradient centrifugation using Lymphoprep tubes and medium (Stemcell Technologies). Isolated PBMCs were wash twice followed by automated viable cell count (Nucleocount, Chemometec) and resuspension in AIM-V medium (Gibco, ThermoFisher Scientific) with 1% penicillin/streptomycin (Pen/Strep) for *in vitro* stimulation.

**In vitro stimulation and Interferon-γ ELISA**

For *in vitro* culture, 2.5 x 10⁵ fresh PBMCs were stimulated in 96-well culture plates (Nuclon Delta Surface, Thermo Scientific) in a total volume of 200µl in AIM-V medium + 1% pen/strep. Stimulation concentration was 5µg per peptide/mL in maximum 0.5 % DMSO. To avoid edge-effect the outermost wells were filled with 250 µl sterile PBS. The assay setup included unstimulated, purified protein derivative (PPD, RT50 “SSI”) (10 µg/mL) and Red Kidney Bean Agglutinin (PHA, Sigma-Aldrich, 2.5µg/mL) stimulated wells. Plates were incubated in a humidified atmosphere at 37°C / 5% CO₂ for five days followed by centrifugation (2000 rpm/10 minutes) and harvest of supernatants. Supernatants were stored at -20°C until use. IFN-γ was quantified by an in-house enzyme-linked immunosorbent assay (ELISA), as previously described. For ELISA analysis, the plasma supernatants were diluted times four and cut-off was set at 150 pg/mL.
**Flow cytometry**

Suspensions of $1 \times 10^6$ PBMCs were incubated in 200 µl AIM-V + 1% Pen/Strep at 37 °C/5 % CO$_2$ for 2 hours, either unstimulated or in the presence of peptide pools (5 µg per peptide/mL), Purified protein derivative (PPD, RT50 “SSI”) (10 µg/mL) or PHA (2.5 µg/mL). After two hours, PBMCs were treated with Brefaldin A (Sigma) and Monensin (Becton Dickinson) and incubated for 6 hours at 37 °C. Cells were stained with Live/Dead-stain-BV510, anti-CD4-AF780 (eBioscience, clone RPA-T4). Anti-CD3-BV421 (BD, clone UCHT1), anti-CD8-PerCP-Cy5.5 (BD, clone SK1), anti-CCR7-PE (BD, clone 150503), anti-CD45RA-BV786 (BD, clone HI100), anti-IFN-γ-AF700 (BD, clone B27), anti-TNF-α-PE-Cy7 (eBioscience, clone Mab11) and anti-IL-2-FITC (BD, clone 5344111). Fluorescence minus one (FMO) and unstimulated controls were included in the panel. Flowcytometric analysis was performed on LSRFortessa™ II (Becton Dickenson). Data was analyzed using FlowJo™ 10.0 with the following gating strategy: singlets $\rightarrow$ lymphocytes $\rightarrow$ viable cells $\rightarrow$ CD3+ $\rightarrow$ CD4+CD8- $\rightarrow$ CD4+ IFN-γ+, CD4+TNF-α+ and CD4+IL-2+. Frequencies of cytokine producing cells in unstimulated samples were subtracted from the frequencies in stimulated samples. Cytokine profiles were calculated using Boolean Gating. Representative gating plots are presented in online supplement 2 and 3.

**Statistical analysis and Functional Differentiation Score calculation**

Data was analyzed using Prism 6 software (Graphpad Prism®, v.7.01) by non-parametric statistical analyses. Group medians values compared by Mann-Whitney or Kruskal-Wallis tests for un-paired data. Paired data was compared by Wilcoxon matched-pairs signed rank and Friedman tests. Functional differentiation score (FDS) was calculated as the proportion of IFN-γ-expressing antigen-specific CD4+ cells divided by the proportion of cytokine producing CD4+ cells not expressing IFN-γ (i.e., expressing TNF and/or IL-2).
### Results

#### Study population

Clinical and demographic characteristics of the study population are listed in Table 1.

<table>
<thead>
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<th>LTBI</th>
<th>BCG vaccinated</th>
<th>Controls</th>
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<td>% (n/n)</td>
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<tr>
<td>BCG vaccination</td>
<td>% (n/n)</td>
<td>69 (11/16)</td>
<td>82 (9/11)</td>
<td>100 (15/15)</td>
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<tr>
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<td>median years (range)</td>
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<td>53 (32-66)</td>
<td>36 (20-58)</td>
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<td>Time from diagnosis**</td>
<td>median weeks (range)</td>
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<td>1.75 (0-8)</td>
<td>-</td>
</tr>
</tbody>
</table>

* two volunteers with Danish ethnicity and TB were born in Greenland
**Time from diagnosis data was only available for 6/11 in the LTBI group

#### MPT70 specific IFN-gamma responses

IFN-γ was measured in MPT70 peptide pool stimulated PBMC cultures from individuals active TB, LTBI, BCG vaccinated and unvaccinated healthy controls (Fig 1a), responders were arbitrarily defined by a cut-off a 150 pg/mL (figure 1).

In the LTBI group, 73% (8/11) of the individuals responded to MPT70 peptide stimulation with IFN-γ responses above the cutoff. Responder rates were 50% (8/16), 40% (6/15) and 13% (1/8) in the TB, BCG vaccinated and control group, respectively (figure 1b). Responses were significantly higher in the LTBI group compared with responses in the TB, BCG vaccinated and control groups (median 977 pg/mL (IQR: 1373 – 5485) vs. 93 (4 – 422), 70 (4 – 344) and 3 (0 – 103), respectively, p= 0.006, Kruskal-Wallis test).

There was no correlation between time from TB diagnosis and MPT70 peptide IFN-γ response among individuals with TB and LTBI (Spearman R = 0.164, p = 0.540 (n = 16) and R = 0.69, p = 0.147 (n = 6), respectively (data not shown)).
Comparison of IFN-γ responses across antigens

We next compared the IFN-γ responses to MPT70 peptides with predicted peptide pools from three other antigens expressed at different stages during Mtb infection.

First, we compared peptide pools from MPT70 with the latency antigen α-crystalline. Both antigens were characterized by a high rate of responders in the LTBI group (73% (8/11) vs 64% (7/11)) and intermediate rate of responders in the TB group (50% (8/16) vs. 31% (5/16) %). When looking at quantitative differences in the LTBI group, median IFN-γ response was twice as high in MPT70 stimulated samples compared with α-crystalline, however the difference was not significant (MPT70 median 979 pg/mL (IQR: 137 – 5485) vs. α-crystalline 453 pg/mL (IQR: 54 – 3146), p = 0.206, Wilcoxon test). Responses were generally low in the among BCG-vaccinated and non-vaccinated controls, however in the BCG vaccinated group, MPT70 responses was slightly higher compared with α-crystalline (69 vs 21 pg/mL, p= 0.013, Wilcoxon test) with 40% (6/15) of MPT70 samples responding above the cutoff vs. 0% (0/16) of the α-crystalline stimulated samples.

We next compared the MPT70 peptide recognition to responses induced by all three antigens expressed early or constitutively during Mtb infection. In the TB group, median IFN-γ responses were significantly higher in in samples stimulated with Ag85B peptides (p = 0.018, Friedman, median levels: 416 pg/mL (IQR: 16 – 1186), 127 pg/mL (IQR: 4 – 519), 14 pg/mL (0.5 – 336) and 29 pg/mL (IQR: 4 – 422) for samples stimulated with of Ag85B, EsxL, α-crystalline and MPT70, respectively). Of note, four of the 16 individuals with TB disease responded to all antigen peptide pools with INF-γ levels > 150 pg/mL. Among individuals with LTBI, all antigen peptide pools induced high IFN-γ responses with median 4060 pg/mL (IQR: 51 – 4691), 2135 pg/mL (IQR: 676 – 4942), 453 pg/mL (54 – 3146) and 977 pg/mL (IQR: 137 – 5485) for Ag85B, EsxL, α-crystalline and MPT70 peptide stimulation, respectively (p = 0.056, Friedman test).

Among BCG- and unvaccinated controls, median responses were below cutoff for all antigen stimulations (data not shown). However, there were 20% positive responders to α-crystalline stimulation and 40% to Ag85B and MPT70. Of note, one donor responded to all antigens except Ag85B with IFN-γ release > 500 pg/mL.
Antigen specific cytokine expression profiles

Next, we proceeded to characterize the phenotypic and functional properties of antigen responding CD4+ T cells. Only a limited number of volunteers had sufficient PBMC yield for ICS flowcytometry, and only individuals with fractions of cytokine producing cells > 0.03% were included in the quantitative analysis (determined as fraction of viable CD3+CD4+CD8- producing either IFN-γ, TNF-α or IL-2 following antigen stimulation, unstimulated fractions subtracted). Numbers of responders above cutoff and total number of available PBMC samples are listed in figure 2.

Among individuals with LTBI, the MPT70 cytokine profile was dominated by IFN-γ, TNF-α and IL-2 single expressers and very low frequencies of polyfunctional cells (figure 2). The Rv2031 profile showed similar characteristics. The profiles of Rv1886 and EsxL tended to present slightly higher frequencies IFN-γ double and triple positive CD4+ T cells compared with MPT70. Of note, all antigen profiles seemed to be dominated by IL-2 single-expressing cells and all cytokine producing cell fractions were generally low.

In the TB group, the MPT70 cytokine profile was dominated by IFN-γ, TNF-α and IL-2 single expressers and very low frequencies of polyfunctional cells – a profile that was somewhat comparable to the Rv2031 profile. Comparison profiles across all antigens did not show any clear patterns, nonetheless, constitutively and latency antigens all seemed to induce a profile dominated by IFN-γ single positive cells, whereas these cells were almost absent in samples stimulated with Rv1886.

Finally, we calculated the FDS-score for all antigens (as defined in Materials and methods section). The FDS score for MPT70 was 1.5 (range 0.13 – 18.83) and 0.64 (range 0.03 – 3.48) for individuals with TB and LTBI, respectively (p = 0.343). For all antigens, FDS scores tended to be lower among individuals with TB compared with individuals with LTBI, and across antigens FDS tended to be highest for latency antigens and lowest for the early expressed antigen Ag85B, but these trends were not significant (data not shown).
Discussion

In this short report, we characterize the human immune response to MPT70 peptides across groups of individuals with TB, LTBI, BCG vaccinated and healthy controls. We found that 2/3 of the LTBI group and approximately half of the TB and BCG vaccinated groups responded to MPT70, while the peptide pools was poorly recognized in non-vaccinated controls. Finally, we compared MPT70 peptide responses with other Mtb antigens expressed at different stages during Mtb infection. Here, we found that the immune profile of MPT70 was dominated by low frequencies of IFN-γ, TNF-α and IL-2 single producing cells, which was different from the profile induced by the early expressed Ag85B, and more similar to the profile of the latency antigen α-crystalline.

We expected predicted MPT70 peptides to induce strong responses in people infected with Mtb, and this was confirmed with responder rates of 73% IFN-γ in the LTBI group and 50% in the TB group. Our findings are in line with previous Kuwaiti studies demonstrating Th1 recognition in these groups. Mustafa et al found positive IFN-γ responses (>5 UI/mL) in 47% and T cell proliferation in 58% (11/19) of TB patients in response to MPB70 (culture filtrate). Later the author did another study in BCG vaccinated and PPD-positive positive individuals and found a high frequency of IFN-γ responders (70% > 1.5 IU/mL) among people who also reacted to ESAT-6 and CFP-10 (as a proxy for Mtb specific latent infection). Compared with this study of predominately Danish participants, the Kuwaiti studies were performed in populations with higher prevalence of TB, which was also demonstrated by strong ESAT-6 responses. However, in a setting more similar to ours, Roche et al found that 50% of Australians with TB and 75% of PPD-positive responded above the assay cutoff, while responses were low among BCG vaccinated (15%) and controls (0%) using a MPB70 lymphocyte proliferation assay.

We would not expect BCG vaccination to be a strong inducer of MPT70 responses in healthy individuals, as the majority of participants were Danes without known Mtb exposure and we would expect them to have received vaccination with BCG Danish/Copenhagen, a strain that has very low/no expression of MPB70 and EsxL. Hence we were surprised to see that 40% of the volunteers in the BCG vaccinated group responded to MPT70 (and EsxL). In line with previous findings, α-crystalline peptides did not induce responses in BCG vaccinated. This unexpected
finding could potentially be explained by exposure to environmental mycobacteria, as MPB70 is shared between various environmental mycobacteria.\textsuperscript{21} This phenomenon was demonstrated by two studies performed in settings with different frequencies and types of environmental mycobacterial exposure including \textit{M. bovis}. In the first study, 9\% of BCG vaccinated Malawian young adults responded to MPB70 (and only 11\% to ESAT6) by whole blood IFN-\gamma assay detection.\textsuperscript{47} In the second study, MPB70 recognition among UK schoolchildren was detected using the same assay, and here recognition was higher (22\% before and 33\% after BCG Glaxo (low/no MPB70) vaccination).\textsuperscript{48} Exposure to environmental mycobacteria could also explain why 13\% of healthy controls in our study responded to MPT70 peptides.

In this study, we used pools of five \textit{in silico} predicted MPT70 peptides. Others have successfully tried similar approaches and found peptide sequences similar to ours. \textit{Al-Attiyah et al} compared responses to MPB70 overlapping peptides in Kuwaiti BCG vaccinated donors and ag-specific T cell lines and found three promiscuously presented (Th1) immunodominant epitopes.\textsuperscript{49} Subsequently, \textit{Mustafa and Shaban} used a matrix-based prediction program (Propred) to identify four promiscuous peptides of MPT70 followed by experimental evaluation using T-cell lines and PBMCs from PPD positive individuals.\textsuperscript{50} The authors identified two C-terminal peptides that showed good responses in proliferation and IFN-\gamma assays. Two of the pooled peptides (peptide 3 and 5) used in our study has overlapping sequences with the epitopes recognized by \textit{Al-Attiyah et al} and \textit{Mustafa and Shaban}. Thus, our findings is consistent with existing data that MPT70 has immunodominant epitopes, which could potentially be used in TB vaccine design.

Finally, we looked at the specific CD4\(^+\) functional profiles induced by MPT70 peptide stimulation. Stimulation with MPT70 peptides induced mainly IFN-\gamma, TNF-\alpha and IL-2 single producing CD4\(^+\) cells in individuals LTBI and active TB. As mentioned, the MPT70 protein is secreted in response to a reductive environment and we would expect a delayed expression during \textit{Mtb} infection.\textsuperscript{24} We therefore hypothesized, that the phenotypical profiles of MPT70 specific would be most likely to resemble those of other latency antigens, and less likely to resemble the profile induced by early and transiently secreted Ag85B. In accordance with our hypothesis, we found that the MPT70 profile was somewhat comparable to the profiles of latency antigens, and less comparable to the Ag85B profile, which was to a higher degree characterized by IL-2 single producing cells. To our
knowledge, only one other study has performed flowcytometry on human cells stimulated with MPT70. Bertholet et al investigated intracellular production of IFN-γ and TNF-α in CD4+CD45RO+ and CD8+CD45RO+ cells in six PPD-positive individuals and found almost equal distributions of single and double producing cells. However, response profile and magnitude varied considerably among donors (0 – 0.2 % and 0 – 0.7 % cytokine producing CD4+ and CD8+ cells, respectively). Our results should be interpreted with similar caution, as they may reflect a low sensitivity and a great deal of variability due to low frequencies of cytokine producing cells in a small study population. For the same reason, we did not investigate cytokine expression in CD8+ T cells or memory T cells, though markers for these subsets were included in the staining panel. We calculated the crude ratio of IFN-γ producing cells over non-IFN-γ cytokine producing cells (FDS, figure 3), as this ratio may represent a less variable estimate of T cell differentiation among CD4+ cytokine producers.

Not surprisingly, early and transiently secreted Ag85B had a low FDS whereas peptides from the constitutively secreted EsxL peptides had a high FDS, the latter probably reflecting a more terminal T cell differentiation induced by consistent antigen pressure. The FDS for MPT70 peptides was comparable to that of α-crystalline, with an FDS around one for both antigens, though with a high variability in the MPT70 TB group. The variability in MPT70 FDS in TB patients may be explained by differences in Mtb antigen availability and pressure, as progression toward symptomatic TB may existed long before clinical TB diagnosis.

