Appraisal of second-generation BCG *ΔureC::hly* experimental vaccine candidates co-expressing human interleukin 7 or interleukin 18.

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Abstract.

Bacillus Calmette-Guérin (BCG), the only approved tuberculosis vaccine, provides only limited protection and can cause undesired side effects. Our laboratory had previously reported recombinant BCG vaccine (BCG *ΔureC::hly*) which secretes the pore-forming toxin listeriolysin O (LLO) of *Listeria monocytogenes* thereby allowing phagosomal escape of pathogenic antigens and improved antigen presentation by host cells. Based on the genetic background of BCG *ΔureC::hly*, two new strains expressing either human interleukin-7 (BCG *ΔureC*::*hly_hIL-7*) or interleukin-18 (BCG *ΔureC*::*hly_hIL-18*) were generated to increase the population of pathogen-specific effector memory T cells post vaccination. Both strains exhibited an uncompromised *in vitro* growth pattern, while inducing a pro-inflammatory cytokine profile upon infecting human dendritic cells (DCs). LLO activity remained intact in both BCG *ΔureC*::*hly_hIL-7* and BCG *ΔureC*::*hly_hIL*-18, as shown by their ability to induce perforation of human red blood cells. Human DCs infected with either strain efficiently promote the secretion of interleukin 2 by autologous T cells in a co-culture system, suggesting superior antigen presentation. BALB/c mice vaccinated with BCG *ΔureC*::*hly*, BCG *ΔureC*::*hly_hIL-7* or BCG *ΔureC*::*hly_hIL-18* develop a more robust Th1 response than parental BCG. Both cytokine-expressing strains provided a significantly better protection than BCG in a murine *Mycobacterium tuberculosis* challenge model but efficacy remained similar to BCG *ΔureC::hly*. We conclude that expression of human cytokines 7 or 18 may not be compatible to improve the protective efficacy of BCG *ΔureC::hly*.

Zusammenfassung

Bacillus Calmette-Guérin (BCG), der einzige zugelassene Tuberkulose Impfstoff, verleiht nur begrenzten Schutz und kann unerwünschte Nebenwirkungen hervorrufen. Unsere Abteilung hatte bereits früher einen rekombinanten BCG (BCG *ΔureC::hly*) Impfstoff entwickelt, der das porenbildende Toxin Listeriolysin O (LLO) aus *Listeria monocytogenes* sekretiert und dadurch phagosomales Entkommen pathogener Antigene und verbesserte Antigen-Präsentation durch Wirtszellen erlaubt. Basierend auf dem genetischen Hintergrund von (BCG *ΔureC::hly*) wurden hier zwei neue Stämme generiert, die entweder humanes Interleukin-7 (BCG *ΔureC*::*hly_hIL-7*) oder Interleukin-18 (BCG Δ*ureC*::*hly_hIL-18*) exprimieren mit dem Ziel, die Population an Effektor T Gedächtniszellen (check whether translated correctly) nach Impfung zu erhöhen. Beide Stämme zeigten ein unauffälliges Wachstumsverhalten in Kultur, waren jedoch in der Lage ein pro-inflamatorisches Cytokinprofil nach Infektion von humanen dendritischen Zellen zu induzieren. LLO-Aktivität konnte in BCG *ΔureC*::*hly_hIL-7* und BCG *ΔureC*::*hly_hIL*-18 durch die Perforation von humanen Erythrozyten bestätigt werden. Humane dendritische Zellen, die mit jeweils einem der beiden Stämme infiziert waren, förderten in einem Ko-Kultursystem die Sekretion von Interleukin-2 durch autologe T Zellen, das auf bessere Antigen-Präsentation hindeutet. Balb/c Mäuse, die mit BCG *ΔureC*::*hly*, BCG *ΔureC*::*hly_hIL-7* oder BCG *ΔureC*::*hly_hIL-18* geimpft waren, entwickelten eine robustere Th1 Antwort im Vergleich zum BCG Parentalstamm. In einem murinen *Mycobacterium tuberculosis* Belastungsinfektionsmodell vermittelten beide Cytokin-exprimierenden Stämme einen signifikant besseren Schutz als BCG, verblieben in ihrer Wirksamkeit jedoch gleichwertig zu BCG Δ*ureC::hly*. Wir folgern, dass die Expression der humanen Cytokine 7 oder 18 möglicherweise nicht adäquat ist, die Schutzwirkung von BCG *ΔureC::hly* zu verbessern.

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INTRODUCTION

1. Overview of tuberculosis.

Infectious agents have evolved with humans since the beginning of civilisation. The interplay between the human immune system and pathogens therefore dictates to a great extent the balance between health and disease. *Mycobacterium tuberculosis* (*M. tuberculosis*), the aetiological agent of tuberculosis (TB) is among the oldest known bacterial pathogens to plague the human race. Pathological analysis of a mummified woman dating back to more than 9000 years showed that a sample of the latter's lung contained *M. tuberculosis* genomic DNA, suggesting co-evolutionary existence of both pathogen and host (Hershkovitz, Donoghue et al. 2008). TB currently ranks as one of the most debilitating respiratory infectious diseases, responsible for one death every 20 seconds (WHO TB Control, 2012).

As a master modulator of immune responses in the host, the intracellular lifestyle of this bacterial pathogen influences the outcome of host defence mechanisms in many ways and consequently complicates the control of infection. The evasive strategies employed by *M. tuberculosis* allows for establishment of a stable and life-long infection, making it one of the most successful and persistent pathogens ever known to man.

1.1 TB as a public health challenge.

It is generally accepted that circa 30% of the world's population is latently infected with *M. tuberculosis*, which is also known as the tubercle bacillus, and accounting for approximately 2.5 billion individuals (WHO Global TB Report 2012). The World Health Organisation (WHO) reported 8.7 million TB cases worldwide with 95% of TB-related deaths occurring in low-to-middle-income or developing nations (WHO Global TB Report 2012). Elevated mortality rates are extensively caused by co-infection with the human immunodeficiency virus (HIV), and the prevalence of this scenario is commensurate with economic instability. In line with this, 1.1 million clinical TB cases have been reported to account for HIV co-infected individuals. In this arena, failure of combinatorial chemotherapy (anti-tubercular as well as antiretroviral) in addition to a heavily weakened immune system realistically allows only a meagre chance of survival, if any. In concert with this detail, circa 500,000 TB-related deaths globally per annum have been associated with HIV co-infection and about 1 million deaths among persons who were diagnosed as HIV-positive (Kaufmann and McMichael 2005; Kwan and Ernst 2011).

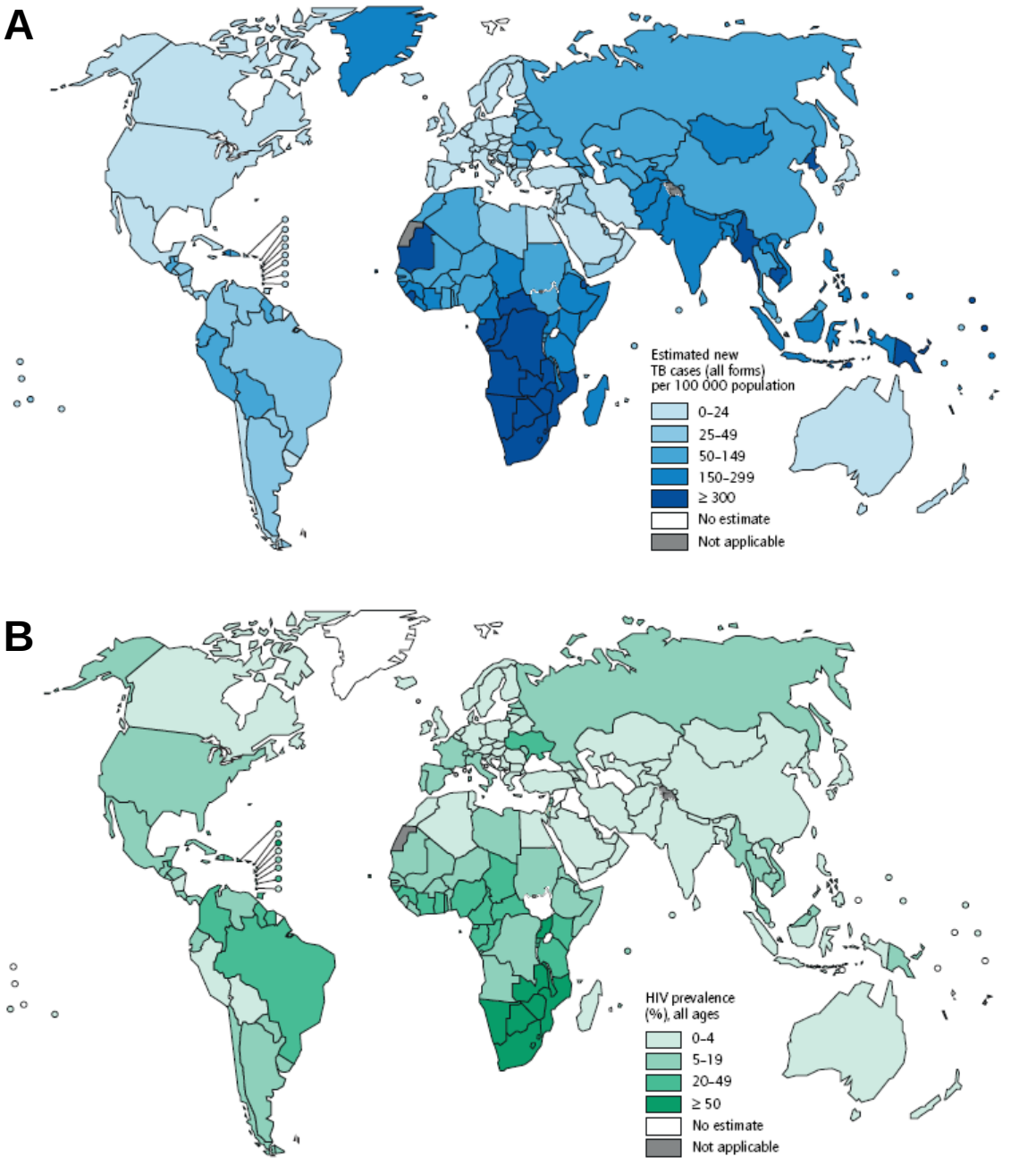

Figure 1. Epidemiology of the worldwide burden of clinical TB. *A*, All forms of TB cases reported in 2011. *B*, Prevalence of HIV infection in all reported cases of TB in 2011. (Source: WHO Global TB Report, 2012)

Fig. 1 shows the global distribution of all reported clinical TB cases (A), along with those with HIV co-infection (B). The most striking message conveyed by both maps is that similar to an array of other human infectious diseases, in particular those regarded as neglected i.e.

leishmaniasis, yellow fever and trypanosomiasis, the burden of TB is directly linked to poverty and lack of infrastructure to handle adverse health problems. This is evident, as much of disease prevalence exists in the Southern hemisphere with the exception of several former Soviet Union nations i.e. Moldova, Belarus and the Russian Federation (Stuckler, King et al. 2008; Skrahina, Hurevich et al. 2013), and almost all low-to-middle income countries are located in this region. Moreover, the inefficient delivery of healthcare services (including unavailability of sufficient supply of medication) as well as very lenient monitoring of TB patients undergoing treatment constitutes one of the major causes for *M. tuberculosis* drug resistance. As a spin-off effect of healthcare system failure in high burden countries, drug resistance also owes to non compliance of patients (or defaulting) with the standard WHO-recommended drug regimen (Castelnuovo 2010).

Furthermore, poverty-stricken communities in high burden countries suffer from poor nutrition, limited access to point-of-care facilities and substandard socio-economic conditions, thus significantly contributing to escalation of clinically drug-resistant TB cases (Lonnroth, Castro et al. 2010; Keshavjee and Farmer 2012; Oxlade and Murray 2012). Adding to this intricacy is the bane of clinical management of TB. Patients presenting with clinical manifestations of TB such as night sweats and chills, as well as dramatic weight loss and in more severe cases, haemoptysis (blood cough) may indicate an advanced disease stage and this complicates treatment (Crofton's Clinical Tuberculosis, 3rd Edition).

1.2 TB diagnostics.

A major contributing factor to inefficient clinical management of TB is suboptimal diagnosis. Sputum smears and microscopy constitute the mainstay of TB diagnosis. In this method, actively-replicating *M. tuberculosis* (or other mycobacteria) present in sputum are spread onto a glass slide, heat-fixed and stained using the Ziehl-Neelsen technique (briefly described in the next section). Since this method offers only an estimated 60 - 70% diagnostic accuracy (culture-confirmed), there exists a sizeable 30 - 40% window of missing treatable cases (Davies and Pai 2008). Where possible, chest X-rays are also performed on patients but this facility is usually cost and labour-intensive (Glaziou 2008; van der Werf, Enarson et al. 2008; McNerney, Maeurer et al. 2012)

Immunodiagnostics of TB, on the other hand relies on the Mantoux or tuberculin skin test (TST) where purified protein derivatives (PPD or tuberculin) of mycobacteria which are administered intra-dermally to observe induration (hardened swelling) on the person's skin. The diameter of

the induration then gives an estimate of the magnitude of cellular immune response to *M. tuberculosis* infection. Although useful, this test only states whether or not an individual has developed antimycobacterial immunity albeit not exclusively to *M. tuberculosis* antigens but also BCG vaccination or infection with non-tuberculous species of the genus such as *M. avium* and *M. abscessus*. Hence, lack of specificity is of noteworthy concern in this regard (Bakir, Dosanjh et al. 2009; Anibarro, Trigo et al. 2012).

Another set of diagnostic tests that employs the same principal are the interferon gamma release assays (IGRAs). The most widely used IGRA is the QuantiFERON skin test, developed by Cellestis, Australia (Streeton, Desem et al. 1998). The QuantiFERON test specifically measures release of interferon gamma (IFN-γ) in response to challenge with PPD, thus providing an estimate of the diagnosed individual's exposure to or infection with *M. tuberculosis*. The TB-specific ELISpot test T-Spot.TB (marketed by Oxford Immunotec Inc.), another IGRA in current use presents a very advanced diagnostic method. *M. tuberculosis*-specific T cell antigens are co-cultured with patient blood samples to obtain an IFN-γ-based read-out (Bakir, Dosanjh et al. 2009). IGRAs were recently proven in a clinical setting to produce more accurate and reliable results in comparison to the TST to predict *M. tuberculosis*-infected individuals who may progress to active disease (Trajman, Steffen et al. 2013). Nonetheless, IGRAs are beyond affordability for many countries with overwhelming disease burden.

Many point-of-care centres in low-resource settings do not offer laboratory facilities to perform molecular diagnostics using ribosomal RNA and/or insertion element sequencing i.e. IS6110 to distinguish between the exact strain of *M. tuberculosis* and any other mycobacterial species a patient maybe infected with, with a major focus on drug susceptibility profile of the *M. tuberculosis* strain (Fluit, Visser et al. 2001). This helps the clinician to decide as to which drug regimen the patient should be treated with. The standard mycobacterial drug susceptibility assay/test (MDST/A) which determines minimum inhibitory concentration (MIC) values is also carried out in clinics and microbiological laboratories to aid prescription of medication (Grosset, Singer et al. 2012).

GeneExpert MTB/RIF is a state of the art detection method of *M. tuberculosis* presence in sputum samples as well as its resistance to rifampicin. This nucleic acid-based assay employs amplification of the *rpoB* gene responsible for genotypic resistance to rifampicin using only mycobacteria recovered from sputa of patients. Laboratories that carry out the GeneExpert assay are required to first confirm presence of *M .tuberculosis* in sputa of patients by standard

culture methods (Löwenstein-Jensen medium / Mycobacteria Growth Indicator Tube, MGIT) as well as secretion of MPT64, an immunogenic protein of *M. tuberculosis*, Effectively, only a few viable bacilli are required to perform this assay (usually less than 100 CFUs) and the rate of detection is at least 70% for cases initially diagnosed as smear-negative (Boehme, Nabeta et al. 2010).

Taken together, TB is not merely an infectious disease which requires immediate and effective intervention but also a serious public health conundrum. As an airborne infection, spread of TB if uncontrolled can reach unmanageable rates, especially with the newly-reported TDR-TB variant reported in Italy, India, Iran and South Africa (Migliori, De Iaco et al. 2007; Velayati, Farnia et al. 2009; Shah, Richardson et al. 2011; Udwadia, Amale et al. 2012). Should this variant become more widespread, then no current drug against TB can help and hence, the very urgent need to develop new interventional strategies or at least improve current ones.

1.3 General microbiology of M. tuberculosis.

M. tuberculosis belongs to the family of mycolic acid-containing actinobacteria (MACA) which also includes the genus *Cyanobacteria*, *Streptomyces* and *Nocardia*. This unique rod-shaped bacillus was discovered by the German microbiologist Robert Koch in 1882 in Berlin (Cullen 2009). The genus *Mycobacterium* enlists of species with immense public health significance: *M. tuberculosis* and other members of the MTB complex (MTBC) i.e. *M. kansasii*, *M. africanum*, *M. avium, M. bovis* that are involved in the pathogenesis of human tuberculosis and TB meningitis (*M .bovis* is the aetiological agent of bovine tuberculosis), whereas *M. leprae* and *M. ulcerans* are aetiological agents of leprosy (or Hansen's disease) and Buruli ulcer, respectively (Borrell and Gagneux 2011; Lawn and Zumla 2011; Bratschi, Njih Tabah et al. 2012).

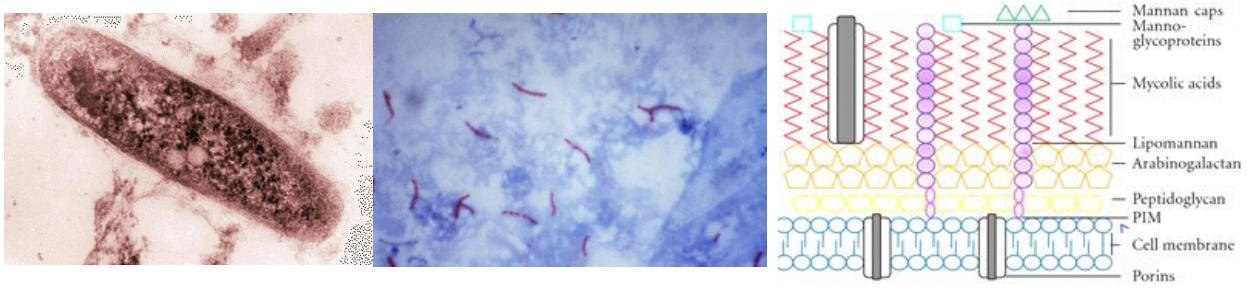

Figure 2. Common microbiological features of *M. tuberculosis*. A, Electron micrograph of *M. tuberculosis*. B, *M. tuberculosis* in a clinical sputum smear sample. C, Cross-section of the mycobacterial acid-fast cell wall. PIM = phosphatidylinositol mannoside (Source: A: http://www.wadsworth.org/databank/mycotubr.htm; B: http://health.state.tn.us/ceds/tb/guidelines.htm; C: http://www.hindawi.com/journals/cdi/2011/405310/fig1/)

Due to their complex cell wall structure (Fig. 2), members of *Mycobacterium sp.* can be microscopically identified via Ziehl-Neelsen staining, a method characterised by the use of carbol fuchsin to stain cell-wall bound mycolic acids while withstanding subsequent decolourisation by organic solvents (Lawn and Zumla 2011). Interestingly, the mycobacterial cell wall comprises morphological features spanning Gram positive and Gram negative eubacteria (Hett and Rubin 2008; Zuber, Chami et al. 2008). In addition to this characteristic and with an array of cellular adaptations, *M. tuberculosis* is rendered refractory to a range of antibiotics, giving rise to an alarming variety of drug-resistant strains circulating in human populations. These range from multidrug-resistant TB (MDR-TB) and extremely drug-resistant TB (XDR-TB) to the afore-mentioned TDR-TB variants, which are resistant to all known antimycobacterial agents in clinical use (Cegielski, Nunn et al. 2012).

Although generally a facultative aerobe, *M. tuberculosis* has evolved very clever ways of persisting in its human host by excogitating a myriad of strategies. In most individuals (ca. 90% of infected persons), the bacterium is able to remain in a paucibacillary state with restricted replication and minimal physiological activity – denoted as 'dormancy' (Wayne and Sohaskey 2001; Boon and Dick 2002; Chao and Rubin 2010). Latent infection of individuals with *M. tuberculosis* without clinical symptoms is termed as latent tuberculosis infection or LTBI.

In contribution to pathogenesis, an array of defence mechanisms has allowed *M. tuberculosis* to evolve alongside its human host and successfully resist not only chemotherapy but also immune clearance. For example, *M. tuberculosis* uses cofactor F_{420}-mediated pathways to neutralise reactive nitrogen and oxygen intermediates (Gurumurthy, Rao et al. 2012). *M. tuberculosis* H37Rv mutants deficient in endogenous F_{420} biosynthesis were found to be highly sensitive to nitrosative stress (Darwin, Ehrt et al. 2003), although the mechanism by which this protection is conferred (or lost) remains unknown. Singh and colleagues showed that the experimental pro-drug PA-824, which requires cofactor F_{420}-dependent mechanisms for activation within the mycobacterial cell eventually kills bacteria by intracellular release of nitric oxide (NO) and terminally modifies DNA as well as lipid moieties in the cell wall (Singh, Manjunatha et al. 2008). The most interesting feature of cofactor F_{420} is that it is not present in any other eubacteria but only in archaea and actinobacteria, which may also shed some light on the evolutionary origins of mycobacteria (Selengut and Haft 2010).

Efflux pumps and porin-like channels of mycobacteria have also been described in the context of excreting drug molecules that the bacterial cell is exposed to (Adams, Takaki et al. 2011;

Balganesh, Dinesh et al. 2012; Machado, Couto et al. 2012). These structures may well have a role to play in steady-state bacterial physiology but this is yet to be fully investigated. Current research investigating the dynamics of the cell envelope of *M. tuberculosis* beckons that transmembrane molecules of the bacterium may participate in detoxification as well as cleavage of certain host proteins to increase their chance of survival in the phagosome (Adams, Takaki et al. 2011). Other bacterial molecules that have been reported to withstand host immune attack include superoxide dismutase (SOD), members of the OxyR-AhpC region, NarGHIJK operon, as well as the electron transport chain (ETC), particularly the quinones (Deretic, Philipp et al. 1995; Dussurget, Stewart et al. 2001; Tan, Sequeira et al. 2010; Kumar, Farhana et al. 2011).

Ability of *M. tuberculosis* to prevent the fusion of phagosomes with the lysosome in the MΦ is another major virulence mechanism which dampens host immune responses (Koul, Herget et al. 2004; Baena and Porcelli 2009). Protein kinase G (PknG) is a well described bacterial molecule that facilitates phagolysosome formation, and mycobacterial mutants lacking this enzymes are efficiently processed and delivered to the exogenous antigen presentation pathway (Walburger, Koul et al. 2004). Another extremely unique feature of mycobacteria is that they are the only known prokaryotes to possess a proteasome which functions not only in degrading wrongly folded proteins but also in detoxification of incoming drug compounds (Darwin, Ehrt et al. 2003; Cerda-Maira and Darwin 2009).

1.4 Immune response to M. tuberculosis infection and immunopathology.

Fig. 3 summarises the general route of TB pathogenesis in humans. Upon inhalation, *M. tuberculosis* survives within alveolar macrophages (AMΦs) following phagocytosis. These sentinel-like innate immune cells that reside in the alveolar lumen of the lung, an immune-privileged organ are in constant contact with inhaled material including pathogens (Lambrecht 2006). Following antigen encounter, AMΦs release cytokines such as interleukin 1 beta (IL-1β), tumour necrosis factor alpha (TNF-α), interleukin 12 (IL-12), interleukin 6 (IL-6) etc. to initiate inflammatory signalling cascades into their microenvironment (Lambrecht 2006). Activated AMΦs also trigger influx of migratory primary immune cells such as monocytes (which, upon activation differentiate into dendritic cells or DCs) and neutrophils downstream of intracellular killing of ingested bacteria (Bitterman, Rennard et al. 1982; Gordon and Read 2002; van Crevel, Ottenhoff et al. 2002). MΦs and DCs, which are professional antigen presenting cells (APCs) then sample the available bacterial antigens , process them and present specific epitopes to

either naïve CD4+ or CD8+ T cells (also known as cytotoxic lymphocytes or CTLs) in the draining lymph nodes (dLNs).

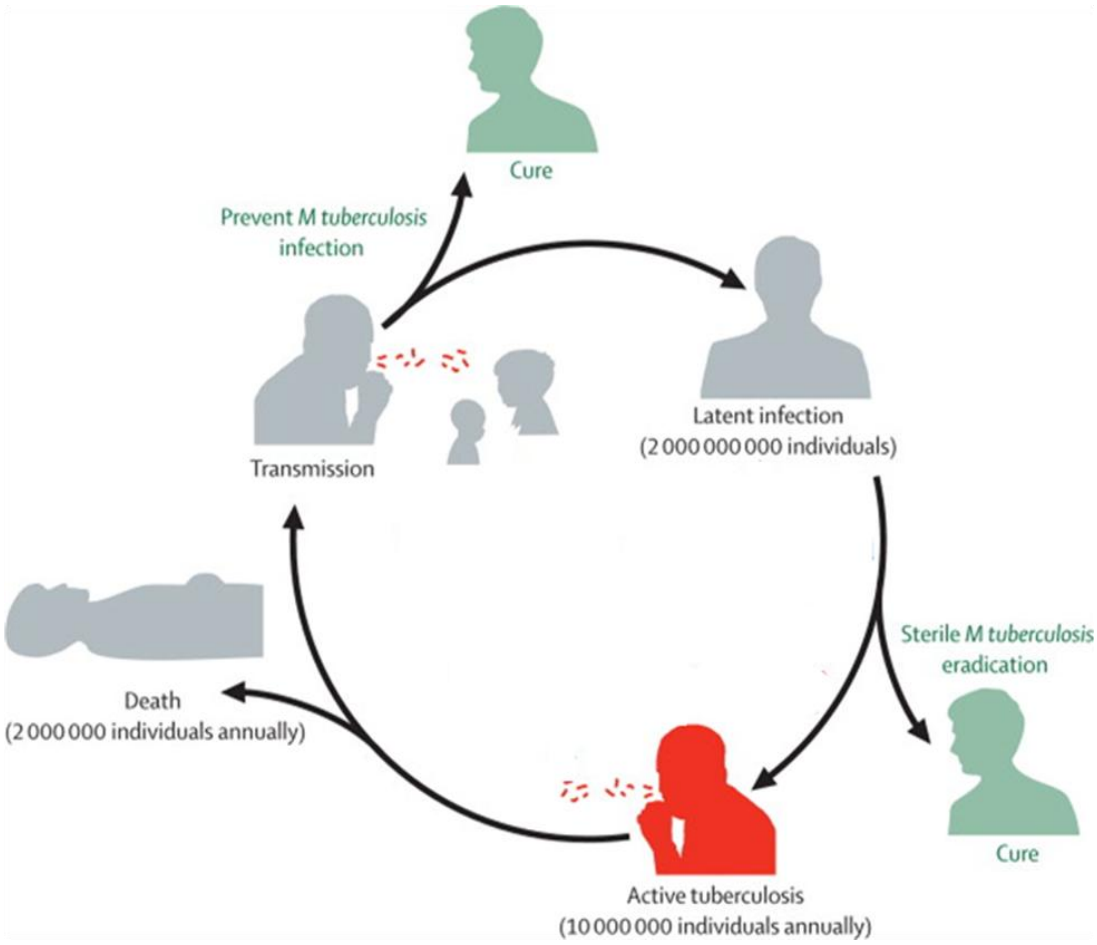

Figure 3. Schematic showing pathogenesis of human TB in the context of vaccination (Adapted from Kaufmann, 2011).

1.4.1 Antigen processing and presentation.

CD4+ T cells recognise epitopes ranging from 15 to 20 amino acids in length presented via the major histocompatibility class II (MHC-II) pathway. Antigen processing via the MHC-II pathway requires pathogen killing and soluble antigen degradation by lysosomal proteins for presenting to CD4+ T cells. Phagolysosomes containing digested exogenous antigens (e.g. engulfed bacteria, toxin) fuse with an endosome carrying the immature MHC-II molecule characterised by the

epitope groove-bound class II invariant chain peptide (CLIP). Upon successful fusion, the appropriate epitopes are exchanged with CLIP, and this process occurs within an endosome.

Conversely, proteasomal processing of endogenously-derived antigens (e.g. viral / cytosolic origin) in the cytosol leads to epitope presentation to CD8+ T cells via the MHC-I pathway, and usually recognises epitopes of 8 – 9 amino acids in length. MHC-I-restricted antigen processing requires activity of the transporter associated with antigen processing (TAP) which is localised on the membrane of the rough endoplasmic reticulum (rER). Proteasomally-degraded cytosolic antigens are subsequently channelled into the rER for loading onto the MHC-I molecule, which in its immature form is bound to calreticulin and tapasin. In both pathways, the endosome-bound MHC-epitope complex is first trafficked to the Golgi apparatus in order to have it translocated to the cell surface (Janeway's Immunobiology, 7th Edition).

Two other subsets of non-canonical T cells that have a role in immune responses to *M. tuberculosis* infection are the gamma-delta (γδ) and natural killer (NK) T cells (O'Garra, Redford et al. 2013). Both subsets recognise antigen presented by the CD1 family of antigen presentation molecules which shares sequence homology and general morphology to the MHC-I molecule (Barral and Brenner 2007). Humans express all the 5 known isomers of CD1 (a to e) while mice only express CD1d (Barral and Brenner 2007). γδ T cells recognise phospholipid (e.g. PIM) and protein antigens (e.g. heat shock proteins, PPD) presented by CD1a, CD1b or CD1c, respectively (Haregewoin, Soman et al. 1989; Barral and Brenner 2007). NK T cells, on the other hand recognise glycolipid antigens presented by the CD1d molecule (Tupin, Kinjo et al. 2007).

Activated effector T cells are primed in dLNs with the afore-mentioned bacterial epitopes and can then traffic to the lung to respond to infected AMΦs and DCs by activating their intracellular antibacterial mechanisms. This programme is largely facilitated by APC-mediated pro-inflammatory cytokine signalling (mainly IL-6, IL-12 and IL-18) leading to IFN-γ, TNF-α, IL-2 and interleukin 17 (IL-17) release by activated CD4+ T cells. CD4+ T cells that secrete IFN-γ, TNF-α, IL-2 are usually referred to as the T helper 1 (Th1) subset while those secreting IL-17 are termed as Th17 cells and require IL-23 for development (Khader and Cooper 2008). Activated CD8+ T cells, on the contrary release cytolytic molecules such as granzyme B, cytolysin and perforin besides the afore mentioned Th1 cytokines to eliminate infected cells to contain infection (Wong and Pamer 2003). Table 1 shows a comparison of the major characteristics of CD4+ T cells and CD8+ T cells involved in immunological control of *M. tuberculosis* infection.

Aspect	CD4+ Th1 cells	CD4+Th17 cells	CD8+ T cells
Major cytokine profile	IFN-γ, TNF-α, IL-2	IL-17	IFN-γ, TNF-α, IL-2
Toxic molecule secretion	N/A	N/A	Perforin, grazyme, cytolysin
Activity during infection	Activation of IRF-1, NOS2, ROI production	Increase monocyte infiltration / macrophage accumulation	Direct killing of infected cells
Immunologic memory	Yes	Yes	Yes
Antigen specificity	Yes	Yes	Yes
Antigen recognition	MHC-II-restricted	MHC-II restricted	MHC-I-restricted

Table 1. The cardinal differences between CD4+ Th1, CD4+ Th17 and CD8+ T cells that contribute to controlling *M. tuberculosis* infection.

It is essential that antigen turn-over, as already mentioned earlier in this chapter occurs very rapidly and efficiently in order to vouch the cytokine milieu sourced by antigen-activated phagocytes. The quality of the immune synapse, which occurs between the epitope-loaded MHC complex (human leukocyte antigen, HLA in human or H2 in mice) of an activated DC or MΦ and its matching TCR on the surface of a T cell, determines priming of the latter. The roles of T cells in antitubercular immunity will be discussed in further detail within the context of vaccination in a later section.

1.4.2 Neutrophils and TB immunopathology.

The chain of events stretching from antigen encounter by innate immune cells to priming of T cell responses is heavily influenced by the rate at which antigen turn-over occurs within the APC – a correlate of how efficient these cells are at processing and presenting pathogen-associated epitopes. Also, neutrophils have been implicated to play a noticeable role in modelling the early transport of mycobacterial antigens to draining lymph nodes due to their phagocytic and antigen-presenting properties (Abadie, Badell et al. 2005). They are capable of activating *M. tuberculosis*-specific CD4+ T cells either indirectly after engulfment by DCs or directly engaging naïve CD4+ T cells (Morel, Badell et al. 2008; Blomgran and Ernst 2011).

Conversely, neutrophils have also been associated with contributing to TB disease. Neutrophil infiltration into the lung early upon *M. tuberculosis* infection increases chances of developing full-blown disease in highly susceptible mice (Eruslanov, Lyadova et al. 2005). In addition, long-term persistence of *M. tuberculosis*-infected neutrophils in the lung can facilitate pathology due to decreased apoptosis (Blomgran, Desvignes et al. 2012).

In effect, successful immune responses initiated to control *M. tuberculosis* infection aim to curb and confine the infection to one part of the lung, where *M. tuberculosis*-infected myeloid cells are surrounded by interacting effector lymphocytes primed in the dLNs. An outermost layer of fibrotic

tissue then forms a cuff, which eventually leads to the formation of a highly organised secondary lymphoid structure called a granuloma. This presents the immunopathological hallmark of *M. tuberculosis* infection in the lung.

1.4.3 The tuberculous granuloma – hallmark of human pulmonary TB.

Granulomas represent a unique immunological means to restrict spread of primary infection with *M. tuberculosis* to unaffected parts of the lung (Fig. 4). Since infections of human with *M. tuberculosis* vary extensively in terms of clinical outcome, one of the best animal models in place for studying TB pathogenesis are cynomolgus monkeys or macaques (Kirschner, Young et al. 2010). Recent evidence from studies conducted in macaques suggests that some granulomas in the lung self-heal, disappearing a few months post-infection even without chemotherapeutic intervention whilst others in the same lung become larger and perpetrate establishment of severe disease (Joann Flynn, EMBO Tuberculosis Conference 2012, Paris; plenary talk).

