Evaluation of novel vaccine candidates against tuberculosis in murine models of persistent and latent infection

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spin the wheel of fortune or learn to navigate

JOAKIM MONTELIUS

Für Moni und Erhard

Content

Abbreviations

aa	amino acid
Ag	antigen
AICD	activation induced cell death
APC	antigen presenting cell
BCG	*Mycobacterium bovis* bacillus Calmette et Guérin
BSA	bovine serum albumin
BSL3	biosafety level 3 (S3/L3 in Germany)
CD#	"cluster of differentiation" international nomenclature for cell surface molecules
cfu	colony forming units
DC	dendritic cell
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immuno spot technique
FACS	fluorescence activated cell sorting, flow cytometry
FCS	foetal calf serum
G	gauge
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hours
H&E	Hematoxylin and Eosin Y
i.m.	intramuscular
i.n.	intranasal
i.v.	intravenous
ICCS	intracellular cytokine staining
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IVR	*in vitro* re-stimulation
LTBI	latent tuberculosis infection
M	mol/L
M.tb	*Mycobacterium tuberculosis*

mAb	monoclonal antibody
mfi	mean fluorescence intensity
MHC	major histocompatibility complex
min	minutes
NO	nitric oxide
$OD_{\#}$	optical density, # indicates wavelength in nm
ORF	open reading frame
p.i.	post infection
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RBC	red blood cell
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
rpm	rounds per minute
RT	room temperature
s.c.	subcutaneous
sec	seconds
TB	tuberculosis
T_H1	T-helper cell type 1
T_H17	T-helper cell type 17
T_H2	T-helper cell type 2
TNF-α	tumour necrosis factor alpha
TST	tuberculin skin test
v/v	volume per volume
w/v	weight per volume

1 Introduction

”Not only does man lack the power to create life but his ability to destroy it, at least at the microbial level, is sharply limited” [McDermott 1959].

Humankind has been suffering from tuberculosis (TB) through millennia. Signs of TB have been detected in prehistoric skeletons and in the bones of Egyptian mummies. TB was commonly known as “White Death” or “Wasting Disease”. Symptoms of disease encompass fever, night sweat, weight loss, fatigue, lassitude, rheumatic pains, coughing, dyspnoea, gastroenteritis and lethargy. Lukas Schönlein realised that all these symptoms were part of the same disease and named it TB [Virchow 1865]. The discovery of *Mycobacterium tuberculosis* as causative agent of TB was reported to the “Physiologische Gesellschaft” in Berlin on 24th of March 1882 by Robert Koch [Koch 1882]. During his lecture on the “Ätiologie der Tuberkulose” Koch pointed out, that it was a specific staining procedure, which visualised characteristic, so far unknown bacteria in tuberculous affected organs. Wilhelm Zopf named the organism *Bacterium tuberculosis* in 1883. Three years later the name was changed to *Mycobacterium tuberculosis* (M.tb), probably because the appearance in culture made it look fungus-like [Lehmann 1896].

Soon after Robert Koch’s discovery vaccine development began. Koch introduced tuberculin as a vaccine [Koch 1891]. However, these bacterial components were not protective [Guttstadt 1891]. The French bacteriologists Albert Calmette and Camille Guérin passaged the cattle pathogen *Mycobacterium bovis* over a period of 13 years in a beef-bile-glycerine-medium, thereby developing an attenuated strain that, when used as a vaccine, provided protection to high risk groups, especially newborns of tuberculous mothers. This vaccine strain was called *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). It was introduced into the clinic in 1921 and is one of the oldest applications of live-vaccination [Calmette 1929]. At first, public acceptance was low, and became worse after the disaster 1930 in Lübeck, where 72 infants died, because the vaccine strain had been

contaminated with virulent M.tb [Moses 1930]. To date a total of four billion doses of BCG have been administered, thereby making it one of the most widely administered human vaccines [Reece 2008]. In the 1920s it was already speculated that TB would be conquered by 1950 [Krause 1926]. This was fuelled by the discovery of antibiotics in the 1940s, especially Selman Waksman's discovery of streptomycin as anti-TB drug [Daniel 2005; Schatz 1944]. However, the ability of M.tb to persist is remarkable. Almost 70 years later the World Health Organization (WHO) had to declare TB a global emergency [WHO 1994].