In conclusion, we found that predicted peptides of MPT70 were strongly recognized in people with LTBI, as previously described in other study populations. The MPT70 immune profile resembled that of latency antigen α-crystalline, and differed from the profile induced by the early expressed Ag85B. These observations support the role of MPT70 as an immunogenic latency antigen and may pose certain immunological implications for the utility of MPT70 in human TB vaccines.
References


Figure legends

Figure 1. IFN-γ responses
(a) IFN-γ responses following 5-day PBMC *in vitro* stimulation with Ag85B, EsxL, α-crystalline and MPT70 peptides pools in unvaccinated (n = 8) and BCG vaccinated (n = 15) individuals, and individuals with LTBI (n = 11) and active TB disease (n = 16). Data is presented as individual mean values of duplicate ELISA measurements (pg/mL) and group medians. Dotted line represent cutoff 150 pg/mL (b) Group median fraction of responders above cutoff (>150 pg/mL).

Figure 2. Profiles of cytokine producing CD4+ T cells
Fresh PBMCs from individuals with LTBI and TB were stimulated with peptide pools from four different antigens and evaluated by ICS flowcytometry, as described in materials and methods. Data is presented as fractions of viable CD3+CD4+CD8- cytokine producing cells by Boolean calculation. Bars represent group median values and error bars represent IQR. Only samples with total fractions of CD4+ cytokine producing cells > 0.03 % were included in the plots. Numbers of available samples/responders > cutoff for individual stimulations are presented below each plot.

Figure 3. Functional differentiation score
Functional differentiation score (FDS) was calculated using the fractions of cytokine producing CD4+ cells represented in Fig 2. See materials and methods for calculation. Bars represent median FDS, error bars represent IQR.

S1. Individual peptide sequences for PBMC stimulation.
S2. Gating strategy for unstimulated fresh PBMCs from a representative donor with active TB.
S3. Gating strategy for PPD-stimulated fresh PBMCs from a representative donor with active TB.
Figure 1.

A. 

B. 

Legend:
- Unvaccinated controls (n = 8)
- BCG vaccinated controls (n = 15)
- LTBI (n = 11)
- TB (n = 16)
Figure 2.

- **Ag85B : TB**
- **EsxL : TB**
- **α-crystalline : TB**
- **MPT70 : TB**

- **Ag85B : LTBI**
- **EsxL : LTBI**
- **α-crystalline : LTBI**
- **MPT70 : LTBI**
Figure 3.
## Online supplement 1.

**MPT70 (Rv2875) peptides:**

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**Ag85B (Rv1886c), EsxL (Rv1198) and α-crystalline (Rv2031c) peptides:**

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Online supplement 2.
Online supplement 3.
A suction blister protocol to study human T-cell recall responses in vivo

A suction blister protocol to study human T-cell recall responses \textit{in vivo}

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\textbf{KEYWORDS:}

Suction Blister, Immunology, Recall, Skin challenge, Antigen, T-cell, Memory T-cell, PPD, Tuberculin skin test, Vaccines, \textit{In vivo} response, Tuberculosis

\textbf{SHORT ABSTRACT:}

In this report, we provide a visual demonstration of the suction blister cutaneous recall model. This model allows for simple access to human \textit{in vivo} adaptive immune responses, e.g. in the context of vaccine development.

\textbf{LONG ABSTRACT:}

Cutaneous antigen recall models allow for studies of human memory responses \textit{in vivo}. When combined with Skin Suction Blister (SB) induction, this model offers accessibility to rare populations of antigen-specific T-cells representative of the cellular memory response as well as the cytokine microenvironment \textit{in situ}.

In this report, we provide a visual demonstration of the practical procedure of cutaneous recall, suction blister induction and harvest of antigen-specific T-cells. We use the Tuberculin Skin Test for antigenic recall in individuals with prior Bacillus Calmette-Guérin vaccination against infection.
with *Mycobacterium tuberculosis*. Finally, we provide examples of multiplex and flowcytometric analyses of SB specimens, illustrating the high fractions of antigen-specific polyfunctional CD4+ T-cells available by this sampling method compared with cells isolated from the blood.

The method described here is safe and minimally invasive and provide the unique opportunity to study both innate and adaptive immune responses *in vivo*. We believe that this method may be beneficial to a broad community of researchers working with cell-mediated immunity and human memory responses, in particular in the context of vaccine development.

**INTRODUCTION:**

A skin suction blister (SB) is an artificially induced blister, which allows for harvest of cells and fluid directly from the skin. The technique of raising a SB by vacuum is a well-known tool within the field of dermatology used to study skin immunology in health and disease.\(^1\)–\(^8\) In this report, we demonstrate how the SB technique combined with the induction of cutaneous antigenic recall (SB cutaneous recall method) can provide direct insights into adaptive immune responses *in vivo*.

The principle behind SB induction is simple: a light vacuum is applied to a small area of the skin. The negative pressure will eventually force the epidermis to separate from dermis creating a local blister filled with fluid and cells.\(^1\), \(^2\), \(^9\) The blister fluid can be harvested by fine needle aspiration and the content used for further study *in vitro*.

In recent years, there has been an increasing interest in the SB method for the study of *in vivo* immune responses other than diseases restricted to the skin.\(^10\), \(^11\) Study of adaptive immunity in humans is limited by the fact that the cells and cytokines of interest are sampled from peripheral blood because invasive sampling of e.g. lymph node or gastrointestinal mucosal tissue may be unacceptable and unethical. An example is the study of long-lived human memory T-cell responses after vaccination.\(^12\) In such trials, sampling of relevant T-cells can be a great challenge, because the relevant population of cells that mediate immunity reside in lymphoid tissue and only a very limited number of specific T-cells circulate in peripheral blood.

The SB technique offers a unique opportunity to study of memory T-cells and other specific cell populations. Following cutaneous inoculation of antigen, T-cells specific for the antigen are recruited from their lymphoid hideaways to the skin and can easily be sampled from the SB. The methodology and research applications of this cutaneous recall model was described by Akbar and colleagues in 2013.\(^2\) A commonly used antigen for skin recall is the commercially available purified protein derivative (PPD) of mycobacteria used to perform a tuberculin skin test (TST)\(^13\) where PPD is injected into the dermal layer of the skin. In individuals with existing immunological memory towards PPD (e.g. individuals with *M. tuberculosis* infection or prior Bacillus Calmette-Guérin (BCG) vaccination), the antigen deposition result in a recall response with migration to the skin and *in vivo* clonal expansion of PPD-specific CD4+ T-cells.\(^2\), \(^11\), \(^14\), \(^15\) As a result, high fractions of PPD-specific polyfunctional CD4+ T-cells accumulate in the skin ready for SB sampling. T-cells collected by this method have proven to be robust and sufficiently abundant to be characterized by a range of immunoassays and by long-term *in vitro* culture.\(^15\) Thus, the
cutaneous recall model and SB induction may prove a valuable method to study in vivo T-cell responses by ex vivo analyses. We believe that increased knowledge of this approach can benefit researchers with interests in cellular immunology and vaccinology.

In this report, we provide the first stepwise video guide on how to induce human skin suction blisters. We demonstrate the cutaneous antigen recall model using the TST in BCG-vaccinated volunteers. Finally, we show examples of ex vivo analysis of cells and cytokines isolated by SB. Phenotypical and functional characteristics of PPD-specific T-cells obtained by the SB method are thoroughly described elsewhere.2, 10, 11, 16–19 Here, we aim to provide a visual demonstration and discuss the extrapolation of this technique into in multiple areas of research within the field of immunology and vaccinology.

PROTOCOL:

All methods described below including the use of human volunteers was been approved by the Danish Committee on Health Research Ethics (H-15002988) and the Danish Data Protection Agency (Jr.Nr. 2015-57-0102). The purified protein derivative (PPD) should be a certified product approved for human use and administered within the correct dosage provided by the manufacturer. Any deviation in dosage or administration may require additional ethical approvals.

1. INTRADERMAL DEPOSITION OF ANTIGEN (PPD)

1.1 Obtain consent
Obtain oral and written informed consent from the volunteer. Prior to injection, ensure that the volunteer understands and accepts the procedure including possible adverse effects.

1.2 Prepare the injection site
Locate the injection site on the ventral side of the volunteer’s forearm. Do this by placing the arm palm-up on a plain surface and chose an area on the upper third of the forearm, approximately 5-10 cm from the elbow joint. Stay clear of scars, veins or damaged skin. Disinfect the area using an alcoholic swab.

1.3 Prepare the PPD-solution
Disinfect the rubber cap of a vial of PPD ready-made solution for human use (2 tuberculin units (TU)/0.1 mL) using an alcohol swab. Use a 1 mL sterile syringe with a 27-30G / short (e.g. 12 mm) fixed needle to gently mix and then aspirate 0.1 mL of the PPD solution.

1.4 Intradermal PPD injection
Stretch the skin and point the needle at a 5-15 degree angle with the bevel facing upwards. Insert the tip of the needle into the dermal layer of the skin and make sure the tip is almost visible through the epidermis. Slowly inject 0.1 mL PPD solution. If administered correctly, a pale papule
of 6-10mm will appear immediately. The papule disappears after approximately 10 minutes.

**NOTE:** This protocol describes how to perform a single PPD deposition, but multiple depositions of PPD in the same arm is possible. However, this may require specific ethical approval. When making two or more depositions, aim for maximum separation (5 – 10 cm) of the injection sites while keeping in a good distance from the elbow and wrist area. This prevents a potential confluence of the skin test response and allow uninterrupted positioning of two suction chambers.

2. **EVALUATION OF THE SKIN REACTION – DAY 3**

2.1 After 48 – 72 hours, note the size of the skin reaction. It is important to measure the induration (the swelling) *not* the erythema (the redness) of the reaction. First, palpate the hard swelling using your fingers and then mark the edges of the induration using a ballpoint pen. Use a ruler to measure the induration diameter and note the result in millimeters.

**NOTE:** Reading of the induration size may guide the choice of orifice diameter for the suction chamber.

3. **SUCTION BLISTER INDUCTION – DAY 7**

3.1 Assemble the suction device unit.

3.2 Determine optimal orifice diameter of the orifice plate relative to the size of the skin reaction, the larger the size of the orifice, the larger the blister.

3.3 Disinfect the orifice plate and the skin of the volunteer using alcohol swabs.

3.4 Attach the suction chamber to the skin. Make sure the hole in the orifice plate is situated at center of the PPD reaction. Secure the chamber loosely using straps: when negative pressure is applied, the chamber will adhere to the skin and maintain its position. Make sure the volunteer’s arm is resting comfortably.

3.5 Turn the suction device on and adjust the pressure to -20 kPa.

3.6 After 30 minutes, increase the negative pressure to -25 kPa.

3.7 After 60 minutes, further increase the negative pressure to -30 kPa.

3.8 Keep the pressure at minus -30 kPa until a blister is fully formed. The time required for induction ranges from 60 to 180 minutes with the actual blister formation occurring within the last 30 minutes. Blistering is often associated with an itchy sensation. If blister rupture or hemorrhage occurs it is advised to terminate the blister induction by slowly releasing the pressure.
3.9 Release pressure and carefully remove the suction chamber from the skin without rupturing the blister.

3.10 Apply a soft dressing on the surrounding skin area and place a hard cap (e.g. from a 50ml plastic tube) over the blister. Secure the cap by non-allergenic surgical tape followed by a soft stretchy bandage. Instruct the volunteer to avoid excessive physical strain on the blister area for the next 12-24 hours.

**Note.** Suction devices are vacuum pumps with attached chambers for suction blister induction. Optimal settings may vary between instruments. The negative pressures provided in this protocol are specific for our customized instrument and it may be necessary to adjust the pressure slightly when applying the protocol in other settings.

### 4. HARVEST OF BLISTER FLUID – DAY 8

4.1 On the following day, gently remove the protective dressing and disinfect the blister (use spray only).

4.2 Use a 2 mL sterile syringe with a 23G needle to harvest the blister content: Insert the needle into the top/lateral side of the blister and slowly aspirate the fluid. Avoid touching the floor of the cavity.

4.3 Apply a dressing on the collapsed blister

4.5 Transfer the blister fluid to a sterile tube. Note the volume.

4.6 Spin down at 600 x g using a tabletop centrifuge for 4 minutes.

4.7 Transfer the supernatant to another tube and resuspend the cell pellet in 500 µL cell medium e.g. AIM V. The cells are now ready for counting and further analysis. Supernatants can be stored at -20 to -80°C until use.

### 5. ANALYSIS OF SUCTION BLISTER CELLS AND FLUID FOR REPRESENTATIVE RESULTS SECTION

In this report, intracellular stain flow cytometry and multiplex analyses were performed to provide a representative description of suction blister cells and fluid. The methodology is described in brief below.

For flowcytometric characterization, suspensions of $1 \times 10^5$ fresh SBCs or $1 \times 10^6$ PBMCs from one BCG-vaccinated volunteer were incubated at 37 °C and 5% CO₂ with 10µg/ml PPD or w/o stimulation. After two hours cells were treated with Brefaldin A and Monensin and incubated for 6 hours at 37 °C. Cells were stained with Live/Dead-stain-BV510, anti-CD4-AF780. Anti-CD3-
BV421, anti-CD8-PerCP-Cy5.5, anti-CCR7-PE, anti-CD45RA-BV786, anti-IFN-γ-AF700, anti-TNF-α-PE-Cy7 and anti-IL-2-FITC. FMO controls were included in the PBMC panel. Flowcytometric analysis was performed on LSRFortessa and analyzed using FlowJo 10.0. Cytokine producing CD3+CD4+CD8- subsets presented in the representative results section was obtained with the following gating strategy singlets → Viable CD3+ → CD4+CD8- → IFN-γ+, TNF-α+ or IL-2+. Individual cytokine profiles were calculated using Boolean Gating. Data on effector memory cell populations was obtained by gating: singlets → Viable CD3+ → CD4+CD8- → CCR7-CD45RO-.

For demonstration of SBC cytokine release, levels of IL-2, IFN-γ, TNF-α, IL-10 and IL-13 were measured by multiplex analysis in suction blister fluid from four volunteers, as well as in supernatants of M. tuberculosis (100 CFU) infected SBCs and PBMCs cultures from one BCG-vaccinated volunteer.

REPRESENTATIVE RESULTS:

For this demonstration, eight healthy adult volunteers (median age 30 years, range 26-43) with documented previous BCG vaccination (median time from BCG-vaccination: 5.5 years, range 1 – 30 years) were included. Participants were challenged intradermally with 2 TU PPD followed by TST evaluation on day 3. SBs were induced on day 7 and harvested on day 8, and all blisters were raised using suction blister chambers with 10 mm orifice diameters. Seven individuals were given two separate PPD inoculations simultaneously in the same arm followed by two parallel SB inductions (please refer to note regarding multiple PPD depositions below point 1.4 in the protocol section). Peripheral blood was drawn on day seven for plasma and PBMCs isolation by density gradient centrifugation. Plasma and fluid supernatants from SBs were stored at -20 °C. Fresh SB cells (SBCs) were counted using nigrosine stain.

Clinical TST responses and SBC yield is presented in Figure 1. Median size of TST induration was 10.25 mm (range: 0 – 20 mm) and median cell number per blister was 50,000 (range: 15,000 – 210,000 cells, number of blisters = 15). Two of the volunteers had no clinical response in either of two TSTs and a corresponding low total cell yield of 15,000 cells/blister. Cell yield was associated with the mean clinical response of TST, (Spearman R = 0.643, p = 0.094).

[Insert Figure 1]

To demonstrate flowcytometric SB characterization, SBCs were obtained from a 43 year old volunteer who had been BCG-vaccinated 30 years earlier and had no known exposure to M. tuberculosis. SBCs were isolated following induction of a single blister (induration 1.4 mm / 100,000 SBCs). Figure 2 shows representative plots of intracellular cytokine staining of SBCs versus PBMCs).