Considering individuals who are latently infected comprise about 90% of all *M. tuberculosis* infections, the granuloma most likely curbs *M. tuberculosis* infection very aptly and successfully prevents progression of disease to its extrapulmonary form. This is further supported by the observation that individuals with LTBI exhibit none of clinical manifestations of active TB and are usually poor or negative responders to the classically-employed TST described earlier, indicating undetectable mycobacteria-specific cellular immunity (Anibarro, Trigo et al. 2012).

A good number of reasons shoulder the phenomenon of LTBI – good nutrition, natural immunity, hygienic residential conditions and vaccination to list a few. As observed in macaques, it could also be that an individual diagnosed via radiography in the clinic had been infected with *M. tuberculosis* at some point in time and in response, formed solid granulomas. However, this individual may never develop clinical disease due to complete clearance of the pathogen in minimal time, a phenomenon recently achieved in a rabbit model of tuberculosis (Subbian, Tsenova et al. 2012). Hence, LTBI has been noted as a very good base for mining the correlates of protection against clinical TB – considering the protective nature of solid, non-progressive granulomas. Inevitably, information arising from such efforts may contribute to rational vaccine design of TB vaccines.

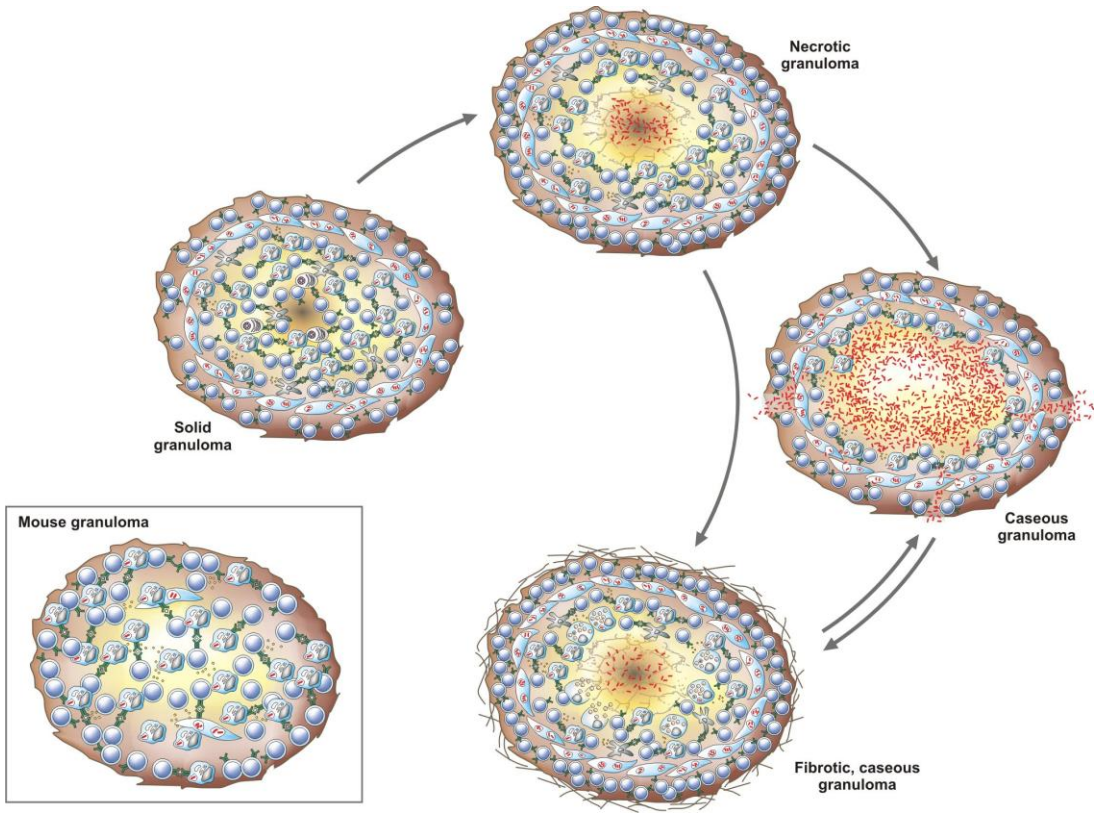

Figure 4. Schematic representation of the dynamic stages of the TB granuloma. Solid granulomas are often associated with protection from clinical TB . An example of a solid granuloma in the mouse is depicted inset (Adapted from Reece and Kaufmann, 2012).

1.4.4 Failure of solid granulomas and reactivated TB disease.

Current opinion has it that the granuloma could also serve as a platform for exacerbating disease although this greatly relies on host-pathogen interactions. The core of the solid granuloma may begin to liquefy and caseate, forming a pulp of necrotic tissue which acts as a nutritious broth for uncontrolled *M. tuberculosis* replication – the pathophysiological stilts for reactivated TB. As bacterial load increases, *M. tuberculosis* gains access across the fibrous cuff walling the granuloma and spills into the airways. At this stage of disease, the infected individual becomes highly contagious due to coughing out of viable bacilli.

When the primary infection in the lung worsens, bacteria disseminate into systemic circulation and go on to colonise other vital organs so as to form miliary TB. The skin, nervous system, liver, kidneys, eyes and testes are the most commonly affected organs with sporadic cases involving the heart (Liu, Hu et al. 2012; Ray, Talukdar et al. 2013). Although symptomatic TB accounts for only 10% of all *M. tuberculosis*-infected individuals, those in close contact with TB

patients are at an extremely high risk of contracting clinical disease during the course of their lifetime (Attamna, Chemtob et al. 2009).

The exact mechanisms by which LTBI can progress to active disease remain unclear. There may not necessarily be definite determinants herein although there are several hypotheses shrouding this phenomenon. Post-surgery immunosuppression, malnutrition, full-blown AIDS and helmith co-infection to name a few have been identified as perpetrators of reactivated TB (Lawn and Zumla 2011).

Just on the contrary, it is also possible that a very intense pro-inflammatory immune response could cause notable damage to lung tissue, unfavourably leading to tissue damage. Matrix metalloproteinase 1 (MMP-1), an enzyme which possess collagenase activity and disrupts parenchyma integrity has been reported to exist at high levels in sera from pulmonary TB patients, and this observation is directly attributed to severe *M. tuberculosis* infection (Elkington, Shiomi et al. 2011). In the same study, primary human blood monocytes which were infected *ex vivo* with virulent *M. tuberculosis* also showed elevated levels of MMP-1 production. MMP-1 has been directly linked with immunopathology and tissue destruction in the lung and this effect was reversed with doxycycline, a known MMP inhibitor (Walker, Clark et al. 2012). There is also evidence of pulmonary neo-vascularisation in late-stage TB patients, where increased levels of vascular-endothelial growth factor (VEGF) are found in pleural fluid (Djoba Siawaya, Chegou et al. 2009; Seiscento, Vargas et al. 2010) samples.

As TB is generally considered a chronic infection which may progress to active lung disease, the physiological state of the human host pronouncedly influences outcome of encounter with *M. tuberculosis* in the primary infection, largely owing to failed or waning immunity (Ernst 2012).

2. Vaccination as a means of handling the global burden of TB.

As already mentioned ~90% of *M. tuberculosis*-infected individuals do not develop clinical TB in their lifetime - afforded by efficient control of bacterial burden by the immune system. Adding to this is the fact that *M. tuberculosis* is a human pathogen, and has evolved alongside its human host for over 11,000 years following zoonotic transmission from cattle (Wolfe, Dunavan et al. 2007; Russell 2013). Quite naturally, this evolutionary process would have established an equilibrium dominated by the immune system to keep the bacterium under check and not cause disease. When immunity wanes, it is possible to revamp protective immune response with

considerable help. Therefore, immunological intervention ranks as a potentially powerful tool to defy the tubercle bacillus and hence, paving the way for vaccines.

Generally aimed at priming the adaptive immune system to specifically fight an infectious disease, vaccines harness the host's ability to recognise and combat an infectious agent while not causing much harm to the former. Also, vaccines are the most cost-efficient method of disease control from a public health point of view as they give rise to herd immunity and extensively keep large populations from vulnerability to disease – antitubercular drugs are more expensive, complication-prone and require greater compliance in order to achieve a close enough feat on a global scale (Glynn, Whiteley et al. 2002; Reece and Kaufmann 2008).

In recognition of the afore-mentioned reasons, much manual and financial effort has been invested in TB immunology and vaccinology to design and formulate the most strategic approaches to developing a highly efficacious TB vaccine. In this section, some of the most recent advances in TB vaccine research are discussed, with a focus on live recombinant vaccine candidates.

2.1 Immunology of vaccination and rational TB vaccine design.

It is crucial to bear in mind that an effective TB vaccine needs to augment the early, pro-inflammatory and subsequent cell-mediated immune responses. Upon injecting a vaccine into the skin of the host (BCG is delivered intradermally in humans; discussed further in section 2.2), highly efficient resident skin DCs called Langerhans cells (LCs) as well as dermal MΦs internalise incoming mycobacteria. This marks one of the first steps in promoting vaccine-induced immunity as DCs and MΦs constitute antigen presenting cells (APCs). The phagocytosed bacteria are then processed - in the case of BCG largely via the MHC-I pathway and transported mainly by DCs to skin draining lymph nodes (dLNs) where antigens are presented to T cells. Neutrophils have also been shown to carry out this duty, and play a critical role in activating DCs and priming T cells in auricular dLNs (Abadie, Badell et al. 2005).

Akin to *M. tuberculosis* infection-mediated immuno-dynamics, successful interactions between DCs and T cells are best achieved in the presence of innate immune pro-inflammatory cytokines such as IL-6, IL-12 and IL-18 as well as stable expression of activation markers such as CD40, CD80 and CD86 by APCs (Schmidt, Nino-Castro et al. 2012). Activated CD4+ T cells then assume a Th1 phenotype and express IFN-γ, TNF-α and interleukin 2 (IL-2) in response to antigen-stimulation (Cooper 2009). In this regard, expression of the CD4+ T cell activation

marker CD40 ligand (CD40L or CD154) is of noteworthy importance (Frentsch, Arbach et al. 2005). There is also evidence that lays a platform for CD8+ T cell identification using CD154 in concert with intracellular cytokine presence (Andreas Thiel, unpublished data). CD154 is a member of the TNF family of proteins and its expression is usually observed after 4 hours of antigen stimulation *in vitro*. Upon encounter with CD40, surface-bound CD154 is internalised and presence of this molecule can only be seen intracellularly. This is also possible by blocking the Golgi apparatus in order to prevent translocation of CD154 to the cell surface, as is done with intracellular cytokine production. However, in the absence of or blockade of access to CD40 (usually achieved via co-incubating activated APCs with an anti-CD40 antibody), CD154 may be retained on the surface of antigen-specific T cells for up to 24 hours post stimulation (Kirchhoff, Frentsch et al. 2007).

Activated T cells then migrate to the spleen where they can proliferate, after which a large proportion enters systemic circulation to home to the lung or any other organ and reside there. Presently, the bone marrow is also being increasingly considered an important reservoir for homing of resting memory CD4+ T cells and CD8+ T cells in mice and human (Becker, Coley et al. 2005; Mazo, Honczarenko et al. 2005; Tokoyoda, Zehentmeier et al. 2009). These T cells that are usually raised against a broad battery of mycobacterial epitopes also express classical memory markers such as CD44, CD62L, CD127 (interleukin 7 receptor, IL-7R) (Krawczenko, Kieda et al. 2005). It is to be stressed that the major subset of memory T cells retain specificity for the most immunogenic epitopes but this does not necessarily guarantee protective immunity to TB.

Resident dermal MΦs may be instrumental in presenting antigens to CD4+ T cells in the dermis of the skin immediately after vaccination since their role in wound healing is indispensable (Mahdavian Delavary, van der Veer et al. 2011). Skin CD4+ T cells are the first effector cell type to respond to APCs, and spark the release of pro-inflammatory cytokines such as IFN-γ and TNF-α. Simultaneously, programmed cell death (PCD) mechanisms such as apoptosis and autophagy are activated, leading to enhanced clearance of bacteria and antigen presentation to T cells (Labbe and Saleh 2008; Jagannath, Lindsey et al. 2009).

Fig. 5 is a schematic representation of a proposed path which requires to be taken by the migrating DCs upon antigen encounter and subsequent activation. Thus, DCs play an integral role in realising the immunological basis of the success of a vaccine by bridging the innate and adaptive immune systems. It is noteworthy that the cytokine milieu present during acute

inflammation in the skin following vaccination has much to influence activation of antigen-loaded DCs. Also shown in this diagram are the CD141+ DCs, which are the human orthologue of murine CD8+ DCs (Bachem, Guttler et al. 2010). CD141+ DCs have been shown to possess the ability to cross-present soluble antigens to CD8+ T cells, as well as necrotic tissue debris derived from human cytomegalovirus-infected cells to both CD4+ T cells and CD8+ T cells (Bachem, Guttler et al. 2010; Poulin, Salio et al. 2010). Cross presentation is an effective mechanism by which extracellular (exogenous) antigens can be taken up by DCs (i.e. via phagocytosis) to process and present epitopes to T cells (Joffre, Segura et al. 2012).

In addition to activation of CD8+ T cells, CD141+ DCs appear to induce antibody-mediated immune responses (Ueno, Palucka et al. 2010). In mice, targeted antigen delivery to C-type lectin 9A (Clec9A) expressed selectively on the surface of CD8+ DCs is able to enhance serum antibody titres in the absence of adjuvants (Caminschi, Proietto et al. 2008). As such, CD141+ DCs may have an important role in orchestrating and amplifying vaccine-mediated immune responses (Ueno, Palucka et al. 2010).

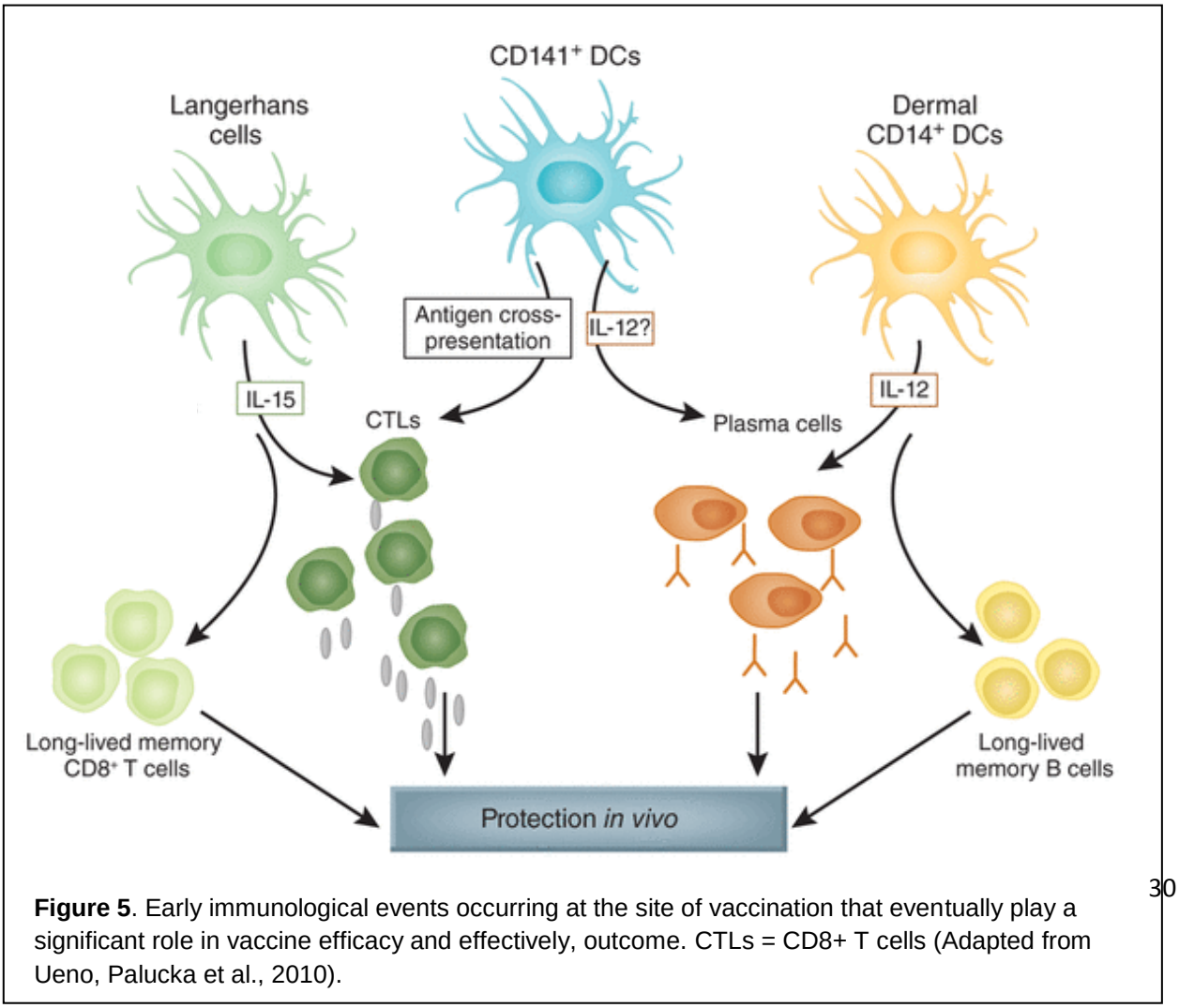

Figure 5. Early immunological events occurring at the site of vaccination that eventually play a significant role in vaccine efficacy and effectively, outcome. CTLs = CD8+ T cells (Adapted from Ueno, Palucka et al., 2010).

As much of vaccine efficacy studies are conducted in mice, MHC-I (equivalent to HLA-A, B or C in humans) –restricted antigen presentation to CD8+ T cells is a key interaction which has been the focus of research. While CD4+ T cells can interact with infected MΦs and DCs to elicit cascades of intracellular events essentially via IFN-γ signalling (which requires interferon gamma receptor 1 or IFNGR1), CD8+ T cells target, engage and directly kill *M. tuberculosis*-infected cells. This possibly applies also to alveolar epithelial cells (AECs) as they can also afford MHC-I-restricted antigen presentation.

Much of our current understanding of how protective antitubercular immunity or at least associated host immune response against development of clinical TB is immensely defined by clues sourced by clinical and basic research. The unifying link from all scientific excavations is inflammation - TB vaccine design is largely characterised by candidates that can successfully give rise to a sizeable pool of memory T cells which can convert to the IFN-γ^+ TNF-α^+ effector arm triggered by *M. tuberculosis* infection (Orme 2001; Reece and Kaufmann 2008; Rowland and McShane 2011; Kaufmann 2012). Therefore, unearthing nascent information concerning immunomodulatory molecules such as cytokines have a rather defining role to play herein. For example, newly-described members of the IL-1 family (summarised in Table 2), the IL-36 isoforms (α, β and γ) have been shown to prime DCs for efficient MHC-II-restricted antigen presentation to CD4+ T cells (Vigne, Palmer et al. 2012). Also, the IL-32 isoforms (α, β and γ) have been implicated in *M. leprae* infection to contribute to NOD2 activation and MHC-I-restricted antigen processing (Schenk, Krutzik et al. 2012).

Name of cytokine	Citation
IL-1α	Sims and Smith, 2010.
IL-1β	
IL-1Ra	
IL-18	
IL-33	
IL-1F5 / IL-36Ra	Dinerello et al., 2010.
IL-1F6 / IL-36α	
IL-1F8 / IL-36β	
IL-1F9 / IL-36γ	
IL-1F7 / IL-37	
IL-F10 / IL-38	Veerdonk et al., 2012

Table 2. Members of the IL-1 family. IL-1F5 to IL-F10 were recently renamed and hence, both the old and new nomenclatures are shown here. Source: (Dinarello, Arend et al. 2010; Sims and Smith 2010; van de Veerdonk, Stoeckman et al. 2012)

Although a gross simplification, Fig. 7 is aimed at summarising the cardinal criteria that need to be addressed in order to raise and maintain stable antitubercular immunity. Again, the trophy in TB vaccinology is to develop a vaccine which can effectively disallow *M. tuberculosis* infection altogether or provide sterilising immunity against the pathogen (Kaufmann 2012).

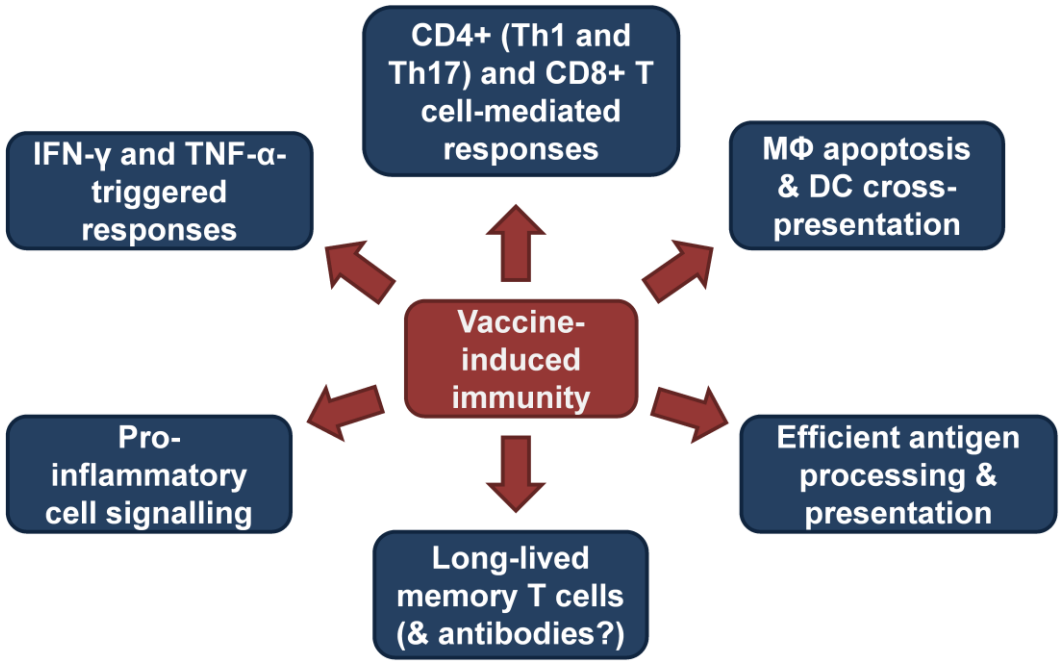

Figure 6. A schematic summary of the various immunological events that contribute to the success of a live TB vaccine in generating a strong and sustained antigen-specific effector memory T cell repertoire against *M. tuberculosis*.

2.2 Bacille Calmette-Guérin (BCG) vaccination.

BCG remains the oldest and most widespread human vaccine in circulation and with respect to TB, has been the best approach to deal with the global burden of disease for more than 80 years although this scenario is gradually ceasing (Doherty 2005; Kaufmann 2011). Developed at the Institute Pasteur de Lille (France) between 1906 and 1921 by French veterinarians Leon Charles Albert Calmette and Jean-Marie Camille Guérin, BCG is an attenuated form of the virulent *M. bovis* which causes bovine tuberculosis in cattle (Hussey, Hawkridge et al. 2007). Over a 13-year period, Calmette and Guérin made more than 230 passages of *virulent M. bovis* isolated from milk of an infected cow (by Edouard Nocard and deposited at the Institute Pasteur Paris in 1901) in glycerinated bile potato medium developed solely for the purpose of attenuating the pathogen (Oettinger, Jorgensen et al. 1999). The degree of BCG attenuation was observed via

avirulence of the resultant strain in a range of animals including guinea pigs, rhesus monkeys and farm cows. In addition, BCG also protected these animals against challenge with virulent mycobacteria after 30 days of vaccination. Finally in 1921, BCG was orally administered with milk ingestion to the first infant in Paris. In the next 5 years alone, an impressive 50,000 infants were administered the BCG vaccine in France and Europe at large (Gheorghiu et al., 2010).

This strain, which eventually became the sole vaccine for TB was then distributed to various laboratories and hospitals across Europe and eventually, the rest of the world. Currently, there are more than 10 existing substrains of BCG as shown in Fig. 8.

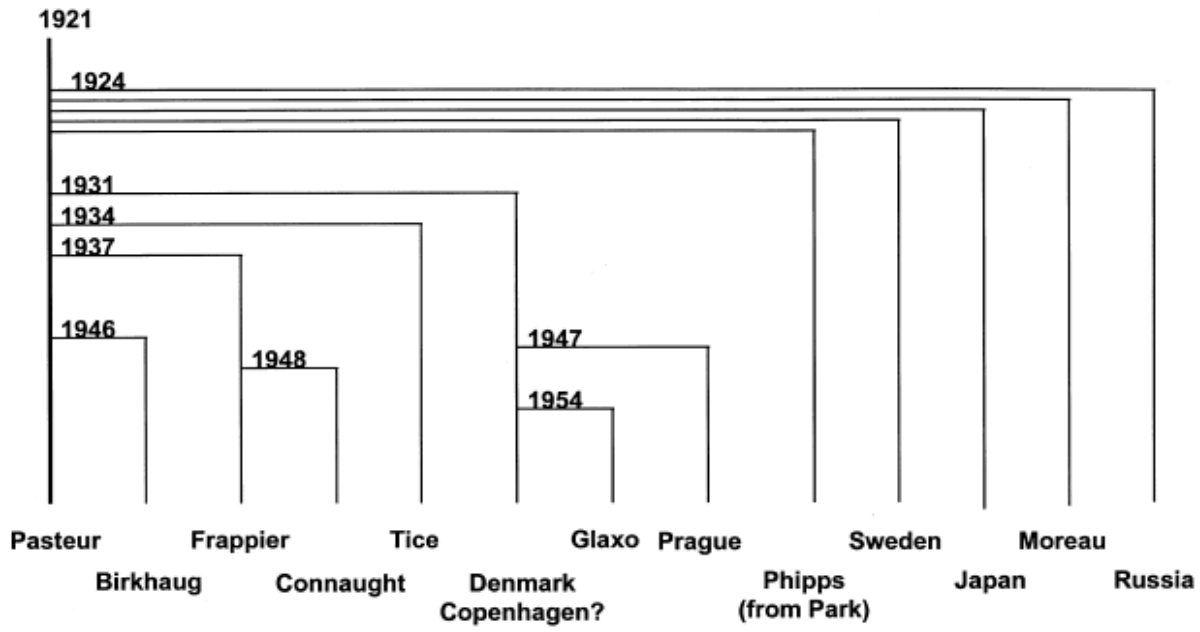

Figure 7. The various BCG strains that have evolved since 1921 after the derivation of the original Pasteur strain in Lille. (Source: Behr and Small, 1999).

As such, genetic differences across the various BCG substrains make some more immunogenic in the host than others. For example, the expression of the secreted protein MPB64 waned with substrains that were derived over time, rather evident after 1926 – BCG Russia, Moreau, Sweden and Japan were derived between 1924 and 1926 and hence, are active producers of MPB64. This has been attributed to the loss of **r**egion of **d**ifference 2 (RD-2) in the post-1926 substrains. Similarly, the insertion element IS6110 which is often used in molecular diagnostics appears to have decreased in genomic copy number although this also applies to BCG Sweden

(Behr and Small 1999). MPB70, another secreted protein and a potential diagnostic antigen is either normally secreted by some substrains (Moreau, Japan and Sweden) or poorly secreted by others (Danish, Montreal, Glaxo, Tice and Pasteur) (Oettinger, Jorgensen et al. 1999).

Since BCG bears 97% genetic homology with *M. tuberculosis* (Brosch, Gordon et al. 2007), many of the antigens present in the vaccine and recognised by the host immune system are shared between the two mycobacterial species, the vaccine and the pathogen. This therefore forms much of the basis for the protection conferred by the BCG vaccine. Some relevant examples are the MPB proteins in *M. bovis* BCG that are identical to the MPT members in *M. tuberculosis* (Baba, Dyrhol-Riise et al. 2008; Reddy, Riley et al. 2009; Mustafa 2011). However, the efficacy of BCG has waned remarkably over time and a plethora of factors have contributed to this - loss of immunogenicity of BCG, inability of BCG to promote long-term antigen-specific T cell memory, co-infection with helminths, co-infection with HIV, malnutrition and exposure non-tuberculous mycobacteria due to shared antigens to list a few (Checkley, Wyllie et al. 2011; Jaganath and Mupere 2012; Pawlowski, Jansson et al. 2012; Rafi, Ribeiro-Rodrigues et al. 2012).

In addition, a considerably large battery of immunogenic protein antigens present in *M. tuberculosis* is absent from BCG i.e. components of the RD-1 (Mahairas, Sabo et al. 1996; Behr, Wilson et al. 1999; Gordon, Brosch et al. 1999). The RD-1 region remains the most significant difference between all BCG substrains and virulent *M. bovis* as well as *M. tuberculosis*. Spanning 9.5 kilobases, the RD-1 locus encodes at least 10 genes, including the Esx secretion system (Berthet, Rasmussen et al. 1998). One of the most immunologically-relevant secreted antigens of *M. tuberculosis*, namely the pore-forming early secreted antigenic target 6 (ESAT-6, also annotated as Esx1) and its chaperone culture filtrate protein 10 (CFP-10, also annotated as Esx2) are exported by *M. tuberculosis* via the Esx secretion apparatus (Smith, Manoranjan et al. 2008). ESAT-6 contains immunodominant epitopes that elicit CD4+ and CD8+ T cell responses *in vitro* (Woodworth, Wu et al. 2008; Dheda, van Zyl-Smit et al. 2009; Reddy, Riley et al. 2009). Importantly, ESAT-6 epitopes form the platform for the T-SPOT-TB and Quantiferon test that is currently in extensive use (Bakir, Dosanjh et al. 2009).

BCG is still capable of contributing to global control of TB and this very hallmark of the vaccine makes it the most promising interventional method against TB. Currently, BCG vaccination is reported to save circa 40,000 infantile lives per annum (Trunz, Fine et al. 2006). Also, vaccines rely on harnessing the immune system's ability to fight infection and thus, represent a natural

means of tackling TB and other infectious diseases in general. Therefore, numerous efforts are underway to improve the BCG vaccine with hope that genetic manipulation, if implemented rationally and appropriately may elevate its efficacy to the effect of complementing parental BCG or even replacing it.

2.3 Measures to improve BCG-mediated immunity.

Various efforts are currently underway to enhance or improve antitubercular immunity afforded by the BCG vaccine. Two main domains form the current scene in TB vaccine research – subunit and live vaccines (Fig. 8). Subunit vaccines are generally aimed at boosting cellular immunity initially raised by BCG vaccination. Live vaccines, on the other hand are developed either to replace BCG itself or complement its efficacy in humans. In this regard, homologous prime-boosts involves priming and boosting immunity using the same vaccine while heterologous prime-boosts employ the use of one vaccine to prime and another to boost later in life (Kaufmann 2011).

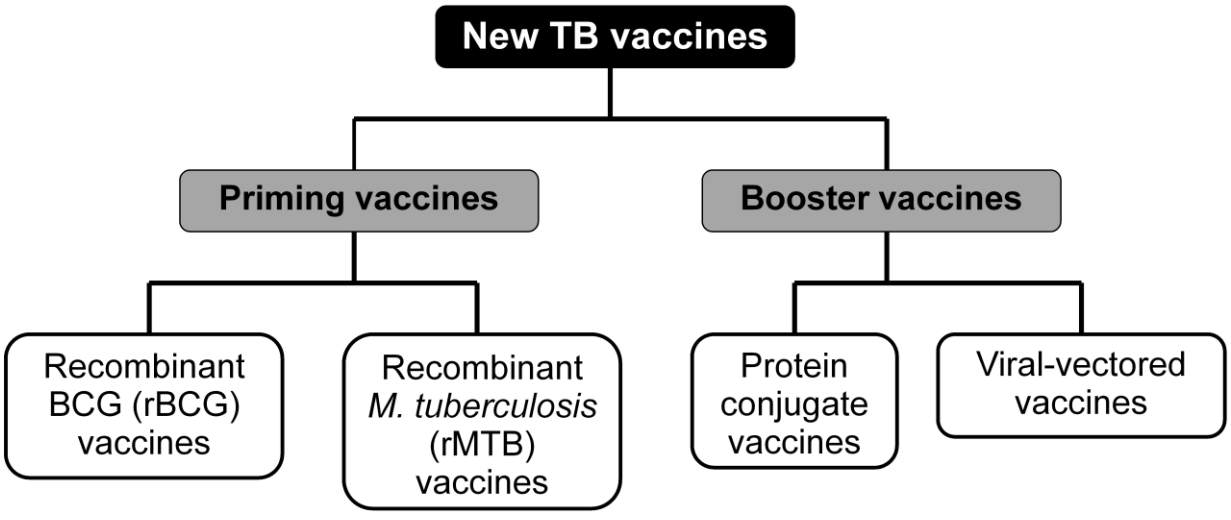

Figure 8. General overview of the current areas in TB vaccine research.

2.3.1 Protein subunit vaccine candidates.

The essence of boosting the immune response elicited by a priming vaccine is to evoke and expand the pool of antigen-specific (or at large, pathogen-specific) T cells that were initially generated and hopefully maintained in a sizeable number. In this regard, the cardinal criterion is to somehow ensure that the afore-mentioned pool of antigen-specific T cells is exposed to a

highly immunogenic TCR epitope that can afford protective immunity to *M. tuberculosis.* Therefore, providing the immune system with only the necessary antigenic molecules or moieties (protein and lipid alike) should theoretically trigger activation of the desired cellular immune response to provide longevity of protection.

In the case of TB, protein subunit vaccines are targeted at boosting immunological memory constituted by BCG-primed antigen-specific Th1 cells by contributing to their proliferation during TB. The Statens Serum Institute (SSI) in Copenhagen champions this area of vaccine research with 3 different candidates in adjuvant undergoing clinical development: H1:IC31 (fusion of Ag85B and ESAT-6) (Weinrich Olsen, van Pinxteren et al. 2001), H4:IC31® (fusion of Ag85B and TB10.4)(Dietrich, Aagaard et al. 2005) and H56:IC31 (fusion of Ag85B, ESAT-6 and Rv2660c) (Aagaard, Hoang et al. 2011). These candidates appear to show superior reduction in *M. tuberculosis* burden in the lungs of vaccinated animals albeit in concert with BCG priming.

Another subunit vaccine that is being clinically tested by GlaxoSmithkline is M72 (*M. tuberculosis* serine proteases *pepA* (*Rv0125*) and *pepD* (*Rv0983*) in a liposome formulation (AS01) (Leroux-Roels, Forgus et al. 2012).