1.1 Tuberculosis: Still a global threat

TB is a contagious disease and, like the common cold, it spreads through the air. When infectious people cough, sneeze, talk or spit, they propel bacteria into the air. Only a small number of inhaled bacteria can cause infection. Inhaled bacteria are deposited in the lung, which becomes the primary site of infection. Left untreated, each person with active TB will infect on average 10 to 15 people every year. Someone in the world is newly infected with M.tb every second. Overall one-third of the world's population is currently infected with M.tb. 5-10% of people who are infected with M.tb become sick and most of these contagious at some time during their life. It is estimated that 1.6 million deaths resulted from TB in 2005. Both the highest number of deaths and the highest mortality per capita are in the Africa Region [WHO fact sheet number 104, revised March 2007]. TB is the leading cause of death among people who are HIV positive and in Africa HIV is the single most important factor contributing to the increase of TB incidences since 1990 [Sonnenberg 2005]. HIV and TB form a deadly alliance, each speeding the other's progress [Kaufmann 2005].

Until 70 years ago, there were no medicines to cure TB. Directly observed therapy, short-course (DOTS) is now an internationally recommended TB control strategy developed in the 1990's [Dye 2005]. The initial regime consists of four drugs: Isoniazid, rifampin, pyrazinamide and ethambutol with a minimum treatment length of six to nine months, which is considered short

course, for the treatment of drug-susceptible TB [Blumberg 2005]. Watching the patient swallow the anti-TB drugs is termed directly observed therapy and can help ensure higher completion rates and prevent the emergence of drug-resistant M.tb strains, thus enhancing TB control.

However, inconsistent or partial treatment lead to the emergence of drug-resistant M.tb strains. Multi drug-resistant TB (MDR-TB) is caused by bacilli which are resistant to at least rifampicin and isoniazid, the two most powerful first-line anti-TB drugs [Zhang 2005]. Drug-resistant TB is generally treatable but requires extensive chemotherapy for up to two years with expensive second-line anti-TB drugs, causing severe but manageable side effects [WHO fact sheet number 104, revised March 2007]. The detection of extensively drug-resistant TB (XDR-TB) in HIV/AIDS infected individuals in South Africa has caused even greater concern. XDR-TB is defined as MDR-TB plus resistance to at least three of the six classes of second-line agents [Singh 2007; Wilson 2007]. The emergence of XDR-TB poses a serious threat to infection control, forcing the WHO in 2006 to launch a new Stop TB strategy, which encompasses expansion of the DOTS scheme, strengthening of health systems as well as the development of improved diagnostics, drugs and vaccines.

1.2 Tuberculosis: A problem with persistence

Latency or persistence describes an *in vivo* situation, where TB infection has become stably established and an immune response developed, which forced bacteria into a quiescent state, referred to as dormancy, resulting in a balance between bacteria and the host without apparent symptoms [Orme 2001]. Persistency refers to M.tb being present despite a vigorous immune response or chemotherapy [Zhang 2004]. Whether persistent organisms are truly dormant, remains to be elucidated. In contrast to patients with active TB, individuals with latent TB do not transmit the disease and do not pose a public health risk [Parrish 1998]. Patients who develop active disease from latent TB infection account for the majority of new cases of TB [Manabe 2000]. Latently infected persons are an important public health problem because they represent a tremendous reservoir for disease. These individuals are also the

major obstacle for eradication of TB by vaccination or chemotherapy. The current anti-TB chemotherapy is effective in killing growing bacteria but largely ineffective in killing persistent or dormant bacteria, leading to prolonged therapy [Wayne 1994b; Zhang 2004]. The resistance of dormant M.tb to conventional anti-TB drugs further complicates treatment efforts. The preferred regimen for the treatment of latent TB infection is nine months of isoniazid [Blumberg 2005].