In this volunteer, the fraction of CD3+CD4+CD8- SBCs increased from 67% in unstimulated cells to >90% upon PPD re-stimulation, whereas the fraction of CD3+CD4+CD8- PBMCs remained constant (~51%, figure 2A-B). Over 92 % of both PPD- and un-stimulated CD3+CD4+CD8- SBCs were of effector memory type while (CCR7-CD45RA-, data not shown). Overall fractions of
specific CD3+CD4+CD8- cells induced by PPD-stimulation were higher in SBCs compared with PBMCs (33.1 vs. 0.2%, unstimulated samples subtracted). In PBMCs, fractions of polyfunctional PPD-specific CD3+CD4+CD8- cells were all < 0.05%. As expected, CD3+CD4+CD8- SBCs were activated \textit{in vivo}, illustrated by a high proportion of cells staining for upregulated cytokines (12%), with the predominant cytokines being TNF-\(\alpha\) and IFN-\(\gamma\) (data not shown). However, upon PPD-stimulation the cytokine secreting cells shifted towards a triple or double positive IFN-\(\gamma\)+TNF-\(\alpha\)+IL-2+ (17%) and IFN-\(\gamma\)+TNF-\(\alpha\)+ (15%) profile (Figure 2C). Cytokine expression profiles for PPD-stimulated effector memory CD4+ T subsets (CD3+CD4+CD8-CCR7-CD45RA-) were comparable to the CD3+CD4+CD8- population presented in Figure 2 (data not shown).

To explore the SB fluid as a source of information on the cytokine microenvironment at the site of skin testing, cytokine levels was measured in fluid harvested from SBs induced 7 days post-TST (Figure 3A). Median levels of IFN-\(\gamma\), TNF-\(\alpha\) and IL-2 were 339 pg/mL, 19 pg/mL and 1 pg/mL, respectively (\(n = 6\)). Plasma levels were generally very low. Cells isolated from SBs produced high levels of pro-inflammatory cytokines \textit{ex vivo} (Figure 3B). Tittrations of SBCs from one volunteer were cultured for four days in the presence of virulent \textit{M. tuberculosis} +/- autologous PBMCs. IFN-\(\gamma\) levels were > 30-fold higher in cultures containing SBCs, irrespective of the presence of PBMCs.

\[\text{Insert Figure 2}\]

\[\text{Insert Figure 3}\]

\textbf{FIGURE AND TABLE LEGENDS:}

\textbf{Figure 1: Suction blister cell yield}
Representative microscopy of nigrasine-stained cells isolated from SBs raised 7 days post-TST (A), and relationships between mean TST induration (mm) and mean cell yield (n/blister) in eight BCG-vaccinated volunteers (number of blisters = 15). Dots represent individual mean measurements, please note that two dots are overlapping as two volunteers both had a TST induration of 0 mm and a cell yield of 15,000.

\textbf{Figure 2: Representative flowcytometry plots of suction blister cells vs PBMCs}
\textit{Top panel:} Representative density plots of CD8+ and CD4+ populations in unstimulated vs. PPD-stimulated SBCs (A) and PBMCs (B). Plots of intracellular cytokine staining in PPD-stimulated CD3+CD4+CD8- cells are displayed below.
\textit{Bottom panel:} Individual profiles for cytokine producing CD3+CD4+CD8- SB cells (C) and CD3+CD4+CD8- PBMCs (D). Bars represent fractions of antigen-specific cytokine profiles in unstimulated cells (white bars) and upon \textit{in vitro} stimulation with PPD (colored bars).
Figure 3: Cytokine levels in SB fluid, plasma and in Mycobacterium tuberculosis infected culture supernatants

(A) Cytokine levels in SB fluid supernatants and plasma from six volunteers. Bars represent median cytokine levels; error bars represent range. (B) Cytokine levels in supernatants from four parallel 600 µl ex vivo four-day cultures of: 1 x 10^6 PBMCs (black bars), 1.75 x 10^5 SBCs (white bars), and 1 x 10^6 PBMCs spiked with either 0.5 or 1.75 x 10^5 SBCs (grey bars) infected with M. tuberculosis. Measurements above assay calculation range were adjusted to the upper calculation limit.

DISCUSSION:

Short summary
This report describes a practical procedure for the study of human immune memory responses in vivo, using cutaneous antigen recall and cell harvest by suction blister induction. TST is used as an example of intradermal antigen deposition and BCG-vaccine recall. Finally, an example of SB specimen characterization by flow cytometric and multiplex cytokine analysis is provided, demonstrating that roughly a third of SB cells are antigen specific polyfunctional T cells of effector memory phenotype.

Critical steps and trouble shooting
The critical steps of this protocol include the intradermal injection technique, the suction blister induction and the blister puncture. Firstly, correct intradermal deposition requires trained personnel and incorrect deposition can lead to suboptimal results. PPD is generally a well-known and safe cutaneous antigen, but its composition may vary between manufacturers limiting comparability.\textsuperscript{13, 20} This report provides instructions for a single intradermal deposition of 2 TU, and optionally two parallel depositions (2 x 2 TU), which may require additional ethical approval. However, other studies have used 10 TU, which increase the likelihood of a strong skin reaction.\textsuperscript{10, 21} During the SB induction step, small stepwise increases in negative pressure will reduce the risk of hemorrhage and blister rupture. Contamination with red blood cells or leucocytes from the bloodstream is generally low.\textsuperscript{2} Aseptic puncture technique prevents microbial contamination, and avoiding contact between the puncture needle and the dermal blister floor reduces impurities of debris or resident skin cells. However, some prefer to harvest SB fluid by applying a rolling pressure to the punctured blister.\textsuperscript{10} It may be necessary to puncture septa within the blister. The SB technique itself is minimally invasive, collapsed SBs heal without scarring and infection is very rare.\textsuperscript{2} However, some degree of hypo-pigmentation may occur and SB induction should be avoided in people with a history of colloid scarring.

Assay limitations
Technical limitations include low total cell yields and consequently limited options for long term storage. Relationships between leucocyte yield, clinical TST responses, blister size and
erythrocyte contamination has been thoroughly described elsewhere. In the BCG-recall experiments presented here (Figure 1), the median total yield from each blister was 50,000 implying a small-scale experimental set-up using these cells, especially if SBCs are studied alone. However, the specific T-cell population in a SB sample mostly exceeds what is found in PBMC samples in both relative and absolute cell counts. In the example presented in Figure 2, the number of triple-positive PPD-specific T-cells in a sample of 100,000 SB cells were more than two times greater than the number found in 1 x 10^6 PBMCs from the same donor (data not shown).

Visual scoring of the TST reaction is the most common clinical method for evaluation of M. tuberculosis memory. Of note, the underlying adaptive immunological reaction does not peak at the same time as the clinical skin reaction (figure 1B). Not all BCG-vaccinated or M. tuberculosis exposed individuals will develop strong TST reactions and strategies for classification and handling of samples from clinical skin reaction non-responders needs to be considered before initiation a recall trial using this method. Also, in both SB sampling and visual scoring, there is a potential for theoretical bias in individuals with reduced skin responses due to global or skin related impaired immunity as seen e.g. in HIV-infection and certain age groups. In addition, immunological boosting of the TST reaction is well known with repeated testing. However, we and others find that the SB cell phenotypes remain rather constant when TSTs are repeated. This observation supports the role of SB recall in longitudinal studies of cellular immune responses.

**SBC subsets and timing of sampling**

For T-cell immunologists, SB recall allows for harvest of exceptionally high fractions of antigen-specific cells. However, the timing of SB for sampling from PPD-deposition is critical as both cellular composition and cytokine microenvironment change over time. Kinetic studies suggest that sampling within the first four days of the skin reaction is optimal for the study of non-specific inflammatory responses, whereas the adaptive response consolidates and dominates later and remains detectable for more than two weeks. In this protocol, blisters were induced seven day post challenge, which has proven optimal for the study of adaptive immunity. The SBCs characterized in this demonstration were primarily CD4+ effector memory T cells including high fractions of cells with simultaneous expression of IFN-γ, TNF-α and IL-2. These observations are in line with previous kinetics studies on cutaneous T-cell activation, proliferation and differentiation in PPD-primed skin. However, in one recent BCG-trial, the adaptive response was reported to peak a little earlier with central memory T-cells present three days post-TST and a shift in phenotype towards effector cells accompanied by a decrease in pro-inflammatory cytokine secretion only five days post-TST. Of note, the study did not include time-points exceeding five days. In our hands, day seven post-TST appears to be the most optimal time-point for collection of memory T cells. Other time-points may of course be relevant depending on the antigen and response of interest.

SB fluid contains high levels of both pro-inflammatory cytokines and other proteins shown to be representative of the skin microenvironment. Kinetics studies have shown that TNF-α and IFN-γ levels in SB fluid peak after three days while IL-2 levels peak after seven days, probably reflecting the dominance of adaptive responses at this later stage. Because SB cells have been activated in vivo they exhibit high spontaneous cytokine release as well as a potential for specific release upon re-stimulation (figure 2C and 3).
Rethinking TB vaccine evaluation?

T-cells are considered essential for immune control of *M. tuberculosis*, however it has been difficult to identify a reliable correlate of protection reflected in adaptive immune response from the blood. This roadblock severely hinders the development of new TB vaccines, as there currently is no alternative to large and very costly efficacy trials. TB vaccine developers determine vaccine immunogenicity by assessing small and transient changes in the vaccine specific T-cell populations in the blood. However, it is questionable whether the small fraction of circulating antigen-specific T cells found in the blood is relevant, i.e. capable of migrating to the site of infection and representative of the T-cell rich microenvironment controlling *M. tuberculosis*.

Based on previous studies and the data presented herein, the SB cutaneous recall model represent an untapped potential for the study of vaccine specific T-cells. Not only does the model enable a recall of a vaccine response generated decades ago, it also allows evaluation of the true memory potential in an tissue-specific context. Novel specific skin tests, which includes antigens also comprised in candidate subunit TB vaccines, suggests new opportunities for vaccine evaluation using this model. Furthermore, transcriptomic analyses suggest that CMI response generated in PPD-challenged skin is similar to the response found in the *M. tuberculosis* infected lung.

While skin punch biopsies also allow for cell harvest from the skin and provide spatial information, compared with SB-sampling, the method is more invasive and requires enzymatic or mechanic processing to prepare single-cell suspensions. Measurements of cytokines and cell markers are comparable between the two methods.

Helicopter perspective – the study of adaptive immunity

The suction blister method has already been applied in many areas of medical research besides dermatology, either alone or in combination with systemic or local skin challenge. Examples include studies of sepsis, Epstein-Barr virus associated lymphoproliferative disease, diabetic neuropathy, effects of glucocorticoid intake and human trials testing therapeutic antibodies or models for T-cell targeted therapies.

From a therapeutic point of view, the SB cutaneous recall method offers unique advantages to study the central T-cell memory potential and - from both a biological and technical point of view - the skin seem to provide a relevant sampling compartment. In particular, compared to traditional passive sampling of circulating PBMCs, the SB cutaneous recall method allows for the study of T-cells that have proven the ability to migrate from the lymph node in response to their relevant antigen, and complete local expansion and differentiation in an tissue-specific in vivo context.

In conclusion, the model demonstrated here could be relevant for researchers within the field of human adaptive immunity and T-cell targeting agents. The TST model applied in this protocol, is of special relevance in the field of TB research. However, the basic concept of this model is highly applicable in other fields of research.
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DISCLOSURES:
None
REFERENCES:


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VIDEO DEMONSTRATION

‘A suction blister protocol to study human T-cell recall responses in vivo’ includes a video demonstration of the practical procedure discussed in the manuscript.

The video demonstration is available for download via the following dropbox link:

https://www.dropbox.com/sh/jrub2va7b6zylua/AABBrAVgBBJl0ouueYhE2eBa?dl=0

The presented video is currently in review in Journal of Visualized Experiments and will only be available via this link while the assessment committee is evaluating this thesis.
FIGURE 1.

A.

B.

Mean cell number/blister

Mean TST induration (mm)
FIGURE 2.
FIGURE 3.

A. Cytokine conc. (pg/mL)

- IFN-γ
- IL-10
- IL-13
- IL-2
- TNF-α

Cytokine conc. (pg/mL)

B. Cytokine conc. (pg/mL)

- Plasma
- SB fluid

- 1x10^6 PBMCs
- 1x10^6 PBMCs + 0.50x10^5 SBCs
- 1x10^6 PBMCs + 1.75x10^5 SBCs
- 1.75x10^5 SBCs
APPENDIX III

Manuscript III

Optimisation of a murine splenocyte mycobacterial growth inhibition assay using virulent

*Mycobacterium tuberculosis*

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Optimisation of a murine splenocyte mycobacterial growth inhibition assay using virulent *Mycobacterium tuberculosis*

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In the absence of a validated correlate of protection or robust animal models for human tuberculosis, Mycobacterial growth inhibition assays (MGIA) aim to assess vaccines ability to inhibit mycobacterial growth *in-vitro*. We optimised a reproducible murine splenocyte MGIA based on *in-vitro* infection with virulent *Mycobacterium tuberculosis (M.tbc)* Erdman. We identified splenocyte viability as a problem in state-of-art MGIA protocols, which can be improved by simple changes in culture conditions (viability increase from 21% to 46% at last day of culture). The growth inhibitory potential in mice immunised with either BCG, H56:CAF01 or H56:CAF01 administered side-by-side with BCG was significantly better compared to placebo in all groups (0.3 log₁₀ CFU [±0.2, p = 0.049], 0.5 [±0.2, p = 0.016] and 0.6 [±0.1, p = 0.0007]), respectively corresponding to the levels of *in-vivo* protection. Unexpectedly the CAF01 adjuvant control group also induced significant growth inhibition of 0.3 log₁₀ CFU ([±0.2, p = 0.047]). Finally, we explored vaccine-associated T cell effector functions. Despite presence of high levels of vaccine-specific T cells, we found no increase in CD4⁺ T cell number or cytokine expression profile, nor a difference in cytokine levels in the supernatant after four days culture with or without *M.tbc*. Spontaneous IFN-γ release correlated with growth inhibition levels (p = 0.02), however the cellular source was not found.

Over the last two centuries, tuberculosis (TB) is estimated to have killed one billion people, and remains the world's most lethal infectious disease. The current tools for controlling TB are insufficient, and without new efficacious TB vaccines the WHO End TB strategic goals of a reduction of TB deaths by 99% and cases of TB disease by 90%, from 2015 and 2035 will not be met. Drug-resistant TB is a growing threat to the epidemic, and since TB vaccines are expected to be equally effective against drug-sensitive and drug-resistant strains, vaccines are key to managing the spread of resistant strains.

A major roadblock in the development of new vaccines for TB is the absence of validated correlates of protection or robust animal models for human TB. Consequently, TB vaccine developers rely on large and expensive trials (more than 3,000 subjects) with long follow-up periods to generate proof of concept efficacy data. Therefore there is a relevant push for further research into animal models and correlates, as well as the integration of exploratory immunological projects nested in the clinical trials.

Mycobacterial growth inhibition assays (MGIA) have been proposed as simple and unbiased tools to evaluate vaccine efficacy *in vitro*. These assays study *in vitro* co-culturing of vaccine-induced cells and mycobacteria followed by a determination of the immune cells capacity of inhibiting mycobacterial growth. Several variations of human and murine MGIA are described in the literature including assays based on human whole blood or PBMCs, and murine assays based on splenocytes or pre-infected bone marrow derived macrophage target cells in splenocyte co-culture assays (BM/SP-MGIA). The predominate organism used for both vaccination and *in vitro* challenge is BCG.

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Encouragingly, several of the murine MGIAs have demonstrated significant in vitro growth inhibition in a BCG vaccination model corresponding to in vivo protection in parallel challenge studies[4,15-17]. Within the last years, there has been a drive towards protocol harmonisation and standardisation in an otherwise heterogeneous field. In particular, a standardised murine MIA based on direct co-culturing of mouse splenocytes with BCG has been proposed as a robust and simpler version of the BM/SP-MGIA[15,16,18]. This protocol was optimised and qualified with particular emphasis on multiplicity of infection (MOI) for low assay variability and widest window of growth inhibition[15]. However, it remains to be demonstrated that the underlying mechanism responsible for the observed growth inhibition is driven by vaccine-specific adaptive immunity, as well as essential assay parameters such as cell viability and T cell effector functions during the four-day culture are unknown[13-15].