2.3.2 Recombinant BCG (rBCG) vaccine candidates.

For over two decades now, availability of very apt tools such as integrative and episomal plasmids encoding mycobacteria-specific promoters and phage-derived integration genes has made the molecular manipulation of mycobacteria much simpler (Stover, de la Cruz et al. 1991; Bardarov, Bardarov Jr et al. 2002). Thus, not only has studying mycobacterial physiology become more feasible but also modifying the vaccine strain has been possible. One of the earliest approaches attempted was to incorporate Th1 cytokines into BCG using specific mycobacteria-optimised protein expression systems in place (Stover, de la Cruz et al. 1991; O'Donnell, Aldovini et al. 1994; Bardarov, Bardarov Jr et al. 2002). The general idea was to generate rBCG strains that were capable of actively secreting pro-inflammatory murine cytokines that could skew the host cellular immune response in a favourable, antitubercular fashion.

The earliest described rBCG strains were ones that secrete the cytokines GM-CSF, IFN-γ, IL-2 and IL-18, enhancing antigen-responsive effector splenic Th1 cells that developed in vaccinated BABL/c mice (O'Donnell, Aldovini et al. 1994; Murray, Aldovini et al. 1996; Biet, Kremer et al. 2002; Young, O'Donnell et al. 2002). To a great extent, the generally-accepted notion was that the greater the T cell response, the higher the chances of achieving effective clearance of *M.*

tuberculosis from the host. This would then directly correlate to a decrease in pathology, leading to improved lung conditions manifested in well-controlled infection but not necessarily sterilising immunity. Nonetheless, there was no follow-up evidence of protection against TB triggered by any of the above-mentioned strains published in later years.

2.3.2.1 BCG *ΔureC::hly.*

Independent early work involving the intracellular gastrointestinal bacterial pathogen *Listeria monocytogenes* pathogenicity in the United States and France showed that listeriolysin O (LLO), encoded by the *hly* gene allows lipid membrane perforation and phagosomal escape of the bacterium within the host MΦ for survival and proliferation in the cytoplasm (Gaillard, Berche et al. 1987; Portnoy, Jacks et al. 1988; Bielecki, Youngman et al. 1990). This cholesterol-dependent toxin was shown to require a pH of 5.5 (achieved inside the phagosome) upon acidification for optimal activity and subsequently, membrane disruption and release of bacterial antigens into the cytosol. Very interestingly, LLO contains a PEST (**p**roline, **g**lutamic acid, **s**erine, **t**heronine) sequence which functions as a protein degradation signal upon its localisation into the cytosol (Decatur and Portnoy 2000).

Also in the United States, Clemens and colleagues showed that the urease enzyme of *M. tuberculosis* is essential for de-acidification of the phagosome for intraphagosomal survival and proliferate in MΦs (Clemens, Lee et al. 1995). Mycobacterial urease metabolises urea to produce ammonia which in turn buffers the acidic environment within the phagosome (Gordon, Hart et al. 1980). This enzyme comprises three subunits – UreA, B and C (encoded by *ureA*, *ureB* and *ureC*, respectively) the latter constituting its catalytic site. *ureC* mutants were rendered incapable of breaking down urea, suggesting that absence of *ureC* disallows *M. tuberculosis* to neutralise the acidic phagosomal microenvironment (Reyrat, Berthet et al. 1995). As BCG also possesses urease activity and encodes all three subunits, molecular biological studies implemented in the laboratory of Zakaria Hmama proved that absence of *ureC* contributed to phagosome acidification and enhanced antigen processing in THP-1 MΦs (Sendide, Deghmane et al. 2004).

Since a BCG *ΔureC* mutant was already in place (Reyrat, Berthet et al. 1995), further modifications were made to express and secrete recombinant LLO using the mycobacteria-specific P_{hsp60}-Ag85BSS secretion apparatus (Hess, Miko et al. 1998). This expression system contains a 240 bp fragment corresponding to the promoter region of the *groEL2* (heat shock protein 60, *hsp60*) chaperone cloned upstream of 120 bp of the signal sequence of fibronectin-

binding protein B (*fbpB*), otherwise known as antigen 85 B (Ag85B). As such, the system is aimed at allowing active secretion of heterologously expressed proteins by recombinant mycobacteria. BCG Δ*ure::hly* was shown to more efficiently induce apoptosis of infected MΦs as well as enhanced MHC-I- and-II-restricted antigen presentation to T cells. With the success given by these conceptual studies, a BCG strain harbouring the mature *hly* gene fused to P_{hsp60} and Ag85BSS to disrupt the *ureC* locus was generated and termed BCG *ΔureC*::*hly* (Grode, Seiler et al. 2005). This mainly served the purpose of stable chromosomally integrating the *hly* expression cassette as opposed to plasmid-encoded. Observations conducted in BCG *ΔureC*::*hly*-infected MΦs using immunofluorescence microscopy revealed enhanced antigen load exposed to the immune system for priming of effector cells leading to better potentiation of cellular immunity, and effectively affording superior protection of BALB/c mice against TB in comparison to parental BCG. The chief characteristic of BCG *ΔureC*::*hly* in this regard is its ability to perforate the phagosome while mediating escape of mycobacterial antigens.

Significant antigen-specific CD4+ and CD8+ T cells responses are observed in experimental murine infections with *L. monocytogenes* (Mittrucker, Kohler et al. 2000; Kursar, Bonhagen et al. 2004). Particularly, CD8+ T cells responses in listeriosis have been attributed to presence of LLO (Kursar, Kohler et al. 2004). Although BCG vaccination also prompts come CD8+ T cell responses, protection against TB is often associated with CD4+ T cells ((Hanekom 2005; Mittrucker, Steinhoff et al. 2007; Soares, Kwong Chung et al. 2013)). Mycobacterial antigens that become cytosolically localised can then access the MHC-I antigen processing pathway (also referred to as the endogenous pathway), allowing epitope presentation to CD8+ T cells. Intrinsically, it is plausible that modulation of intracellular processes within MΦs and DCs is affected by BCG *ΔureC*::*hly*. Phagosomal escape and subsequent cytosolic localisation of *M. tuberculosis*, as opposed to BCG has been directly linked to the afore-mentioned Esx-1 secretion system (van der Wel, Hava et al. 2007; Simeone, Bobard et al. 2012). This pinpoints the need for membrane perforation, a property possessed by ESAT-6 which is actively secreted by the Esx-1 system together with the chaperone CFP-10 (Smith, Manoranjan et al. 2008).

Interestingly, ESAT-6 appears to inhibit autophagy of the host cell and hampers phagosome maturation (Romagnoli, Etna et al. 2012; Zhang, Zhang et al. 2012). LLO, on the contrary is capable of inducing autophagy within *L. monocytogenes* infected MΦs (Meyer-Morse, Robbins et al. 2010). Autophagy is an important homeostatically-regulated programmed cell death mechanism which degrades damaged cellular organelles for renewal and maintenance (Shintani

and Klionsky 2004). In the context of host interaction with mycobacteria, autophagy has been reported to facilitate and enhance antigen presentation in BCG-infected murine DCs, thus elevating vaccine efficacy (Jagannath, Lindsey et al. 2009), and in general influencing the cytokine milieu governing protective immunity to *M. tuberculosis* infection (Harris, Hope et al. 2009). BCG *ΔureC*::*hly* may well be capable of performing autophagy to achieve a similar feat and experiments are underway in our laboratory to address this question.

A recent study showed that BCG *ΔureC*::*hly* affords superior protection against *M. tuberculosis* infection by augmenting Th1 and Th17 responses (Desel, Dorhoi et al. 2011). Multiples cytokine analysis revealed that this vaccine strain enhances pro-inflammatory responses in mice, demonstrated by the manifold increase in IFN-γ, IL-17, IL-6 and GM-CSF levels in serum after 83 days post vaccination.

In terms of safety, the endogenous PEST-like sequence encoded within LLO does not permit the latter's survival in the cytosol but rather promotes proteasomal degradation. Hence, LLO only exerts minimal activity, if any under standard physiological conditions (pH 7.2- 7.5). BCG *ΔureC*::*hly* has shown an excellent safety profile in two Phase I studies and is currently undergoing Phase IIa clinical trials in South Africa, and generates high numbers of IFN-γ-secreting Th1 cells (Grode, Ganoza et al. 2013), (FDA trial no: NCT01479972).

2.3.2.2 rBCG30.

Antigen 85B (Ag85B, or fibronectin-binding protein B, FbpB encoded by *fbpB*) is a 30-kDa secreted protein of *M. tuberculosis* which is involved in transporting mycolic acids from the mycobacterial cytosol to its cell wall (mycolyl transferase activity). It is one of the most abundant extracellular proteins in culture filtrates of *M. tuberculosis* and belongs to a family of proteins comprising other members, namely Ag85A (*fbpA*) and Ag85C (*fbpC*). All 3 proteins are expressed by BCG with 100% identity and contain many known T cell epitopes (Reddy, Riley et al. 2009). Using this as basis, a team of researchers lead by Marcus Horwitz constructed a BCG strain harbouring stable integration of a second copy of *fbpB* to over-express this protein (Horwitz, Harth et al. 2000). Although BCG naturally synthesises and secretes Ag85B, the generation of rBCG30 was fuelled by the idea that providing more of an immunogenic antigen may raise a greater T cell response and hopefully maintain the number of antigen-specific cells in the host. Despite encouraging *in vivo* data and a promising Phase I clinical trial in the United States, further clinical development of rBCG30 is currently on hold due to undisclosed reasons (Kaufmann 2012).

2.3.2.3 Aeras-422.

Another recombinant BCG strain which was shortly in Phase I clinical trials is Aeras-422 (Sun, Skeiky et al. 2009). This vaccine candidate expresses a mutant form of perfringolysin O (PFO_{G137Q}) derived from *Clostridium perfringens* as well as *M. tuberculosis* Ag85A, Ag85B and TB10.4 – which is also encoded in genome of BCG in its native form. PFO_{G137Q} was placed under the control of Ag85B promoter and signal sequence, and introduced into BCG via homologous allelic exchange with the *ureC* gene. PFO was selected as an effector molecule although it does not possess a pH optimum to unleash its activity nor a PEST sequence and hence, can perforate lipid membranes under standard physiological conditions. This candidate is no longer in clinical development due to safety issues.

2.3.3 Recombinant *M. tuberculosis* (rMTB) vaccine candidates.

BCG has lost a good number of antigenic properties which *M. tuberculosis* still possesses, and these cannot be exposed to the host immune system with BCG vaccination. Genetic manipulation of *M. tuberculosis* to attenuate the pathogen has been a new avenue of constructing live, recombinant TB vaccines with greater potential. There is currently only one such vaccine candidate in clinical development termed MTB *ΔphoP ΔfadD26* (MTBVAC) (Cardona, Asensio et al. 2009; Kaufmann and Gengenbacher 2012). This vaccine candidate lacks activity of the crucial PhoPQ two-component transcriptional regulatory system which controls circa 4% of metabolic processes in *M. tuberculosis* as well as fadD26 (a fatty-acid Co-A ligase), which is involved in polyketide synthesis, and was developed at the University of Zaragosa (Perez, Samper et al. 2001).

The rMTB vaccine candidate currently under preclinical testing is MtbΔRD1ΔpanCD (Sambandamurthy, Derrick et al. 2006). This mutant strain of *M. tuberculosis* lacks the RD-1 locus as well as *panCD*, two genes essential for *de novo* synthesis of pantothenic acid (vitamin B5) in mycobacteria (Sambandamurthy, Wang et al. 2002).

2.3.4 Viral-vectored vaccine candidates.

The most clinically-advanced TB vaccine candidate at present is MVA85A (Aeras-485). Developed by a team headed by Helen McShane at the Jenner Institute, Oxford University, this booster vaccine candidate consists of a modified vaccine Ankara virus (MVA) which was engineered to express Ag85A, termed as MVA85A. Preclinical assessment of MVA85A displayed very encouraging *in vivo* results (experiments conducted in mice, guinea pigs and macaques) - manifest in strong antigen-specific CD4+ and CD8+ T cell responses besides

desirable safety features (McShane, Brookes et al. 2001; Williams, Hatch et al. 2005; Verreck, Vervenne et al. 2009). MVA85A enjoyed highly successful early-phase clinical studies (McShane, Pathan et al. 2004; Hawkridge, Scriba et al. 2008), although recently published data did not reveal superior protection in infants despite its impressive safety profile (Tameris, Hatherill et al. 2013).

Other viral-vectored vaccine candidates in the clinical pipeline are Ad35/Aeras-402, an adenoviral construct expressing Ag85A, Ag85B and TB10.4 (Radosevic, Wieland et al. 2007) and Ad5, another adenoviral vector expressing Ag85A, and developed by McMaster University (Wang, Thorson et al. 2004).

3. Aim of this PhD project.

3.1 Improvement of BCG ΔureC::hly by incorporation of human cytokine expression.

Despite the current success attributed to BCG *ΔureC*::*hly* in the clinic, additional factors that aid in further fortifying antimycobacterial immunity for increasing the protective efficacy this strain can offer. As already known and in use, adjuvants (especially those administered with subunit vaccines) certainly amplify the primary innate and cellular immune responses projected by the host and these immensely dictate how the ensuing immunological scaffold is being shaped. Adjuvant-like effects can be developed by elevating immunogenicity via gene deletions in BCG *ΔureC*::*hly* e.g. removing anti-apoptotic genes or alternatively, introducing new ones. The strategy employed by our laboratory is to integrate naturally-occurring adjuvants such as cytokines and immunomodulatory molecules into BCG *ΔureC*::*hly* in order to enhance the already encouraging vaccine profile of this strain. Therefore, my task through this PhD project has been to further modify this strain in order to create larger pools of antigen-specific Th1 effector memory cells that respond to antigenic stimulation long after vaccination.

3.1.1 Interleukin 7 (IL-7).

Produced mainly by stromal cells in the bone marrow, spleen and liver, IL-7 has been implicated in vaccine efficacy due to its involvement in recalling adaptive immune responses (Appasamy 1993). Along with IL-15, IL-7 can augment T cell immunologic memory in humans and both B and T cells in the mouse (Krawczenko, Kieda et al. 2005; Melchionda, Fry et al. 2005). IL-7 signalling occurs via the phosphorylation of the transcription factor signal transducer and activator of transcription 5 (STAT5) in humans as well as mice (Johnson, Shah et al. 2005). The IL-7 receptor (IL-7R or CD127) is constituted by the IL-7Rα chain and the IL-2Rγ chain, which is

also shared by IL-2 (Takada and Jameson 2009); the latter already a well-known inducer of T cell proliferation (Robb, Munck et al. 1981). Binding of IL-7 to CD127 triggers activation of Janus kinases 1 (JAK1) and 3 (JAK3) which in turn lead to the phosphorylation of STAT5. Fig. 9 schematically represents the essential intracellular events in lymphocytes triggered by IL-7 signalling. STAT5 phosphorylation leads to expression of two anti-apoptotic proteins with a role in T cell survival, namely B cell lymphoma 2 (BCL-2) and myeloid cell leukemia sequence 1 (MCL1) (Takada and Jameson 2009) It is important to note that human IL-7 is functional in the mouse (Melchionda, Fry et al. 2005).

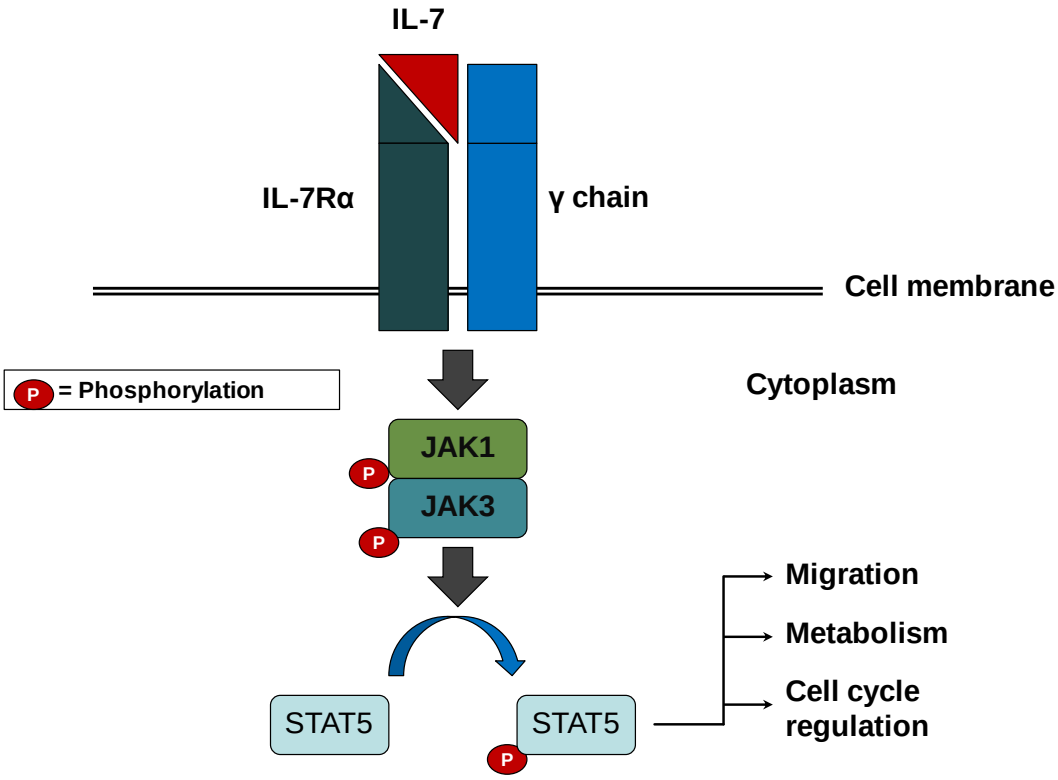

Figure 9. IL-7 signalling in B and T lymphocytes via sharing of the IL-2R gamma chain (γ chain). (Adapted from Takada and Jameson, 2009).

Experimental assessment of IL-7 in a therapeutic context has for the most part involved testing it as an adjuvant to boost protective immunity. In a mouse β-islet cell tumour model, recombinant murine IL-7 exogenously delivered following LCMV vaccination showed improved antitumour responses mediated mainly by CD8+ T cells (Pellegrini, Calzascia et al. 2009). Yet another study reports metastatic cancer patients suffering from lymphopenia who, after receiving

subcutaneously receiving recombinant human IL-7 showed an increase in antigen-specific CD4+ and CD8+ T cell pools (Rosenberg, Sportes et al. 2006). Collectively, these studies provide a decent basis for the activity of IL-7 in expanding the pool of memory T cells with desirably potent effector functions.

In the context of *M. tuberculosis* infection, intraperitoneal administration of either IL-7 or IL-15 was shown to increase the life span of *M. tuberculosis*-infected mice when treated 3 weeks post infection (Maeurer, Trinder et al. 2000). Relevant to TB vaccine research, co-administration of murine IL-7 and IL-15 with parental BCG in mice showed long-term protection against *M. tuberculosis* infection, indicating establishment of CD4+ T cell-mediated memory to *M. tuberculosis* antigens (Singh, Gowthaman et al. 2010). In TB diagnostics, IL-7-treated T cells from HIV and TB-infected patients tested in a TB ELISpot assay have been reported to produce more IFN-γ in response to antigen compared to the untreated control (Feske, Nudelman et al. 2008).

I therefore hypothesised that BCG *ΔureC*::*hly* secreting hIL-7 (rBCGIL-7) may be able to facilitate expansion of mycobacteria-specific to CD4+ and CD8+ T cells upon establishing immunological synapses with infected APCs. An adequate proportion of these cells would then acquire a memory phenotype and home to the spleen.

3.1.2 Interleukin 18 (IL-18).

IL-18, also known as interferon gamma-inducing factor (IGIF) is a non-canonically processed and secreted cytokine which belongs to the IL-1 family and requires caspase 1 activity for release of the mature protein (Ghayur, Banerjee et al. 1997). It is largely produced and secreted by a range of myeloid-derived cells, particularly MΦ and DCs (Okamura, Tsutsi et al. 1995). IL-18 is rather unique in that it does not contain an endogenous signal sequence for secretion, and is directly involved in programming of Th1 responses, along with co-priming by IL-12 (Gracie, Robertson et al. 2003).

IL-18 signals via the IL-18 receptor complex (IL-18R), composed of alpha (IL-18Rα) and beta (IL-18Rβ) chains although it is the IL-18Rα chain which effectively binds mature IL-18 on the surface of T cells (Gracie, Robertson et al. 2003). Binding of mature IL-18 to IL-18R on the surface of T cells triggers recruitment of myeloid differentiation primary response gene 88 (MyD88), an important adaptor molecule in innate immunity (Ohnishi, Tochio et al. 2012). Attachment of MyD88 with the cytoplasmic tail of IL-18R then engages the IL-1 receptor-

associated kinase (IRAK), leading to autophosphorylation of the latter. Following this, phosphorylated IRAK dissociates from the MyD88-IL-18R complex and binds to tumour necrosis factor receptor-associated factor 6 (TRAF6) in the cytoplasm, a process which allows phosphorylation of TRAF6. TRAF6 can then activate transcription factors nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) in order to ultimately program the T cell to express and secrete IFN-γ (Chung, Park et al. 2002; Gracie, Robertson et al. 2003). This process is schematically represented in Fig. 10.

Besides this, IL-18 has been shown to be directly involved in IL-8 secretion by human lung epithelial cells (Lee, Kim et al. 2004) and CD14+ monocytes (Puren, Fantuzzi et al. 1998). IL-8 plays an instrumental role in neutrophil recruitment to tissue and as mentioned earlier, these cells play an integral role in antigen transport to dLNs. It has also been shown that the human orthologue of IL-18 is functional in mice (Skopinski, Rogala et al. 2005).

Interestingly, IL-18 has been implicated in pulmonary diseases ranging from bacterial infections to asthma and sterile inflammatory pathologies of the lung which eventually lead to tissue destruction (Kawayama, Okamoto et al. 2012). Also, patients with inflammatory dermatological disease appear to produce more IL-18 which in effect leads to highly pronounced local inflammatory responses in the skin, orchestrated largely by recruited skin MΦs (Kanda, Shimizu et al. 2007). However, in the context of TB, this may instead be beneficial. C57BL/6 mice lacking IL-18 or MyD88 have been shown to be more susceptible to *M. tuberculosis* infection compared to the wild type strain and exhibit extensive polymorphonuclear cell-driven inflammation (Schneider, Korbel et al. 2010). Of particular interest is that IL-18 has been found to circulate in high quantities in sera of patients with pulmonary TB (Yamada, Shijubo et al. 2000). Thus, IL-18 seems to play an indispensable role in the generation of effector T cell pools upon antigen encounter to evoke a strong pro-inflammatory cellular immune response.

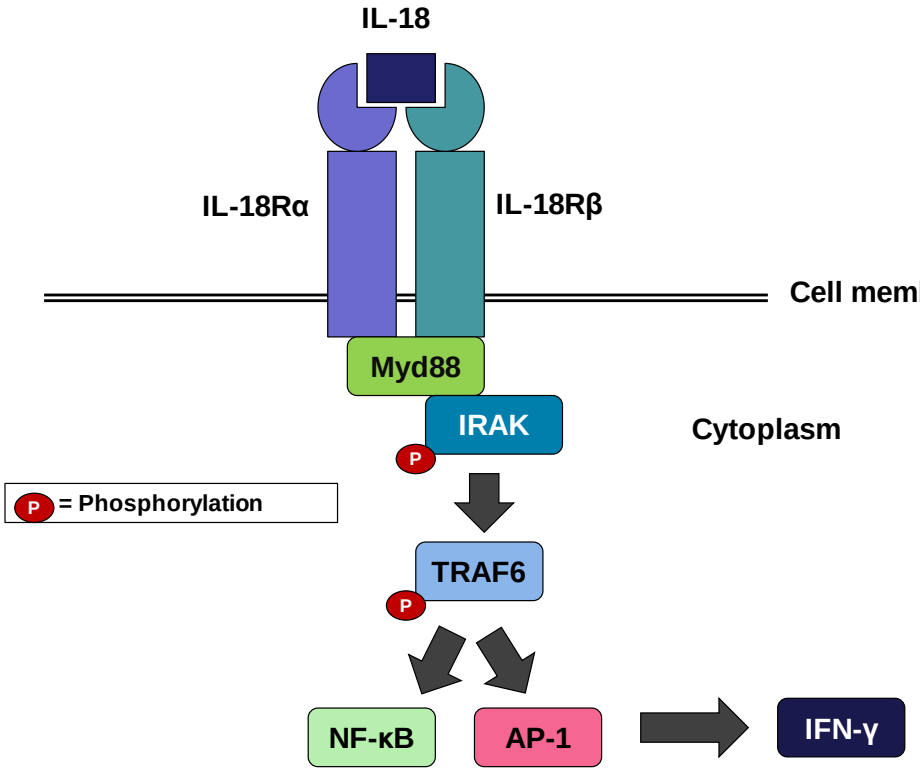

Figure 10. IL-18R-mediated IL-18 signalling. Involvement of NF-κB and well as IFN-γ secretion by T cells is shown here. (Source: http://www.genego.com/map_2747.php).

About a decade ago, recombinant BCG secreting bioactive IL-18 was shown to be capable of generating IFN-γ-secreting splenocytes in mice, indicating a favourable Th1-polarised lymphocyte response to vaccination (Biet, Kremer et al. 2002; Luo, Yamada et al. 2004). The rationale behind developing BCG *ΔureC*::*hly* secreting hIL-18 (rBCGIL-18) is to enable APCs to present a wider array of mycobacterial antigens to T cells while polarising them to assume a Th1 phenotype. In addition, mature IL-18 synthesised and secreted by BCG *ΔureC*::*hly* might potentially access the endogenous secretion pathway of host MΦs and thus, contribute to higher bioavailable IL-18 in the draining lymph nodes. Table 3 is a summary of the major biological effects exerted by IL-7 and IL-18.

Cytokine	Activity/effects	References
IL-7	Bcl-2 expression, T cell survival VDJ recombinantion in B cells Generation of CD8+ T cells, NK cells Recall responses to *M. tuberculosis* infection	Takada and Jameson, 2009 Krawczenko et al., 2005 Maurer et al., 2000; Singh et al., 2010
IL-18	IFN-γ secretion by T cells NF-κB activation NK cell activity IgE expression by B cells TNF-α, IL-6 and CCL2 production by MΦs Enhanced immune responses to *M. tuberculosis* infection	Gracie et al., 2003 Schneider et al., 2010; Sugawara et al., 1999

Table 3. General biological effects of IL-7 and IL-18.

3.1.3 Interferon regulatory factor 1 (IRF-1).

Reported as an anti-tumour / anti-viral transcription factor, expression of IRF-1 is dominantly dependent on phosphorylation of the transcription factors JAK-STAT1 triggered by IFN-γ signalling (Taniguchi, Ogasawara et al. 2001). IRF-1 is encoded among the gamma-activated sequences (GAS) and hence, found mainly in MΦs and DCs. Although IFN-γ signalling is the major contributor to IRF-1 expression, type I interferons (IFNs) such as IFN-α/β are also able to initiate this process via nuclear factor kappa-B (NF-kB) and interferon-stimulating gene factor 3 (ISGF3) and IFN-α-activated factor (AAF) (Pine, Decker et al. 1990; Decker, Lew et al. 1991). IRF-1 itself can induce expression of type I IFNs (Fujita, Kimura et al. 1989). IRF-1 is involved in expression and activation of inducible nitric oxide synthase (iNOS) which in turn leads to nitric oxide 2 (NOS2) synthesis. The expression of this molecule is critical for killing of intracellular pathogens by production of nitric oxide (NO) and furthermore to up-regulate MHC-II-restricted antigen processing and presentation to CD4+ T cells (Bogdan, Rollinghoff et al. 2000). Fig. 11 schematically represents this process.

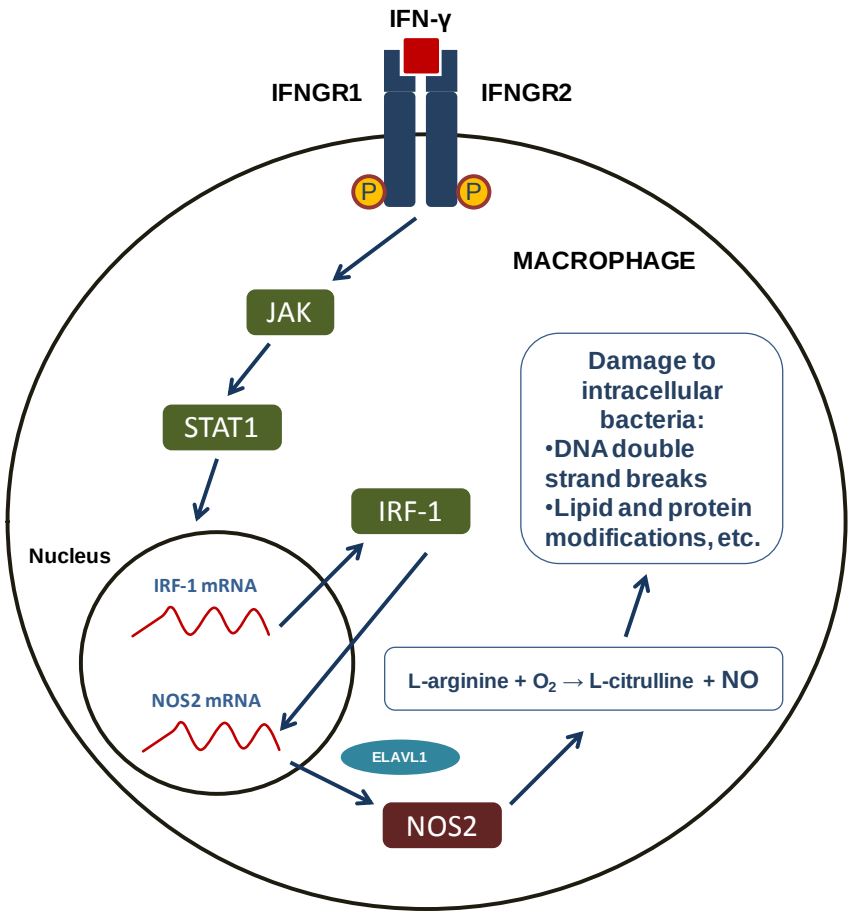

Figure 11. JAK-STAT-mediated IRF-1 expression and NOS2 production in MΦs leading to killing of intracellular bacteria.. (Source:Adapted from Lowenstein and Padalko, 2004)

IRF-1 expression has been rather closely linked to *M. tuberculosis* infection and enhanced MHC-I antigen presentation, and aberrant or reduced IRF-1 activity may have a direct effect on recruitment and activation of CD8+ T cells and hence, minimal killing of *M. tuberculosis*-infected host cells (Pine 2002). The idea behind including IRF-1 as a candidate for improving BCG *ΔureC::hly* is as follows: increasing intracellular expression of this protein would enhance proteasomal antigen processing and subsequent presentation to T cells. This may consequently drive a more robust clearance of *M. tuberculosis*-infected cells to avoid onset of clinical disease, provided the pools of antigen-specific CD4+ and CD8+ T cells are maintained throughout the vaccinee's lifetime and can be expanded upon boosting with a mycobacterial protein conjugate, for instance. However, secreted IRF-1 has first to access the host cells' cytoplasm through LLO-induced pores on the phagosomal membrane to localise into the nucleus. Therefore, the scientific rationale of this project is to investigate the effect of human cytokines on the modulation of T cell response to *M. tuberculosis* infection when heterologously expressed by BCG *ΔureC::hly*.

MATERIALS AND METHODS

Materials and methods – *in vitro*

1. Bacterial strains

The recombinant Bacille Calmette-Guerin *hly*$^{+}$ *ΔureC* Prague (BCG *ΔureC*::*hly*)(Grode, Seiler et al. 2005) was used in all molecular manipulation experiments. For infection experiments, parental BCG Prague was used as a control alongside BCG *ΔureC*::*hly*. All bacterial strains were grown and stocked in-house at the Department of Immunology, Max-Planck Institute for Infection Biology (MPIIB), Berlin. *Escherichia coli* (*E. coli*) TOP 10 chemically-competent cells were purchased from Invitrogen (USA).

2. Bacterial growth media

2.1 Mycobacterial growth media.

2.1.1 Middlebrook 7H9 complete liquid medium.

To prepare 1 litre of medium, 4.7 g of 7H9 Middlebrook broth base (Becton Dickinson, USA) is dissolved in 900 ml of distilled water by magnetic stirring. 2.0 ml of 100% glycerol is then added and residual glycerol is removed by repeatedly but gently pipetting the medium. After further stirring, 2.0 ml of sterile 25% Tween 80 is added to the medium mixture and stirred until a homogenous solution is achieved. This is then autoclaved at 121^{o}C for 10 minutes. The mixture is cooled to room temperature, following which 100 ml of sterile albumin-dextrose-catalase (ADC) supplement is added and magnetically stirred. The complete medium is then filter sterilised and incubated at 37^{o}C overnight to ascertain that the medium is contamination free. The medium can then be stored at 4^{o}C for up to one month.

2.1.2 Sauton's liquid medium.

To prepare 1 litre of medium, the following ingredients are first added to 500 ml of distilled water while magnetically stirring: 0.5 g potassium dihydrogen phosphate (KH_2PO_4), 0.5 g magnesium sulphate ($MgSO_4$), 4.0 g L-asparagine, 60 ml glycerol, 0.05 g ferric ammonium citrate ($FeC_6H_5O_7{\cdot}NH_4OH$), 2.0 g citric acid, 0.1 ml 1% (w/v) zinc sulphate ($ZnSO_4$). Volume is then topped up with distilled water to 900 ml, followed by pH adjustment to 7.0. Medium is then autoclaved and cooled to room temperature prior to adding 2.0 ml of 25% Tween 80.

2.1.3 Middlebrook 7H11 solid medium.

To prepare 1 litre of medium, 21.0 g of 7H11 Middlebrook agar base (Becton Dickinson, USA) is dissolved in 900 ml of distilled water by magnetic stirring. Once the base has dissolved, the

mixture is autoclaved at 121^{o}C for 10 minutes. Following this, the autoclaved mixture is cooled to 55^{o}C in a pre-warmed water bath. Upon cooling, 100 ml of sterile oleic acid-albumin-dextrose/glucose-saline (OADC) supplement and 4.0 ml of sterile 50% glycerol are sequentially added and magnetically stirred for about 5 minutes. Plates can then be prepared in a Class II bio safety cabinet, each containing 24 ml of molten agar. Solidified agar plates can be stored at 4^{o}C up to 1 month.