Therefore, we aimed to characterise and optimise a murine splenocyte MIA to study the growth inhibitory potential of experimental TB vaccines in vitro. We based our assay on the current state-of-art protocol[15,18] and aimed to describe fundamental parameters and estimate variability of the assay. Under the assumptions that Mycobacterium tuberculosis (M.tb) is an intracellular pathogen in vivo and in vitro and that cellular immunity is essential for host control of infection, we focused on the adaptive immune responses. Instead of using BCG as the in vitro infectious organism as in the previously described murine splenocyte MIGAs[13,15], the virulent mycobacterial strain M.tb Erdman was used.

Materials and Methods

Animals. Six- to eight-weeks old female CB6F1 mice (BALB/c × C57BL/6, Envigo, Horst, Netherlands) rested 1-week were housed and handled in Biosafety Level 2 (BSL2) animal facilities at Statens Serum Institut, Denmark and were provided standard food and water ad libitum. The handling of mice was conducted in accordance with the regulations set forward by the national animal protection committee in compliance with European Community Directive 2010/63. In agreement with the Danish Animal Welfare Act all experimental methods including protocols involving animals were carried out in accordance with relevant guidelines and regulations. All protocols were reviewed prior to the start of the experiment by an independent ethical review board at Statens Serum Institut and approved to be in accordance with our license for animal experiments issued by The Animal Experiments Inspectorate (License no. 2014-15-2934-01065) under the Ministry on Environment and Food of Denmark.

Immunisation. The mice were immunised subcutaneously (s.c.) three times at 2-week intervals with either Tris HCl buffer or CAF01 (dose 250 µg/50 µg (DDA/TDB)) alone or CAF01 mixed with 5 µg H56 protein, produced as previously described[19]. Positive control mice received a single dose 200 µl of 2.5 × 10⁶ Colony Forming Units (CFU)/ml BCG Danish 1331 (Statens Serum Institut).

When H56 was used as a BCG booster vaccine (H56:CAF01 side-by-side (SBS) with BCG), mice were vaccinated with 200 µl 2.5 × 10⁶ CFU/ml BCG the first day and then with 0.1 µg H56 in CAF01 the next day, followed by two H56:CAF01 immunisations, 2-weeks apart. In the first vaccination round, mice were vaccinated with 100 µl BCG or H56:CAF01 s.c into the left and right side of the base of the tail. Unless specified otherwise, splenocytes were isolated one week after last immunisation.

Cell culture optimisation. Single splenocyte suspensions were prepared by homogenisation through 100 µm cell strainers followed by washing in RPMI 1640 (Invitrogen) and adjustment to 5 × 10⁶ splenocytes per 600 µl in MIA media. MIA media were either standard media (RPMI-1640, 10% heat-inactivated FCS (Biocohm GmbH) + 10 mM Hepes (Invitrogen) + 2 mM L-Glutamine (Invitrogen)) or enriched media (standard media + 1 mM Natriumpyruvate (Invitrogen) + 1 × Non-essential amino acids (MP Biomedicals, LLC) + 5 × 10⁻⁵ M 2-mercaptopethanol (Sigma-Aldrich)). The cell suspensions were cultured in 2 ml screw cap tubes (Sarstedt) on a 360° tube rotator (Intelli-Mixer Rm-2L, ELMI) or in a rack at 37°C for four days. At different time points, the splenocytes were counted with an automatic Nucelocounter™ (Chemotec) or manually using Nigrosine. All cell work pre-M.tb infection was done in BSL2.

Mycobacteria and culture conditions. For in vitro infection a frozen vial of M.tb Erdman (ATCC strain, grown in 7H9 broth stored at −80°C) was thawed in a water bath followed by 5 minutes sonication. Any clumps were removed by three times of syringe aspiration. Mycobacterial suspensions for infection inoculum and BACTEC MGIT standards were prepared in enriched media by serial 10-fold dilutions. All work involving M.tb infected samples was done in BSL3.

In vitro mycobacterial growth inhibition assay. M.tb Erdman was prepared in enriched media aiming at a concentration of 167 CFU/ml (unless specified otherwise). Within one hour from preparation, 300 µl mycobacterial suspension was added to 5 × 10⁶ splenocytes prepared in 300 µl enriched media (corresponding to an inoculum of 50 CFU per sample tube). M.tb-splenocyte co-cultures were incubated in a rack at 37°C for four days followed by 10 min centrifugation at 12,000 rpm in a bench-top microcentrifuge. One-hundred µl supernatant was removed for multiplex cytokine assays and the remaining 500 µl were resuspended, added to a MGIT tube (BD Biosciences) and incubated until registered positive (BACTEC MGIT liquid culture system (BD Biosciences)). The resulting time to positivity (TTP) was converted to bacterial numbers (CFU) using a linear regression of a standard curve comprised of TTP values from inoculated M.tb Erdman 10-fold dilutions against CFUs obtained from plating aliquots of M.tb Erdman onto Middlebrook 7H11 agar plates (BD Biosciences). Direct-to-MGIT controls were included, defined as 50 CFU M.tb Erdman directly placed in the BACTEC MGIT system without any pre-incubation (at day 0). Data are presented as total number log₁₀ CFU per sample tube. To compare the growth inhibition between experiments, delta log₁₀ CFU was calculated by subtracting the individual log₁₀ CFU values in the immunised group from the mean of the control group.
For examination of intracellular growth, splenocyte-mycobacteria co-cultures were incubated for three hours, then treated with 0 or 100 μg/ml gentamicin (Gibco, Life Technologies) for one hour followed by three times wash and placement in the BACTEC MGIT system. Samples without splenocytes were cultured and treated in parallel without wash before TTP assessment.

**Intracellular cytokine staining assay.** A total of 1–2×10⁶ splenocytes were stimulated *in vitro* in V-bottom 96-well plates at 37°C in media containing anti-CD49d (1 μg/ml) and anti-CD28 (1 μg/ml) without antigen or in the presence of 2 μg/ml H56 protein for 1 hour, plus 6 hours in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich), after which cells were maintained at 4°C until staining.

Cells were stained for the surface markers using anti-CD4-BV786 (clone GK1.5; BD Biosciences), anti-CD44-FITC (clone IM7; eBioscience, USA) and anti-Fixable Viability Dye-APC-Cy7 (eBioscience, USA) before fixation and permeabilisation using Cytoperm/cytofix kit (BD Biosciences) as per manufacturer’s instructions, and subsequently stained for intracellular cytokines with anti-IFN-γ-PeCy7 (clone XMG1.2; eBioscience, USA), anti-TNF-α-Pe (clone MP6-XT22; eBioscience, USA) and anti-IL-2-APC (clone JES6-544; eBioscience, USA). Non-specific background cytokine values were determined for each combinatorial Boolean gate and subtracted. Gates for surface markers were based on fluorescence-minus-one controls. All flow cytometry analyses including Boolean analysis were performed with FlowJo Software v.10 (Tree Star, Ashland, OR, USA).

**Multiplex cytokine assay.** The Proinflammatory panel 1 (Mouse) 7-plex cytokine assay (Meso Scale Discovery (MSD)) measuring IFN-γ, IL-1β, IL-6, KC/GRO, IL-10, IL-12(p70) and TNF-α was performed according to the manufacturer’s instructions. The plates were read on the Sector Imager 2400 system (Meso Scale Discovery) and calculation of cytokine concentrations in unknown samples was determined by 4-parameter logistic non-linear regression analysis of the standard curve.

**Statistical analysis.** Prism 6 software (Graphpad v6.05) was used for all statistical analyses. Mean values and parametric tests were used under the assumption that data are normally distributed. Unpaired two-tailed t-tests were used to compare control and vaccinated groups in the MGIAs. Cytokine levels detected with MSD were analysed using one-way ANOVA with Dunnett’s multiple comparisons test. Associations between growth inhibition and cytokine responses were measured using Spearman’s rank correlation coefficient. A p-value of p < 0.05 was considered significant. Statistically significant differences are marked by asterisks in figures and explained in the figure legends.

**Results**

**Assay optimisation and fundamental parameters in the splenocyte MGIA.** The TTP was closely related to the number of CFUs per millilitre suspensions of *M. tb* Erdman. In three independent experiments, TTP values were found to be highly reproducible with a duplicate CV <6% in all titrations (5 to 1 × 10⁸ CFU) (Fig. 1a). A low inter-assay variability was also detected, with a CV <6% across experiments in a concentration at 50 CFU per 600 μl culture media. Given the importance of viable functional effector T cells in TB vaccine immunology, we next focused on splenocyte viability during four-day culture. We initially described viability under standard culture conditions wherein 5 × 10⁶ splenocytes from naive mice were isolated and cultivated in standard media (RPMI, FCS, Hepes and L-Glutamine) and incubated at 37°C with 360° rotation for four days. These conditions led to a rapid and substantial loss of viability with only mean 21% (range 17–25%) viable cells at day four (Fig. 1b). Enrichment of the culture media by addition of nutrients (Natriumpyruvate, Non-essential amino acids, and 2-mercaptoethanol) and incubation without rotation, increased cell viability at day four to 46% (43–49%) (Fig. 1b). Nutrient enrichment and no rotation were studied separately and demonstrated day four viability of 19% (17–21%) and 36% (35–37%), respectively (data not shown). Based on the assumption that viable splenocytes are an essential component in the study of vaccine-induced growth inhibition in this type of assay we advanced our experiments using nutrient enrichment without rotation.

We next focused on describing the co-culture of *M. tb* Erdman and vaccine naive splenocytes. Under the assumption that the lowest reproducible CFU inoculum demonstrates potential growth inhibition best, we titrated the *M. tb* Erdman inoculum, determined the delta log growth from day zero to day four, and demonstrated a fairly consistent growth window of 1.6 log₁₀ CFU with inoculum above 50 CFU per 600 μl culture media, which was used in the subsequent MGIAs (Fig. 1c). Finally, to verify that the mycobacteria grow intracellularly, splenocytes and mycobacteria were co-cultured for three hours to allow infection, followed by addition of 100 μg/ml gentamicin; an antibiotic which is not transported across the eukaryote cell membrane killing only extracellular bacteria. *M. tb* Erdman growth was unaffected by gentamicin in the extracellular environment when splenocytes were present, while there were no live mycobacteria in samples without splenocytes, indicating that the mycobacteria were indeed intracellular (Fig. 1d).

**Assay variability.** Next, we assessed the sample variability of our optimised MGIAs. Groups of four mice were immunised with either BCG, H56:CAF01 or placebo, and splenocytes were assayed one week after the last immunisation (Fig. 2). We observed low within mouse duplicate variability in the placebo group (Coefficient of Variability (CV) 4%) (Fig. 2a) while duplicate variability was higher in the vaccinated mice (CV 19% and 21% in BCG (Fig. 2c) and H56:CAF01 (Fig. 2e), respectively). The variability within groups was also higher in the vaccinated groups with CV of 2%, 2% and 14% for placebo, BCG and H56:CAF01 (Fig. 2b,d and f), respectively.

**H56:CAF01 and BCG immunisation induced mycobacterial growth inhibition in murine splenocytes.** To determine whether the optimised MGIAs could demonstrate growth inhibition *in vitro*, we selected a panel of experimental vaccines developed at Statens Serum Institut, which previously have shown protective in *in vivo* challenge experiments: BCG and H56:CAF01 (both ~1 log₁₀ CFU protection⁰, and unpublished) and...
H56:CAF01 SBS with BCG (~1.3 log_{10}, Aagaard unpublished and ref. 21). Groups of eight mice were immunised with the three vaccines and compared to a placebo and CAF01 adjuvant control (Fig. 3). Significant growth inhibition was observed in all groups, where H56:CAF01 SBS with BCG induced the strongest growth inhibition with a reduction of 0.6 (SEM ± 0.1) log_{10} CFU compared to the placebo group (p = 0.0007; t-test). Splenocytes from H56:CAF01 or BCG immunised mice induced growth inhibition with a reduction of 0.5 (± 0.2, p = 0.016; t-test) and 0.3 (± 0.2, p = 0.049; t-test) log_{10} CFU compared individually to placebo, respectively. Unexpectedly the CAF01 adjuvant control group mediated significant growth inhibition of 0.3 (± 0.2, p = 0.047; t-test) log_{10} CFU on a level comparable to BCG. The application of one-way ANOVA with Dunnett’s adjustment for multiplicity resulted in significant growth inhibition in splenocytes from H56:CAF01 (p = 0.005) and H56:CAF01 SBS with BCG (p = 0.0005) vaccinated mice compared to the placebo group, supporting these vaccines as most potent in the system. Of note, we observed a larger within group variability also in the placebo group compared to the earlier variability assessment CV <12%.

Mycobacterial growth inhibition is reproducible, allowing for comparison between experiments. We compared the between run variability in two separate H56:CAF01 vaccination experiments (data from Figs 2 and 3) after adjustment for different M.tb Erdman inocula (68 and 70 CFU) by subtraction of the log_{10} CFU growth in direct-to-MGIT controls from the individual sample values (Fig. 4a). H56:CAF01 immunisation induced a reproducible difference of 0.5 log_{10} CFU compared to the respective placebo group in both experiments (p = 0.92; t-test) (Fig. 4b) supporting the use of this subtraction method to allow comparison between experiments.
We then proceeded to investigate the temporal aspects of the vaccine-induced growth inhibition potential in splenocytes obtained 1, 5 and 29 weeks after BCG immunisation (Fig. 5). Significant growth inhibition in the BCG immunised versus the respective placebo group was observed 1 week (0.4 ± 0.04 log_{10} CFU; p < 0.0001; t-test) and 5 weeks (0.3 ± 0.2 log_{10} CFU; p = 0.049; t-test) after BCG immunisation; however, interestingly this

**Figure 2.** Assay variability. Groups of mice were immunised three times s.c. with H56 in CAF01 (e,f), one time with BCG (c,d) or placebo (Tris buffer) (a,b) with 2-week intervals. Splenocytes from individual mice (n = 4) were isolated one week after the last immunisation and co-cultured with 50 CFU of *M. tuberculosis* Erdman in the four days MGIA. The mean log_{10} CFU of measurements of individual mice done in duplicates (a,c,e), group means (b,d,f).
response was not detected 29 weeks after immunisation (0.2 ± 0.2 log10 CFU; p = 0.4; t-test) (Fig. 5). We also explored H56:CAF01 growth inhibition at 29 weeks, in line with the findings of BCG also this vaccine failed to control at the late time point (0.05 ± 0.3 log10 CFU; p = 0.9; t-test, data not shown).

**In vitro infection does not drive detectable change in T cell functionality.** As an initial and crude control of the role of cellular immunity in the MGIA, we explored the mycobacterial growth in parallel cultures of live splenocytes from H56:CAF01 vaccinated mice compared to heat killed splenocytes from H56:CAF01 vaccinated mice (20 minutes at 60 °C) and found no indication of growth inhibition in the heat killed culture (data not shown). Therefore, under the hypothesis that MGIA measures a vaccine-specific T cell dependent mechanism, we investigated IFN-γ, TNF-α and IL-2 expression in CD4+ T cells following H56 stimulation in splenocytes from H56:CAF01 immunised mice using intracellular stain flow cytometry before and after four days culture with or without M.tb Erdman infection. In agreement with the literature, H56:CAF01 induced a CD4+ T cell profile dominated by TNF-α⁺ IL-2⁺, and IFN-γ⁺ TNF-α⁺ IL-2⁺ polyfunctional CD4+ T cells (Fig. 6a), which did not change on day four (Fig. 6b and c). There was no indication of a change in frequency or phenotype of the CD4+ T cell population on day four comparing M.tb Erdman-infected and uninfected splenocyte-cultures (Fig. 6b and c).

**Association between polyfunctional T cells and mycobacterial growth inhibition.** Next, we investigated whether the demonstrated vaccine-induced growth inhibition correlate with the cytokine flavour of the vaccine-induced CD4+ T cells at day zero in splenocytes from a separate experiment comparing responses in groups of eight H56:CAF01 and CAF01 control vaccinated mice. We observed a significant inverse correlation between IFN-γ⁺ TNF-α⁺ IL-2⁺ polyfunctional CD4+ T cell frequency and log10 CFU (Spearman r = −0.738; Figure 3).
exercise for all IFN-γ positive and TNF-α+ T cells, which after exclusion rendered the slope null. We repeated this through T cells, we explored vaccine-associated CD4+ T cells that an efficacious TB vaccine (at least in part) control infection driven expansion of vaccine-specific CD4+ T cells, seemingly was driven by the same outlier (data not shown).