2.2 *Escherichia coli* growth media.

2.2.1 Luria-Bertani (LB) liquid medium (lysogeny broth).

To prepare 1 litre of medium, 25.0 g of LB broth base (Becton Dickinson, USA) is dissolved in 500 ml of distilled water and magnetically stirred. Following this, the volume is topped up to 1 litre with distilled water and the medium is autoclaved at 121^{o}C for 15 minutes.

2.2.2 Luria-Bertani (LB) solid medium.

To prepare 1 litre of medium, 40.0 g of LB agar base (Becton Dickinson, USA) is dissolved in 500 ml of distilled water and magnetically stirred until all traces of powder are gone. Following this, the medium is autoclaved at 121^{o}C for 15 minutes. 15 ml of molten agar is poured into each plate. Plates are allowed to cool at room temperature, after which they can be stored at 4^{o}C up to 1 month.

3. Bacterial growth conditions.

3.1 Mycobacterial growth conditions.

Glycerol seed stock of mycobacteria (adjusted to OD_{600} 1.0) is inoculated into Middlebrook 7H9 complete medium in a 1 litre plastic roller bottle (Corning Inc., USA) at 37^{o}C under slow rolling. The working OD_{600} at the time of inoculation is hypothetically 0.02, but can vary slightly (± 0.005). Experimental work usually requires bacteria to be grown to mid-log phase OD_{600} (between 0.6 and 0.8) unless otherwise stated.

3.2 Mycobacterial growth condition after electroporation.

Competent cells for transformation are prepared by harvesting mid-log phase cells (OD_{600} 0.60 – 0.80) via centrifugation at 4000 rpm for 20 minutes. After discarding the supernatant, the cell pellet is re-suspended in an equal volume of sterile TG buffer (0.05% Tween 80 + 10% glycerol in distilled water) with a sterile serological pipette. This is repeated twice. Following the third and final wash, the cell pellet is re-suspended in 1 ml of TG buffer and 200 µl of this concentrated

suspension is pipetted into a 0.2 cm electrocuvette (Bio-Rad, USA) for transformation. 0.5 – 1 µg of plasmid DNA is required for transforming 200 µl of concentrated culture in a Gene Pulse Xcell electroporator (Bio-Rad, USA) under these conditions: 2.5 kV voltage, 25 µF capacitance and 1MΩ resistance. Transformed cells are recovered overnight at 37^{o}C in 10 ml of fresh 7H9 complete medium with rotation at 120 rpm. Recovered cells are then transferred to a 50 ml Falcon tube and centrifuged at 4000 rpm for 5 minutes. The resulting supernatant is discarded, and the cell pellet re-suspended in 1ml of fresh 7H9 complete medium. 200 µl of the concentrated suspension is then evenly plated onto 7H11 agar plate containing 20 µg/ml of kanamycin, sealed with Parafilm and wrapped in aluminium foil to be incubated in a 37^{o}C microbiological incubator for 2 – 3 weeks.

3.3 Selection of legitimate mycobacterial clones following transformation.

Legitimate mycobacterial clones were selected by a multistep process:

a) Six mycobacterial colonies from each transformation experiment are inoculated into 10 ml of 7H9 complete medium containing 25 µg/ml of kanamycin and grown in a rotary incubator at 37^{o}C and 120 rpm for 5 – 6 days, until high turbidity is reached.

b) A 200 µl aliquot of the culture is aseptically transferred to a fresh 1.5 ml microcentrifuge tube, and centrifuged at 4000 rpm for 5 minutes at room temperature.

c) The supernatant is removed by pipetting, and the cell pellet dislodged in 50 µl of distilled water and incubated at 95^{o}C for 5 minutes.

d) The culture is once again centrifuged at 4000 rpm for 5 minutes at room temperature. The resulting supernatant (which contains genomic DNA) is then transferred to a fresh 1. 5 ml microcentrifuge tube. 2 – 5 µl of this supernatant is used for colony PCR reactions with primers specific for either hIL-7 or hIL-18. Clones that give a positive signal are noted as legitimate clones.

e) Cultures of three of the legitimate clones are then transferred to a sterile 50 ml Falcon tube and centrifuged at 4000 rpm for 5 minutes at room temperature.

f) Upon removing the supernatant, the cell pellet is resuspended in 10 ml of sterile PBS + 0.05% Tween 80 buffer and centrifuged again under the same conditions.

g) The cell pellet is then resuspended in 20 ml of sterile Sauton’s medium and transferred to a sterile 100 ml Erlen-Meyer flask, and incubated in a rotary incubator at 37^{o}C and 120 rpm for 2 – 3 days.

h) Once high turbidity is reached, the culture is equally separated into two sterile 50 ml Falcon tubes and centrifuged at 4000 rpm for 5 minutes at 4^{o}C.

i) The resulting supernatant is then syringe filtered using an Omnifix 50 ml plastic syringe (Braun, Germany) and Millex GP 0.22 µm filter unit (Millipore, USA) into a fresh 50 ml Falcon tube and stored on ice. An Amicon Ultra-15 centrifugal filter unit (Millipore, USA) is used according to manufacturer's instructions for concentrating the supernatant to a 100-fold (e.g. 20 ml of supernatant is concentrated down to 200 µl).

j) On the other hand, cell pellet in the two separate Falcon tubes are used for the following purposes:

- RNA extraction – 1 ml of TRIzol is added to the pellet, thoroughly resuspended and stored at -80^{o}C.
- Whole cell lysate preparation – Cell pellet is washed once with 10 ml PBS. Upon decanting the supernatant, 500 ul of extraction buffer (50 mM Tris-HCl at pH 7.1 in distilled water) and 2 ul of protease inhibitor cocktail solution (Roche, Germany) are added to the cell pellet and thoroughly resuspended. The cells are then transferred to LyseTube B (MP Biomedicals, USA), placed in a BIO101/Savant FastPrep FP120 cell disrupter (MP BIomedicals, USA) and disrupted for 40 seconds at speed 6.0.
- The liquid phase is then carefully removed and applied onto a Spin-X column (Costar, USA) and spun at 13000 rpm for 15 minutes at 4^{o}C. The flow-through is transferred to a fresh 1.5 ml microcentrifuge tube and stored at -20^{o}C.

3.4 *E. coli* growth conditions.

For all experiments involving *E. coli*, a 1:100 inoculation of glycerol seed stock is made in LB liquid medium and grown to the required turbidity unless otherwise stated.

3.5 *E. coli* growth conditions after chemical transformation ('heat-shock treatment').

The following protocol is used for transformation and growth of *E. coli* TOP 10 cells:

- 30 minutes on ice
- 30 seconds in a 42^{o}C water bath
- 2 minutes on ice
- Addition of 250 µl of S.O.C medium and incubation in a 37^{o}C heating block for 1 hour with vigorous shaking at 950rpm for post-transformation recovery of cells.
- Two volumes i.e. 40 µl and 120 µl plated on LBA + 50 µg ampicillin + 40 µg/ml X-gal and incubated in a 37^{o}C microbiological incubator overnight.

4. Molecular biology techniques.

4.1 Polymerase chain reaction (PCR).

Two types of PCR reactions were carried based on the application:

- Cloning PCR reactions: for amplification of DNA fragments to clone into plasmid vectors.
- Confirmatory PCR reactions: for confirming the legitimate insertion of cloned DNA fragments into plasmid vectors.

The conditions used for ALL PCR reactions are listed in Table 1:

Step	Temperature (°C)	Time
Pre-heating	95	3 min
Denaturing	95	20 sec
Annealing	variable	30 sec
Extension	72	60 sec
Final extension	72	5 min
Cooling	4	∞

Table 1. Conditions for PCR reactions performed in this project.

Annealing temperatures used in this project are listed in Table 2:

PCR reaction	Length (bp)	Annealing temperature
hIL-7 (1)	477	52.0
hIL-7 (2)		54.7
hIL-7 (3)		57.4
hIL-7 (4)		58.8
hIL-18 (1)	472	54.7
hIL-18 (2)		57.4
hIL-18 (3)		58.8
hIL-18 (4)		62.0
hIRF-1 (1)	1000	56.2
hIRF-1 (2)		58.2
hIRF-1 (3)		60.2
hIRF-1 (4)		62.2
Secretion apparatus (1)	431	56.1
Secretion apparatus (2)		58.4
Secretion apparatus (3)		60.3
Secretion apparatus (4)		61.8

Table 2. Annealing temperatures used in specific PCR reactions performed in this project.

The recipe used for all cloning PCR reactions are listed in Table 3:

Ingredient	Volume (µl)	Final concentration
Distilled water	30.0	N/A
10x PCR buffer	5.0	1x
25 mM MgCl2	1.0	0.5 mM
10 µM dNTPs	1.0	0.2 µM
100 pmol forward primer	0.5	1pmol
100 pmol reverse primer	0.5	1pmol
5x Q-solution	10.0	1x
Template DNA	1.0	1 - 5 ng/µl
Taq polymerase (5 units/µl)	1.0	0.5 units/µl
TOTAL	**50.0**	

Table 3. Recipe for all cloning PCR reactions performed in this project.

The recipe used for all confirmatory PCRs are listed in Table 4:

Ingredient	Volume (µl)	Final concentration
Distilled water	10.1	N/A
10x PCR buffer	2.0	1x
25 mM MgCl2	1.0	1.25 mM
10 µM dNTPs	1.0	0.5 µM
100 pmol forward primer	0.2	1pmol
100 pmol reverse primer	0.2	1pmol
5x Q-solution	4.0	1x
Template DNA	1.0	1 - 5 ng/µl
Taq polymerase (5 units/µl)	0.5	0.8 units/µl
TOTAL	**20.0**	

Table 4. Recipe for all analytical restriction enzyme digestions performed in this project.

All primers used in this project were synthesised by and purchased from Eurofins MWG Operon, Germany. Primers were designed in-house using either Clone Manager 9 (Sci-Ed Central) or Vector NTI (Invitrogen). The primers used in this project are listed in Appendix 1.

4.2 Restriction enzyme (RE) digestion.

Restriction enzymes were purchased from New England BioLabs Inc. (USA). RE digestions were performed for 1- 2 hours in water baths set at 37^{o}C. RE digestions were carried out for one of two purposes:

- Cloning RE digestion: restriction and ligation of clones DNA fragments
- Analytical RE digestion: confirmation of cloned DNA fragments in vector

All restricted products were electrophoretically analysed on 1.3% agarose gel. The recipe used for all cloning RE digestions is listed in Table 5:

Ingredient	Volume	Final concentration
Distilled water	16.0	N/A
10x reaction buffer	2.0	1x
Restriction enzyme	1.0	Enzyme-dependent
DNA	1.0	1 - 5 ng/µl
TOTAL	**20.0**	

Table 5. Recipe for all cloning restriction enzyme digestions performed in this project.

The typical recipe used for all analytical restriction enzyme digestions is listed in Table 6:

Ingredient	Volume	Final concentration
Distilled water	30.0	N/A
10x reaction buffer	5.0	1x
Restriction enzyme	5.0	Enzyme-dependent
DNA	10.0	10 - 50 ng/µl
TOTAL	**50.0**	

Table 6. Recipe for all analytical restriction enzyme digestions performed in this project.

When double digestions were carried out, the final volume of distilled water was the only parameter modified. The volume of restriction enzyme used varied according to the stock concentrations provided by the manufacturer. Some enzymes were used in excess volume due to their mediocre activity in comparison with others.

4.3 Agarose gel electrophoresis.

All PCR, RE-digested and ligated products were electrophoretically analysed on 1.3% agarose gels. Typically, 5 - 10 ul of sample is added to 1 – 2 ul of 5x loading buffer and loaded onto the gel. GeneRuler 1kb DNA Ladder (Fermentas, Lithuania) was used as marker in all cases.

4.4 DNA ligation.

DNA ligations (unless otherwise mentioned) were performed using the Rapid DNA Ligation Kit (Roche, Mannheim; cat #: 11635379001) and used according to the manufacturer's instruction. Briefly, RE-digested insert and vector DNA are added to a 200 µl-reaction tube to a ratio of 1:3 and topped up with distilled water to 10 µl. This is followed by the addition of 10 µl of ligation buffer and finally, 1 µl of Rapid T4 DNA ligase and incubated at room temperature for 5 - 7 minutes. 2 µl of this reaction is then used for transforming *E. coli* TOP 10 chemically-competent cells.

4.5 Minipreps and maxipreps.

The QIAPrep Spin Miniprep Kit (QIAGEN, cat #: 27104) was used for all experiments involving isolation, purification and RE analysis of plasmids after propagation in *E. coli*. The Nucleospin Maxiprep Kit (Machery-Nagel, cat #: AX 500) was used for all experiments involving isolation and purification of plasmids after propagation in *E. coli* for transformation of mycobacteria.

4.6 Cloning of the secretion apparatus from pBMG11.

A 490-bp DNA fragment comprising mycobacterial heat shock protein 60 promoter (Phsp60), Ag85B 45-bp upstream region and signal sequence was amplified from pBMG11 using primers SecApp.fwd and RPSecAppV2 (Appendix 1).

4.7 Cloning of human IL-7 (hIL-7) cDNA.

455-bp mature hIL-7 cDNA was amplified via PCR using primers FPIL7.fwd and RPIL7.rev (Appendix 1) from a pCMV-SPORT6 construct harbouring a cDNA insert of 1.5 kb and spanning the entire hIL-7 ORF including its native signal sequence (ATCC-10436529) from the American Type Culture Collection (ATCC, USA).

4.8 Cloning of human hIL-18 (hIL-18) cDNA.

450-bp mature hIL-18 cDNA was amplified via PCR using primers FPIL18.fwd and RPIL18.rev (Appendix 1) from pEN::hIL-18 (F. Biet, INRA). This constructs harbours the mature hIL-18 ORF downstream of the mycobacterial heat shock protein 60 promoter (P_{hsp60}) and fused to the mycobacterial antigen 85 B signal sequence (Ag85BSS).

4.9 Cloning of codon-optimised human IRF-1 cDNA.

978-bp codon-optimised hIRF-1 cDNA was amplified via PCR using primers FPIRF1.fwd and RPIRF1.rev (Appendix 1) from pMK-RQ-hIRF1 (Life Technologies, USA). This construct harbours synthetically assembled hIRF1 based on the codon usage of *M. bovis* BCG as well as a kanamycin resistance gene. Codon-optimisation was selected for this gene due its size as well as failed previous attempts to produce the full-length recombinant protein in mycobacteria.

4.10 Codon optimisation of hIRF-1 cDNA.

Native hIRF-1 sequence of 978 bp was submitted to the following software for codon-optimisation: http://www.jcat.de/Start.jsp and cross-checked with http://bips.u-strasbg.fr/EMBOSS/.

4.11 Use of pCR TOPO 2.1 as a cloning vector.

TOPO TA Cloning Kit was purchased from Invitrogen (USA) and used according to the manufacturer's instruction. Briefly, 0.5 to 4.0 µl of PCR product is added to a 200- µl reaction tube followed by 1 µl of salt solution. The volume of the reaction is topped up to 5 µl with distilled water if necessary. Finally, 1 µl TOPO vector mix (pCR TOPO 2.1 cloning vector + Topoisomerase I) is added to the reaction mix and incubated at room temperature for 5 minutes. At the end of the incubation period, 2 µl of this reaction is used for transforming 50 µl of *E. coli* TOP 10 chemically-competent cells. The plasmid map of pCR TOPO 2.1 is attached in Appendix 2.

4.12 Use of pBMG11 as an intermediate cloning vector.

pBMG11 is a construct harbouring the listeriolysin O (LLO) ORF (encoded by *hly*) downstream of the mycobacterial hsp60 promoter and fused to Ag85BSS. The entire *hly* gene was substituted with either mature hIL-7 or hIL-18 cDNA flanked by *Nsi*I (5`) and *Kpn*I (3`) in order to generate full-length expression cassettes for the respective cytokines.

4.13 Use of pMV306 as destination vector.

The final expression cassette encompassing the following components: P_{hsp60}-Ag85BSS-hIL-7 OR hIL-18 was inserted into the *E. coli*-mycobacterial shuttle vector pMV306 (available at the MPIIB, Berlin) after RE digestion using the Rapid DNA ligation kit. The plasmid map of pMV306 is attached in Appendix…

4.14 Protein concentration measurements.

Protein concentrations of BCG whole cell lysates or culture supernatants were measured using the standard Bradford method which employs the Bradford reagent (Thermo Pierce, USA). Briefly, 5 – 10 µl of sample was added to a 96-well microtitre plate followed by 100 µl of Bradford reagent. The plate is then placed in the dark for approximately 5 minutes before reading absorbance at 595 nm in a SpectraMax 100 Luminometer using the SoftMax V5 software (Molecular Devices, USA).

4.15 Western blotting.

The Invitrogen NuPAGE Novex gel system was used in all western blot experiments. Briefly, samples to be analysed and 5 µl of PageRuler Pre-stained protein ladder (Fermentas) were mixed with 5 µl of 4x LDS sample buffer, 2 µl of 10x NuPAGE reducing agent (where necessary) and distilled water to a final volume of 20 µl and boiled at 70^{o}C for 10 minutes in a heating block (Eppendorf, Germany). After this, the boiled samples are quickly centrifuged in a microcentrifuge (Heraeus Microfuge) and then loaded onto a NuPAGE 10-well 10% Bis-Tris SDS-PAGE. Gels were typically electrophoresed at 200V for 35 minutes in an electrophoresis chamber (Bio-Rad, USA) containing running buffer (1x MES SDS buffer). Upon completion of electrophoresis, the gel is removed from its cassette and carefully placed on a nitrocellulose membrane (Bio-Rad, USA) already laid onto a blotting paper, both pre-soaked in transfer buffer (0.3% Tris + 1.4% Glycine + 0.1% SDS + 20% methanol in distilled water). Another piece of pre-soaked blotting paper in then placed on the gel and the components evenly pressed together by rolling a serological pipette over the surface. Transfers are performed in a Hoefer Semi Phor semi-dry transfer unit (Amersham Pharmacia, USA) at 55 mA per membrane for 2 hours. At the end of the transfer, the membrane is then removed and placed in a pipette tip box lid for blocking with 1x fluorescent western blotting blocking buffer (Rockland) for 90 minutes at 4^{o}C. Following this, the primary antibody diluted in the same blocking buffer, and the membrane is covered with an aluminium foil for overnight incubation at 4^{o}C in the dark under slow rocking. At the end of the incubation period, the membrane is washed 3 times with PBST buffer (0.05% Tween 20 in 1x PBS) with 10 minute-intervals on a slow rocking machine at room temperature. After the final wash, the second antibody already diluted in 1% blocking buffer is poured onto the membrane, covered with an aluminium foil and incubated on a slow rocking machine for 1 hour at room temperature. This is succeeded by another washing step same as the previous one. Upon completion, the membrane is read on an Odyssey Li-Cor Image Scanner (Li-Cor, USA) using the

Li-Cor imaging software. The fluorescence intensity settings for scanning the membrane are manipulated as necessary.

4.16 Preparation of mycobacterial total messenger RNA (mRNA).

Cell pellets are resuspended in 1 ml TRIzol (Invitrogen, USA) and stored overnight at -80^{o}C. Upon thawing, the suspension is transferred to Lyse Tube B (MP Biomedicals, USA) and disrupted in a FastPrep FP120 bead-beater (MP Biomedicals, USA) for 40 seconds at speed 6.0. After this, the disrupted suspension is transferred to a fresh 1.5 ml microcentrifuge tube and 200 µl of chloroform is added. The tube is vigorously shaken by hand following which it is left to stand at room temperature for 2 -3 minutes. The tube is then centrifuged at 10000 rpm for 15 minutes at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIzol Reagent used for homogenisation. The resultant aqueous phase is transferred to a gDNA Eliminator column (Qiagen, USA), centrifuged for 30 seconds at 10000 rpm at room temperature. The flow-through is immediately transferred to an RNase-free 15 ml Falcon tube on ice, and an equal volume of room temperature RNAse-free 70% ethanol at room temperature is added. The mixture is homogenously pipetted and transferred immediately to a mini spin column (Qiagen, USA), and centrifuged at 10000 rpm for 15 seconds at room temperature. A maximum of 700 µl is applied at one time and if the sample volume exceeds 700 µL, successive aliquots are centrifuged in the same RNeasy spin column. The flow-through is discarded after each centrifugation. The column must be re-centrifuged should there be liquid remaining on the surface of the filter. Next, 700 µl of buffer RW1 (Qiagen, USA) is added to the RNeasy spin column and centrifuged at 10000 rpm for 15 seconds to wash the spin column membrane. Upon discarding the flow-through, 500 µl of buffer RPE containing 70% ethanol (Qiagen, USA) is pipetted into the mini column, and centrifuged at 10000 rpm for 15 seconds to wash. The flow-through is discarded and collection tube reused. Another 500 µl of RPE buffer is pipette into the RNeasy spin column and centrifuged at 13000 rpm for 2 minutes at room temperature to dry the RNeasy membrane. The flow-through is discarded, and the spin column is centrifuged at 13000 rpm for 1 minute at room temperature to eliminate any chance of possible RPE buffer carryover. The spin column is then transferred to a fresh RNase-free 1.5 ml microcentrifuge tube and 30 µl of RNase / DNase free water is added directly to the spin column membrane. The spin column is then centrifuged at 10000 rpm for 1 minute at room temperature. This step is repeated once to

make a 60 µl collection volume. After the final centrifugation, the filter is discarded and the mRNA in suspension stored at -80°C.

5. In vitro cell culture and infection techniques.

5.1 RPMI complete (cRPMI) medium preparation.

The following ingredients are added to 500 ml of sterile RPMI medium 1640 (Cat #: 31870-025; Gibco, USA):

- 5 ml of 200 mM L-glutamine (Cat #: M11-004; PAA, USA)
- 5 ml of 1M HEPES (Cat #: S11-001; PAA, USA)
- 500 µl of 50 mM β-mercaptoethanol (Cat #: 31350-010; PAA, USA)
- *Optional*: 5 ml of 10000 U/ml penicillin- 10000 µg/ml streptomycin solution (Cat #: P11-010; PAA, USA)
- 50 ml of heat-inactivated fetal bovine serum (Cat #: 10270-106; Gibco, USA)

Upon thorough shaking, the medium is filtered through a 0.22 µm filter unit (Cat #: SCGPS05RE; Millipore, USA) and stored at 4^{o}C.

5.2 Growing THP-1 monocytes.

One seed stock vial containing 5 x 10^6 THP-1 cells is thawed, transferred to a sterile 50 ml Falcon tube and resuspended in 5 ml of sterile cRPMI. The cells are washed once via centrifugation at 1500 rpm for 5 minutes at room temperature. The resulting supernatant is carefully aspirated using a sterile Pasteur pipette attached to a motor pump, leaving no more than 50 – 100 ul of residual volume in the tube. The pellet is then resuspended in 20 ml of sterile cRPMI, transferred to a T_{75} culture flask using a serological pipette and incubated at 37^{o}C with 5% CO_2 for humidity. Culture is growth is monitored daily and upon reaching a density of 1x 10^6 cells per ml, the cells are passaged in sterile cRPMI at a ratio of 1:2.

5.3 Differentiation of THP-1 monocytes into MΦs for infection.

Cells are transferred to a sterile 50 ml Falcon tube and centrifuged at 1500 rpm for 5 minutes at room temperature. Upon aspirating the supernatant, the cell pellet is gently resuspended in a maximum volume of 10 ml of cRPMI. 20 µl of the resuspended culture is then mixed with 180 µl of 4% Trypan Blue solution and 10 µl of this diluted culture is then applied onto a

haemocytometer for microscopic counting, usually at 20x magnification. Based on the cell count, a dilution of the cells is first made in a sterile 50 ml Falcon tube using sterile cRPMI and then seeded in a 96-well plate at the required density per well (max: 1 x 10^5 cells) in a maximum volume of 100 µl per well. Following this, 100 µl of 100 ng/ml phorbol 12-myristate 13-acetate (PMA) in cRPMI is added to each well to achieve a final concentration of 50 ng/ml and incubated overnight at 37^{o}C with 5% CO_2 to allow adherence of the cells to the surface of the well upon differentiating into MΦs. Following the incubation period, the cells are washed twice with sterile cRPMI and left to further incubate in 200 µl of the same medium until they ready to be infected. NOTE: Cells can survive up to 6 days post-differentiation.

5.4 Infection of THP-1 monocytes with bacteria.

PMA-differentiated THP-1 MΦs in a 96-well plate are washed once with sterile 1x PBS at 1500 rpm for 5 minutes following which the supernatant is aspirated using a sterile Pasteur pipette. Following this, 200 µl of bacteria at the required density (usually 1 x 10^6 CFUs / ml for MOI 10) is added to each well and centrifuged at 1500 rpm for 5 minutes to increase contact between the MΦs and bacteria. Following this, the plate is incubated at 37^{o}C with 5% CO_2 for 4 hours. At the end of the incubation period, the plated is washed twice with sterile 1x PBS and aspirating the supernatant between washes. After the final wash, 200 µl of fresh cRPMI is added to each well and the plate re-incubated at 37^{o}C with 5% CO_2. The infection for each condition is implemented in duplicates.

5.5 Isolation of peripheral blood mononuclear cells (PBMCs).

50 ml of venous blood is drawn from healthy human donors with EDTA as anti-coagulant, and diluted 1:1 with 1x PBS. 35 ml of this diluted blood is very carefully layered onto 15 ml of Ficoll solution (Biochrom AG) in a sterile 50 ml Falcon tube and centrifuged at 1800rpm for 25 minutes at 15^{o}C without brake. All diluted blood is used in order to maximise the number of PBMCs to isolate. The resulting buffy layer, largely comprising PBMCs is then gently transferred into a fresh 50 ml Falcon tube using a sterile Pasteur pipette, and the volume topped up to 50 ml with sterile 1x PBS. The cell suspension is centrifuged at 1500 rpm for 10 minutes with brake. After aspirating the resulting supernatant, the cell pellet is thoroughly washed twice with 50 ml of sterile 1x PBS and centrifugation at 1500 rpm for 10 minutes. A final centrifugation at 1200 rpm for 10 minutes is done to remove platelets and cellular debris. The resulting supernatant is then aspirated and the pellet resuspended in 10 ml of sterile 1x PBS.

20 µl of this PBMC suspension is diluted with 180 µl of 0.4% Trypan blue for haemocytometry. The suspension is then centrifuged at 1500 rpm for 5 minutes at 15^{o}C, after which the supernatant is aspirated and cell pellet resuspended in 5 ml of sterile MACS buffer for cell separation on an LS MACS magnetic column. This was carried out using the CD14^{+} positive selection kit from Miltenyi Biotec (Cat # 130-050-201) according to the manufacturer's instructions.

5.6 Differentiation of primary human CD14^{+} monocytes into macrophages (hMoMΦs) and dendritic cells (hMoDCs).

CD14^{+} primary human monocytes were purified as mentioned in subsection 5.1.1 of this chapter. Cell pellets of purified CD14^{+} human monocytes were then resuspended in 20 ml of sterile cRPMI and 20 µl of the suspension is used for counting. After this, the suspension is divided into two parts of equal volume (10 ml each) and transferred to sterile T75 cell culture flasks. Monocytes in one flask are differentiated into MoDCs as described in subsection 5.1.1 of this chapter. In the second flask, MoMΦs are generated using 50 ng/ml of hGM-CSF and 25 ng/ml of human interleukin 4 (hIL-4) on the day of purification. Upon addition of appropriate cytokines, the cultures are incubated at 37^{o}C with 5% CO_2 for 6 days.

5.7 Isolation of human T cells.

The previously-described procedure for PBMC isolation is followed, and T cells are purified via magnetic separation on an LS column using the pan T cell isolation kit (Cat # 130-091-156) according to the manufacturer's instruction.

5.8 Preparation of hMoMΦs and hMoDCs for infection with mycobacteria.

After 6 days of differentiation, hMoMΦs and hMoDCs are harvested by first transferring the cultures into sterile 50 ml Falcon tubes. Cells that are adhering to the bottom inner surface of the culture flask are carefully scraped using a 25 cm cell scraper (Sarstedt, USA), and washed with 20 ml of sterile PBS buffer containing 5 mM EDTA. The washings are transferred to the 50 ml Falcon tubes (to make a final volume of 30 ml per tube) and centrifuged at 1500 rpm for 5 minutes at room temperature. Upon discarding the resulting supernatant, the cell pellet is resuspended in fresh cRPMI and 20 µl of this suspension is mixed with 180 µl of 4% Trypan Blue for counting. The cell number is then adjusted to 1 x 10^{6} cells per 200 µl of culture (equalling 5 x 10^{6} cells / ml) and added to a 48-well plate, and stored at 37^{o}C with 5% CO2 until they are ready to be infected with mycobacteria.

5.9 Preparation of mycobacteria for infection.

Mycobacterial cultures are grown to mid-log phase (OD_{600} 0.6 – 0.8) starting with the seed stock in 16% glycerol followed by a maximum of 6 passages. 5 ml of passaged culture is transferred to a sterile 50 ml Falcon tube and centrifuged at 4000 rpm for 5 minutes at room temperature. The resulting supernatant is decanted while the cell pellet in washed in 5 ml of sterile 1x PBS under the afore-mentioned centrifugation conditions. Upon decanting the supernatant, the cell pellet is evenly dislodged in sterile 1x PBS using a P1000 pipette. The culture is then passed through a 23G needle (Braun, Germany) attached to a 2 ml plastic syringe (BD Diagnostics, Germany) into a sterile 1.5 ml capped microcentrifuge tube to prepare a single cell suspension. This process is repeated twice. 50 µl of the resulting suspension is then added to 950 µl of 1x PBS in a cuvette and mixed thoroughly to measure cell density at OD_{600}. The numbers exhibited on the spectrophotometer are multiplied by a factor of 20 to get the actual cell density after which the following formula is used to calculate the number of bacterial cells required for the infection:

OD_{600} value x [5 x 10^8] = bacterial CFUs/ml (A); where 5 x 10^8 is the number of CFUs/ml at OD_{600} 1.0

An infection stock of 5 x 10^6 CFUs / ml is prepared in sterile cRPMI in order to have 1 x 10^6 CFUs per 200 ul which be used for the infections. The usually used multiplicity of infection (MOI) is 10 – 10 bacteria infecting 1 cell.

5.10 Co-culturing autologous T cells with infected hMoDCs.

Purified T cells were co-incubated with infected hMoDCs at a ratio of 1:20 (20 T cells per infected DC) over a 24-hour period at 37^{o}C with 5% CO_2. Following this, the cells are centrifuged at 1500 rpm for 5 minutes at room temperature and cell-free supernatants are prepared by eliminating cellular debris using Costar Spin-X columns (Corning, USA; Cat #: CLS8163-100EA). The flow-through is then stored at -20^{o}C for ELISA experiments.

5.11 Processing of infected THP-1 MΦs.

5.11.1 Collection of culture supernatants.

At designated time points, the culture supernatant from each well with MΦs infected with bacteria is removed and passed through a Costar Spin-X column at 1500 rpm for 5 minutes to sieve out cellular debris. The flow-through is stored at -20^{o}C for cytokine analysis.

5.11.2 Plating of intracellular bacteria.

The infected MΦs are washed once with 1x PBS at 1500 rpm for 5 minutes after supernatant collection. Upon aspirating the washing, 100 µl of sterile lysis buffer (1% Triton X-100 in 1x PBS, pH 7.0) is added to each well and incubated at room temperature for 5 -10 minutes. After this, the lysates are resuspended thoroughly and 10-fold serial dilutions are made in a sterile 48-well flat bottom microtitre plate (Nunc, Denmark) by adding 100 µl of the lysate to 900 µl of sterile TG buffer. 100 µl of each dilution is plated on fresh 7H11 plate and sealed with parafilm, and wrapped with aluminium foil for incubation at 37^{o}C for 3 – 4 weeks.

5.12 Haemolysis assay.

In this assay, the first requirement is preparation of human red blood cells (RBC) freshly isolated from human venous blood. The RBC fraction is washed 3 times in sterile 5 mM PBS-EDTA buffer by centrifugation at 1500 rpm for 10 minutes subsequent aspiration of the resulting supernatant. Following the final wash, a 1:100 dilution of the RBC fraction is prepared in the same buffer, and a final 1:10 dilution of this suspension is performed with 0.4% Trypan Blue for haemocytometry. The undiluted RBC fraction can be stored at 4^{o}C for up to one week.

After obtaining the cell count, RBCs are adjusted to 10^8 cells / ml in PBS-EDTA buffer and transferred to a sterile 96-well microtitre plate for the assay. Each well requires 200 µl of RBCs (accounting for 2 x 10^7 cells) + 25µl samples of the following components:

- 0.1% Triton X-100 in 1x PBS (positive control)
- PBS-EDTA buffer (negative control)
- 25 ng of purified recombinant LLO from Listeria monocytogenes () in 1x PBS.
- Highly concentrated mycobacterial culture supernatants

The plate is then incubated at 37°C with 5% CO2 for one hour. After this, the plate is centrifuged at 1500 rpm for 5 minutes at room temperature, and 100 ul of the resulting supernatant is transferred to a fresh set of wells. Since the read-out of the assay is haemoglobin release, absorbance is read at 412 nm for optimal results. The data obtained here is then normalised to reflect percentage of haemolysis. For this purpose, PBS-EDTA signifies 0% haemolysis while 0.1% Triton X-100 represents 100% haemolysis.

5.13 Cignal hIRF-1 luciferase reporter assay (SA Biosciences, Qiagen, USA).

5.13.1 Establishment of the reporter-transduced THP-1 cell line (r-tTHP-1; with assistance from Dr. Pedro Alves, Department of Immunology, MPIIB).

This reporter assay employs the use of a Renilla (firefly) luciferase gene fused to a DNA fragment which contains a high-affinity binding site for hIRF-1. hIRF-1 enables transcription of the luciferase gene upon binding to its target, leading to light production. This output is measured using relative light units (RLUs) to account for luminescence intensity.

5.13.2 Assay setup.

Differentiated r-tTHP-1 MΦs in a 96-well plate are first washed with sterile PBS via centrifugation at 1500 rpm for 5 minutes at room temperature. Upon aspirating the supernatant, fresh cRPMI is added to the wells, followed by mycobacteria (suspended in cRPMI) according to the multiplicity of infection (MOI) required. The plate is then centrifuged at 1500 rpm for 5 minutes and then incubated at 37^{o}C with 5% CO_2 for 4 hours. At the end of the incubation period, the plate is once again centrifuged at 1500 rpm for 5 minutes and the resulting supernatant removed by aspiration. Fresh cRPMi is added the wells and the plate is again left to incubate for 24 hours under standard conditions.

5.13.3 Processing and reading the plate.

Following the 24-hour incubation period, the plate is centrifuged at 1500 rpm for 5 minutes and the supernatant removed afterwards via aspiration. The cells are washed once with PBS and then, lysed with 30 µl 1x Passive Lysis Buffer prepared from a 5x stock solulation (Part #: E194A; Promega, USA) by incubating at room temperature for 5 minutes, without exposure to light. 15 µl of the lysate is transferred to a fresh 96-well plate to be read on a luminometer. The remaining 15 µl of the lysate is used for measuring protein concentration using the standard Bradford assay. The latter is required for normalising the data based on RLUs produced per unit of protein concentration.

6. Immunological techniques.

6.1 Cytokine measurements.

6.1.1 Enzyme-linked immunosorbent assays (ELISAs).

The various ELISA kits used in this project are listed in Table 7:

ELISA kit	Manufacturer	Cat. #
Human Interferon gamma (hIFN-γ) DuoSet	R & D Systems	DY285
hIFN-γ ELISA Ready-SET-Go	eBioscience	88-7316-88
Human Interleukin 2 (hIL-2) DuoSet	R & D Systems	DY202
Human Interleukin 7 (hIL-7) DuoSet	R & D Systems	DY207
Human Interleukin 18 (hIL-18) Antibody pairs	eBioscience	BMS267/2MST
Human tumour necrosis factor alpha (hTNF-α)	Peprotech	900-K25

Table 7. List of all commercially available ELISA kits used in this project.

ELISAs were performed according to manufacturers' instructions. In all cases, BD OptEIA Solutions A and B (BD Biosciences, USA) mixed at a ratio of 1:1 were used as substrate. NOTE: samples were not diluted in any of the experiments unless otherwise stated.

6.1.2 Multiplex cytokine analysis.

The Bio-Rad Pro Human Cytokine 27-plex Panel (Cat #: M50-0KCAF0Y) was used for these experiments. For analysis of mouse serum cytokines, the Bio-Rad Mouse Cytokine 23-plex Panel (Cat #: M60-009RDPD) was used. In all multiplex assays, the volume of the coupled beads, detection antibodies and streptavidin-PE conjugate was halved and topped up with the appropriate buffer. Otherwise, the assays were performed according to the manufacturer's instructions. Assay plates were read using the Bio-Plex 200 system (Bio-Rad, USA).

6.2 Flow cytometry.

All flow cytometric experiments were performed on the BD LSRII FACS machine (BD Biosciences, USA) while analyses were carried out using the FlowJo software (Tree Star Inc., USA). All antibodies used in this project are listed in Table 8:

FACS antibody	Fluorophore	Clone	Catalogue no.	Lot no.	Manufacturer
Anti-Mouse CD3ε	Alexa Flour 700	17A2	56-0032-82	E08933-1634	eBioscience
Anti-Mouse CD4	AmCyan (V500)	RM4-5	560782	2111669	BD Horizon
Anti-Mouse CD8α	PerCP	169	N/A	N/A	in-house
Anti-Mouse CD44	Pacific Blue	IM7	N/A	N/A	in-house
Anti-Mouse CD154	APC	MR1	17-1541-82	E07226-1630	eBioscience
Anti-Mouse Interferon gamma (IFN-γ)	PE-Cy7	XMG1.2	557649	24389	BD Pharmingen
Anti-Mouse Interferon gamma (IFN-γ)	PE-Cy7	XMG1.3	505810	B153999	BioLegend
Anti-Mouse Tumour Necrosis Factor alpha (TNF-α)	FITC	XT-22	N/A	N/A	in-house

Table 8. List of all flow cytometry antibodies used in this project.

6.2.1 Flow cytometic analysis of activated CD4+ T cells from vaccinated BALB/c mice.

1) Spleens, lungs and draining lymph nodes (dLNs) of mice are surgically removed and put in separate 15 ml Falcon tubes containing 2 ml of cRPMI with penicillin and streptomycin (cRPMI+p/s) on ice. Spleen and dLNs per mouse are individually mashed using a sterile 40 µm

cell strainer (BD) and 2 ml syringe plunger in a petri dish to prepare single-cell suspensions (SCS). The cell strainer is then washed with 12 ml of cRPMI+p/s and the flow through transferred to the respective 15 ml Falcon tube. All tubes are then centrifuged at 1300 rpm for 5 minutes at 4^{o}C.

2) For the lungs, they are first transferred to a sterile petri dish and cut into small pieces with a pair of surgical scissors. Next, 10 ml of a collagenase digestion stock (Collagenase IV (0.7mg/ml) + Collagenase D (0.3mg/ml) in cRPMI+p/s) is pipetted into the petri dish and incubated at 37^{o}C with 5% CO_2 for 30 minutes. At the end of the incubation period, the digested pieces of lung are transferred to a 40 µm cell strainer (BD) and mashed using a sterile 2 ml syringe plunger in the same petri dish in which the digestion was performed. The cell strainer is then washed with 12 ml of cRMPI+p/s and the washing transferred to the 15 ml Falcon tube in which the organ was first put. All tubes are then centrifuged at 1300 rpm for 5 minutes at 4^{o}C.

3) After carefully decanting the supernatant (SN), the resulting cell pellets are loosened by vigorous tapping on the bottom surface of the tube. After this, 2 ml of red blood lysis buffer (0.0083% NH_4Cl, 0.001% $KHCO_3$ and 0.037% EDTA in distilled water) is added to the tubes containing lung and spleen only, and incubated at RT for 2 minutes. Following this, 12 ml of PBS + 0.2% BSA buffer is added to the respective tubes to stop the reaction, after which the tubes are centrifuged at 1300 rpm for 5 minutes at RT. Meanwhile, the dLN tubes are tapped for dislodging the pellets.

4) After carefully decanting the SN, the dLN and lung cell pellets are resuspended in 500 µl while spleen in 3 ml cRPMI+p/s medium. 5 µl of the respective suspensions is added to 200 µl of 1x AccuCheck bead suspension (Invitrogen; 1:5 diluted) for flow cytometric counting. 500 µl of each cell suspension is then seeded in a 48-well plate. Each well is then re-stimulated with 2.65 µl of 1.866 mg/ml of *M. tuberculosis* H37Rv whole cell lysate (final concentration: 10 µg/ml) for 6 hours at 37^{o}C with 5% CO_2. 5 µg/ml of sterile Brefeldin A is added to the cells for the entire duration of culture.

5) At the end of the incubation period, the cells are transferred to a 15 ml Falcon tube and washed by centrifugation at 1300 rpm for 3 minutes at RT. After carefully decanting the resulting SN, the cell pellets are resuspended in enough FACS buffer to have a final suspension volume of 90µl. 10 µl of a 10x surface stain master mix in FACS buffer containing the following

antibodies (each at 1:20; 4.0 µl / Ab) is then added to the cell suspensions in the 96-well plate and stained for 20 minutes on ice:

- CD4-V500
- CD44-Pacific Blue
- CD3-Alexa Fluor 700
- CD8-PerCP

6) The cells are then washed with 200 µl of FACS buffer by centrifugation at 1300 rpm for 3 minutes. The SN is then removed by quickly and gently flicking the plate.

7) The washed cells are then resuspended in 60 µl of BD Cytofix / Cytoperm solution and left to incubate on ice for 20 minutes, followed by one wash with 150 µl perm wash at 1300 rpm for 3 minutes.

8) The resulting cell pellet is resuspended in 50 µl of perm wash containing 1:200 diluted antibodies for intracellular staining (CD40L-APC (diluted at 1:20), TNFα-FITC, IFNγ-PECy7) and stained on ice for 30 minutes. This is followed by one wash with 150 µl perm wash at 1300 rpm for 3 minutes. The stained cells are resuspended in 200 µl FACS buffer and applied to a 35 µm cell strainer capped onto a 12 x 75 mm tube (BD) for a quick spin followed by flow cytometry.

Materials and methods – *in vivo*.

1. Protective efficacy study.

Female BALB/c mice aged between 7 and 8 weeks were vaccinated as follows:

a. PBS (sham)
b. BCG SSI 1331 (Control 1)
c. BCG *ΔureC::hly* (Control 2)
d. rBCGIL-7
e. rBCGIL-18

f. rBCGIL-7 and rBCGIL-18

Each mouse received 100 ul of either PBS or vaccine subcutaneously 1 cm above the tail base. The mice were challenged via the aerosol route with 200 CFUs of *M. tuberculosis* H37Rv 90 days post vaccination. At the designated time points, lungs and spleen of mice were aseptically harvested and put in an organ collection bag containing 1 ml of sterile PBS + 0.05% Tween 80. They were then individually placed between a plastic disposable bag and thoroughly mashed using the lid of a tip box for homogenate prepation. 100 µl of the homogenate was then used for performing 10-fold dilutions in a 48-well plate containing 900 µl of sterile PBS + 0.05% Tween 80 (dilutions plated per time point are indicated in parantheses):

Day 1 post challenge: Lungs only (250 µl of neat homogenate on two plates)

Day 30 post challenge: Lungs (unvaccinated mice: 10^{-2}-10^{-5}; all vaccinated mice: 10^{-1}-10^{-4}) and spleen (unvaccinated mice: 10^{-1}-10^{-4}; all vaccinated mice: 10^{0}-10^{-3}).

Day 90 post challenge: Lungs (unvaccinated mice: 10^{-2}-10^{-5}; mice vaccinated with BCG SSI: 10^{-1}-10^{-4}; mice vaccinated with rBCG strains: 10^{0}-10^{-3}) and spleen (unvaccinated mice: 10^{-1}-10^{-4}; mice vaccinated with BCG SSI: 10^{-1}-10^{-4}; vaccinated with rBCG strains: 10^{0}-10^{-3}).

Day 200 post challenge: Half of lung (unvaccinated mice: 10^{-1}-10^{-4}; mice vaccinated with BCG SSI: 10^{0}-10^{-3}; mice vaccinated with rBCG strains: 250 µl of neat homogenate on two plates) and spleen (unvaccinated mice: 10^{-1}-10^{-4}; mice vaccinated with BCG SSI: 10^{0}-10^{-3}; mice vaccinated with rBCG strains: 250 µl of neat homogenate on two plates). The remaining half of the lung was used for histopathology.

2. Assessment of clearance of rBCG strains from vaccinated mice.

Female BALB/c mice between 7 and 8 months of age were separated in groups of 3 to be treated as follows:

1) BCG SSI 1331

2) BCG *ΔureC::hly*

3) rBCGIL-7

4) rBCGIL-18

Each mouse was subcutaneously vaccinated (1 cm above the tail) with 1 x 10^6 CFU of bacteria suspended in 100 µl of sterile PBS. At designated time points post vaccination - days 7, 14, 30, 60, 90 and 120, spleen and dLNs of mice were surgically removed and put in 1 ml of 1x PBS-0.05% Tween 80 buffer, homogenised and added to a sterile 48-well plate to perform serial dilutions in the same buffer. On days 7, 14 and 30, 100 µl of dilutions 10^{-1} to 10^{-4} were plated on 7H11 medium for both organs whereas on days 60, 90 and 120, undiluted homogenate along with dilutions 10^{-1} to 10^{-3} were plated for both organs. The plate were sealed with Parafilm, wrapped in aluminium foil and incubated in a 37^{o}C microbiological incubator for 3 - 4 weeks.

RESULTS

1. Generation of rBCGIL-7, rBCGIL-18 and rBCGIRF1.

1.1 Construction of the pMVhIL-7mat / pMVhIL-18 / pMVhIRF1 integrative expression vectors.

This project was initiated by amplification of four genes using the Qiagen Taq Polymerase PCR system:

1) secretion apparatus comprising a fusion of the mycobacterial heat shock protein 60 promoter (P_{hsp60}) and antigen 85B 45-bp upstream region in addition to its endogenous signal sequence (hereafter termed 'Ag85BSS'), flanked by *Xba*I (5') and *Nsi*I (3')

2) mature hIL-7, flanked by *Nsi*I (5') and *Kpn*I (3')

3) mature hIL-18, flanked by *Nsi*I (5') and *Kpn*I (3')

4) codon-optimised hIRF-1, flanked by *Nsi*I (5') and *Kpn*I (3')

hIL-7, hIL-18 and hIRF-1 PCR products were 16 nucleotides longer on the 3' flank due to the addition of a second restriction enzyme site, namely *Xho*I and dinucleotide shoulders (Fig.1A). However, *Xho*I was not used afterwards due to technical reasons. This was followed by cloning of the amplicons into the commercially-available intermediate cloning vector pCR TOPO 2.1 and automated sequencing to confirm legitimacy. The LLO expression/secretion cassette, which was used to generate BCG *ΔureC::hly* (Hess, Miko et al. 1998; Grode, Seiler et al. 2005) was then cloned into pBMG11 using *Xba*I and *Nsi*I to generate pBMG11V3 while removing the first 30 nucleotides of mature Ag85B downstream of Ag85BSS. Subsequently, the *hIL-7*, *hIL-18* and *hIRF-1* genes were cloned into pBMG11V3 using *Nsi*I and *Kpn*I to replace the *hly* gene from *Listeria monocytogenes* to generate pBMG11V3hIL7, pBMG11V3hIL18 and pBMG11V3hIRF1, respectively. This newly-derived expression/secretion cassette flanked by *Xba*I (5') and *Kpn*I (3') was subsequently digested with the afore-mentioned restriction enzymes for insertion into pMV306 to generate the final destination vectors pMVhIL7mat, pMVhIL18 and pMVhIRF1, respectively (Fig. 1). pMV306 is a commonly used mycobacterial integrative vector which allows stabile insertion into the *attB* site of mycobacterial genomes via activity of mycobacteriophage L5 integrase (Lee *et al*., 1991). These vectors were then transformed into *E. coli* TOP 10 for propagation and later isolated for confirmation by restriction enzyme analysis with *Xba*I and *Kpn*I (Fig. 2) as well as PCR prior to transforming mycobacteria.

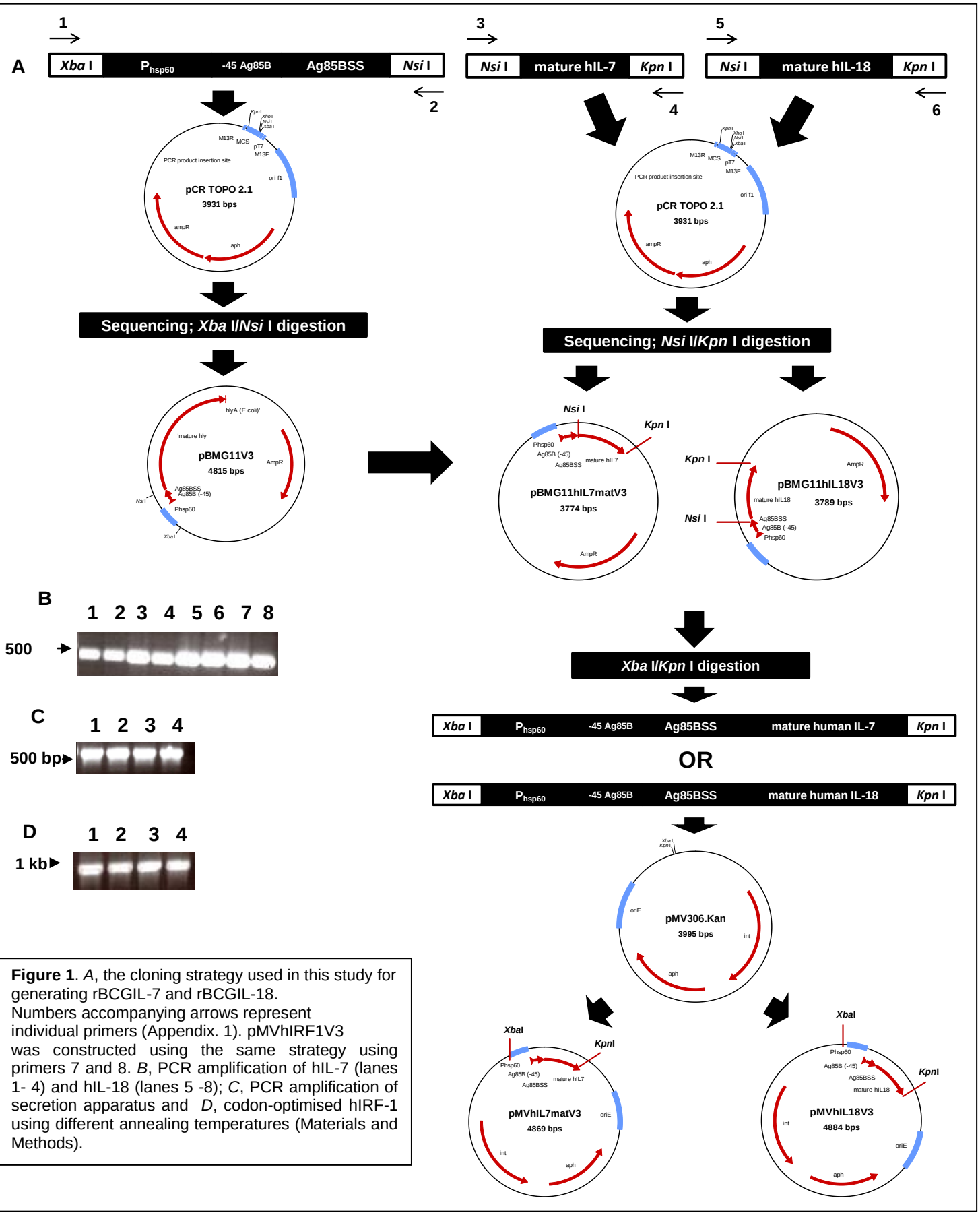

Figure 1. *A*, the cloning strategy used in this study for generating rBCGIL-7 and rBCGIL-18. Numbers accompanying arrows represent individual primers (Appendix. 1). pMVhIRF1V3 was constructed using the same strategy using primers 7 and 8. *B*, PCR amplification of hIL-7 (lanes 1- 4) and hIL-18 (lanes 5 -8); *C*, PCR amplification of secretion apparatus and *D*, codon-optimised hIRF-1 using different annealing temperatures (Materials and Methods).

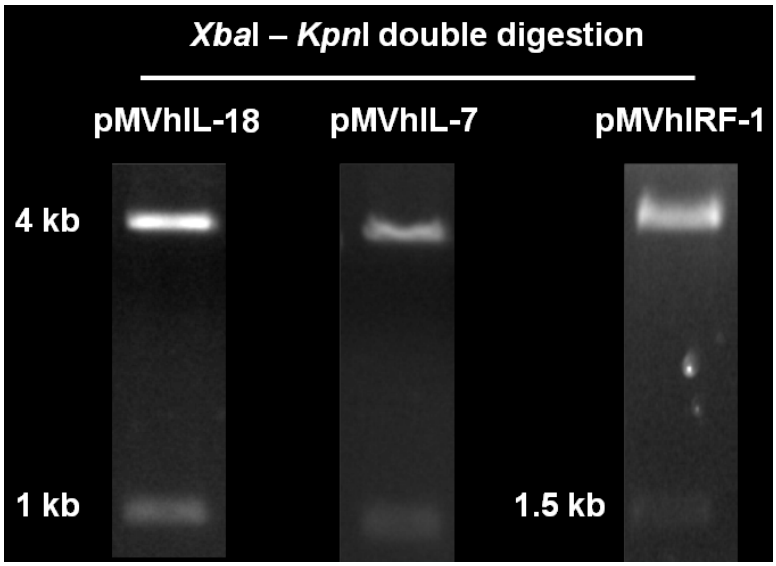

Figure 2. Restriction enzyme analysis of pMVhIL-18, pMVhIL-7 and pMVhIRF-1. Shown are representative gel photos of each destination vector.

Several prior attempts to genetically manipulate BCG *ΔureC*::*hly* were not met with success (personal observation). In general genetic manipulations in BCG are harder to achieve as compared to *M. tuberculosis* and the project described here was no exception. However, after several months of trying with multi-replicate transformation experiments, rBCGIL-7, rBCGIL-18 and rBCGIRF-1 were derived.

Although I was finally successful in deriving the intended modified strains, much of the technical conundrums faced at this stage involved identification of correct mycobacterial clones. 4 - 5 new transformants that were screened turned out to be illegitimate, due to non-intended recombination events. As pMV306 confers resistance to kanamycin via expression of the *aph* (amino phosphotransferase) gene, only legitimate transformants harbouring the plasmid should grow as they are plated on antibiotics-containing medium for selection. However, in practice, spontaneous phenotypic mutations may occur due to false recombination of only the kanamycin resistance cassette, thus generating illegitimate clones.

1.2 Confirmation of legitimate rBCGIL-7, rBCGIL-18 and rBCGIRF1 clones.

1.2.1 PCR analyses to confirm expression cassette integration and heterologous cytokine gene transcription by the respective rBCG strains.

The first point of confirming expression of the hIL-7, hIL-18 and hIRF-1 genes was to perform PCR reactions with primers listed in Appendix 1. Complementary DNA prepared from mRNA of the respective rBCG strains was used as template. Cultures were harvested between mid and late-log phase optical density (OD_{600} 0.6 – 1.0) of growth in Middlebrook 7H9 complete medium. As depicted in Fig. 3, the recombinant genes were expressed by rBCGIL-7, rBCGIL-18 and rBCGIRF-1, respectively. I also checked if further genetic manipulation of BCG *ΔureC*::*hly* might affect normal expression of the *hly* gene (encoding listeriolysin O) using internal primers. Alongside this control measure, *pdx1* (encoding pyridoxine synthase) was included as a loading control to confirm presence of DNA in all lanes shown.

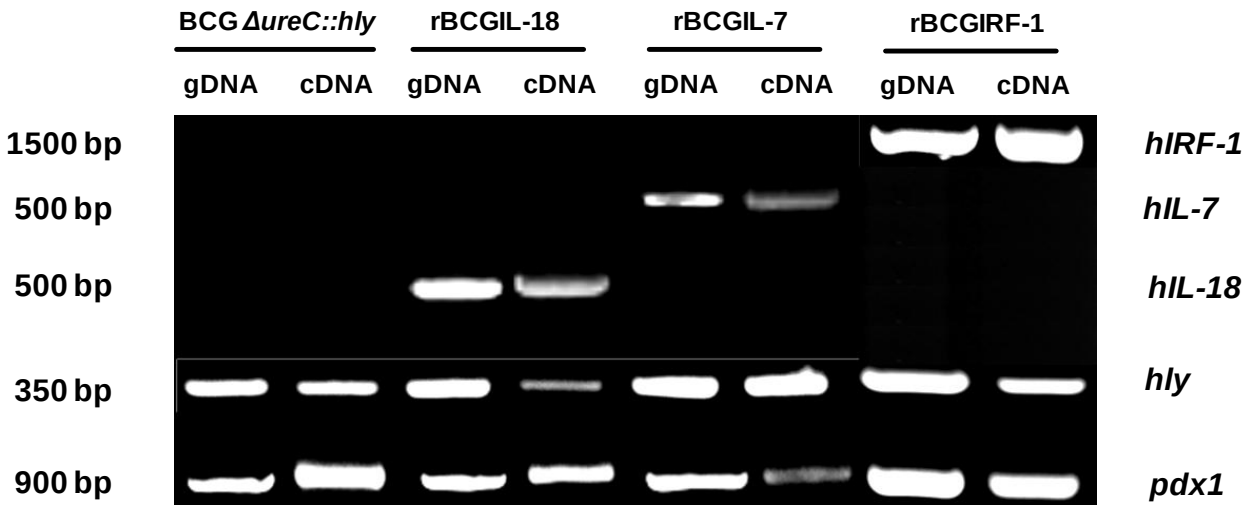

Figure 3. RT-PCR analyses of rBCGIL-7 and rBCGIL-18 to verify integration and transcription of respective cytokine expression cassettes. *hly* transcription was not compromised in both strains and *pdx1* was used as an internal control of gene expression. Similar PCR reactions performed by using respective RNA preparations did not result in detectable PCR products thereby confirming the absence of DNA contaminations. gDNA denotes genomic DNA while cDNA denotes complementary DNA prepared from total mRNA.

1.2.2 Confirmation of heterologous protein expression by rBCGIL-7, rBCGIL-18 and rBCGIRF-1.

The next step was to examine whether the gene products of the respective recombinant genes were synthesized by the rBCG strains. In order to do this, culture supernatants (CSN) and whole cell lysates (WCL) of the respective rBCG strains were analysed by ELISA to check for presence of hIL-7 or hIL-18. Human IRF-1 expression, on the other hand was verified using Western blotting as no ELISA kits were available to assess presence of this molecule. As mid- to late-log phase cultures were used in each case, the number of mycobacterial cells contributing to total protein content was ~10^8 CFU/ml lysate.

Figure 4. Western blot of mycobacterial WCL and CSN samples. Primary anti human IRF-1 monoclonal antibody (BD Biosciences) was used at a dilution of 1:1000 as recommended by the manufacturer.

Recombinant human IRF-1 expression could be detected in neither whole cell lysates nor culture supernatants of rBCGIRF-1 using Western blotting after several attempts (Fig. 4). As opposed to encouraging cDNA analysis data (Fig. 3), protein expression analysis returned with no positive signal whatsoever. One major point was whether or not the amount in which this protein was synthesized by the mycobacteria was sufficient to be detected via Western blotting. Therefore, I resorted to using a highly sensitive luciferase reporter assay, which employs the use of THP-1 cells (acute monocytic leukaemia cell line) harbouring a luciferase reporter gene fused to a hIRF-1 binding element. The multiplicity of infection (MOI) used in all experiments was 10, and is the standard procedure employed in our laboratory for *in vitro* infections with BCG.

Upon stable IRF-1 expression and protein synthesis in MΦs, the transcription factor would bind irreversibly to a consensus sequence encoded upstream of the luciferase gene. This interaction results in activation of the transcription machinery, which in turn switches on luciferase expression. Source of IRF-1 is mainly indigenous in this assay; IFN-γ is used as a positive

control to naturally induce the expression of this molecule in the cytoplasm. Fig. 4A is schematic representation of this method.

In this assay, I hypothesised that following phagocytosis of rBCGIRF-1 by MΦs, mycobacterial antigens would escape into the cytosol along with heterologously expressed proteins, hIRF-1 in this case – pertinent to the mode of action of BCG *ΔureC*::*hly* . Cytosolically-localised hIRF-1 would then translocate into the nucleus and bind to the IRF-1 consensus sequence, subsequently triggering luciferase expression and light production. Disappointingly, my results showed that fold induction of hIRF-1 by rBCGIRF-1 was not comparable even to that of wild type BCG or BCG *ΔureC*::*hly* (Fig. 5B). However, a difference seen here need not necessarily mean that the effect was induced by actively-secreted native-like hIRF-1 by rBCGIRF-1 – mycobacterial infection of MΦs on its own elicits intracellular signalling processes culminating in endogenous IRF-1 activation (Pine 2002). Considering this observation, I concluded that the rBCGIRF-1 mutant was able to express transcripts of human IRF-1 but not synthesise the mature protein.

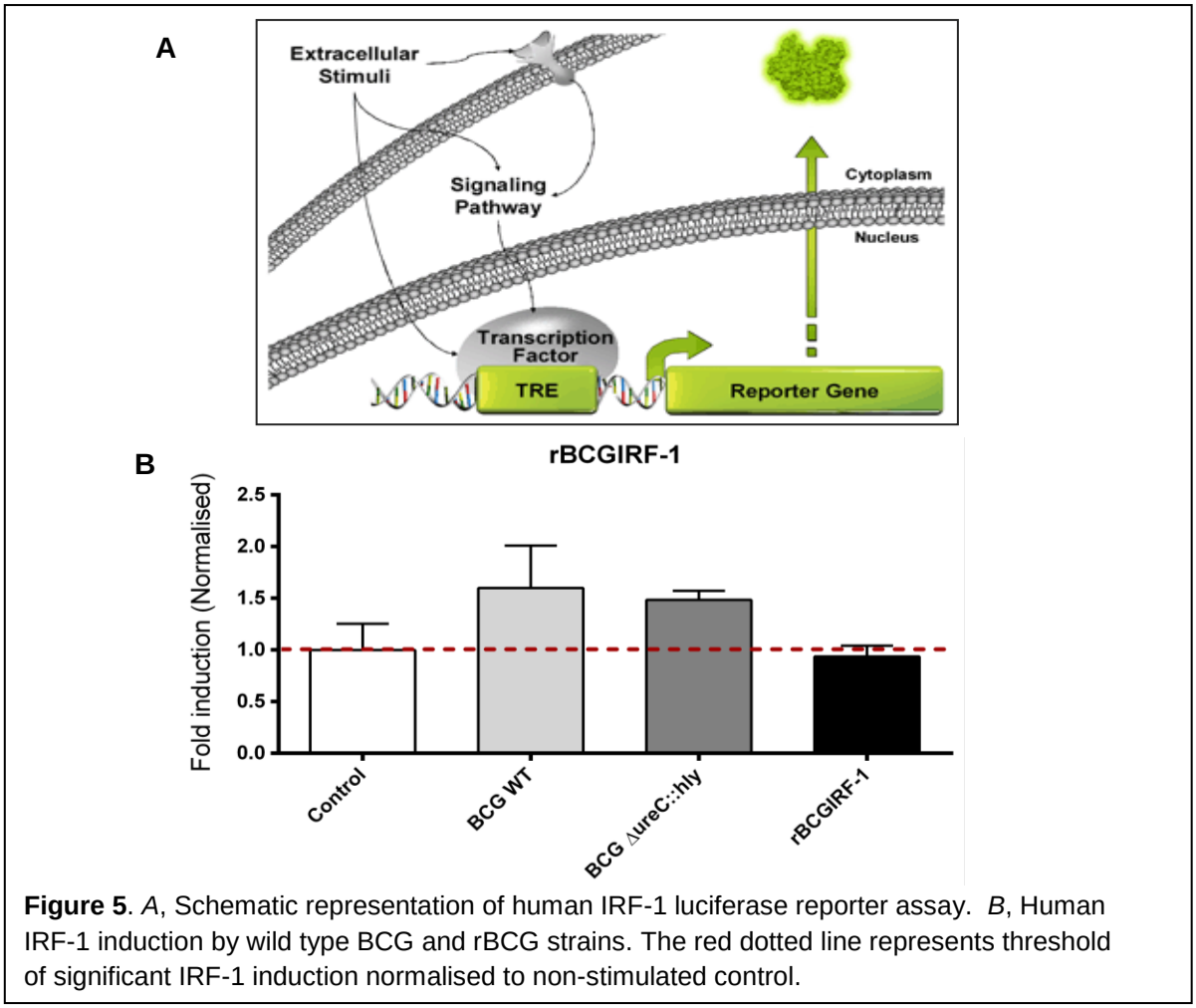

Figure 5. *A*, Schematic representation of human IRF-1 luciferase reporter assay. *B*, Human IRF-1 induction by wild type BCG and rBCG strains. The red dotted line represents threshold of significant IRF-1 induction normalised to non-stimulated control.

To determine presence of mature hIL-7 and hIL-18 in mycobacterial sample preparations, highly sensitive human ELISA kits were used (refer to Materials and Methods). Fresh samples were prepared per replicate experiment in order to ensure quality of assay.

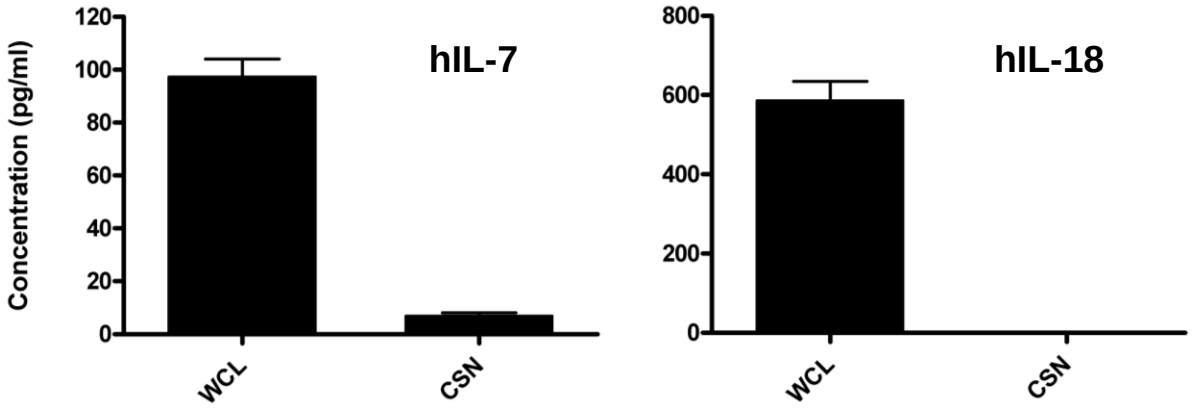

Figure 6. ELISA analysis of human IL-7 (left panel) and IL-18 (right panel) expressed by rBCGIL-7 and rBCGIL-18, respectively. (WCL = whole cell lysate; CSN = culture supernatant).

As shown in Fig. 6, both hIL-7 and hIL-18 were detected in whole cell lysate preparations of rBCGIL-7 and rBCGIL-18, respectively. These observations lead to the conclusion that both cytokines were produced in decent quantities within the mycobacterial cell but likely secreted to a lesser amount, based on cytokine detection in culture supernatants. A noteworthy phenomenon which significantly affects active secretion of foreign proteins by mycobacteria is the mechanism by which the protein of choice is transported across the cell envelope. Stable export of endogenous Ag85B and LLO by BCG *ΔureC::hly* might subdue additional transport of hIL-7 or hIL-18. Inaccurate cleavage of the signal sequence resulting in cytoplasmic retention of the cytokine, and intracellular misfolding of the protein as well as the preceding signal sequence may also influence suboptimal secretion of the mature cytokines by the recombinant bacteria.

Since preliminary validation of rBCGIL-7 and rBCGIL-18 turned out more promising than rBCGIRF-1, I decided to proceed with further characterisation of the former two strains.

2. *In vitro* evaluation of rBCGIL-7 and rBCGIL-18.

2.1 Perforating activity of rBCGIL-7 and rBCGIL-18.

Since the defining characteristic of the BCG *ΔureC*::*hly* strain is its ability to secrete bioactive LLO, I decided to confirm as to whether this feature was retained in rBCGIL-7 and rBCGIL-18. This was mainly to ascertain that further genetic modification did not hamper the pre-existing perforating activity of both strains. I therefore performed a haemolysis assay using freshly isolated human erythrocytes in PBS-EDTA buffer.