By subtracting the individual log10 CFU values in the immunised BCG groups from the mean of the respective placebo group. Solid lines represent the mean delta log10 CFU of 4, 8 and 4 mice, for 1 week, 5 weeks or 29 weeks, respectively, measured in duplicates. t-test between the BCG group and the respective placebo group.

\( p = 0.046; \) Fig. 7) in the H56:CAF01 group, however, the data suggests that this association is driven by one outlier with the highest number of polyfunctional T cells, which after exclusion rendered the slope null. We repeated this exercise for all IFN-γ positive and TNF-α+ T cells identifying a similar weak association where the slope seemingly was driven by the same outlier (data not shown).

**Cytokine release associated with vaccination but not infection.** As we identified no detectable infection driven expansion of vaccine-specific CD4+ T cell populations during the four day culture (Fig. 6), we proceeded to investigate whether we could detect infection specific cytokine response (IFN-γ, IL-1β, IL-6, IL-10, IL-12p70 and TNF-α) in the culture supernatant. Of note, we observed differences in the magnitude of cytokine release between the vaccines, with BCG containing combinations driving the highest levels; in particular H56:CAF01 SBS with BCG immunisation primed significant IFN-γ, IL-6, IL-10 release compared to placebo while BCG immunisation induced significant IL-6 and IL-10 responses (Fig. 8a,b and c (grey bars)). IL-1β and IL-12p70 expression followed the same pattern as IFN-γ, IL-10 and IL-6 however, levels were low (<30 pg/ml) (data not shown). There were no vaccine-specific differences in the magnitude of TNF-α release (stable between 55–60 pg/ml for all vaccines). As suggested by the flow cytometry data earlier, M.tb infection did not induce a difference in cytokine responses in any of the investigated vaccine groups (Fig. 8a,b and c (grey bars)).

Next, we explored the association between vaccine-primed cytokine release during four-day M.tb splenocyte co-culture and the observed growth inhibition by correlating the mean level of cytokine release and mean growth inhibition in the same group. There was strong significant inverse correlation between IFN-γ release and log10 CFU (Spearman r value = −1.0; \( p = 0.02; \) Fig. 8d) but not between IL-6 or IL-10 and log10 CFU (Spearman r value = −0.7; \( p = 0.2; \) Fig. 8e, Spearman r value = −0.5; \( p = 0.5; \) Fig. 8f).

**Discussion**

In this project, we optimised a reproducible murine splenocyte MGIA using virulent M.tb Erdman as the target bacteria. Poor splenocyte viability was identified as a problem in the standard protocol, which could be overcome with simple changes in the culture conditions. Using our optimised MGIA protocol, BCG, H56:CAF01 and H56:CAF01 SBS with BCG induced M.tb growth inhibition in vitro corresponding to the relative in vivo protection20. Of note, the adjuvant control also mediated significant growth inhibition at the level of BCG. Assuming that an efficacious TB vaccine (at least in part) control M.tb through T cells, we explored vaccine-associated CD4+ T cell effector functions, but failed to identify a T cell associated mechanism to explain observed growth inhibition. Spontaneous IFN-γ release in the co-culture supernatant correlated with mycobacterial growth inhibition levels, but the cellular source was not identified.

There remains an incomplete understanding of the host factors that determine why some individuals are protected from M.tb infection while others fail to contain infection and progress to active TB. The absence of a protective marker has driven the development of MGIA as a potential correlate of protection encompassing a range of immune mechanisms and their complex interactions5. It is a heterogeneous field and a diverse range of assays have been proposed for both humans, mice and cattle. Recently, there has been a move towards protocol harmonisation and standardisation in the field, including the publication of a murine MGIA protocol based on direct co-culturing of mouse splenocytes with BCG13,15,18, which we and others have used as foundation for murine as well as human PBMC based MGIA studies22.

In the MGIA, M.tb very rapidly infects cells and becomes intracellular, wherefore it has been an overriding aim of this project to describe the health of the splenocytes and in particular the subpopulation of vaccine-specific CD4+ T cells potentially capable of mediating intracellular kill or growth inhibition during the four-day co-culture. We initially explored splenocyte survival under the culture conditions described in the standard protocol.

**Figure 5.** Kinetic in BCG-induced mycobacterial growth inhibition. Groups of mice were immunised with a single dose BCG or given Tris buffer (Placebo) three times s.c. with 2-week intervals. In different immunisation experiments, splenocytes were isolated 1 week, 5 weeks or 29 weeks after BCG immunisation and used and MGIA. Delta log10CFU values represent the BCG-induced mycobacterial growth inhibition and were calculated by subtracting the individual log10CFU values in the immunised BCG groups from the mean of the respective placebo group. Solid lines represent the mean delta log10 CFU of 4, 8 and 4 mice, for 1 week, 5 weeks or 29 weeks, respectively, measured in duplicates. t-test between the BCG group and the respective placebo group.

* \( p < 0.05; \) **** \( p < 0.0001. \)

p = 0.046; Fig. 7) in the H56:CAF01 group, however, the data suggests that this association is driven by one outlier with the highest number of polyfunctional T cells, which after exclusion rendered the slope null. We repeated this exercise for all IFN-γ positive and TNF-α+ T cells identifying a similar weak association where the slope seemingly was driven by the same outlier (data not shown).
splenocyte protocol\textsuperscript{15,18}, and were surprised to find that even in the absence of \textit{M. tb} in the culture, there was a substantial, rapid and reproducible splenocyte death; which could be prevented with simple modifications of the assay (no rotation and use of enriched media). Elaborate explorations with manual and automated counting, other rotators, varying rotator speeds, and reproduction in parallel studies using human PBMCs (Holm personal communication) underpin that the shear forces brought on by rotation negatively affects cell survival\textsuperscript{23}. It could be speculated that the cells who encounter their relevant antigen would be more prone to survive. However, we found no indication of a relative increase in the number of specific T cells compared to unspecific T cells on day four. To our knowledge, there is no published data demonstrating the benefit of rotation in the MGIA, and until now there is no studies describing cell viability in these assays\textsuperscript{13,15}. These sobering findings raise concern and call for independent confirmation.

In line with other groups\textsuperscript{17,24}, we used the virulent \textit{M. tb} Erdman as the target bacteria in the MGIA. We consider virulent \textit{M. tb} more relevant than BCG as it expresses more vaccine candidate antigens and allow for better comparison to the \textit{in vivo} challenge experiments we used to benchmark the MGIA assay\textsuperscript{25,26}. Under the assumption that vaccine-induced control of mycobacterial growth may be overwhelmed at higher inoculi\textsuperscript{8,15}, we used \textasciitilde{}50 CFU \textit{M. tb} Erdman which in our hands had low variability and comparable growth as higher inoculi.\textsuperscript{8,15} This assay was reproducible and had comparable or lower variability compared to similar splenocyte MGIA described in the literature\textsuperscript{3–15,17}.

**Figure 6.** \textit{In vitro} infection does not drive detectable change in T cell functionality. Groups of mice were immunised three times s.c. with 2-week intervals with H56 in CAF01 or adjuvant control (CAF01). One week after the last vaccination, splenocytes were isolated and used for intracellular cytokine analysis by flow cytometry at day 0 (a) or after four days culture with or without 50 CFU of \textit{M. tb} Erdman (b). Splenocytes were stimulated with H56 \textit{in vitro} before the frequencies of antigen-specific CD4\textsuperscript{+} cells (CD44\textsuperscript{high}) producing IFN-\textgamma, TNF-\alpha and IL-2 were measured by gating for singlets, lymphocytes and live CD4\textsuperscript{+} cells. All possible combinations of cytokine expression were tabulated by Boolean gating analysis, and, after subtracting the background (non-stimulated) samples, the results for the seven combinations expressing at least one of the cytokines were shown. Bars represent mean \pm SEM of eight mice. (c) Pie charts over the polyfunctional CD4\textsuperscript{+} cells shown in Fig. 6a and b.

**Figure 7.** Association between polyfunctional T cells and mycobacterial growth inhibition. Scatter plots of the frequency of IFN-\textgamma\textsuperscript{+}TNF-\alpha\textsuperscript{+}IL-2\textsuperscript{+} polyfunctional CD4\textsuperscript{+} cells at day 0 from the experiment shown in Fig. 6a versus H56:CAF01 induced growth inhibition data from the same experiment. Spearman’s rank *p < 0.05.
We and others have assessed the MGIA potential in splenocytes of BCG-vaccinated mice. Recently Zelmer et al. compared the ability of splenocytes from BCG Danish (Statens Serum Institut) and BCG Pasteur (Aeras) vaccinated C57BL/6 mice to mediate growth inhibition of the vaccine BCG in vitro using the standard rotator based splenocyte MGIA protocol. Of note, both BCG Pasteur and BCG Danish were protective in vivo, but only the BCG Pasteur model was capable of mediating growth inhibition in vitro (0.7 log10 CFU, CV 23%). BCG Pasteur has also proven capable of mediating growth inhibition of M.tb Erdman in the more complex BM/SP-MGIA with pre-infected bone marrow derived macrophage target cells in seven-day splenocyte co-culture. In our assay, BCG Danish mediated a significant growth inhibition of 0.3–0.4 log10 CFU with a CV < 8%, calling for further studies to elucidate whether BCG Pasteur vaccinated mice or a switch from virulent M.tb to the slower growing BCG as target organism would mediate a superior growth inhibition in our model.

As in other studies, we demonstrated an association between individual vaccines ability to control growth in vitro and protect in vivo - an essential positive control supporting the concept of MGIA as a correlate of protection. CD4+ T cells are fundamental components of both host control and successful vaccination against TB, and a central role for CD4+ T cell-mediated growth inhibition has previously been demonstrated in the MB/SP-MGIA model. In the standard splenocyte MGIA model, such a link has only been indicated by an upregulated inflammatory mRNA signature, wherefore we attempted to demonstrate it directly. In agreement with the literature, H56:CAF01 immunisation induced a strong polyfunctional CD4+ T-cell profile in our study. Vaccine-specific CD4+ T cells in H56:CAF01 immunised mice traffic more efficiently to the M.tb infected lung than infection-driven responses and would be a potential correlate to study in this assay. However, in spite of significant growth inhibition, we failed to demonstrate changes in activation, cytokine expression profile or clonal expansion of vaccine-specific CD4+ T cells during four-day co-culture with M.tb. These findings do not preclude that a T-cell mediated effect could be demonstrated by using a higher MOI or a preculture step as in the BM/SP-MGIA where more antigen should be available for T-cell recognition. However, in our attempt to describe the mechanisms at play under the conditions which can control infection in the assay, the data suggest that the splenocyte based MGIA rely on a growth inhibitory mechanism(s) which is either very subtle requiring more sensitive assays for detection, or that the mechanism is simply independent of vaccine-specific T cells recognising their antigen on infected cells. The latter interpretation echoed by the significant growth inhibition observed in the negative control (CAF01 adjuvant) group combined with the observation that the vaccine-mediated

Figure 8. Cytokine release associated with vaccination but not infection. Groups of mice were immunised three times s.c. with 2-week intervals with H56 in CAF01, H56:CAF01 SBS with BCG or given placebo (Tris buffer) or CAF01 as controls. At the same time, as the first vaccination, a group of mice received a single dose BCG. Splenocytes were isolated one week after the last immunisation and used in the MGIA. Culture supernatants were analysed for the released cytokines IFN-γ (a), IL-6 (b) and IL-10 (c). Black bars indicate the levels of cytokines released from splenocytes before in vitro culture, while grey bars represent the levels of cytokines measured in the MGIA cultures after four days infection and white bars represent cytokines measured in cultures without infection. Bars represent mean ± SEM of eight mice (CAF01 n = 10). For the groups of mice where growth inhibition and MSD data was available (n = 5), scatter plots of mean log10 CFU values versus mean levels of IFN-γ (d), IL-6 (e) and IL-10 (f) measured in the same MGIA samples were drawn. One-way ANOVA with Dunnett’s multiple comparisons test was used to compare cytokine levels between vaccinated and placebo control groups (a–c). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. (d–f) Spearman’s rank *p < 0.05.
growth inhibition only could be demonstrated early (1 and 5 weeks post vaccination) and not late (29 weeks post vaccination).

The only investigated factor which correlated with growth inhibition was the level of IFN-γ released in the culture supernatant from day zero to day four. Of note, the IFN-γ levels were comparable in the presence and absence of M.tb in the culture, suggesting that the IFN-γ levels do not derive from vaccine-specific T cells recognizing their relevant antigen. IFN-γ can be induced by other cells e.g. antigen presenting cells, NK cells and/or neutrophils, cell populations which role in this assay remains to be studied.

In conclusion, we have optimised a murine splenocyte MGIA with M.tb Erdman as target organism. The association between vaccine-induced in vitro growth inhibition and in vivo protection suggested that this assay could represent a relevant tool to compare vaccines and study correlates. However, after failing to demonstrate a direct link between vaccine-induced T cells and growth inhibition, we call for caution drawing firm conclusions on vaccine effects using the splenocyte MGIA before the involved mechanisms are better understood.

References

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Author Contributions
C.J., L.H., C.A. and M.R. designed the experiments. C.J. performed laboratory work and analysed the results supervised by L.H., M.R.; and L.H. participated in some of the MGIA experiments and mycobacteriology work. E.S. contributed reagents/materials/analysis tools. C.J. wrote the first draft of the manuscript supervised by M.R. and L.H. All authors reviewed and approved the final version of the manuscript.

Additional Information
Competing Interests: C.A. is co-inventor on a patent disclosing the use of H56 and CAF01 in vaccines. All rights have been assigned to Statens Serum Institut, a Danish non-profit governmental institute. The other authors have no financial conflicts of interest.

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APPENDIX IV

Manuscript IV

Development and qualification of a human Mycobacterial growth inhibition assay using

*Mycobacterium tuberculosis*

Ready for submission, PlosOne
Title
Development and qualification of a human Mycobacterial growth inhibition assay using
Mycobacterium tuberculosis

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Abstract

Introduction: Mycobacterial growth inhibition assays (MGIAs) are emerging tools for assessment of tuberculosis (TB) vaccine efficacy. We aimed to describe and qualify a human MGIA based on infection of peripheral mononuclear cells (PBMCs) with *Mycobacterium tuberculosis* (*Mtb*) H37Rv.

Method: Study volunteers were Bacille-Calmette-Guérin (BCG) vaccinated or unvaccinated Danes, and individuals with active TB or latent *Mtb* infection (LTBI). Standard MGIA setup: 1x10^6 PBMCs were infected with 100 CFU mycobacteria and cultured for four days in RPMI media +/-autologous plasma or pooled human serum. Mycobacterial numbers were quantified using Mycobacterial Growth Indicator Tubes and Time to detection – Log_{10} CFU conversion plots.

Results: PBMC viability was improved by exclusion of continuous rotation from the MGIA protocol (22% vs. 59% viable cells after four days, p = 0.012). MGIA variability was low within-run (CV 4 %, range 0 – 17 %) and between-run (CV 8 %, range 0 – 28 %). We did not find superior mycobacterial control in groups of individuals with BCG vaccination, active TB or LTBI when compared with healthy controls. Interferon-γ (IFN-γ) levels were elevated in BCG vaccinated and *Mtb* infected cultures containing autologous plasma. However, IFN-γ release did not correlate with mycobacterial numbers in individual cultures, nor did culturing with autologous plasma improve discrimination of mycobacterial numbers between groups. Addition of high frequencies of purified protein derivative (PPD)-specific cells from skin suction blisters and CD4+ T cells isolated from PBMCs did not reduce mycobacterial numbers in MGIA cultures from BCG vaccinated individuals.

Conclusion: Despite high assay reproducibility, we did not observe reduced mycobacterial numbers in groups of individuals with BCG vaccination, active TB or LTBI. Attempts to augment
adaptive growth inhibitory mechanisms by introduction of PPD-specific T cells or autologous plasma, were unsuccessful. Our systematic assessment of core assay variables sheds light on strengths and weaknesses of the MGIA methodology.
Introduction

Tuberculosis (TB) is a major global health threat with a quarter of the world’s population being latently infected with *Mycobacterium tuberculosis* (*Mtb*) and more than 1.5 million deaths annually [1]. The sole licensed TB vaccine, the Bacillus Calmette-Guérin (BCG) vaccine, has limited protection in adults and development of new and more effective TB vaccines is a global priority [1–5].

To date, immune parameters associated with immunity against TB have failed to predict vaccine protection *in vivo* and consequently, there is presently no substitute for efficacy testing by large and lengthy clinical trials [6–12].

The Mycobacterial growth inhibition assays (MGIAs) have been suggested as unbiased tools for assessment of vaccine efficacy and recently there has been a move towards harmonization and qualification of a standardized MGIA for use as an early vaccine triage tool [13–15]. Encouragingly, several studies have demonstrated that TB vaccine candidates that provide *in vivo* protection in animal models also control infection *in vitro* as determined by murine MGIA [16–19]. Additionally, MGIA provide an opportunity to study immune mechanisms and vaccine specific effects against one or several mycobacterial strains in one assay. Due to biosafety concerns, most MGIA use *Mycobacterium bovis* BCG as the infectious agent. However, a MGIA based on virulent *Mtb* would be highly attractive because novel TB vaccine constructs may contain ESAT-6 and other *Mtb* specific antigens not expressed in BCG [20].

In this project, we aimed to develop and qualify a human peripheral mononuclear cell (PBMC) MGIA using the virulent *Mtb* H37Rv for *in vitro* infection and, based on previously described principles, the Bactec MGIT system for evaluation mycobacterial numbers measuring time to detection of growth [21]. Our first objectives were to describe the basic assay parameters,
optimize and evaluate variability. Secondly, we wanted to compare mycobacterial numbers MGIA in cultures using PBMC samples from individuals with BCG-vaccination, latent TB infection (LTBI), active TB disease and healthy unvaccinated controls. Finally, we tried to explore possible growth inhibitory mechanisms by addition of autologous plasma or high numbers of CD4+ T-cells or purified protein derivative (PPD)-specific skin suction blisters cells to MGIA cultures.

Materials and Methods

Study populations
The majority of study participants were healthy Danish adult volunteers without any history of TB exposure or LTBI. Other study groups comprised adults diagnosed with either active TB disease or LTBI recruited from the TB outpatient clinic at Gentofte Hospital, Copenhagen, Denmark. All volunteers provided information on age, BCG vaccination status, TB exposure and previous Quantiferon or tuberculin skin test results. Individuals with active TB or LTBI provided additional information on TB/LTBI diagnosis, duration of anti-TB treatment (if any), comorbidities and concurrent medication. All volunteers provided verbal and written informed consent. The study was approved by the Danish Committee on Health Research Ethics (H-15002988 and H-17006335) and registered at the Danish Data Protection Agency (Ref. 2015-57-0102 and 2015-54-0102).

PBMCs
Peripheral whole blood from study volunteers was collected in heparinized tubes and PBMCs were isolated by density gradient centrifugation using Lymphoprep tubes and medium (Stemcell
Technologies, Oslo, Norway) followed by twice wash in RPMI-1640 + L-glutamine (RPMI) medium (Gibco, ThermoFisher Scientific). PBMCs (5x10⁶/mL) were stored in liquid nitrogen suspended in a medium consisting of 75% RPMI, 15% Fetal Bovine Serum (Sigma Aldrich, F2442, USA) and 10% Dimethyl Sulfoxide (Sigma Aldrich) until use. For some experiments, CD14+ monocytes and CD4+ T cells were isolated from thawed PBMCs by negative magnetic selection according to the manufacturer’s protocol (EasySep, Stemcell Technogies). All medium used for washing, cryopreservation and culture of specimens was free of antibiotics, unless specified in the results section.

**Plasma and serum**

Autologous plasma from study volunteers was isolated from heparinized whole blood after centrifugation. Sterile filtrated pooled human serum (Sigma Aldrich, H4522) and autologous plasma were stored at -20 °C until use. Prior to use, thawed plasma and serum were heat inactivated at 56 °C for 30 minutes and sterile filtrated.

**Cells from skin suction blisters**

Skin suction blisters were induced as described in Holm et al, (JoVE, in review). In brief, cells were harvested from skin blisters eight days after intradermal injection of two tuberculin units of PPD (SSI). Freshly harvested cells were stained with nigrosine, counted by light-microscopy manual count and re-suspended in RPMI for MGIA.
**MGIA principle and definitions**

The basic principle of the PBMC MGIA applied here, is to measure and compare mycobacterial numbers in cultures of PBMCs from immunized versus non-immunized individuals. In short, PBMCs are infected with a fixed number of mycobacteria and cultured for four days. Then samples are then transferred to Mycobacterial Growth Indicator Tubes (MGIT; Becton Dickinson, Franklin Lakes, NJ) and placed in a Bactec 960 (Becton Dickinson) MGIT incubator that measure oxygen-sensitive fluorescent induced by metabolizing mycobacteria [22]. In the Bactec 960 MGIT, samples are registered as positive when a preset threshold for fluorescent signal is reached, and hence, the initial MGIA readout is given as Time to detection (TTD, hours). For translational purposes, TTD was following converted to \( \log_{10} \) colony forming units (CFU) using conversion plots.

In this paper, we refer to converted \( \log_{10} \) CFU values in as mycobacterial numbers. The number of mycobacteria used for infection is referred to as inoculum. In some experiments, we also calculated the change in mycobacterial numbers over the four-day culture period by subtracting the inoculum \( \log_{10} \) CFU value from the day-four \( \log_{10} \) CFU value, \( \Delta \) bacterial numbers.

**Mycobacteria**

The virulent \( Mtb \) strain H37Rv (ATCC 27294) was used for all MGIA *in vitro* infections. In one experiment, BCG Danish 1331 (Batch BCG.SSL.02) serial suspensions were used for H37Rv conversion plot comparison. Mycobacteria were grown to mid-log phase in 7H9 broth (produced in house, Statens Serum Institut) with BBL Middlebrook ADC enrichment (Becton Dickinson) and stored at \(-80 \, ^\circ C\) until use. Prior to use in MGIA, frozen mycobacterial stock vials were thawed in a water bath at room temperature, sonicated for five minutes and aspirated thrice by syringe for clump removal. Mycobacterial suspensions for conversion plots and PBMC infection were
prepared in RPMI by 10-fold serial dilutions immediately before sample inoculation. All work with mycobacteria was done in Biosafety level 3 facility.

**MGIA protocol**

Cryopreserved PBMCs were thawed, washed thrice and rested for 2 hours at 37 °C / 5 % CO₂ in RPMI with DNase. After resting, PBMCs were washed twice and counted by automated Nucleocount (Chemometec, Allerod, Denmark). PBMCs were infected by mixing 300 µl mycobacterial suspension (100 CFU / 300 µl) with 300 µl PBMC suspension (1x10⁶/300 µl), aiming for PBMC density of 1.67x10⁶ PBMCs/mL and a multiplicity of infection of 1 mycobacterium per 1000 PBMCs (corresponding to approximately 8 – 50 mycobacteria per 1000 monocytes). Three different culture media were assessed in parallel: RPMI-1640 (RPMI), RPMI + 10 % pooled human serum (RPMI-PS) and RPMI + 10 % pooled autologous plasma (RPMI-AP). If not specified otherwise, all MGIA cultures (600 µl) were incubated in duplicate 2 mL tubes (Sarsted, Germany) with closed lids standing in a rack at 37°C for four days. After four days, tubes were spun at 12.000 rpm for 10 minutes in a benchtop centrifuge. Supernatants (100 µl) were removed and stored at -20 °C until use. Cell pellets were re-suspended in the 500 µl remaining medium and transferred to MGIT tubes containing fluorescent indicator and readymade culture medium. The tubes were supplemented with 800 µl BBL MGIT PANTA and BACTEC MGIT growth supplement mixture (Becton Dickinson), according to the manufacturer’s instructions. MGIT tubes were incubated in a Bactec MGIT 960 automatic incubator until registering positive (TTD, hours). Any deviations from the MGIA protocol are described in the results section where relevant.
**Quantification of mycobacteria**

In order to convert TTD to mycobacterial numbers, TTD - log_{10} CFU conversion plots were created for individual experiments by parallel assessment for mycobacterial viable count and TTD in 10-fold serial mycobacterial RPMI suspensions in. For viable counting, 100 µl of mycobacterial suspensions were spread on Middlebrook 7H11 plates (Beckton Dickinson), followed by incubation for 14 days at 37°C and mycobacterial colony count (CFU). Mean duplicate CFU counts were multiplied by 3 (to correspond to the mycobacterial number in the volume used for PBMC infection) and log_{10} converted. For TTD assessment, 300 µl of the same mycobacterial suspensions were added to duplicate MGIT tubes until registering positive in the Bactec MGIT. Matched log_{10} CFU and TTD values were plotted into an x-y coordinate system, followed by linear regression to produce standard curves for TTD- log_{10} CFU conversion in infected PBMC samples.

**ELISA**

IFN-γ was measured in thawed culture supernatants by an in-house enzyme-linked immunosorbent assay (ELISA), as previously described [23]. Supernatants were treated with 10% 1M Sodium Azide (NaN₃) overnight to decontaminate mycobacteria prior to cytokine measurement. All samples were run in duplicates.

**Multiplex cytokine assay**

Measurement of IFN-γ, TNF-α, IL-2, IL-10 and IL-13 in plasma, pooled serum and culture supernatants was performed by validated multiplex cytokine assays according to the manufacturer’s protocol (V-plex, Meso Scale Discovery). Cytokine levels above assay range were
set to the highest level of detection. Cytokine measurements levels below assay range were set to zero.

Statistical analysis

Mycobacterial numbers are reported as log$_{10}$ CFU converted from TTD by linear regression. Numerical values are reported as mean of duplicates, unless specified in the text. Data was analyzed in Prism 6 software (GraphPad Prism, version 7.01) by non-parametric statistical analyses. Group median values were compared by the Mann-Whitney or Kruskal-Wallis test for un-paired data and by the Wilcoxon matched pairs signed rank or Friedman tests for paired data. Coefficient of variation (CV) was calculated as standard deviation divided by the mean in individual replicates or experiment group.

Results

PBMC viability and optimization of culture conditions

First, we aimed to establish optimal conditions for the human effector cells during the MGIA four-day incubation step. PBMC viability was evaluated under different culture conditions using fixed numbers of $1 \times 10^6$ uninfected PBMCs suspended in 600 μl medium ($1.67 \times 10^6$ PBMCs/mL in 2 mL tubes with closed lids). Viable PBMCs were counted after one and four days of culture (Fig 1). Viability was low in cultures incubated with 360 ° continuous rotation (10 rpm/min) but increased significantly without rotation (median 22 % (range 13 – 22 %) viable cells vs. median 59 % (range 35 – 79%) after 4 days, $p = 0.012$). Reduced rotation speed and change of other culture
parameters including medium composition, addition of antibiotics and allowing for gas exchange did not significantly affect viability (S1 Fig). Consequently, all subsequent MGIA experiments were performed without rotation.

(FIG 1)

*Mtb inoculum variability*

Next, we assessed the reproducibility of the mycobacterial inoculum. Assuming that infection with a low, but reliable and reproducible, CFU number would be preferable [21], we aimed for an inoculum of 100 CFU per 1x10^6 PBMCs suspended in 600 µl RPMI medium. Within-run variability (CV) for inoculum suspensions (n = 10) was 9% (range 2 – 22 %) and 2 % (range 0.2 – 4 %), evaluated by duplicate agar plate viable counts and TTD, respectively. Across all optimization experiments, median inoculum was 93 CFU (range 50 – 128, mean CV 24 %) corresponding to a median TTD of 280 hours (range 247-311, CV 7 %) (S2 Fig).

Mycobacterial numbers were comparable in 10-fold serial dilutions of H37Rv and BCG (S3A Fig). Addition of 10 % pooled human serum to the suspension medium seemed to slightly reduce mycobacterial numbers in H37Rv serial dilutions when compared with dilutions without pooled serum (S3 Fig). The effect of pooled human serum on BCG growth was not assessed.

*Characterization of mycobacterial numbers according to medium*

In a series of experiments, we evaluated mycobacterial numbers and Δ bacterial number (bacterial number after four days minus inoculum) in MGIA four-day cultures using three different media (individual experiment data is presented in S4 Table). Median mycobacterial numbers among
controls were 3.2 (Δ1.2), 3.5 (Δ1.5) and 3.0 (Δ1.0) Log_{10} CFU in RPMI, RPMI-AP and RPMI-PS medium, respectively, based on five experiments with parallel cultures including all three media. Mycobacterial numbers were not affected by changes in autologous plasma concentration (5 – 20%) in the culture medium (S5 Fig).

Finally, we evaluated the effect of penicillin and streptomycin on mycobacterial number in four-day PBMC cultures. Addition of penicillin and streptomycin (0.25%) to the culture medium fully eliminated mycobacteria from the system. Addition of penicillin and streptomycin (1%) to the PBMC cryopreservation medium resulted in a 0.4 Log_{10} CFU reduction of mycobacterial numbers despite three times wash in antibiotic free medium after thawing (S6 Fig).

**MGIA variability**

Within-run variability was evaluated in sample replicates from 17 volunteers who participated in more than one of 10 independent MGIA experiments (S4 Table). Overall, replicate variability among volunteers was 4% (CV range 0 – 17%). Composition of the culture medium did not affect within-run variability (CV = 3, 4 and 5% in replicate samples cultured in RPMI, RPMI-PS and RPMI-AP, respectively). A representative plot of MGIA variability among controls using different culture media is presented in Fig 2.

(FIG 2)

To explore the platform for pooling of data between independent experiments, we calculated between-run variability for 11 individuals who participated in two or more of 10 MGIA
experiments (eight BCG-vaccinated volunteers and three controls). On average, between-run variability of mycobacterial numbers ($\log_{10}$ CFU) was 8% (CV range: 0-28% between individuals) with the lowest variability in samples cultured in RPMI without pooled serum or autologous plasma (4%, 8% and 14% for RPMI, RPMI-PS and RPMI-AP, respectively).

**Pooling of experimental data for proof of concept**

Next, we pooled data from independent experiments stratified by culture medium in order to establish early proof of concept for possibly growth control by BCG vaccinated individuals (Fig 3). We used this approach, since our experiments only comprised few volunteers and we had found low between-run variability in the optimization experiments. Data was assigned to the pooled analysis starting from the first optimization experiment and onwards. In cases where volunteers participated in more than one experiment, only data from the first experiment was used. There was no significant difference in mycobacterial numbers between BCG vaccinated individuals and controls for cultures in RPMI ($p=0.357$) and RPMI-PS medium ($p=0.734$). However, in RPMI-AP there was a discrete trend of reduced mycobacterial numbers in the BCG vaccinated group ($p=0.065$, Fig 3).

(Fig 3)

**Exploring possible growth inhibitory effects of autologous plasma**

Based on the trend to reduced mycobacterial numbers in BCG-group cultures enriched with autologous plasma, we precedent to explore whether this reduction was associated with an increase in T cell cytokines - as a surrogate of specific T cell activation. We measured IFN-$\gamma$, TNF-$\alpha$,
IL-2, IL-10 and IL-13 in plasma samples and MGIA supernatants from six of the eight BCG-vaccinated individuals and two randomly selected controls. Cytokine levels were below detection limit in all supernatants from baseline samples (culture day zero), control group cultures (day four) and uninfected cultures (day four). In the BCG vaccinated group, however, we observed a trend to increased IFN-γ (p= 0.065) and IL-2 levels (p=0.008) in four-fay MGIA cultures containing 10% autologous plasma compared with cultures without plasma (S7 Fig).

To explore if possible growth inhibitory effects of autologous plasma effects could be transferred or augmented, we performed a crossover experiment using plasma from a BCG vaccinated volunteer who had persistently low mycobacterial numbers in RPMI-AP cultures (S4 Table, ID no. 7). However, we did not detect donor specific changes in mycobacterial numbers in MGIA cultures exposed to 10% plasma from this volunteer, control plasma and autologous plasma, respectively (n = 4 BCG vaccinated and 3 unvaccinated volunteers, S8 Fig).

**BCG vaccinated individuals versus controls**

Having optimized culture conditions, characterized assay variability and observed some degree of growth inhibition using pooled MGIA data, we proceeded to look for BCG-specific growth inhibition in an MGIA with increased sample size (n = 26, Fig 4).