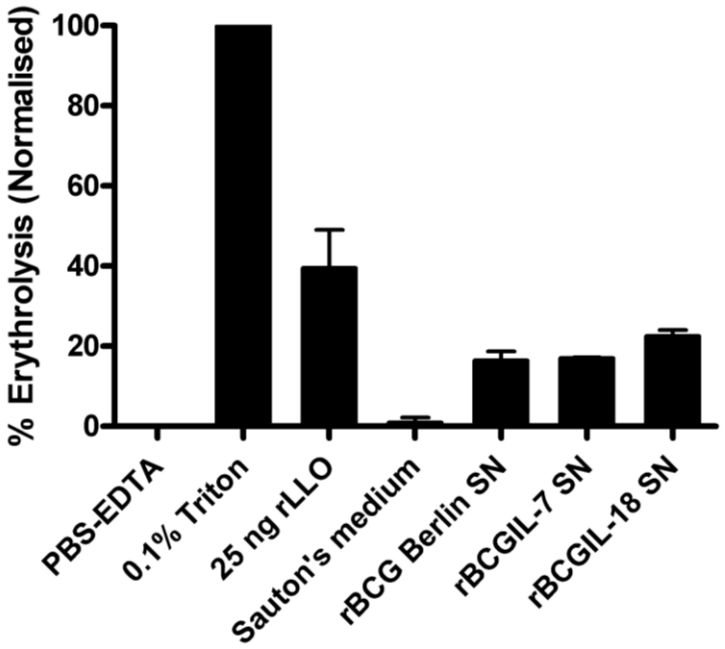

Figure 7. Rate of erythrolysis of human red blood cells measured by haemoglobin release. Erythrolysis was normalised to the 0.1% Triton control. Shown are means ± SEM.

Both rBCGIL-7 and rBCGIL-18 are capable of lysing human red blood cells as efficiently as BCG *ΔureC*::*hly* (Fig. 7). All CSN samples were harvested from bacterial cultures grown to similar optical densities (late log phase; ca. OD_{600} 1.0). 25 ng of purified recombinant LLO which was used as a second positive control did not exhibit as high a percentage of erythrolysis in comparison to the rBCG strains probably due a high content of Tween 80 present in the CSN samples. Being a potent detergent, Tween 80 can lyse lipid membranes when present in high concentrations (ca. 200x concentrated in the CSN samples). However, Sauton's medium alone

did not cause significant lysis of red blood cells. This is due to the fact that there is only 0.05% Tween 80 present in non-concentrated medium, and this is not sufficient to exert such a dramatic haemolytic effect. As opposed to Tween 80, Triton X-100, another commonly used detergent is a much more superior lytic agent as only 0.1% in PBS is required for almost 80% lysis of red blood cells. I could thereby conclude that both rBCGIL-7 and rBCGIL-18 remained uncompromised in their perforating ability.

2.2 Phenotype of rBCG strains in THP-1 monocyte-derived macrophages, primary human monocyte-derived macrophages (hMoMΦs) and dendritic cells (hMoDCs).

A desirable feature of TB vaccine candidates is to induce a controlled pro-inflammatory innate immune responses largely orchestrated by IL-12, TNF-α and IL-18, all secreted by MΦs and/or DCs in humans and mice. These cytokines participate in the initiation of a series of downstream signalling events that lead to effector T cell activation directed towards a specific pathogen. Although not solely responsible, pro-inflammatory cytokines i.e. IFN-γ, TNF-α, IL-18 are one of the mainly assessed biomarkers of protection to evaluate vaccine efficacy against TB. Very importantly, these molecules are part of the milieu that constitutes the earliest immune response at the site of vaccination.

In order to assess the above-mentioned phenomena, I decided to resort to using the *in vitro* human cell culture system. Since TB vaccine development is meant for human use, primary human cells would be able to provide a preliminary insight into how rBCGIL-7 and rBCGIL-18 may behave in its intended host cell. Although IL-7 and IL-18 possess more of a systemic role in immunological responses, the human *in vitro* model would allow a decent understanding of some fundamental feature of the vaccine candidates themselves, mainly in terms of intracellular survival and initiation of innate immune responses.

I first decided to use the previously mentioned well-established human acute monocytic leukaemia cell line THP-1. My choice was largely determined by the minimal variability in the THP-1 cell line and that results obtained should be reproducible. Cells were infected with either BCG *ΔureC::hly*, rBCGIL-7 or rBCGIL-18 at a multiplicity of infection (MOI) of 10. Intracellular bacterial survival was monitored via CFU enumeration at various time points. The former was evaluated to obtain an idea of how the individual rBCG strains survived within the phagocyte and whether their growth dynamics affected the outcome of response. Mature IL-18 content on day 6

post infection was also measured to examine if MΦs infected with rBCGIL-18 were capable of secreting more of this cytokine due to contribution from intracellular bacteria.

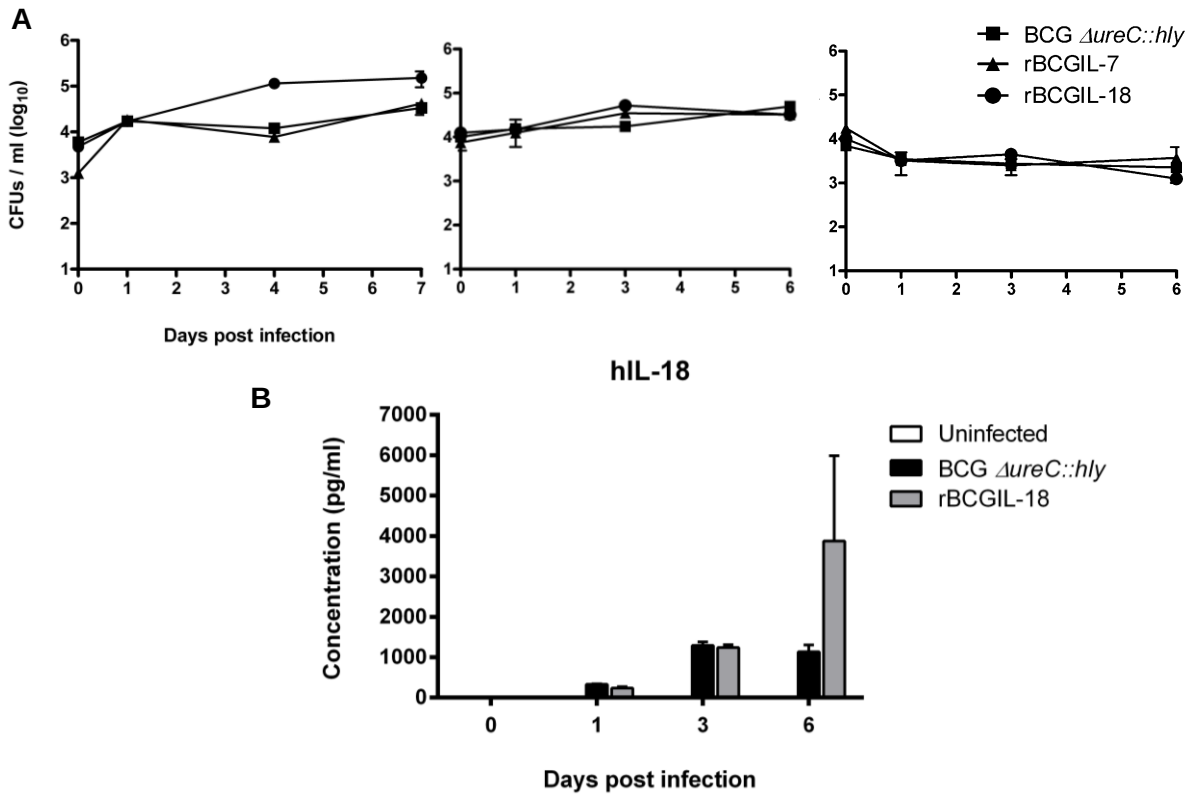

Figure 8. *A*, Growth curves of the respective rBCG strains in THP-1 MΦs (left), hMoMΦs (middle) and hMODCs (right). CFUs were monitored between 6 and 7 days in a 48-well plate. *B*, Presence of mature hIL-18 in culture supernatants of hMOMΦs. Shown are means SEM analysed using one way ANOVA and Tukey's multiple comparison test.

Surprisingly, DCs (Fig. 8A, right panel) seemed to be superior at controlling the intracellular proliferation of mycobacterial cells over the 6-day period of culture compared to THP-1-derived and primary human MΦs (Fig. 8A, left and middle panels). All the tested rBCG strains show a similar and sustained growth pattern over the culture period. My data suggests that under given *in vitro* conditions, the rBCG strains are able to persist in MΦs for a specific time frame but maybe better controlled in DCs. DCs play a very pronounced role in amplifying downstream immune responses (Ueno, Palucka et al. 2010). In addition, I also noticed that rBCGIL-18 contributed to higher levels of mature hIL-18 in culture supernatants of infected MΦs, especially

6 days post infection (Fig. 8B). This suggested that mature hIL-18 heterologously expressed by rBCGIL-18 might access the endogenous secretion pathway in MΦs.

Next, I designed a pilot study to identify the major myeloid cell-derived pro-inflammatory cytokines induced by infection with the respective rBCG strains. This comprised a large-scale, multiplex cytokine assay characterised by a panel of 27 different human cytokines that were checked for presence in culture supernatants of infected human cells – simulating multiple ELISA assays performed simultaneously. Fig. 9 depicts a selected number of cytokines secreted by the infected cells showing noteworthy differences.

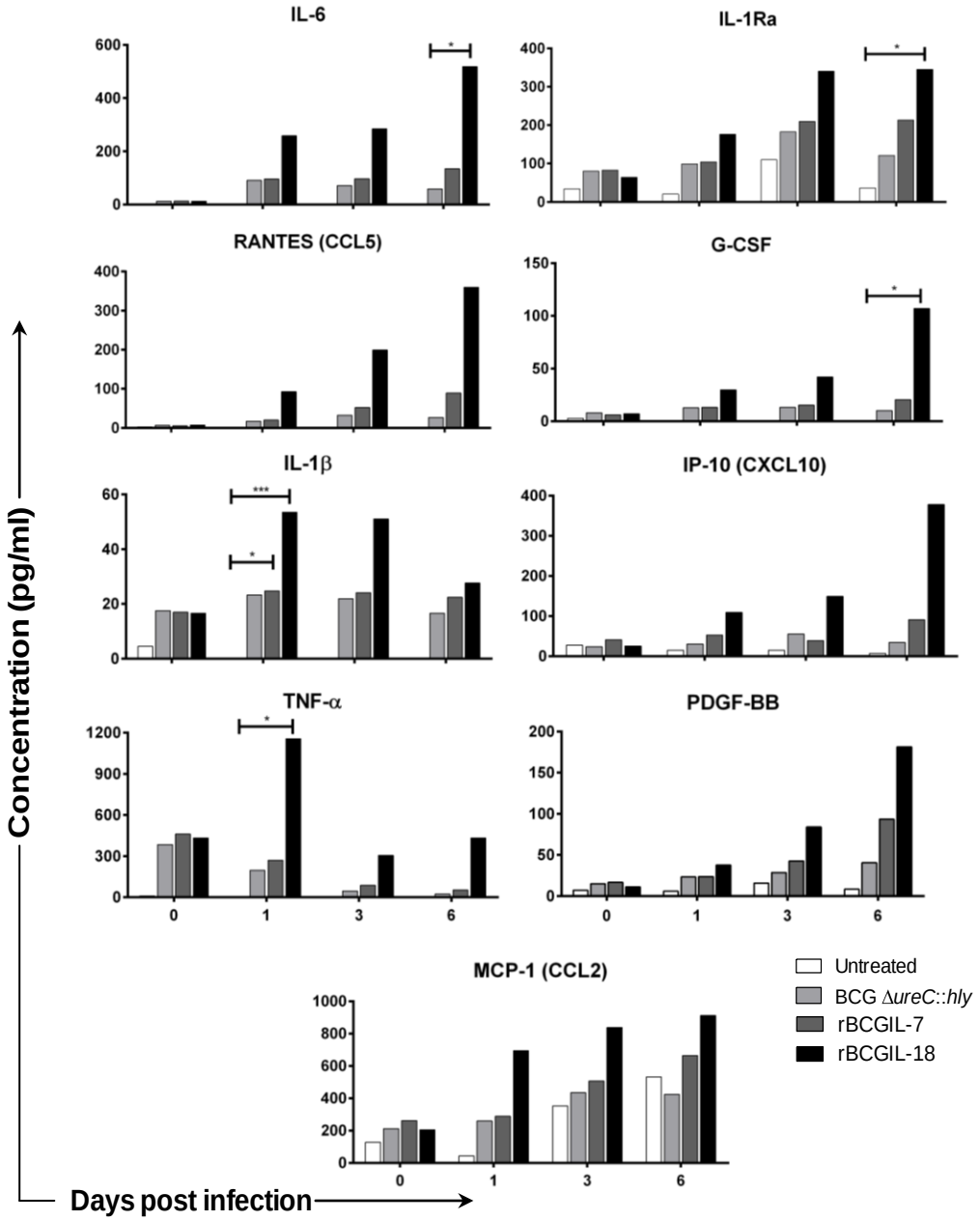

Figure 9. Cytokine profile of huMoDCs infected with the various rBCG strains. Day 0 samples were processed 4 hours post infection. Shown are mean values analysed using one-way ANOVA and Tukey's multiple comparison test, ***p<0.001; *p<0.05.

The array of cytokines secreted by primary human DCs was differentially modulated almost exclusively by rBCGIL-18. For the most part, the milieu of cytokines with a marked difference here are associated with pro-inflammatory signalling and chemotaxis – IL-1β), and its receptor antagonist (IL-1Ra), IL-6, inducible protein 10 (IP-10), **R**egulated **a**nd **n**ormal **T** cell **e**xpressed and **s**ecreted (RANTES), TNF-α and granulocyte colony-stimulating factor (G-CSF). Also upregulated were platelet-derived growth factor – beta-beta chain (PGDF-BB) and monocyte chemotactic protein 1 (MCP-1; also known as chemokine C-C ligand 2, CCL2).

IL-1β and IL-1Ra seem to be expressed in tandem although antagonistically. IL-1β release demonstrates activation of caspase 1, which, as already mentioned is required for secretion of mature IL-18. However, the increasing amount of IL-1Ra after 3 days of infection seems to overlay that of IL-1β and at much higher concentrations (ca. 5-fold more). This suggests that there may be activation of homeostatic stabilisation of intense inflammatory responses by IL-1Ra-mediated blocking of IL-1β interaction with its receptor (IL-1R).

IL-6 is regarded as a potent activator of innate immune antimicrobial defences, and may have a role in fever induction due to its upregulation by prostaglandin PGE_2 release (Hinson, Williams et al. 1996). In addition, IL-18 has been shown to trigger IL-6 release in a recombinant A549 type II pneumocyte cell line expressing IL-18 receptor beta chain (IL-18RB) (Lee, Kim et al. 2004).

IP-10 (also known as chemokine C-X-C motif ligand 10, CXCL10) is induced upon type I IFN (IFN-α/β) or type II IFN (IFN-γ)-mediated signalling initiated by myeloid and lymphoid cells, respectively and have a significant role to play in chemoattraction of monocytes and Th1 CD4+ T cells (Mihm, Schweyer et al. 2003). This is significant in this case as IL-18, together with IL-12 promotes IFN-γ release by Th1 cells and this in turn induces IP-10 secretion by myeloid cells to attract more antigen-specific T cells to sites of mycobacterial infection especially in the lung.

The ability of IL-18 to induce TNF-α by CD14+ cells is already known (Puren, Fantuzzi et al. 1998), and my result with rBCGIL-18 is consistent with this finding. It is however striking that while the amount of TNF-α secreted by rBCGIL-18-infected DCs increases 1 day after infection, the levels appear to decrease in DCs infected with the other rBCG strains. Although a noticeable drop exists in TNF-α concentration secreted by rBCGIL-18-infected DCs three days post infection, the presence of this cytokine is maintained until 6 days post infection, hinting at prolonged inflammation. G-CSF on the other hand is largely responsible for the proliferation of neutrophils and has been shown to be transcriptionally upregulated in mouse splenic T cells

upon IL-18 treatment (Ogura, Ueda et al. 2001). As already mentioned in the introductory chapter, neutrophils may have a role to play in antigen presentation early upon vaccination, thus nominating a role for G-CSF.

RANTES (also called chemokine C-C motif ligand 5, CCL5) is the unique cytokine in the series presented here. Albeit a chemokine, CCL5 is more pronouncedly involved in recruiting granulocytes into tissue, mainly eosinophils and basophils, and these cell types have been extensively shown to mediate airway hyperreactivity (AHR) and pulmonary distress (Rothenberg and Hogan 2006). There is not much described for these cells in alleviating inflammation triggered by *M. tuberculosis* infection but rather helminths and allergenic challenge (Rothenberg and Hogan 2006). However, since CCL5 also facilitates migration of activated T cells into tissue, it can be associated with *M. tuberculosis* Th1 specific responses in the lung.

Therefore, the data obtained thus far suggests that rBCGIL-18 somehow appears to induce natural secretion of endogenous innate immune modulators compared to rBCGIL-7 as well as BCG *ΔureC*::*hly* following infection of primary human DCs. This provided an important clue of its potential as a driver of pro-inflammatory immune signalling with a focus on the early events following vaccination. Moreover, IL-7 primarily and more pronouncedly acts on T cells (Krawczenko, Kieda et al. 2005). In my hands, infected primary MΦs did not appear to exhibit any observable differences in the context of cytokine secretion when analysed via the multiplex platform.

2.3 Growth restriction of intracellular rBCG in MΦs by DCs.

DCs, as previously mentioned have an indispensable position in bridging innate and adaptive immune response and one of their cardinal functions is to sample products from dying cells – due to infection or physiological homeostasis (Schmidt, Nino-Castro et al. 2012). With regard to vaccination, BCG-infected MΦs which undergo apoptotic cell death ultimately release mycobacterial antigens in vesicles which in turn are taken up by patrolling DCs. Such antigens are cross-presented by DCs and the efficiency of this process is yet another determinant of the quality of vaccination outcome (Ueno, Schmitt et al. 2010)

When I assessed the growth of intracellular bacteria in MΦs that were in close and constant contact with DCs, survival of all BCG strains was attenuated. As presented in Fig. 10, a 1-log drop in rBCG CFUs occurs within 24 hours of infection. On the contrary, as already shown in

Fig.8B, bacteria in MΦs can proliferate when DCs are absent despite the simultaneous presence of high amounts of TNF-α.

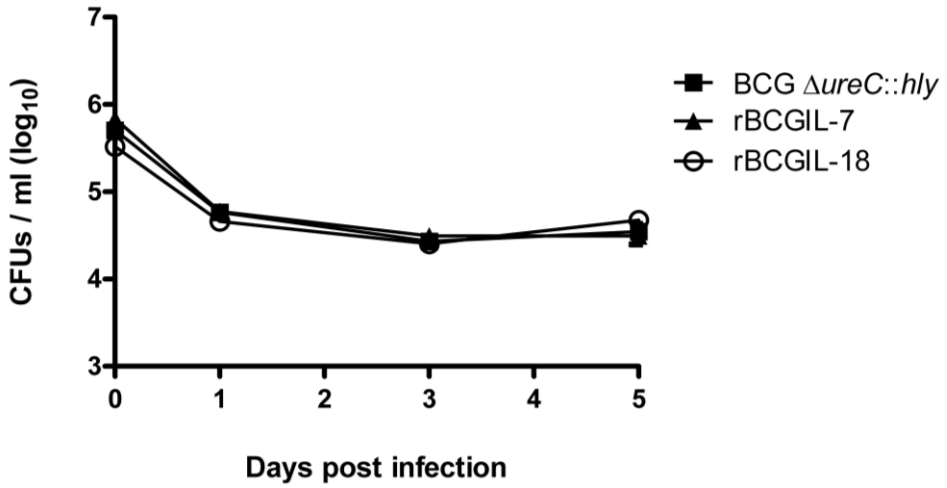

Figure 10. Intracellular growth dynamics of wild type BCG and the respective rBCG strains in hMoMΦs in the presence hMoDCs. Day 0 counts were after 4 hours of infection and washing.

2.4 Effect of rBCGGIL-7 and rBCGIL-18 on proliferation of T cells.

In order to assess whether rBCGIL-7 and rBCGIL-18 were superior at enhancing antigen presentation, I adapted a protocol published by Johansen and colleagues in 2011 (Johansen, Fettelschoss et al. 2011). In this study, the authors prepared bone marrow-derived DCs (BMDCs) from mice and infected them with 3 different recombinant BCG vaccine candidates. Infected BMDCs were then co-cultured with an MHC-matched T cell hydridoma expressing TCR specific for an epitope of Ag85A (namely $Ag85A_{241-260}$), a major secreted mycobacterial antigen. The degree of successful DC-T cell interactions formed in this regard will determine the degree of expansion of T cell numbers, evaluated by measuring IL-2 secretion by the proliferating T cells.

For the purposes of the *in vitro* section of this study, I focused more on the human system to gain a better and more true-to-life idea of how unconditioned immune cells would respond to antigenic challenge. As a positive control, DCs were also treated with PPD at the same time as

when infections were performed. On the same day, autologous T cells were isolated and co-cultured with the infected autologous DCs for 20 hours under the same incubation conditions following the infection period. After the 20-hour incubation period was over, cell-free culture supernatants were prepared to measure presence of human IL-2 by ELISA.

BCG-vaccinated, PPD^+ donors were selected for this assay owing to their T cells which are mycobacterial antigen-experienced. This feature is therefore very useful in evaluating the degree to which an individual may mount an immune response or simply react when posed with the appropriate antigenic challenge.

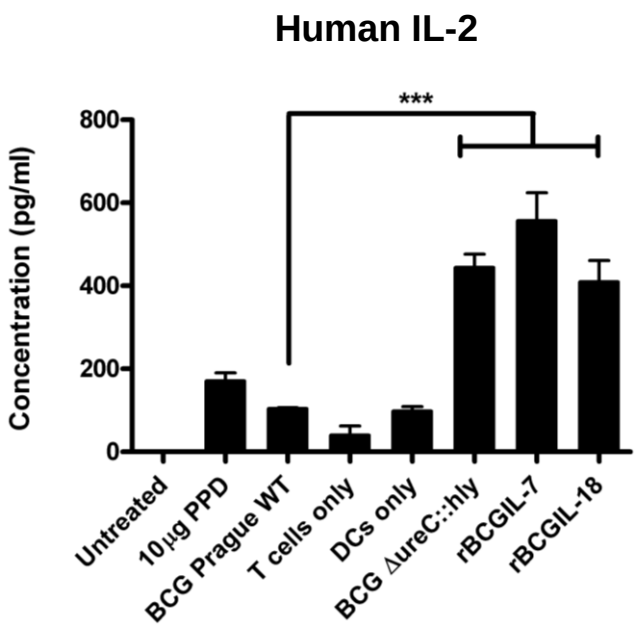

Figure 11. Human IL-2 levels in culture supernatants of infected DC and T cell co-culture. Shown are means SEM analysed using one way ANOVA and Tukey's multiple comparison test, ***p<0.001.

In theory, I expected to see higher degree of T cell proliferation driven by DCs infected with the rBCGIL-7 strain due to the sharing of the IL-2 receptor by IL-7. Furthermore, IL-7 elevates IL-2 expression and release, as a means of expanding the pool of cells upon stimulation with antigen (Appasamy 1993). My human IL-2 ELISA result supports the working hypothesis of this project, and the effect exerted by rBCGIL-7 was seen to be greater than that of PPD itself (Fig. 11). In as far as the bacterial numbers are concerned, the variability observed was within 1-log CFUs between wild type BCG and BCG *ΔureC*::*hly*. However, a clear 10-fold difference exists between

wild type BCG and rBCGIL-7 as well as rBCGIL-18, an observation which also holds true when comparing BCG *ΔureC*::*hly* and rBCGIL-18. In the latter case, this difference cannot be associated with having an impact on IL-2 secretion by T cells since DCs infected with BCG *ΔureC*::*hly* are shown to secrete about as much cytokine into the culture supernatant as those infected with rBCGIL-18. Therefore, rBCGIL-7, when compared with BCG *ΔureC*::*hly* did not seem to have a significantly pronounced effect on IL-2 secretion by activated human T cells.

It is also interesting to note that non-primed T cells also produce a baseline amount of IL-2, most probably for homeostatic regulation of viability in culture. Various DC subsets can produce IL-2 upon antigen stimulation of TLR4 and CD40L-CD40 interactions with T cells (Zelante, Fric et al. 2012) . Therefore, the possibility that infected DCs themselves serve as a source for human IL-2 in this assay cannot be excluded..

I decided not to delve too much into elucidating the intracellular events that occur upon infection of MΦs and DCs with the various rBCG strains owing to the mechanism of action of both IL-7 and IL-18. Instead, my future experiments were based on the mouse model to rather focus on the systemic immunomodulatory effect(s) of rBCGIL-7 and rBCGIL-18.

3. *In vivo* clearance of rBCGIL-7 and rBCGIL-18 from vaccinated mice.

A staunchly sustained immune response is most often attributed to the prolonged presence of antigen in the environment. However, the ensuing memory response is rightly characterised by presence of antigen- or pathogen-specific immune cells that linger in the host especially in the absence of antigen encounter. In the case of BCG, it is highly desirable that a live BCG vaccine does not remain in the host for too long to avoid vaccine-induced BCGosis at a later point, particularly in newborns and infants co-infected with HIV (Hesseling, Marais et al. 2007)

In order to assess whether rBCGIL-7 and rBCGIL-18 were removed from the host's systemic circulation within minimal time, I isolated inguinal (draining) lymph nodes (annotated as dLNs) and spleens from vaccinated mice at various time points between 7 and 120 days post vaccination to enumerate CFUs.

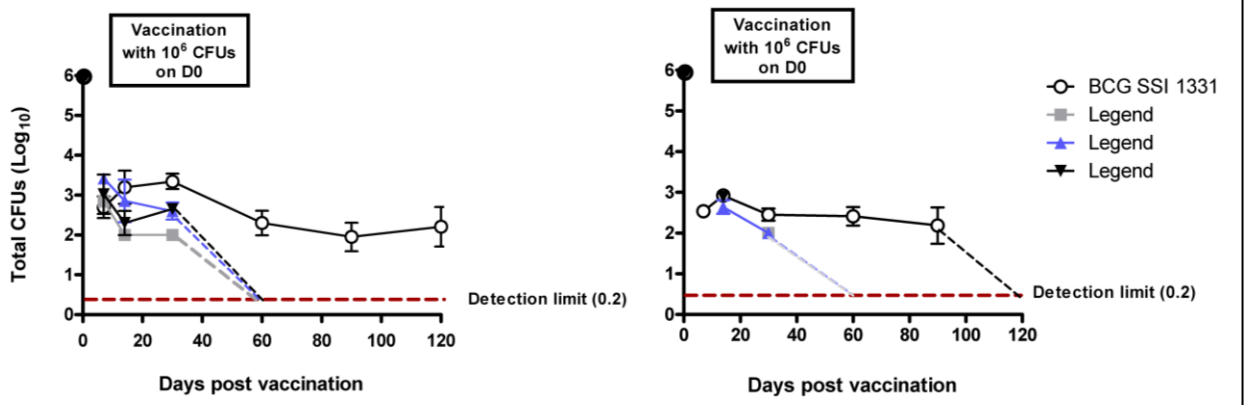

Figure 12. Assessment of bacterial clearance from spleens (right) and draining lymph nodes (left) of vaccinated mice (n=3 per group). Mice were vaccinated via the subcutaneous route at the tail base.

As shown in Fig. 12, all rBCG strains were cleared from spleens of vaccinated mice within 60 days. Although most of the vaccinated mice did not exhibit any presence of live bacteria in the spleen by day 30, some outliers appear to harbour circa 200 bacteria – likely due to individual differences between mice. BCG SSI 1331, the standard vaccine strain used worldwide seems to circulate in the draining lymph nodes of the host beyond 120 of vaccination. This result proves that the rBCG strains, including rBCGIL-7 and rBCGIL-18 while uncompromised in their ability to grow axenically *in vitro* are however subject to rapid clearance *in vivo*, thus fulfilling an important requirement of a live vaccine candidate of choice.

4. Modulation of antimycobacterial CD4+ T cells responses by rBCGIL-7 and rBCGIL-18.

In order to investigate whether co-administration of IL-7 and IL-18 with BCG *ΔureC*::*hly* could enhance the quality of the cellular immune response, CD4+ T cells responding to *M. tuberculosis* antigens in whole cell lysate were assessed via flow cytometry. The major criterion applied in this analysis was expression of the afore-mentioned CD40L, a T cell activation marker observed only upon antigenic stimulation. Lungs, spleen and dLNs of vaccinated mice were used for this purpose, to examine the population of tissue-resident antimycobacterial T cells up to 120 days post vaccination.

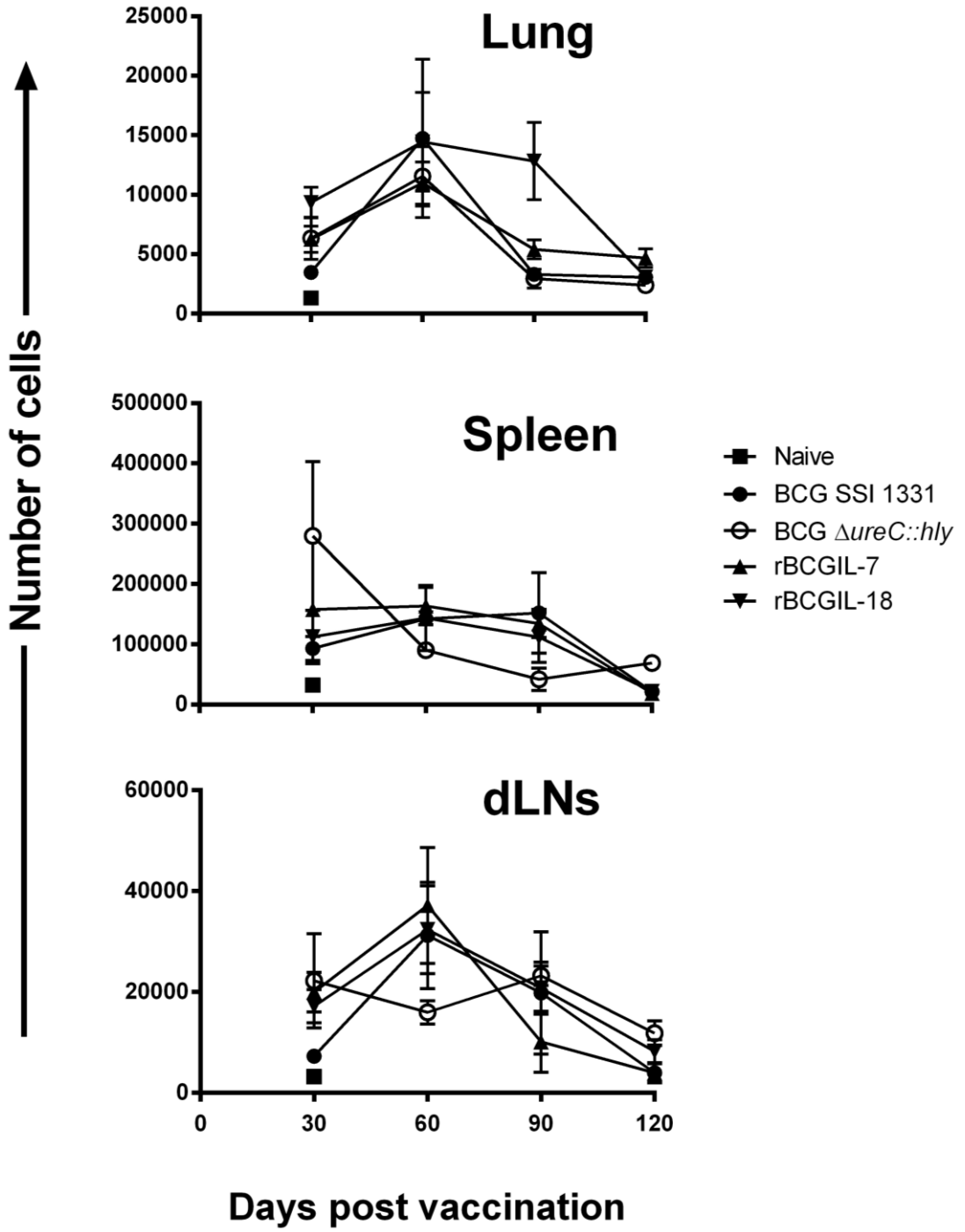

Figure 13. Total numbers of CD4+ T cells expressing CD40L+ in lungs, spleen and draining (inguinal) lymph nodes of vaccinated mice upon stimulation with *M. tuberculosis* H37Rv whole cell lysate. Shown are means ± SEM of 3 mice per group.

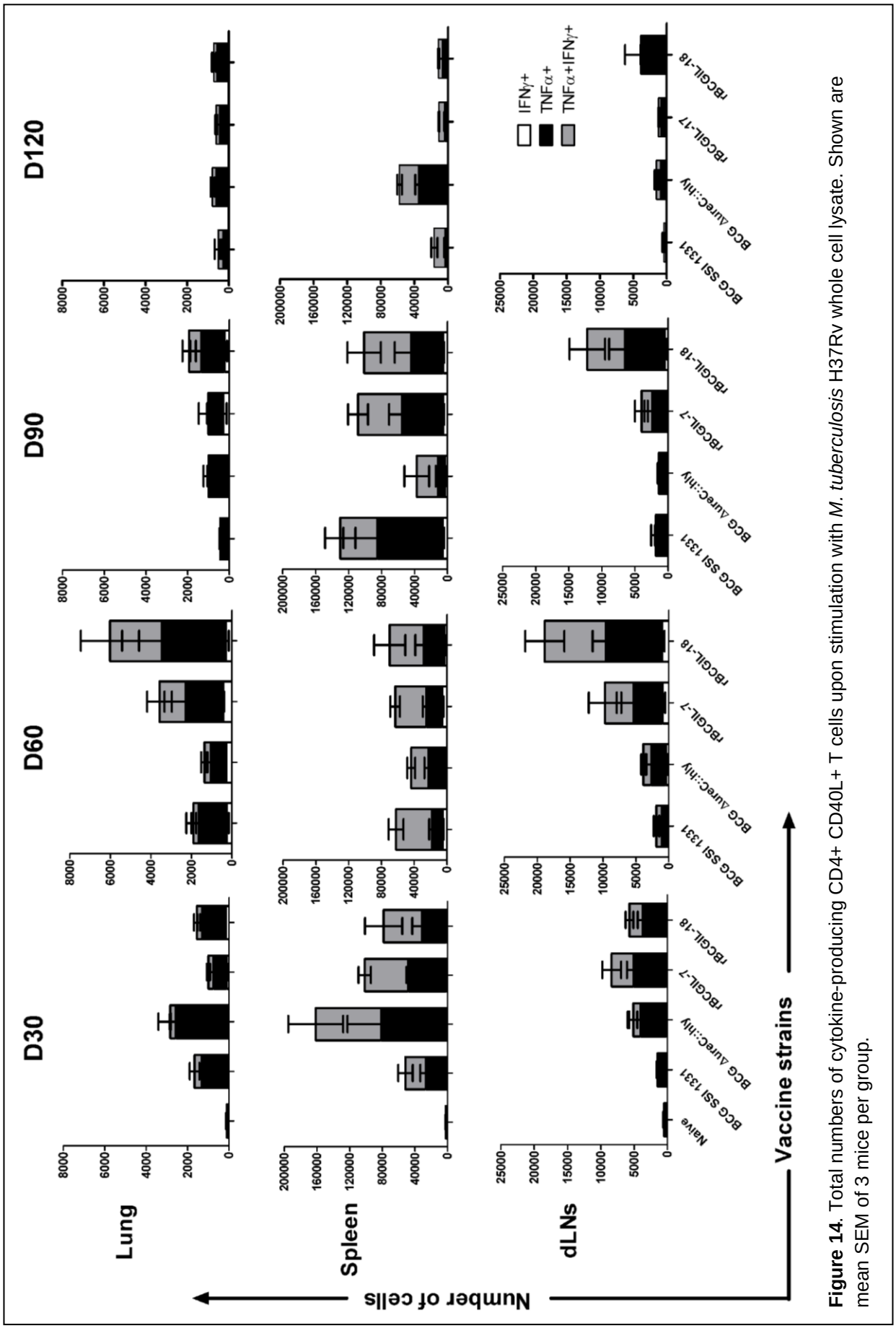

Figure 14. Total numbers of cytokine-producing CD4+ CD40L+ T cells upon stimulation with *M. tuberculosis* H37Rv whole cell lysate. Shown are mean SEM of 3 mice per group.

At first glance, no differences can be observed concerning CD40L+ CD4+ T cells between mice vaccinated with BCG *ΔureC::hly*, rBCGIL-7 or rBCGIL-18 (Fig. 13). Although rBCGIL-18-vaccinated might appear to have slightly more CD4+ CD40L+ T cells in the lung 30 days post vaccination, this number is not significantly higher than the rest. The triple-positive population (for CD40L+TNF-α^+IFN-γ^+) and double positive (CD40L+TNF-a^+) populations seem to be major subsets induced by all the vaccine strains tested in this study (Fig. 14). However, more TNF-α- and IFN-γ-producing CD4+ CD40L+ T cells appear in the lungs of mice 60 days post vaccination with rBCGIL-18. Also, rBCGIL-18 seems to promote generation of the same triple positive population in the lymph nodes up to 90 days post vaccination. In any case, due to the high variability between mice in every group, statistically-significant conclusions cannot be made unless a much larger study cohort is employed.

5. Serum cytokine responses elicited by rBCCIL-7 and rBCGIL-18 in vaccinated mice.

The presence of circulating cytokines in sera of vaccinated mice was also investigated to obtain a global view of modulation of systemic immune responses over time. This was carried out using a mouse multiplex assay comprising 23 cytokines.

The results shown in Fig. 15 represent data obtained on day 7 post vaccination as no observable differences were seen for the rest of the time points (Days 14 to 120). Although rBCGIL-18 appears to induce higher secretion of G-CSF, IL-2, IL-6 and eotaxin compared to BCG *ΔureC::hly*, there is absence of significant difference in the cases of CCL5, TNF-α and KC, the murine equivalent of IL-8. In this study, BCG *ΔureC::hly* is considered the reference or parental strain and only the first 4 cytokines mentioned here show significant differences between rBCGIL-18 and BCG *ΔureC::hly*. IL-2 is important for T cell proliferation, and can be co-induced by IL-7 secretion. However, rBCGIL-7 does not seem to exert this effect as opposed to rBCGIL-18. Eotaxin, on the other hand is a chemoattractant of eosinophils with noteworthy involvement in airway hyperreactivity. Intratracheal administration of IL-18 to cockroach allergen-sensitised mice was shown to increase the eosinophil population in the lung, and this effect was abrogated in the eotaxin-deficient strain (Campbell, Kunkel et al. 2000).

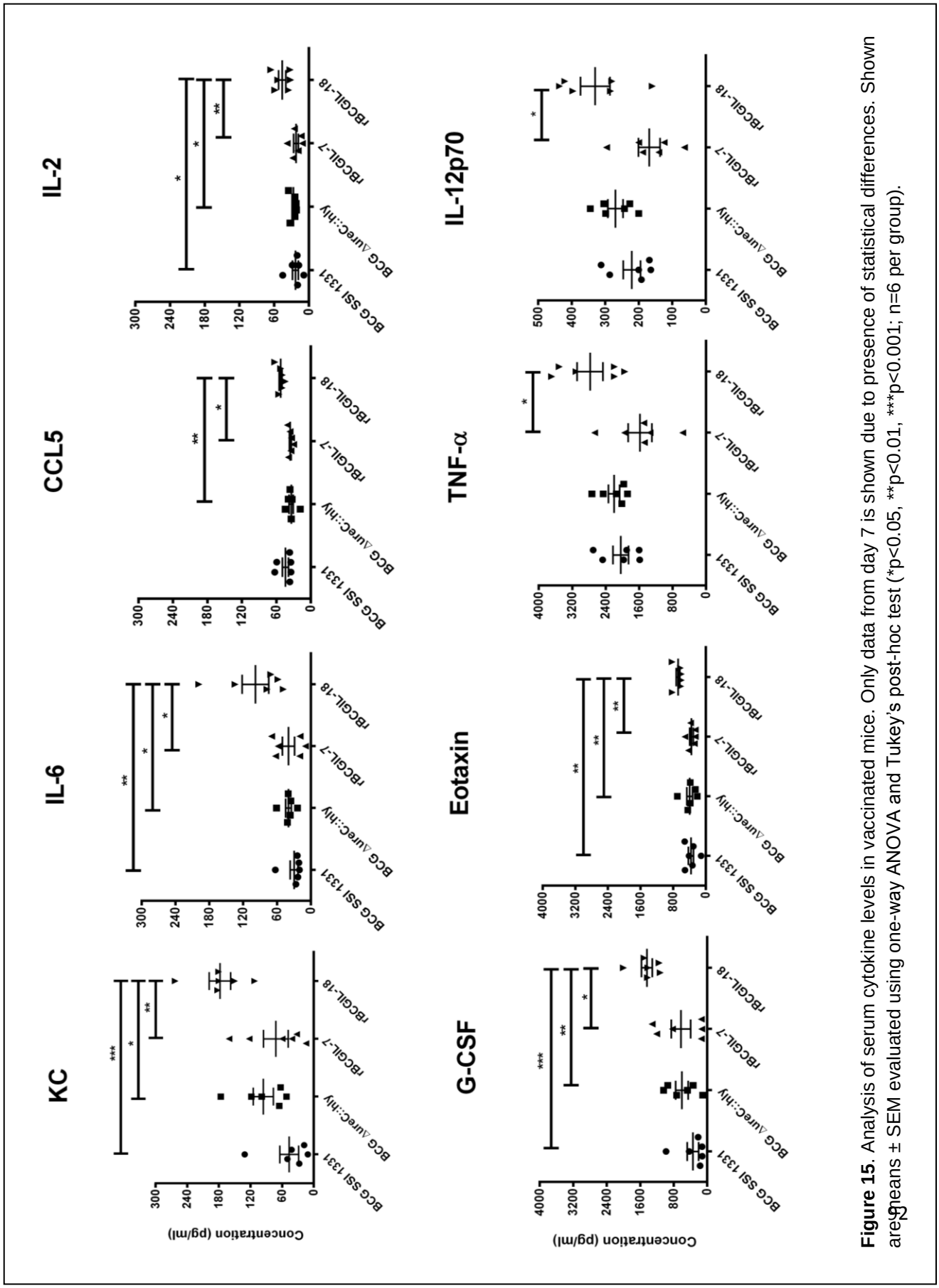

Figure 15. Analysis of serum cytokine levels in vaccinated mice. Only data from day 7 is shown due to presence of statistical differences. Shown are means ± SEM evaluated using one-way ANOVA and Tukey's post-hoc test (*p<0.05, **p<0.01, ***p<0.001; n=6 per group).

6. Protective efficacy of rBCGIL-7 and rBCGIL-18 in a murine aerosol challenge model of *M. tuberculosis.*

The goal of this work was to investigate whether co-expression of hIL-7 or hIL-18 by BCG *ΔureC::hly* could contribute to better *in vivo* protection against *M. tuberculosis* infection. Using a murine aerosol challenge model, BALB/c mice were vaccinated and allowed 90 days for establishment of memory antimycobacterial T cell responses. On day 91 post vaccination, mice were aerosolically challenged with 200 CFUs of virulent *M. tuberculosis* H37Rv. At various time points post aerosol challenge, mice were sacrificed and their lungs as well as spleens were processed for CFU enumeration. A summary of the experimental procedure is schematically shown in Fig. 16.

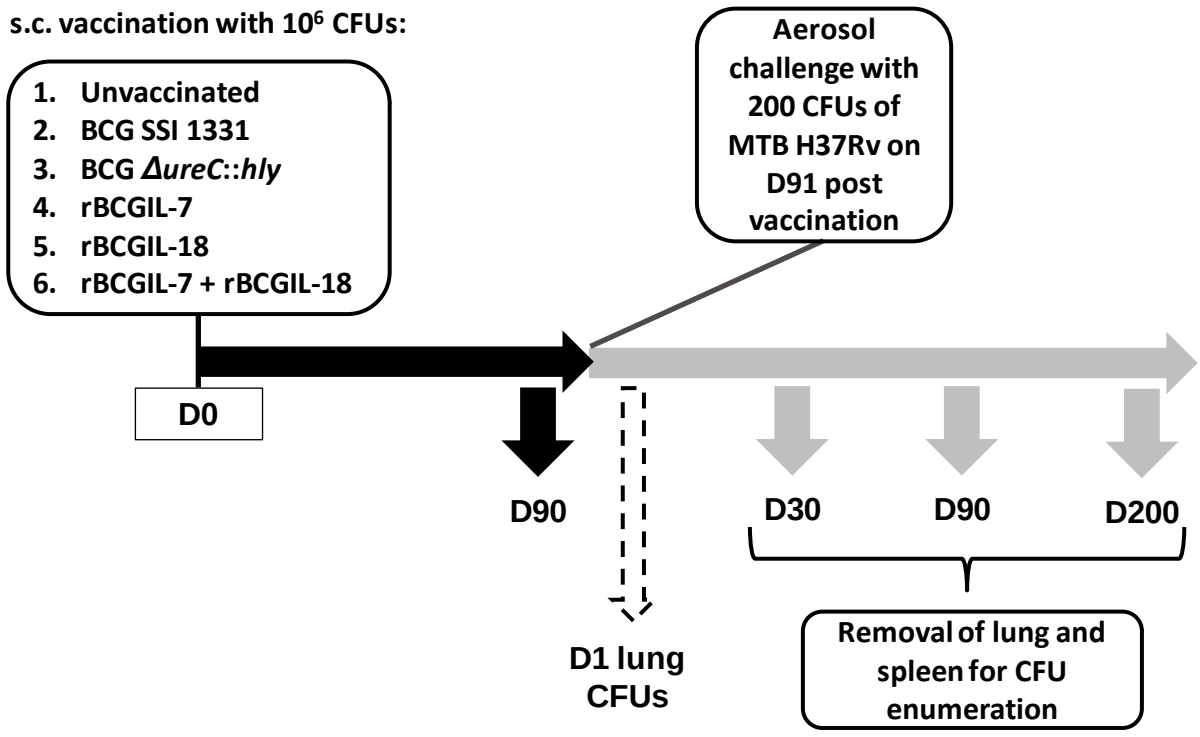

Figure 16. Schematic of the mouse TB challenge model used for investigating protective efficacy of rBCGIL-7 and rBCGIL-18.

No significant difference was observed in reduction of bacterial load afforded by either rBCGIL-7 or rBCGIL-18 in comparison with BCG *ΔureC::hly*, (Fig. 17). Mice that were simultaneously vaccinated with rBCGIL-7 and rBCGIL-18 also did not show any better protection compared to BCG *ΔureC::hly*, disproving the possibility of synergy between the two experimental strains. Nevertheless, I did observe stagnation of bacterial burden in the lungs of mice vaccinated with either rBCGIL-7 or rBCGIL-18 between 90 and 200 days post aerosol challenge with *M. tuberculosis* H37Rv. To my knowledge, ours is one of the few laboratories to employ long term protective efficacy study in mice and as shown in Fig. 17, BCG *ΔureC::hly* affords an impressive 2.5-fold reduction in *M. tuberculosis* H37Rv burden in the lungs compared to BCG SSI 1331 which only provides 1.5-fold reduction in relation to unvaccinated (naïve) mice.

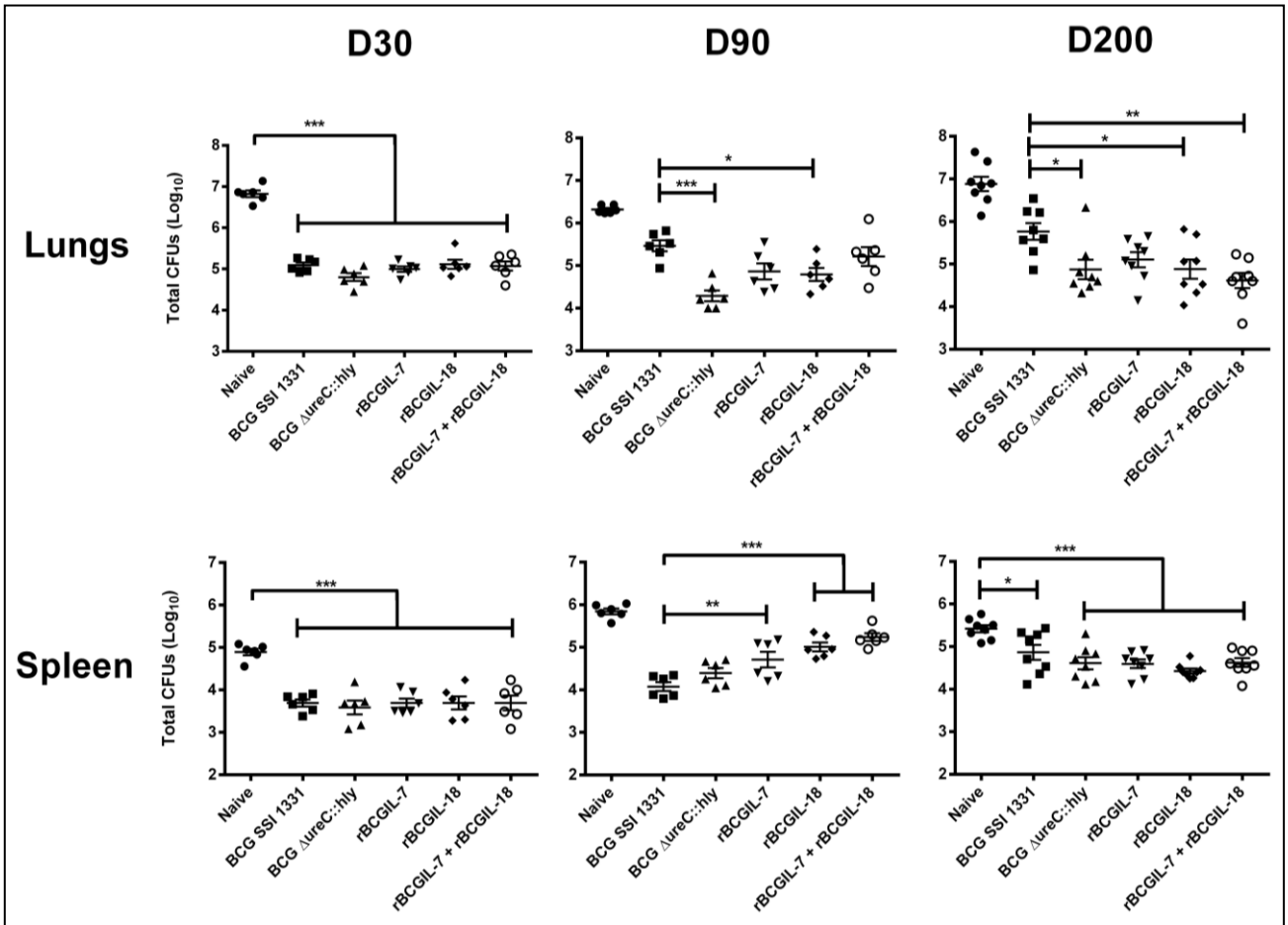

Figure 17. Total CFUs of *M. tuberculosis* in lungs and spleens of vaccinated mice. Shown are means ± SEM, evaluated using one-way ANOVA and Tukey's post-hoc test (*p<0.05, **p<0.01, ***p<0.001; n=6-8 per group).

In summary, the data obtained suggests that incorporation of human cytokines IL-7 or IL-18 into BCG *ΔureC::hly* does not improve the quality of protective immunity to *M. tuberculosis* infection. Nonetheless, rBCGIL-18 is still capable of activating TNF-α and IFN-γ-producing mycobacterial-specific CD4+ T cells in the inguinal lymph nodes of vaccinated mice. Also, DCs infected with this strain appear to secrete more pro-inflammatory cytokines compared those infected with either BCG *ΔureC::hly* or rBCGIL-7. The results presented in this thesis therefore open up new avenues to investigate with respect to rBCGIL-18 from an immunological perspective.

DISCUSSION

Generation of rBCGIL-7 and rBCGIL-18: two new, second generation BCG ΔureC::hly strains.

In this PhD project, I designed and constructed experimental second-generation recombinant BCG (rBCG) vaccine strains based on the genetic background of BCG *ΔureC*::*hly*, a TB vaccine candidate currently in clinical trials. BCG *ΔureC*::*hly* already show superior protection over BCG SSI 1331 against *M. tuberculosis* infection in the BALB/c mouse model (Grode, Seiler et al. 2005). The main idea behind generation of this strain was to increase access of mycobacteria–derived antigens to the host's antigen processing and presentation machinery to improve control of *M. tuberculosis* infection. Over the years, BCG has been cultured and passaged by various laboratories across the globe, giving rise to at least 13 strains to date (Behr and Small 1999). BCG seems to protect against early disseminated TB meningitis in children but not against more severe pulmonary form in adults especially in high burden settings (Andersen and Doherty 2005; Lalvani and Sridhar 2010). This is jointly affected by exposure to environmental mycobacteria and high disease prevalence, thus altering herd immunity (Marais, Obihara et al. 2005).

As such, my overall aim was to improve the already excellent protective efficacy of BCG *ΔureC*::*hly* by incorporating genes encoding human IL-7 and IL-18, respectively. The goal was to capitalise on the phenotypic advantages of BCG *ΔureC*::*hly* - enhanced apoptosis of infected host cells and increased escape of protective antigens into the cytosol. Here after, I sought to investigate whether co-expression of human cytokines would have a pronounced effect on expansion of mycobacteria-specific T cells primarily induced by BCG *ΔureC*::*hly*. I therefore derived two new rBCG strains capable of stably co-expressing human IL-7 (rBCGIL-7) or IL-18 (rBCGIL-18). Hence, my target was to imply that administering BCG *ΔureC*::*hly* with cytokines that act as natural adjuvants would result in the development of more sustained and efficient antitubercular immunity, reflected in generation and maintenance of memory T cells. By doing so, long term protection against *M. tuberculosis* may be achieved with T cells recognising mycobacterial antigens expressed in chronic infection. To the best of my knowledge, my attempt was the first to successfully result in the generation of a recombinant BCG strain expressing human IL-7.

rBCGIL-7 and rBCGIL-18 shows in vitro phenotypes – uncompromised intracellular growth, T cell activation and induction of cytokine secretion by infected DCs.

Presence of both recombinant human IL-7 and IL-18 was detected by ELISA in whole cell lysates of rBCGIL-7 and rBCGIL-18, respectively. However, I could not find cytokine content in bacterial culture supernatants since it was below the detection limit. It has already been shown that mycobacterial antigens can access the cytosol of BCG *ΔureC*::*hly*-infected murine bone marrow-derived MΦs, thus proving the activity of LLO-mediated phagosomal membrane perforation (Grode, Seiler et al. 2005). Therefore, I hypothesised if the cytokines (IL-18 in particular) could also access the host cell's endogenous secretion pathway, an effect may eventually be observed resulting from increased secretion of IL-18. Secretion of host-derived mature IL-18 (both in mouse and man) is dependent on the activity of caspase-1, which is also responsible for the processing and release of mature IL-1β (Gracie, Robertson et al. 2003). IL-18, similar to IL-1β is non-conventionally secreted and lacks a signal peptide for protein export (Dinarello, Novick et al. 1998). Upon receiving highly potent stimuli i.e. LPS, the inactive form of this cytokine, pro IL-18 is constitutively produced especially in the MΦ (Swain 2001). However, the *bona fide* secretion pathway through which mature IL-18 is released upon caspase-1 cleavage has yet to be identified. Since BCG *ΔureC*::*hly* promotes escape of processed mycobacterial molecules into host cell cytosol, I speculated that recombinant human IL-18 expressed by rBCGIL-18 could do the same and eventually be secreted along with host-derived IL-18. Fig. 1 schematically shows the scientific rationale behind co-expression of either human IL-7 or IL-18 by BCG *ΔureC*::*hly*.

Human IL-18 has been previously shown to exert its activity in the murine dermis by inducing neovascularisation (Skopinski, Rogala et al. 2005). As such, heterologously expressed recombinant human IL-18 even when endogenously secreted by murine MΦs should have a pronounced biological effect on T cell as well as phagocyte activation. The vastly pro-inflammatory activities orchestrated by IL-18 generally results in strong adaptive immune responses i.e. triggering of IL-17A, IFN-γ and TNF-α secretion by CD4+ T cells (Puren, Fantuzzi et al. 1998; Nakajima and Owen 2012). This is a reason why IL-18 is pathogenic in autoimmune and allergic diseases, where blocking IL-18 protein has proven to mediate rheumatoid arthritis, eosinophilic airway hyperreactivity and dermatitis (Campbell, Kunkel et al. 2000; Kanda, Shimizu et al. 2007; Marotte, Tsou et al. 2011). In the scenario of BCG vaccination and TB, the early inflammatory response triggered by IL-18 may on the contrary culminate in shaping of robust cell-mediated adaptive immunity in the long run.

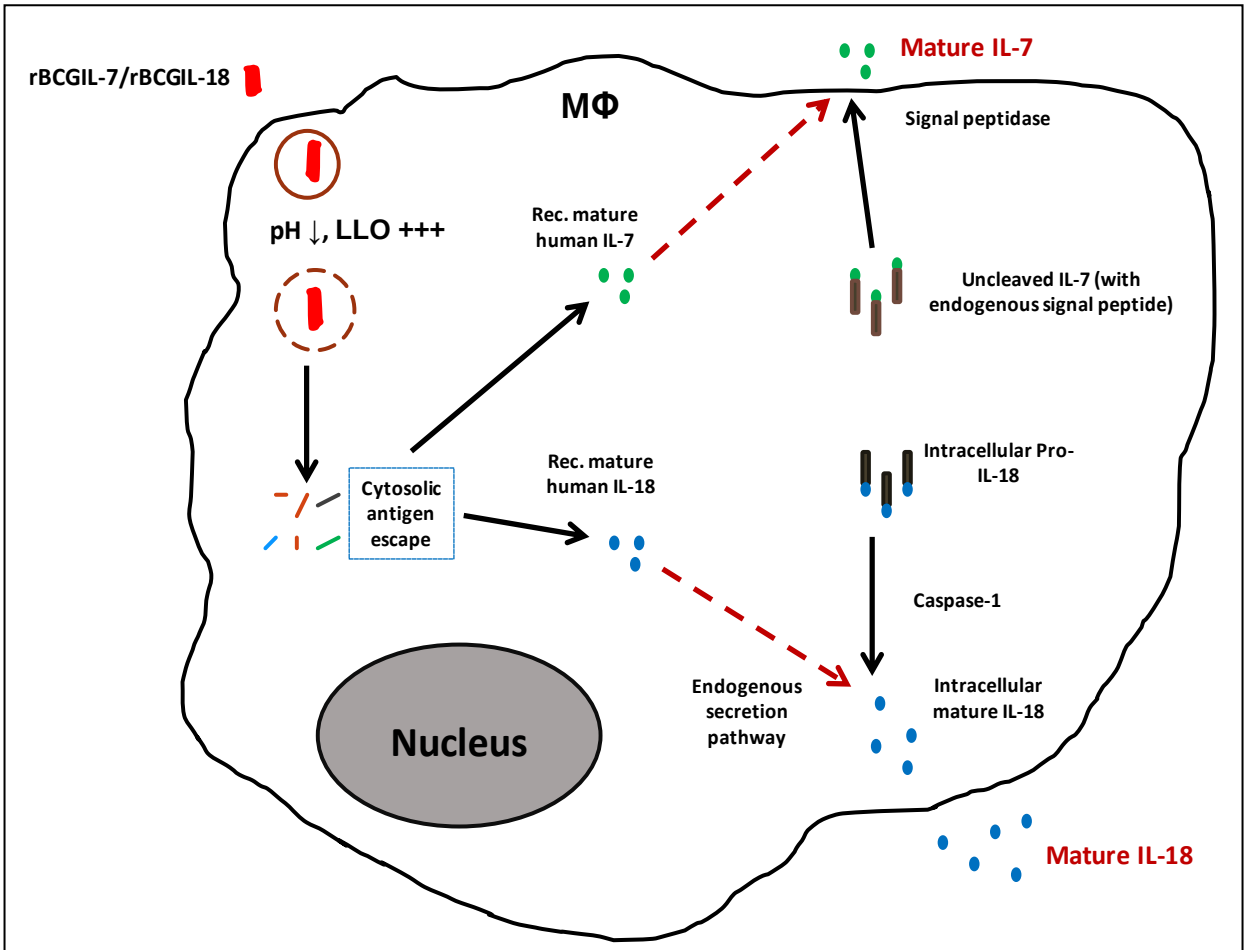

Figure 1. Schematic representation of the hypothesis supporting design of rBCGIL-18 and rBCGIL-7 based largely on escape of mycobacterial antigens into the host cell cytosol. Note that release of mature IL-7 requires cell membrane-bound signal peptidase activity. *MΦ = macrophage*

It was rather challenging to determine the biological effects of human IL-7 and IL-18 expressed by the respective rBCG strains due to abundant presence of mycobacterial proteins in both whole cell lysates and culture supernatants. I used bacterial infections as well as whole cell lysates and supernatants as stimulants that subsequently yielded only convoluting results: NF-κB and IRF-1 activation in THP-1 MΦs, IFN-γ secretion by as well as p38 phosphorylation in human PBMCs and T cells. However, cell debris of wild-type BCG alone was capable of orchestrating activation of all the above-mentioned pathways via TLR signalling.

Assessment of intracellular survival dynamics of rBCGIL-7 and rBCGIL-18 in human MΦ and DCs showed no compromise in survival, indicating that genetic manipulation additionally introduced into BCG *ΔureC*::*hly* did not disrupt normal bacterial growth (Results, Fig. 8A). I

found it rather intriguing that growth of the rBCG strains was more restricted in DCs than MΦs, and that the pro-inflammatory cytokine milieu was again sourced by the DCs. The noticeable increase of IL-6, G-CSF and RANTES content in culture supernatants of DCs infected with rBCGIL-18 suggested that this strain is capable of eliciting expression of highly potent pro-inflammatory immunomodulatory molecules early in host-pathogen interaction (6 days post infection; Results, Fig. 9). The differences in cytokine levels produced by infected human DCs showed that rBCGIL-18 exerted a biological effect in addition to that of BCG *ΔureC*::*hly*. As such, I concluded that rBCGIL-18 might possess the capacity to orchestrate successful T cell interactions with APCs, which was addressed in the *in vitro* antigen presentation / T cell activation assay.

When human DCs were infected with the respective rBCG strains and co-cultured with T cells from the same donor, superior T cell stimulation was observed, as reflected by IL-2 production (Results, Fig. 11). Since donors were PPD^+ and BCG-vaccinated, they represent antigen-experienced hosts whose antigen-specific CD4+ T cells would readily respond to re-challenge with mycobacterial ligands. I chose to incubate the cells over 20 hours as this provides at least one replication cycle of intracellular bacteria in DCs, with an impact on antigen turnover, processing and presentation to T cells. In this regard, it is imperative that significant difference was only seen in comparison with wild type BCG but not among the rBCG strains themselves. Considering that comparisons in this experiment were made between the newly-derived rBCG strains and BCG *ΔureC*::*hly*, phenotypic differences were yet to be found at this juncture. TCR activation is known to trigger IL-2 secretion by and STAT5 phosphorylation in T cells (Welte, Leitenberg et al. 1999; Malek 2008). Therefore, mycobacterial debris as TCR agonists may have this effect and in turn, lead to IL-2 secretion.

Immunomodulatory properties of rBCGIL-7 and rBCGIL-18 in vivo.

Since my prime aim was to improve T cell responses to a wide array of mycobacterial antigens, the critical experiment in this project was to evaluate the modulation of effector CD4+ T cells co-expressing CD40L, IFN-γ and TNF-α in response to whole cell lysate of *M. tuberculosis* H37Rv. I chose to perform *ex vivo* stimulation of mouse organ-derived cell suspensions with whole cell lysate instead of single protein antigens because the former encompasses a plethora of immunogenic stimuli for CD4+ T cells – TCR-specific antigens, TLR agonists comprising a range of pathogen-associated molecular patterns or PAMPs (MacLeod and Wetzler 2007).

The major *M. tuberculosis* antigen Ag85B which is also expressed in BCG is a very noteworthy immunogen that enhances antitubercular immune responses (Horwitz, Harth et al. 2000). It was however recently shown that *M. tuberculosis* downregulates Ag85B expression in the chronic phase of infection in the mouse model (Wolf, Desvignes et al. 2008). It is therefore plausible that antigens found in whole cells lysates of axenically-grown *M. tuberculosis* might be differentially expressed in the host and thus, could escape immune surveillance. The pore-forming antigenic protein ESAT-6 for instance, which is commonly used for *in vitro* stimulation of T cells, is not even expressed in BCG. This is due to deletion of the RD-1 encoding genes necessary for expression and secretion of ESAT-6 and its chaperone CFP-10 from all known strains of BCG owing to attenuation by continuous passaging in broth culture (Behr, Wilson et al. 1999; Brosch, Gordon et al. 2007).

Cytokine production in response to TLR2 activation could potentially be considered masking the aftermath of TCR-mediated T cell activation, thus contrasting antigen-specific stimulation (MacLeod and Wetzler 2007). However, CD40L expression is exclusive to TCR signalling and accentuates antigen-specific host CD4+ T cell response as seen in impaired antiviral and antitumour immunity of mice lacking CD40L (Grewal and Flavell 1998). $CD40L^+$ CD4+ T cells are equally indicative of Th1 cells that help activate DCs to enhance antigen processing and presentation (Seder, Darrah et al. 2008). Moreover, since CD40L is expressed by almost all T cell types including CD8+ T cells and γδ T cells, I envisaged to capture a larger pool of antimycobacterial CD4+ T cells responding to all types of *M. tuberculosis* antigens. This is also in concert with the ability of BCG *ΔureC*::*hly* to generate a wider repertoire of antigen specificity due to its superior apoptotic potential (Grode, Seiler et al. 2005). I therefore assume that $CD40L^+$ CD4+ T cells co-expressing Th1 cytokines constitute the *bona fide* effector T cell subsets recognising *M. tuberculosis* antigens.

Antigen-experienced and activated CD4+ T cells normally exit the lymph nodes via the lymphatics and traffic to the spleen, forming a memory repertoire with antigen specificity (Janeway's Immunobiology, 7^{th} Ed.). In my hands, this phenomenon is not seen with BCG *ΔureC*::*hly*, rBCGIL-18 or rBCGIL-7 - there is no accumulation but rather maintenance of a mycobacteria-specific CD4+ T population which may have migrated from the inguinal lymph nodes between days 30 and 60 post vaccination (Results, Fig. 13). Also in the spleen, the highest proportions of $CD40L^+$ CD4+ T cells belong to the TNF-α^+ IFN-γ^+ subset on days up to 60 days post vaccination (Results, Fig. 14). There is absence of noticeable differences in

proportion sizes between the groups although wild type BCG-vaccinated mice undergo expansion of the CD40L$^+$ TNF-α$^+$ IFN-γ$^+$ and CD40L$^+$ TNF-α$^+$ CD4+ T cell populations 90 days post vaccination commensurate with a slight contraction of both populations in the inguinal lymph nodes.

In the lungs, vaccination of mice with either BCG *ΔureC*::*hly* or rBCGIL-18 appear to facilitate gradual expansion in tissue-resident mycobacteria-specific TNF-α$^+$ CD4+ T cell proportions from days 30 to 90 post vaccination but this number contracts by day 90 (Results, Fig. 14). A similar trend is observed in mice vaccinated either with wild type BCG or rBCGIL-7. Expansion of lung-resident CD40L$^+$ TNF-α$^+$ CD4+ T cells is obvious only 60 days post vaccination although this number decreases after 90 days. Singh and colleagues observed T cell responses of mice to short-term culture filtrates of BCG and *M. tuberculosis* H37Rv as well as PPD 240 days after co-administrating BCG with IL-7 and IL-15 (Singh, Gowthaman et al. 2010). However, since T cells from lymph nodes, spleen and lungs were pooled in this study, organ-specific antimycobacterial CD4+ and CD8+ T cell proportions were not determined separately.