(INSERT FIG 4)

In this experiment, we found no growth inhibition in the BCG vaccinated individuals compared with unvaccinated controls (p = 0.990 and 0.870 for RPMI and RPMI-PS, respectively). In cultures with 10% autologous plasma, mycobacterial numbers were actually significantly higher in the BCG
vaccinated group compared with the control group (0.1 Log_{10} CFU difference, p = 0.039). Individuals with BCG vaccination were older (median 40 vs. 33 years, p = 0.018) compared with controls and predominantly of the male sex (73% vs. 27%, p = 0.111). Median time from vaccination was 30 years (IQR: 1.5 – 50) and time from vaccination was inversely correlated with mycobacterial numbers in RPMI cultures (Spearman R = -0.83, p = 0.001) and RPMI-AP (R = -0.58, p = 0.041), but not in RPMI-PS (R = -0.44, p = 0.135) (S9 Fig). Of note, as in the optimization experiments, mycobacterial numbers were generally reduced in RPMI-PS compared with RPMI or RPMI-AP cultures (p = 0.002 for BCG-vaccinated and 0.004 for controls). Group variability (CV) was below 7% in all groups.

**MGIA in latent and active TB infection**

Since we did not see growth inhibition among BCG-vaccinated individuals, we proceeded to investigate the growth inhibitory capacity in groups of individuals with active TB (n = 8), latent TB infection (LTBI, n = 10), healthy BCG-vaccinated (n = 8) and unvaccinated controls (n = 8). Baseline information on study participants is presented in Table 1.
### Table 1. Baseline information, MGIA experiment B

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<th>BCG-vaccination</th>
<th>Time from vaccination (years / months)</th>
<th>Time from diagnosis (years / months / weeks)</th>
<th>Anti-TB treatment</th>
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</table>

* LTBI group: preventive treatment completed. TB-group: treatment initiated

We did not find differences in mycobacterial numbers between groups in this experiment

\[(p = 0.351, 0.274, 0.914, \text{Kruskal-Wallis comparison of control, BCG, LTBI and TB groups using RPMI, RPMI-AP and RPMI-PS medium, respectively}). \text{MGIA data is presented in Fig 5. Group variability was highest in the control group (highest CV 19% in RPMI-AP) and in the TB group (highest CV 19% in RPMI-PS). Time from TB diagnosis was inversely correlated with mycobacterial growth in samples cultured in pure RPMI and RPMI-AP (Spearman R = -0.75 (p = 0.045) and -0.85 (p = 0.016), respectively. S12 Fig).}\]
Median age for volunteers was 36, 32, 50 and 41 years for controls, BCG, TB and LTBI, respectively ($p = 0.011$). The BCG group comprised individuals who had received BCG several years prior of inclusion (median time from BCG: 12 years (IQR: 3.50 – 28.25)) and in this experiment, time from vaccination did not correlate with MGIA mycobacterial numbers (S10 Fig). In the LTBI group, median time from diagnosis was 4 years (IQR: 0.10 – 10.25) with no correlation between time from diagnosis and MGIA mycobacterial numbers (S11 Fig). Also, 3/10 LTBI volunteers had received preventive treatment. All volunteers in the TB-group had verified pulmonary TB and had initiated anti-TB treatment at the time of diagnosis, usually a few weeks prior of inclusion (median 2.5 weeks (IQR: 1 – 4)).

**IFN-γ release in MGIA cultures**

In order to assess the influence of T cell activation on mycobacterial numbers, we measured IFN-γ release as a surrogate of T cell activation in supernatant from the two MGIA experiments presented in Figs 4 and 5. In the samples from MGIA experiment A (Fig 4), we found significantly increased IFN-γ levels in the BCG vaccinated group compared with controls in the RPMI and RPMI-AP four-day cultures (median 47 vs. 1 pg /mL ($p = 0.013$), 53 vs. 12 pg /mL ($p = 0.012$), Fig 6 and S13 Table). IFN-γ release was highest in the BCG group samples (159 pg / mL ) cultured in the presence of autologous plasma; however, under these conditions IFN-γ levels were also elevated, but to a lesser degree, in the control group (47 pg / mL, $p = 0.079$). Baseline IFN-γ levels (culture time = 0) were low in all samples, indicating that IFN-γ release was associated with the presence of antigen specific T cells (S13 Table). We explored the kinetics of the IFN-γ release during culture in a
subgroup of 7 individuals from the BCG group and found only low release after the first 24 hours of culture (0 pg/mL, 16 pg/mL and 0 pg/mL in RPMI, RPMI-AP and RPMI-PS cultures, Table S13).

(FIG 6)

Interestingly, there were no significant differences in IFN-γ levels between controls and any of the clinical groups after four days of culture in MGIA experiment B (Fig 7 and S14 Table), although there was a trend of high release in the LTBI group across culture media and high release in the BCG-vaccinated group in RPMI-PS cultures. Baseline levels were very low in all groups (< 6 pg/mL). Samples from 15 of the study volunteers were cultured for four days without mycobacteria (S14 Table) and generally, IFN-γ levels were lower in uninfected than in infected samples (p = 0.0005 and 0.0009 in RPMI and RPMI-PS cultures), except in RPMI-AP cultures (p = 0.49).

IFN-γ levels did not correlate with mycobacterial growth in any of the experiments (data not shown).

(FIG 7)

**MGIA with isolated monocytes and CD4+ T cells**

Next, we explored if elimination of non-CD4+ T cells and increased ratio of effector to infected target cells would open the readout window of the assay. To achieve this, we isolated CD4+ T cells and CD14+ monocytes from BCG vaccinated volunteers and controls by magnetic negative selection. Monocytes or PBMCs were infected with mycobacteria (100 CFU) for one hour followed
by addition of T cells in different ratios and four-day MGIA culture. Although this assay also had acceptable variability on duplicate measurements (data not shown), we observed no indication of growth inhibition in BCG vaccinated individuals by increasing numbers of T cells or monocytes, and we did not pursue this avenue beyond initial proof of concept experiments (S15 Fig).

**MGIA with skin suction blister cells**

In a final attempt to provoke adaptive growth inhibitory mechanisms in the MGIA, we conducted a range of MGIA experiments using cells isolated from suction blisters (SB-cells) raised over PPD skin test reactions. Since SB-cells are activated and expanded *in vivo*, and comprise approximately 30 % PPD-specific polyfunctional CD4 T cells [24] (Holm et al, JoVe in review), we hypothesized they would be good candidates to augment the adaptive immune response in the MGIA. We spiked MGIA PBMC cultures from eight BCG-vaccinated volunteers with autologous fresh SB-cells (median 80,000 (range 30,000 – 175,000) SB-cells / 1 x 10⁶ PBMCs), but found no difference in mycobacterial numbers between SB-cell spiked PBMC cultures and PBMC cultures without SB-cells (p = 0.563, Wilcoxon Matched Pairs, Fig 8). Mycobacterial numbers did not correlate with total SB-cell count (Spearman R = 0.099, p = 0.814, n = 8) and increasing the number of SB-cells did not alter mycobacterial growth in individual cultures (20,000 – 175,000 SB-cells in replicate MGIA cultures from one BCG-vaccinated donor, data not shown). Finally, in an attempt to eliminate potential confounding by unspecific PBMCs in the culture, we changed the MGIA setup to include only isolated monocytes combined with SB-cells or CD4+ T cells isolated from peripheral blood (S16 Fig). In this small experiment comprising two donors, one donor had a 0.42 log₁₀ CFU reduction of mycobacterial numbers in monocyte cultures with SB-cells compared with monocyte
cultures without any effector cells. However, overall, there was no clear growth inhibitory effect of adding either SB-cells or PBMC-derived T cells, and we did not pursue this further.

(Fig 8)

Discussion

The MGIA has reemerged in the field of TB vaccinology as an unbiased tool for early triage of vaccine candidates directly linked to killing of \textit{Mtb} [13]. Standardized MGIA protocols based on BCG infection of murine and human cells have been proposed, and recent studies suggest these protocols may provide reliable and relevant results in a BCG vaccine model [13,14]. The reliance on BCG as the challenge organism limits the assays’ relevance for vaccines based on antigens not expressed by BCG, and our aim was to develop and qualify a human PBMC MGIA based on infection with \textit{Mtb}.

In this study, we characterized individual assay parameters and optimized a PBMC MGIA performing with very high reproducibility. We sought to establish proof of concept for vaccine induced growth inhibition by head-to-head comparison of individuals with distant BCG vaccination and unvaccinated controls. Despite finding IFN-\(\gamma\) release suggesting preexisting immunity to \textit{Mtb}, we did not see growth inhibition in BCG-vaccinated individuals. Growth inhibition was also absent between groups in the subsequent experiment, which also included samples from individuals with active TB disease and LTBI. In an attempt to augment adaptive anti-mycobacterial mechanisms, we introduced T cells isolated from PBMCs and suction blister cells isolated from PPD-primed skin into
the MGIA. However, also with these approaches, we were not able to induce growth inhibition in relevant groups.

*Positive lessons from assay optimization*

A strength of this study is the demonstration of low variability within-run and between-run (3–5 % and 4–14 %, respectively, depending on the culture medium) under optimized assay conditions and the thorough description of individual assay components. By changing incubation conditions from continuous 360° rotation to still-culture, PBMC viability increased, probably due to elimination of the shear forces applied to the cells using this method [25]. We have recently reported similar findings in a murine splenocyte MGIA wherein we successfully showed growth inhibition in BCG and subunit vaccinated mice [16]. We consider still-cultures more relevant as they allow for migration of specific cells under more physiological conditions, and there is even evidence of formation of in vitro granuloma-like aggregations in *Mtb* infected monocyte and T cell short time cultures [26]. Although the demonstration of IFN-γ release in MGIA supernatants suggests that specific cell mediated immune responses occur in the still-culture assay, further work is needed to explore whether the failure of the MGIA in our hands, could be explained by an altered infection or immune recognition caused by absence of continuous rotation applied by others [21,27].

*BCG for proof of concept*

Disappointingly, we failed to confirm other groups’ successful discrimination between BCG-vaccinated and unvaccinated individuals in PBMC based MGIA [21,27–30]. The majority of positive studies demonstrate growth inhibition directly following primary or secondary BCG
vaccination with optimal time-points for PBMC sampling suggested at 4 months following primary BCG-vaccination in children or 8 weeks in adults and no significant growth inhibition at later follow-up time-points [21,27]. In that light, our choice of studying individuals with distant BCG vaccination was not ideal. To our knowledge, only one previous PBMC MGIT study have included adults with distant BCG vaccination, and here the authors found significant growth inhibition ($p = 0.05$) in 10 UK individuals with BCG-vaccination compared with 9 unvaccinated controls [21]. In that study, PBMCs were infected with BCG Pasteur (250 CFU) and incubated with continuous 360° rotation, but apart from these differences, assay setup and study populations were fairly similar to ours.

Detailed focus on assay optimization and demonstration of improved cell viability, high assay reproducibility and intact CMI responses in relevant groups, should preclude a technical problem in our assay. Nevertheless, we failed to reproduce earlier studies, suggesting that either $Mtb$ is too difficult an organism to control in this assay, or that the BCG induced immunity had waned in the majority of our study subjects. To assess the latter confounder, we correlated time from BCG-vaccination with mycobacterial numbers in the MGIA and actually found an inverse correlation in samples cultured in the presence of autologous plasma, however this association was not reproduced in the subsequent MGIA experiment or using other culture media.

**LTBI and active TB infection in MGIA**

Unsuccessful with BCG, we tried to establish proof of concept using samples from individuals with LTBI or active TB infection, under the hypothesis that $Mtb$ specific immune mechanisms induced by natural infection are superiorly protective to BCG as demonstrated in early studies [31,32]. However, we did not see inhibition of mycobacterial numbers in either of the experiment groups
(LTBI, active TB and BCG-vaccination) when compared with healthy controls. Limitations of this experiment included low sample size, increased group variability (probably caused by singlet measurements) and importantly, a high frequency of long-term infection in LTBI group, since strong growth control in MGIA only seems to be associated almost exclusively with very recent Mtb exposure (Joosten et al, JCI, in press). In the TB group, we found a negative correlation between time from diagnosis and mycobacterial numbers in RPMI medium +/- autologous plasma, which could potentially reflect the T cell anergy observed in individuals with active TB [33,34].

**IFN-γ release**

Although IFN-γ is important in the killing of Mtb in infected host cells during natural infection, the majority of studies do support IFN-γ as being the key mediator of mycobacterial killing in human MGIAs [13]. In the first BCG proof of concept experiment, we observed increased IFN-γ levels after four days in Mtb infected cultures from BCG-vaccinated individuals (Figs 4 and 6). We also found low levels of IFN-γ at baseline and one day of culture, suggesting that the release occurred after processing and presentation of Mtb by infected cells. Of mention, we did not observe increased IFN-γ levels in the BCG-group in the subsequent, smaller experiment (Figs 5 and 7). In the LTBI-group, however, there was a non-significant increase in IFN-γ responses. In line with previous studies, IFN-γ release in both experiments did not correlate with mycobacterial numbers in the MGIA [21,29,35,36]. While IFN-γ release in relevant groups do suggest the presence of viable T cells capable of producing specific responses in MGIA cultures, the role of these responses is difficult to interpret in the absence of growth inhibition.
Exploring the role of autologous plasma in MGIA

Antibodies have become a topic of interest in TB immunology and also in the context of MGIAs [13,37]. BCG vaccination has shown to induce B cell responses and treatment with post-BCG-vaccination sera has been shown to reduce BCG growth in cultures of both THP-1 cells and macrophages/neutrophils [38–42]. While whole blood MGIAs include the humoral component [21,29,35,36,43], PBMC-based MGIAs lack e.g. antibodies which could preclude important Mtb control mechanisms. We therefore cultured all MGIA samples in parallel replicates using RPMI medium with either no plasma or 10 % heat-inactivated autologous plasma. We also included a third medium consisting of RPMI with 10 % pooled human serum, as this has been applied by others [21,27]. Initially, we found a tendency towards growth inhibition in samples from BCG-vaccinated individuals in cultures with 10 % autologous plasma using pooled data from a series of optimization experiments (Fig 3). However, we were not able to reproduce our findings in larger MGIA experiments or when transferring plasma from the strongest responder to several donors, and we interpret this initial indication of growth inhibition in the presence of plasma samples as bias introduced by the non-weighed pooling of cases and controls from several small experiments. Of note, variability and overall mycobacterial numbers increased in the presence of autologous plasma in the cultures. In addition, we found IFN-γ release in both uninfected and infected cultures exclusively when autologous plasma was present. Together this may just reflect natural variability caused by introduction of additional active factors to the culture mix, but it could also reflect increased complexity in the immune response induced by antibodies - again calling for further exploratory work. Conversely, we found reduced mycobacterial numbers in both BCG- and control cultures as well as in H37Rv standard dilutions in the presence of 10 % pooled human serum (Sigma Aldrich), possibly suggesting a direct and rapidly occurring antibody-bacterium
binding by this serum batch. Global reduction of mycobacterial numbers narrows the MGIA detection window and possibly the chance to observe growth inhibition between groups, making these medium specific observations interesting in an optimization context; however, our observations may reflect a batch effect non-transferable to other commercially available sera. As we did not establish proof-of-concept using either media, the effect of both autologous plasma and pooled human serum on growth inhibition in the MGIA remains unclear.

*T cells and skin suction blister cells in MGIA*

Having failed to demonstrate growth inhibition by the PBMC MGIA protocol, we attempted to increase the likelihood of T cell induced killing by altering the MGIA set-up. First, we increased the ratio of isolated T cells to infected monocytes and next, we added high numbers of activated PPD specific suction blister T cells to the MGIA cultures. Despite positive results from other MGIA studies using somewhat similar approaches with addition of non-stimulated lymphocytes [29,44] and lymphocytes stimulated *ex vivo* with BCG or *Mtb* lysate [28,29] to infected monocyte cultures, we saw no convincing growth inhibitory effect of increased ratios of specific T cells to infected target cells to warrant further pursuit.

We hypothesized that addition of SB-cells would induce a growth inhibitory signal in MGIA cultures, since SB-cells are *in vivo* activated and have high frequencies of memory phenotype PPD-specific polyfunctional CD4+ T cells [24]. Whereas typical recently BCG vaccinated adults have approximately 0.04 % PPD specific CD4+ T cells available in PBMC MGIA cultures, adding SB-cells increases this number by 6-fold on average, which we would expect to be sufficient to tease out a T cell dependent mechanism [24,45] (Holm et al, JoVE in review). Importantly, neither T cell cytokine expression profiles nor growth inhibition have been associated with human BCG vaccine
protection in vivo [7,46]; nevertheless, specific polyfunctional T cell frequencies was recently correlated with in vitro growth inhibition following primary BCG vaccination of UK infants [27]. As we found no growth inhibitory capacity by SB-cells in our MGIA, our results rather echoes recent findings in South African and Dutch settings. Baguma et al explored growth inhibition in whole blood from PPD-positive and PPD-negative South African children and adults and found no correlation between BCG-specific cytokine producing T cell frequencies and growth inhibition [35]. Similarly, Joosten et al found no association between polyfunctional T cell subsets and growth inhibition in cohorts of recent TB contacts and following BCG vaccination (Joosten et al, JCI, in press). Conversely, the authors discovered a strong association between mycobacterial growth inhibition and CXCL10-producing non-classical CD14dim monocytes, and concluded that in vitro growth inhibition was caused by trained immunity (Joosten et al, JCI, in press).

These reports shed some interesting light on current understandings of protective immunity in vivo and ex vivo. Their findings are not directly comparable to our study, since we primarily aimed to investigate growth inhibition in the context of distant BCG vaccination. Most candidate TB vaccines seek to induce long-term protection, often by boosting specific T cell responses against vaccine antigens, and hence we had hoped to see an SB-cell dependent signal to validate the detection of such cells in MGIAs [7,9,46,47]. Further assessment of our approach, i.e. in populations where PBMC MGIA can demonstrate growth inhibition, are needed. However, in line with recent reports, our unexpected results could suggest that other mechanisms such as trained immunity or nonspecific effects of BCG vaccination might explain at least some of the growth inhibition seen in other BCG MGIA studies [48].
In conclusion, despite thorough attempts to qualify and optimize an MGIA based on *Mtb* infection, we did not prove a growth inhibitory capacity in people with distant BCG vaccination by either PBMCs, CD4+ T cells or *in vivo* expanded skin suction blister cells. We cannot exclude that this detection failure was due to unrecognized technical error in our assay or choice of study population. However, this and other recent reports suggest that more work is needed before this type of assay can be used as endpoint in clinical TB vaccine trials.
Acknowledgements

The authors would like to thank Dr. Helen Fletcher, Dr. Andrea Zelmer, Dr. Simone Joosten, Dr. Rachel Tanner and Prof. Helen McShane for sharing protocols and indispensable advice on setting up the core MGIA. This project was funded by European Commission H2020 program [grant number TBVAC2020 643381], and we would like to thank all consortium partners, especially those working with MGIA in WP5, for productive discussions. Finally, we would like to thank the staff at Gentofte TB outpatient clinic and all the volunteers for participating in the study.
References


**FIGURE CAPTURES**

**Fig 1. PBMC viability during four-day uninfected culture**
Duplicate suspensions of $1 \times 10^6$ uninfected PBMCs from 8 volunteers were incubated for 1 – 4 days in 2 ml closed tubes (600µl RPMI-PS/tube, 37° C) with ($n = 3$) and without continuous rotation ($n = 8$). Symbols represent viable fraction of baseline PBMC count (group median, %), error bars represent range between donors.

**Fig 2 a-c. Representative MGIA optimization experiment**
Mycobacterial number (log$_{10}$ CFU) in MGIA cultures from 3 healthy controls (C1-C3). All samples were cultured in parallel in (A) RPMI, (B) RPMI-PS and (C) RPMI-AP. Left panel: bacterial number in individual donor cultures (duplicate values, mean). Right panel: control group bacterial numbers (individual donor means, group median). Dotted lines represent inoculum mycobacterial number. Group CVs = 2.5% (A), 5.0% (B) and 12.2% (C).

**Fig 3. MGIA plot with pooled data**
Mycobacterial number (log$_{10}$ CFU) in MGIA cultures from healthy controls and BCG-vaccinated individuals (BCG+). The figure contains pooled data from 10 independent experiments for samples cultured in (A) RPMI (n = 17), (B) RPMI-PS (n = 14) and (C) RPMI-AP (n = 16). Symbols represent duplicate means. Lines represent group medians.

**Fig 4. MGIA experiment A**
Mycobacterial number (log$_{10}$ CFU) in MGIA cultures from healthy controls ($n = 13$) and BCG-vaccinated individuals (BCG+, $n = 13$). All samples were cultured in parallel in (A) RPMI, (B) RPMI-PS or (C) RPMI-AP. Symbols represent duplicate means. Lines represent group medians.
**Fig 5. MGIA experiment B**

Mycobacterial number (log$_{10}$ CFU) in MGIA cultures from healthy controls (n = 8), BCG-vaccinated individuals (BCG+, n = 8), individuals with confirmed latent TB (LTBI, n = 10) and active TB disease (TB, n = 8). All samples were cultured in parallel in (A) RPMI, (B) RPMI-PS or (C) RPMI-AP. Symbols represent bacterial number in singlet cultures. Lines represent group medians. Note: All PBMCs in this experiment were exposed to 0.5% Penicillin/Streptomycin in the medium used for cryopreservation. Thawed PBMCs were washed thrice before MGIA. All culture media was free of antibiotics, as described in the Materials and methods section.

**Fig 6. IFN-γ levels in MGIA supernatants, experiment A**

IFN-γ levels (pg / mL) in four-day culture supernatants from MGIA experiment A (fig 4) measured by ELISA (n = 13 controls and 13 BCG-vaccinated individuals). Symbols represent duplicate means, lines = group medians.

**Fig 7. IFN-γ levels in MGIA supernatants, experiment B**

IFN-γ levels (pg / mL) in four-day culture supernatants from MGIA experiment B (fig 5) measured by ELISA. There were no significant differences in median mycobacterial number between controls (n = 8) and the BCG (BCG+, n = 8), LTBI (n = 10) and TB (n = 8) groups by individual comparison or Kruskal-Wallis test (p = 0.064, 0.495 and 0.543 in RPMI, RPMI-PS and RPMI-AP, respectively). Symbols represent means of duplicate measurements, lines = group medians.

**Fig 8. MGIA with cells from PPD-induced suction blisters**

Mycobacterial numbers in RPMI MGIA cultures from BCG-vaccinated volunteers (n=8). Cultures of 1x10$^6$ PBMCs were spiked with fresh autologous cells from PPD-induced succion blisters (SB-cells) with individual SB-cell yields ranging from 30,000 – 175,000 (median 80,000 SB-cells/MGIA culture). Standard MGIA cultures (1x10$^6$ PBMCs w/o SB-cells) were cultured in parallel for individual comparison. Symbols represent donor ID, (SB-cell yield). The figure comprise pooled data from four individual experiments.
Fig 1.

![Graph showing viable cells (%) over culture time (days) with and without rotation.](image-url)
Fig 2.

A. RPMI

B. RPMI + 10% pooled human serum

C. RPMI + 10% autologous plasma

log CFU / culture vs. Groups
Fig 3.

A. RPMI

B. 10% autologous plasma

C. RPMI + 10% pooled human serum

* p = 0.357

* p = 0.065

* p = 0.734

151
Fig 4.

A. RPMI

B. RPMI + 10% autologous plasma

C. RPMI + 10% pooled human serum
Fig 5.
Fig 6.
Fig 7.
Fig 8.

Log_{10} CFU / culture

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S1 Fig. PBMC viability under different incubation conditions
Duplicate suspensions of 1x10^6 uninfected PBMCs from 11 study volunteers were cultured for 1 – 7 days. Samples were cultured in different media (RPMI, RPMI-PS, RPMI-AP and AIM-V), with and without penicillin/streptomycin, with and without gas exchange (tube caps on/off) and with and without continuous rotation. All cultures were incubated in 2 ml tubes (600µl medium/tube, 37º C). Number of duplicate sample ranged from 1 – 11 per culture condition tested. Symbols represent viable fraction of baseline PBMC count (group median, %), error bars represent range between donors. Data from Fig 1 is included for comparison.

S2 Fig. 10-fold serial dilutions of H37Rv in RPMI
Plot of matched log_{10} converted viable counts (log_{10} CFU) and Time to detection (TTD, hours) obtained by parallel culture of 10-fold serial H37Rv dilutions on agar plates and in MGIT. Data from 10 independent MGIA experiments. Values are presented as log_{10} CFU /TTD in 300µl dilution. Symbols represent mean value and range of duplicates. Lines represent linear regression. Dotted line represents the log_{10} converted target inoculum value (100 CFU / 300 µl).

S3 Fig. 10-fold serial dilutions using different mycobacteria and media
(A) 10-fold serial dilutions of H37Rv in RPMI (Y = -50.9*X + 364.1) and BCG in RPMI (Y = -54.3*X + 355.5). (B) 10-fold dilutions of H37Rv in RPMI (Y = -50.37*X + 360) and RPMI-PS (Y = -37.83*X + 307.5). Values are presented as log_{10} CFU /TTD in 300µl dilution. Symbols represent mean value and range of duplicates. Lines represent linear regression.
**S4 Table. Experiment raw data for variability analyses**

MGIA data from 17 volunteers participating in 10 independent optimization experiments. Mycobacterial numbers are presented as median of replicates ($\log_{10}$CFU). $\Delta$ mycobacterial number was calculated by subtracting the inoculum from mycobacterial numbers in individual MGIA cultures. ND = no available data. Eleven volunteers participated in more than one experiment. Variability calculations are based on control mycobacterial growth values in experiment no. 2,4,5,7 and 10, as these experiments comprise complete datasets using all three media.

**S5 Fig. Autologous plasma titration**

Mycobacterial numbers in replicate MGIA cultures ($1\times10^6$ PBMCs) from two BCG-vaccinated volunteers. Culture medium was RPMI spiked with 0 – 20 % autologous plasma. Symbols represent mean and range of duplicates.

**S6 Fig. Effect of antibiotics on MGIA mycobacterial numbers**

Mycobacterial numbers in replicate MGIA cultures ($1\times10^6$ PBMCs) from two BCG-vaccinated volunteers. Culture medium was RPMI +/- penicillin/streptomycin. All thawed PBMCs were washed thrice. (A) No antibiotics in either storage or culture medium. (B) Penicillin/streptomycin present in the medium used for PBMC cryopreservation (0.5%), no antibiotics in the culture medium. (C) Penicillin/streptomycin present in both the medium used for PBMC cryopreservation (0.5%) and in the culture medium (0.25%). Symbols represent mean and range of duplicates.

**S7 Fig. Cytokine levels in MGIA culture supernatants +/- autologous plasma**

Levels of IFN-γ and IL-2 (pg. / ml) in MGIA culture supernatants measured by multiplex analysis. Study volunteers were six BCG vaccinated individuals. Black bars represent median cytokine levels in RPMI cultures. White bars represent median cytokine levels in RPMI-AP cultures. Error bars represent range. One IFN-γ measurement was above assay range and corrected to the highest detection limit value (1410 pg. / ml).
S8 Fig. MGIA plasma crossover
Mycobacterial numbers in replicate MGIA cultures (1x10^6 PBMCs) from 4 BCG-vaccinated individuals (A) and 3 controls (B). MGIA culture medium was RPMI enriched with either 10% autologous plasma (Aut. plasma), 10% plasma from BCG-vaccinated donor no. 7 (○) who had the best growth inhibitory potential based on previous experiments (Best plasma) or 10% plasma from an unvaccinated healthy control (Control plasma) (Δ). Symbols represent mean and range of duplicates.

S9 Fig. Correlations between MGIA growth and time from vaccination, MGIA experiment A
Time from vaccination with BCG (years) plotted against mycobacterial number (median of duplicates) for 13 adult volunteers in MGIA experiment A (figure 4). Spearman R= -0.83 (p = 0.001), -0.58 (p = 0.041) and -0.44 (p = 0.135) for cultures in RPMI, RPMI-AP and RPMI-PS, respectively.

S10 Fig. Correlations between MGIA growth and time from vaccination, MGIA experiment B
Time from vaccination with BCG (years) plotted against mycobacterial number for 8 BCG-vaccinated volunteers in MGIA experiment B (figure 5). Spearman R= 0.20 (p = 0.632), -0.11 (p = 0.808) and 0.01 (p = 0.990) for cultures in RPMI, RPMI-AP and RPMI-PS, respectively.

S11 Fig. Correlations between MGIA growth and time from diagnosis in LTBI
Time from diagnosis (years) plotted against mycobacterial number for 10 volunteers diagnosed with latent TB infection (LTBI) in MGIA experiment B (figure 5). Spearman R= 0.39 (p = 0.265), 0.55 (p = 0.106) and 0.29 (p = 0.403) for cultures in cultures in RPMI, RPMI-AP and RPMI-PS, respectively.

S12 Fig. Correlations between MGIA growth and time from diagnosis in active TB
Time from diagnosis (years) plotted against mycobacterial number for eight volunteers diagnosed with pulmonary TB disease in MGIA experiment B (figure 5). Patients were started on anti-TB drugs at the time of diagnosis. Spearman R= -0.75 (p = 0.045), -0.85 (p = 0.016) and -0.45 (p = 0.324) for cultures in RPMI, RPMI-AP and RPMI-PS, respectively.
S13 Table. IFN-γ levels in MGIA culture supernatants, MGIA experiment A
IFN-γ (pg. / ml) in supernatants from MGIA experiment A, measured by ELISA. Data is presented mean of duplicate measures for individual volunteers (n = 13 controls and 13 BCG-vaccinated volunteers) at baseline (culture time = 0) and after one and four days of MGIA culture. All samples were cultured in RPMI, RPMI-AP and RPMI-PS.

S14 Table. IFN-γ levels in MGIA culture supernatants, MGIA experiment B
IFN-γ (pg. / ml) in supernatants from MGIA experiment B, measured by ELISA. Data is presented as mean of duplicate measures for individual volunteers (n = 8 controls, 8 BCG-vaccinated, 10 LTBI and 8 volunteers with active TB, respectively). IFN-γ was measured at baseline (culture time = 0) and after four days of culture with and without H37Rv infection. All samples were cultured in RPMI, RPMI-AP and RPMI-PS.

S15 Fig. MGIA with monocytes and CD4+ T cells
Mycobacterial numbers in MGIA cultures (log_{10} CFU, mean of duplicates) with monocytes and CD4+ T cells from BCG-vaccinated (Δ, n = 1 in experiment A-B, n = 3 in experiment C) and non-vaccinated volunteers (○, n = 1 in experiment (A) and (B), n = 3 in experiment (C)). Monocytes and CD4+ T cells were isolated from cryopreserved PBMCs by negative magnetic selection and combined in different ratios for MGIA culture. Ratios are presented as cell number x 10^3. PBMC cultures (1x10^6) are included for comparison in experiment A. Time from BCG vaccination was 5, 6 and 31 years among BCG-volunteers.

S16 Fig. MGIA with monocytes, T cells and cells from skin suction blisters
Fresh cells from skin suction blisters (SB-cells) were harvested from two BCG-vaccinated donors. Monocytes and CD4+ T cells were isolated from cryopreserved PBMCs by negative magnetic selection. MGIA cultures of either 50,000 autologous monocytes (A) or 1x10^6 autologous PBMCs (B) were infected with 100 CFU H37Rv for one hour followed by addition of either 50,000 SB-cells or 50,000 T cells and standard four-day MGIA incubation in RPMI + 10% autologous plasma. Symbols represent mean and range of mycobacterial numbers in duplicates. In one donor, SB cell yield was only sufficient for a singlet specimen in experiment A.
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S5 Fig.

S6 Fig.
S7 Fig.

S8 Fig.
### MGIA Experiment A

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S15 Fig.

A. Fixed monocyte:T-cell ratio

B. Decreasing monocyte:T-cell ratio

C. Increasing monocyte:T-cell ratio
A. Monocytes spiked with T cells or SB cells

B. PBMCS spiked with T cells or SB cells

S16 Fig.