Another interesting observation after 60 days of vaccination is that wild type BCG seems most capable of inducing CD40L$^+$ TNF-α$^+$ IFN-γ$^+$ CD4+ T cells in the lung among the 4 strains. In a South African study lead by Willem Hanekom, whole blood of newborns drawn after various time points post BCG vaccination was stimulated *in vitro* with the vaccine strain itself to identify cytokine-producing CD4+ T cells. The highest proportion was the TNF-α$^+$ IFN-γ$^+$ T cell population, peaking after 10 weeks (ca. 70 days) of vaccination (Soares, Kwong Chung et al. 2013). However, these were circulating CD4+ T cells and it is not possible to determine whether they had been resident in the lung at some point in time.

rBCGIL-18 seems to contribute to a rather sizeable increase in the proportion of CD40L$^+$ TNF-α$^+$ and CD40L$^+$ TNF-α$^+$ IFN-γ$^+$ CD4+ T cells in the inguinal lymph nodes until 60 days post vaccination (Results, Fig. 14). Although contraction of this population is observed on day 90, the finding itself was somehow consistent with my observations *in vitro* and the initial hypothesis as rBCGIL-18- vaccinated mice harbour the highest proportions of these CD4+ T cell subsets. As already mentioned in the Results chapter, IL-18 is a potent inducer of TNF-α expression due to activation of NF-κB and TRAF6 (Chung, Park et al. 2002).

Day 90 post vaccination is significant as a time point in this experiment since it is at this juncture that vaccinated mice are challenged with virulent *M. tuberculosis* H37Rv via the aerosol route for

vaccine efficacy studies in our laboratory. Three months post BCG vaccination generally is considered sufficient for contraction of effector T cell responses to mycobacterial antigens and formation of immunological memory (Soares, Kwong Chung et al. 2013). I observed that all rBCG bacteria were cleared from the host by day 90 while wild type BCG was still detected in the draining lymph nodes (Results, Fig. 12). BCG is generally regarded to provide protection against TB meningitis in children although the protective temporal window lasts between 10 and 20 years post vaccination (Andersen and Doherty 2005).

In South African newborns, BCG-induced effector CD4+ Th1 cell populations (producing either IFN-γ or TNF-α) peaked after 10 weeks of vaccination but gradually waned by week 52 post vaccination (Soares, Kwong Chung et al. 2013). One of the major hypotheses in short-lived protective immunity owing to BCG vaccination is loss of T cell memory. BCG is rather efficient at promoting early effector functions in CD4+ T cells but fails to establish central memory to mycobacterial antigens (Orme 2010). In addition, exposure to environmental mycobacteria such as *M. vaccae*, *M. avium*, *M. scrofulaceum* ('pre-sensitisation') is capable of eliciting T cell activation and proliferation (Brandt, Feino Cunha et al. 2002). Consequently, this leads to antimycobacterial immune responses to BCG itself due to shared antigens, disallowing sufficient time for the vaccine to proliferate in the host to establish protective immunity to TB (Andersen and Doherty 2005; Orme 2010).

I also did not observe BCG-induced CD40L+ CD4+ T cells lasting beyond 90 days post vaccination in the lung (Results, Fig. 13), suggesting better immunogenicity attributable to BCG *ΔureC*::*hly*, rBCGIL-7 and rBCGIL-18. Since inbred mice are not naturally prone to exposure to environmental mycobacteria, this phenomenon was not noticed in my experiments. Also, BCG was able to persist in vaccinated mice for a much longer period than any of the rBCG strains.

Arguably, increased titres of IFN-γ were not seen in sera of mice 7 days post vaccination with rBCGIL-18 (Results, Fig. 15). Being the potent cytokine it is, constant presence of IFN-γ in circulation may unfavourably promote hyperactivation of MΦs and hence, a constant level of inflammation which may well be unfavourable to the host. The potency of IL-18 to induce generation of greater number of IFN-γ^+ TNF-α^+ CD40L+ CD4+ T cells in the host is better seen with *in vitro* re-challenge with an appropriate stimulus (*M. tuberculosis* H37Rv whole cell lysate, in this case) as this subset more rightly indicates antigen specificity.

Elevated levels G-CSF, IL-6 and IL-2 were observed 7 days post vaccination with rBCGIL-18, indicating early inflammatory responses in the inguinal lymph nodes (Results, Fig. 15). G-CSF is commonly associated with neutrophil recruitment, and as mentioned in the introductory chapter, neutrophils might have a role to play in amplifying BCG-induced cellular immune response in mice (Abadie, Badell et al. 2005). However, it was as intriguing to note that G-CSF levels were elevated in sera of rBCGIL-18-vaccinated mice. Boneberg and Hartung observed suppression of IL-18 and IL-1β in LPS-treated human whole blood samples which had been co-incubated with G-CSF (Boneberg and Hartung 2002). G-CSF was then found to inhibit caspase-1 activity, and the downstream effects also resulted in reduced TNF-α secretion. In another study, murine spleen and liver cells from mice administered with G-CSF were reported to secrete lesser amounts of IL-18 upon *in vitro* stimulation with LPS then after (Shaklee, Guo et al. 2004).

Although IFN-γ and TNF-α form an important basis for activating MΦ antimycobacterial defence mechanism (Pitt, Blankley et al. 2012), these cytokines alone need not necessarily promote establishment of protective immunity to TB. Gallegos and colleagues observed unchanged protection to *M. tuberculosis* Erdman in *ifnγ*$^{-/-}$ and *nos2* $^{-/-}$ mice after 21 days post aerosol challenge (Gallegos, van Heijst et al. 2011). T cells were adoptively transferred from C7 mice (with transgenic TCR for ESAT-6) to those lacking IFN-γ, TNF-γ or iNOS expression and found that the control of lung *M. tuberculosis* burden remain the same as in wild type mice. There may be other effector mechanisms attributable to CD4+ T cells that may confer protective immunity against *M. tuberculosis* infection.

I envisaged to observe higher titres of IL-2 in sera of mice vaccinated with rBCGIL-7, since IL-7 also stimulates IL-2 production and secretion by activated T cells (Appasamy 1993). However,no significant difference was observed between rBCGIL-7-vaccinated mice and the other groups (Results, Fig. 15). IL-2 is also regarded as a pro-inflammatory Th1 cytokine along with IFN-γ and TNF-α (Pitt, Blankley et al. 2012). Active secretion of IL-2 by T cells usually occurs after TCR-specific antigenic stimulation, with a possible role in improving CD8+ T cell memory and quality of response provided by natural killer cells against viral pathogens (Sallusto, Geginat et al. 2004; Seder, Darrah et al. 2008). Therefore, IL-2 can be employed as readout for T cell activation and proliferation.

The expression of CD127 (IL-7R) on the surface of T cells is required for IL-7-dependent signalling (Takada and Jameson 2009). However, this parameter was not considered in my experiment since CD127 is commonly associated with homeostasis of central memory CD4+

and CD8+ T cells and often downregulated on activated T cells (Schluns, Kieper et al. 2000). Having mentioned so, it may be worthwhile to further characterise the CD40L-expressing CD4+ T cells that appear after 90 days of vaccination with rBCGIL-7, while extending the length of the experiment.

The focus in this project was to expand the pool of mycobacteria-specific CD4+ T cells in the vaccinated host. However, IL-7 is also known to robustly contribute to B cell development in mice (Appasamy 1993; Johnson, Shah et al. 2005; Krawczenko, Kieda et al. 2005). As of recent times, more light has been shed on the possible role played by B cells and antibodies in providing lasting immunity to TB (Achkar and Casadevall 2013). Sebina and colleagues showed long-lived mycobacterial-specific B cell populations in BCG-vaccinated individuals even 20 years after vaccination (Sebina, Cliff et al. 2012). Serum antibody responses to PPD were 40 – 60% higher in healthy adults vaccinated with BCG *ΔureC*::*hly* (clinically termed as VPM1002) compared to BCG SSI 1331 (Grode, Ganoza et al. 2013). Given that antibodies can mediate protection against intracellular pathogens, future vaccination strategies against TB should also incorporate induction of antibody titres in serum (Achkar and Casadevall 2013; O'Garra, Redford et al. 2013)..

However, there is convincing evidence in literature regarding host protection orchestrated by IL-18 especially in the context of mycobacterial infections. Elevated levels of IL-18 have been found in patients with active pulmonary TB (Yamada, Shijubo et al. 2000; Song, Lee et al. 2002), while mice with a disrupted IL-18 gene tend to form larger granulomas compared to the wild type strain in response to *M. tuberculosis* infection (Sugawara, Yamada et al. 1999). Experimental *M. tuberculosis* infections with C57BL/6 mice lacking IL-18 expression also showed increased susceptibility to disease; IL-18-/- mutant mice did not survive beyond 30 days post aerosol challenge while the wild type strain did (Schneider, Korbel et al. 2010). With respect to tuberculous leprosy (TL, caused by *M. leprae*), IL-18 in conjunction with IL-12 was shown to contribute to IFN-γ-secreting CD4+ T cells to mediate protection (Garcia, Uyemura et al. 1999). In experimental infections of mice with virulent *M. avium* which also belongs to the *M. tuberculosis* complex, lower transcripts of IL-18 (IGIF) corresponded with lower IFN-γ levels and hence, altered protection against intravenous challenge with *M. avium* (Kobayashi, Nakata et al. 1997). I therefore have reliable basis for selecting IL-18 as a candidate.

A previous study from our laboratory has shown that IL-17-producing CD4+ T cells may play a role in the superior protective efficacy conferred by BCG *ΔureC*::*hly* (Desel, Dorhoi et al. 2011).

In my PhD project however, I sought to investigate whether the biological effects of IL-18 and IL-7 could be seen in expansion of CD40L-expressing CD4+ T cell populations with respect to IFN-γ and TNF-α production, the two cytokines that are directly influenced by IL-18 while forming the signature for Th1 effector functions. Therefore, IL-17 was excluded as a study criterion in this regard.

In the same way, I expected rBCGIL-18 to also stimulate IFN-γ production by activated T cells in concert with host-derived IL-12. Although I did notice some induction of IL-12p70 in sera of rBCGIL-18-vaccinated mice (Results, Fig. 15), the overall detected levels were rather low and only significantly higher than those induced by rBCGIL-7. One important point to ponder upon is the very refractory nature of BCG *ΔureC::hly* to further genetic manipulation. In any case, BCG is generally more resistant to genetic modification when compared to *M. tuberculosis* itself. For example, BCG transformed with plasmid DNA can take up to 4 weeks to present with colonies on the agar while *M. tuberculosis* subjected to the same procedure yields results in circa 2 weeks. Several attempts to establish expression of heterologous proteins implemented in our laboratory were not met with much success. Since LLO is secreted by the same system in BCG *ΔureC::hly*, systemic overload could have compromised Ag85B dependent protein secretion.

rBCGIL-7 and rBCGIL-18 emulate the protective efficacy of BCG ΔureC::hly.

Protective immunity to human pulmonary TB is shrouded by a myriad of factors, accentuated by ongoing discovery of correlates of protection to clinical disease. BCG-induced immunity generally lasts between 10 and 20 years post vaccination (Andersen and Doherty 2005). However, in the case of BCG *ΔureC*::*hly*, its excellent safety profile and superior immunogenicity in healthy adults suggests that rational genetic manipulation of wild type BCG may potentially give rise to a better vaccine candidate for human use (Grode, Ganoza et al. 2013).

The noteworthy early immunomodulatory properties of rBCGIL-18 in particular did not reflect in superior protective efficacy against *M. tuberculosis* infection in mice (Results, Fig. 17). Animals vaccinated with rBCGIL-18 that were subjected to aerosol challenge with virulent *M. tuberculosis* H37Rv only showed comparable protection to that afforded by BCG *ΔureC*::*hly*. After 90 days of infection, bacterial burden in lungs of the rBCGIL-18 and BCG *ΔureC*::*hly* groups are similar to each other. Previous studies employing vaccination of mice with recombinant BCG secreting murine IL-18 only reported elevated numbers of IFN-γ-producing splenocytes but not protection against *M. tuberculosis* infection (Biet, Kremer et al. 2002; Luo, Yamada et al. 2004).

Concluding note.

Observations from vaccination studies performed in this project underline the need to further investigate the immunomodulatory properties of rBCGIL-7 and rBCGIL-18. Proliferation was observed when culture supernatants of *hIL7* cDNA-transfected COS-7 cells containing recombinant human IL-7 were added to murine pre-B cells (Goodwin, Lupton et al. 1989). However, heterologous expression of human IL-7 by a recombinant bacterium and at much lower concentrations may not be sufficient to elicit a significant effect on naïve and/or activated T cells. This is relevant to the immunological synapses occurring in the inguinal lymph nodes, where such an effect is envisaged for rBCGIL-7.

Active protein export in mycobacteria can be affected either translationally or post-translationally (Feltcher, Sullivan et al. 2010). For example, the secretion apparatus which translocates mature Ag85B across the mycobacterial cell wall could experience an overload of proteins to be exported and hence, result in an overall lower export rate on the single protein level. Importantly, Ag85B functions as a mycolyl transferase which transports and transesterifies mature mycolic acids during cell wall biogenesis (Belisle, Vissa et al. 1997). As such, the secretion system of Ag85B is of great physiological importance to mycobacterial cell viability and survival at large, thus underlining the efficiency with which this system should operate. Nonetheless, export of non-mycobacterial proteins which maybe differentially charged or possess non native-like conformation to mycobacterial gene products could potentially be recognised in a variable manner. In any case, export of biologically-active LLO was uncompromised in neither rBCGIL-7 nor rBCGIL-18 (Results, Fig. 7) and this effectively retained the phenotypic background of BCG *ΔureC::hly* in both strains.

The quintessence of appraising an experimental vaccine strain lies in testing its protective efficacy in a suitable animal model (McShane 2011). Marcus Horwitz and co-workers who designed and evaluated rBCG30, a recombinant BCG vaccine candidate which overexpresses Ag85B and was preclinically developed observed better protection against *M. tuberculosis* infection in a guinea pig challenge model as opposed to the conventional mouse model (Horwitz, Harth et al. 2000). It must be mentioned that guinea pigs naturally develop human-like hypoxic tuberculous granulomas characterised by pimonidazole staining in the course of lung infection with virulent *M. tuberculosis* (Via, Lin et al. 2008). This makes them an attractive model to study tuberculosis and for vaccine efficacy testing (Williams, Hall et al. 2009). Indisputably, the best available TB animal model remains the cynomolgus monkey, which almost entirely mimics the

dynamics of human TB, including latency (Via, Lin et al. 2008). Reasons supporting suitability of *in vivo* models, therefore include and are not limited to mimicry of diseases states, antigen turnover influencing bioavailability of immunoprotective TCR epitopes as well as architecture of the target organ.

A recently reported *in vivo* model for BCG vaccine efficacy testing employs dermal delivery of BCG in the murine ear as opposed to subcutaneous injection at the tail base (Minassian, Ronan et al. 2011). The murine ear dermis holds resemblance to the skin of a human newborn baby, thus providing a more true-to-life scenario for evaluating early immunological events and bacterial replication dynamics at the site of vaccination. Thus, it is possible that rBCGIL-7 and rBCGIL-18 could have a more pronounced effect on molding of antimycobacterial T cells responses which may translate to better protection against *M. tuberculosis* infection if investigated in a different *in vivo* challenge model.

Taken together, my data suggests that more work is required in order to elucidate mechanisms which contribute to protective immunity against *M. tuberculosis*. Efforts are currently underway in our laboratory to better understand the molecular basis of immunomodulation driven by BCG *ΔureC::hly*. This may lead to development of novel strategies to further improve the quality of protective immune responses elicited by BCG *ΔureC::hly*.

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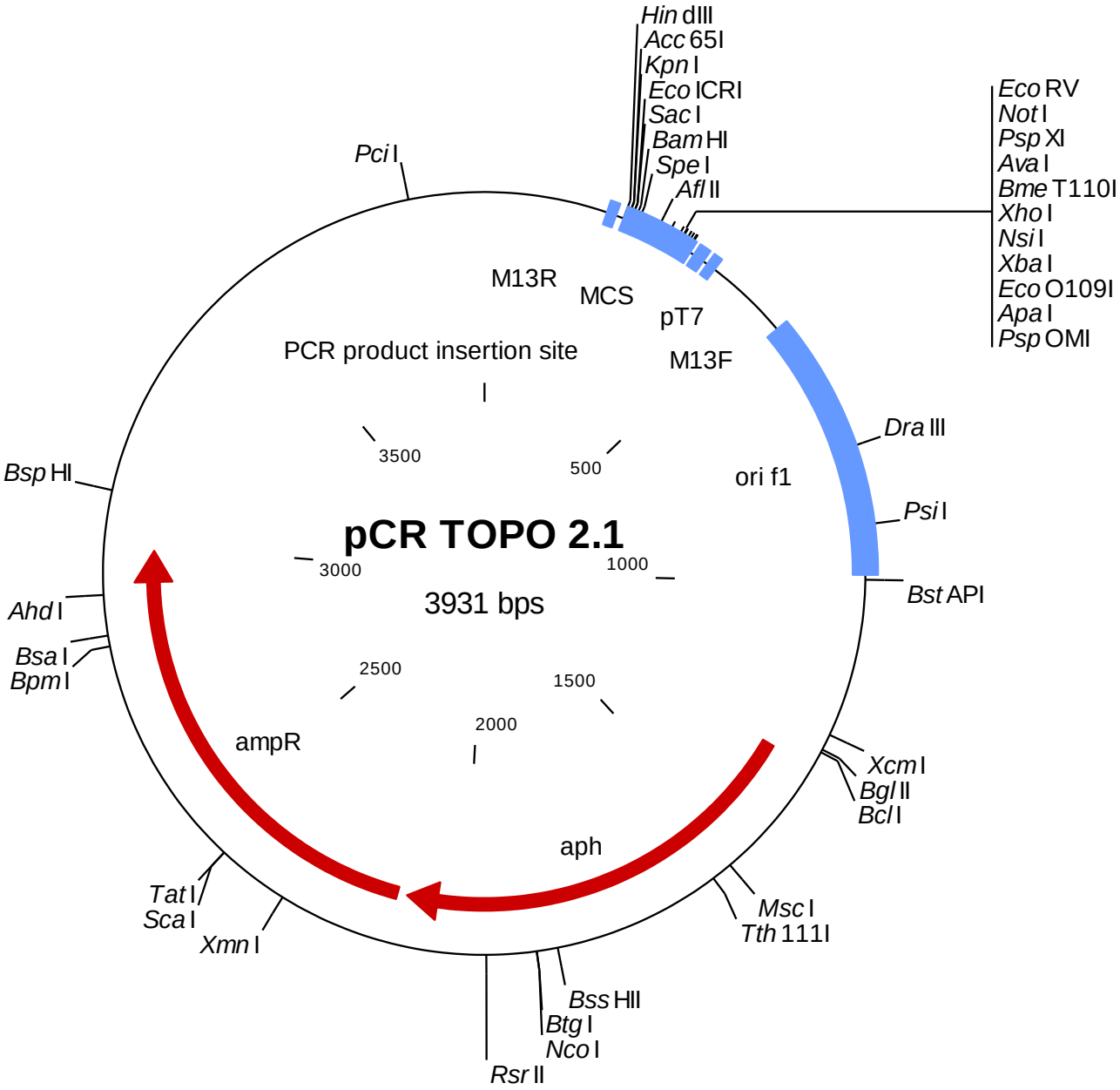
APPENDICES

Appendix 1. List of primers, plasmids and bacterial strains used in this study.

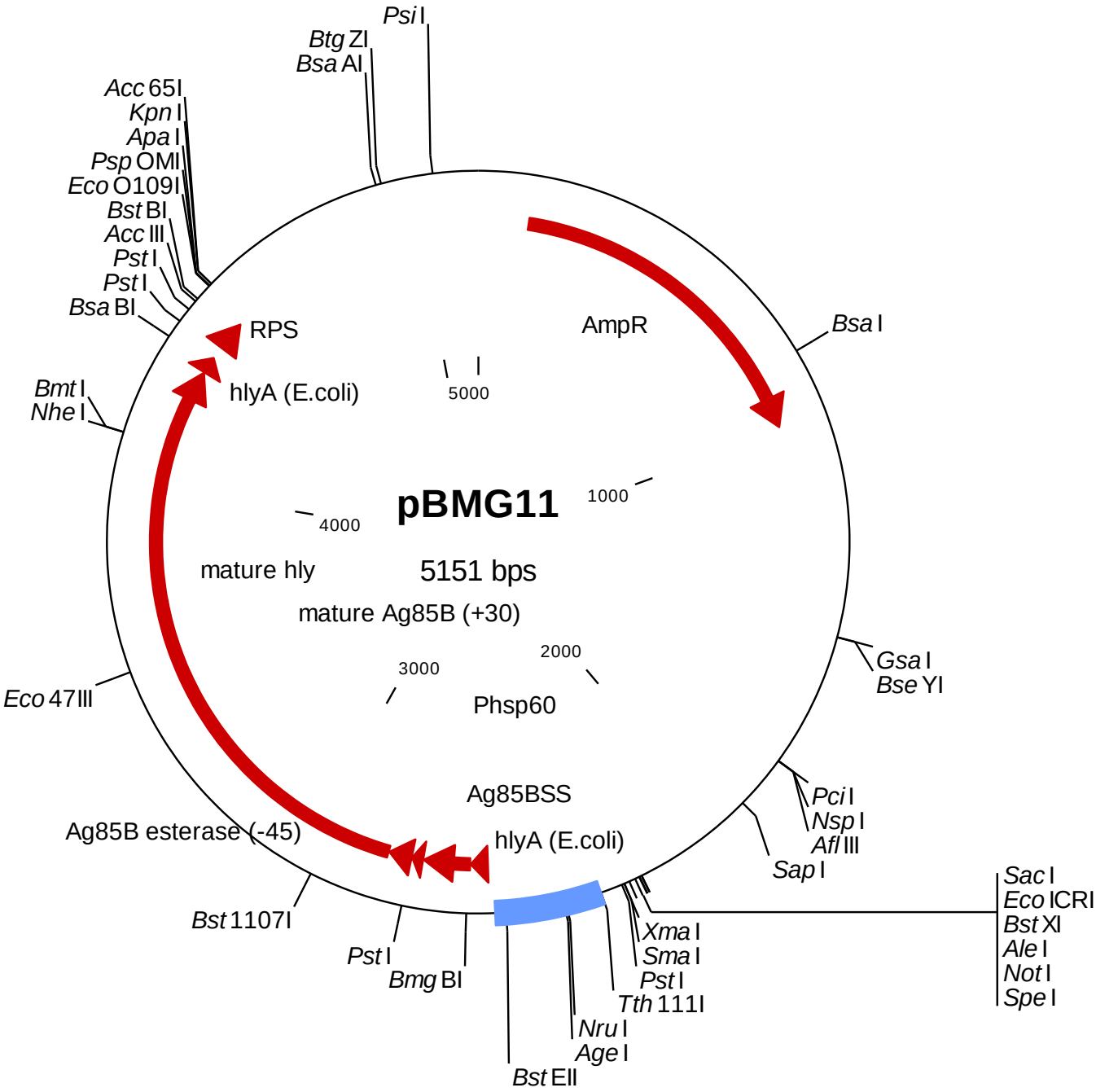
LIST OF PLASMIDS, BACTERIAL STRAINS AND PRIMERS

Plasmids	***Description***	***Reference***
pCR TOPO 2.1	Cloning vector for *E. coli*; M13 forward & reverse priming sites; ampicillin & kanamycin resistance; α-lacZ gene	Invitrogen
pBMG11	Intermediate cloning vector; BCG hsp60 promoter & Ag85B signal sequence; ampicillin resistance; *hly* gene	This study
pMV306	Mycobacterial integrative vector; *int* gene; *attP* site; kanamycin resistance	Bardarov *et al*., 2002
Primers	***Sequence***	
1. FPSecApp	5'-TATCTAGACAAGGTCGAACGAGGGGCA-3' (*Xba* I)	This study
2. RPSecApp	5'-ATATGCATCGCGCCCGCGGTTG-3' (*Nsi* I)	This study
3. FPIL7.fwd	5'-TTATGCATGATTGTGATATTGAAGGTAAAG-3' (*Nsi* I)	This study
4. RPIL7.rev	5'-TTGGTACCCTCGAGTCAGTGTTCTTTAG-3' (*Kpn*I)	This study
5. FPIL18.fwd	5'-TTATGCATTACTTTGGCAAGCTTGAATCTAA-3' (*Nsi*I)	This study
6. RPIL18.rev	5'-ATGGTACCCTCGAGCTAGTCTTCGTTTTG-3' (*Kpn* I)	This study
7. FPIRF1.fwd	5'-TTATGCATATGCCGATCACCCGCATG-3' (*Nsi* I)	This study
8. RPIRF1.rev	5'-TAGGTACCCTCGAGTTACGGGGCGCA-3' (*Kpn* I)	This study
seqhIL7F	5'-TGAAGGTAAAGATGGCAAACA-3'	This study
seqhIL7R	5'-TCAGTGTTCTTTAGTG-3'	This study
seqhIL18F	5'-CTTTGGCAAGCTTGAAT-3'	This study
seqhIL18R	5'-AGCTAGTCTTCGTTTGA-3'	This study
seqCOhIRF-1F	5'-CCGCTCGTGGGCCATCCAC-3'	This study
seqCOhIRF-1R	5'-CGAGGTCGACGGCATCGGC-3'	This study
M13 forward (-21)	5'-TGTAAAACGACGGCCAGT-3'	Eurofins MWG
M13 reverse (-29)	5'-CAGGAAACAGCTATGACC-3'	Eurofins MWG
pMV306F	5'-GATAACCGTATTACCGCCTT-3'	This study
pMV306R	5'-CCAGTCTTTCGACTGAGCCT-3'	This study
Phsp60FWD	5'-TATCTAGACAAGGTCGAACGAGGGGCAT-3'	This study
hlyFWD2	5'-ATTCATTAACACTCAGCATT-3'	This study
hlyREV2	5'-AGATATATGCAGGAGGATTT-3'	This study
pdx1loc.fwd	5'-ATGGATCCTGCAGGTAACCCAGC-3'	This study
pdx1loc.rev	5'-ATGACCGACAGCGATCTCGTCC-3'	This study
Bacterial strains	***Characteristics***	
BCG	*Mycobacterium bovis* BCG; wild type strain	Lab strain
BCG SSI 1331	*Mycobacterium bovis* BCG Danish 1331; wild type strain	SSI, Copenhagen
BCG *ΔureC*::*hly*	urease-deficient recombinant BCG expressing listeriolysin O (rBCG)	Grode *et al*., 2005
One-shot TOP 10	Chemically-competent *Escherichia coli* for transformation	Invitrogen
rBCGIL-7	BCG *ΔureC*::*hly* expressing human interleukin-7	This study
rBCGIL-18	BCG *ΔureC*::*hly* expressing human interleukin-18	This study
rBCGIRF-1	BCG *ΔureC*::*hly* expressing human interferon regulatory factor 1	This study

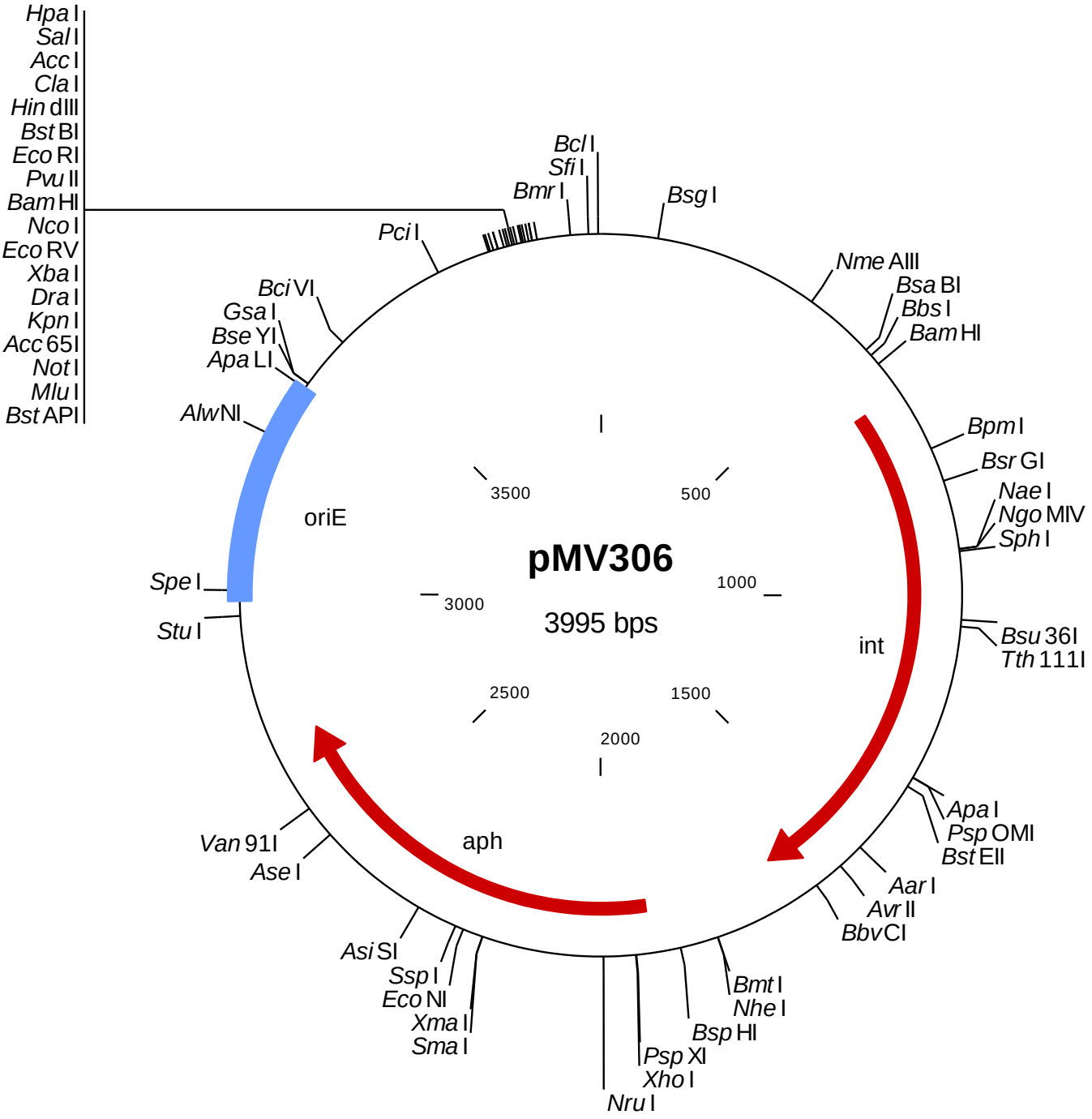
Appendix 2. Plasmid map of pCR TOPO 2.1 (Invitrogen).



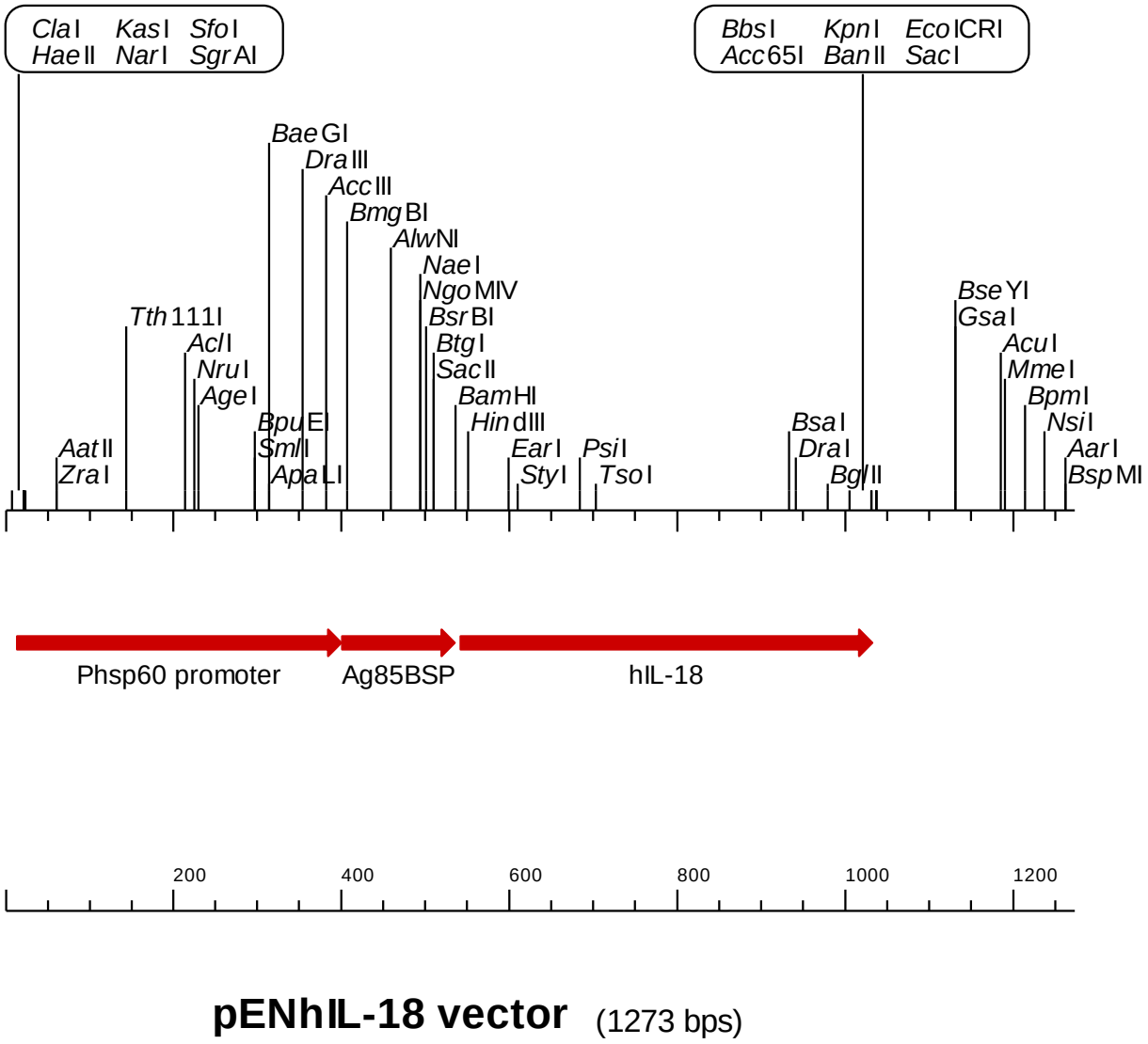
Appendix 3. Plasmid map of pBMG11



Appendix 4. Plasmid map of pMV306.



Appendix 5. Plasmid map of pENhIL-18



pENhIL-18 vector (1273 bps)

Appendix 6. Plasmid map of pMK-RQ-hIRF1.