For more than 100 years the only test to identify latent TB infection (LTBI) was the tuberculin skin test (TST) [Blumberg 2005]. The TST was developed as a result of Robert Koch's vaccination attempts with tuberculin [Koch 1891]. Even though tuberculin was not protective against TB, it proved useful as a diagnostic tool. In this test, a standard preparation of tuberculin, so-called purified protein derivative (PPD) of M.tb, is injected into the skin. PPD induces a delayed-type hypersensitivity reaction that is considered to reflect cell mediated immunity against M.tb. The size of this TST reaction is used to classify individuals according to their likelihood of infection [Whalen 2005]. However, false negative as well as false positive results are common and it is not possible to distinguish BCG vaccination from TB infection [Fine 1999]. Recent tests for the diagnosis of TLBI, based on IFN-γ secretion upon re-stimulation with antigens exclusively expressed by M.tb, namely ESAT-6 and CFP-10, are available and considered to be more reliable [Detjen 2007]. However it has been reported that a response against 16-kDa alpha crystalline, also called heat shock protein X (hspX) [Demissie 2006], or heparin-binding hemagglutinin (HBHA) [Temmerman 2005] may be better markers for latent TB infection than response to PPD or ESAT-6.

1.3 Granuloma formation and the immune response against *Mycobacterium tuberculosis*

Only around 5-10% of infected individuals will develop active TB, half of those within the first few years after infection, the rest later in life as a result of re-infection or reactivation of infection [Styblo 1980; Young 2006]. The other 90% of infected individuals are able to contain the initial infection, due to a vigorous

cell mediated immune response, and become latently infected. However they cannot eradicate the bacilli, hence the statement “once infected, always infected” became well known [Robertson 1933]. Even humans or animals persistently infected with TB or cured by chemotherapy can be super- or re-infected [Chiang 2005; Jung 2005].

TB infection can be divided into three stages: After inhalation of infectious aerosol M.tb replicates unimpeded in alveolar macrophages, then the onset of cell mediated immunity causes bacteriostasis and infection containment inside so-called granulomas. This primary infection site is often referred to as the Ghon complex and is usually found in the mid-region of the lung. These lesions are particularly common in children and are characterised by an apparently healed granulomatous structure in the lung and draining hilar lymphnode [Ghon 1912]. From this primary infection site, bacteria can spread through the lymphatic system to the blood stream and finally re-infect the lungs where secondary lesions form in order to contain the infection [Boshoff 2005]. But if the immune response fails, due to old age, malnutrition or HIV infection, bacteria will resuscitate and start to replicate exponentially, resulting in highly contagious active disease. Given that HIV progression in humans is strongly associated with TB disease is evidence for an important role for CD4 T cells in TB control [Kaufmann 2005]. T cell mediated immunity is critical for protection against TB, mice and humans who lack T cells are very susceptible to lethal disease [Andersen 2005; North 2004].

Experiments with knock-out mice have verified important roles for CD4 T cells [Caruso 1999], CD8 T cells [van Pinxteren 2000], IFN-γ [Flynn 1993b], TNF-α [Bean 1999], IL12 [Cooper 1997] and CD40 [Lazarevic 2003] in protection against TB. NK cells [Vankayalapati 2002] as well as $\gamma\delta$ T cells have been implicated in TB control [D'Souza 1997]. However, B cells and antibodies are generally not considered important in TB [Turner 2001]. IL-4 and IL-10 are thought to have a negative effect on TB control, but knock-out mice do not show altered pathology in response to challenge [North 1998]. Recently, IL-23 has been identified as a new member of the IL-12 cytokine family [Oppmann 2000]. IL-23 can confer protection in the absence of IL-12 [Khader 2005] and is required for the generation of IL-17 secreting CD4 T

cells [Harrington 2005]. Distinct regulatory and inflammatory roles for IL-12 and IL-23 have been proposed [Hoeve 2006]. Clearly, these cytokines are important in maintaining the balance between containment of bacteria and tissue destruction.

Following inhalation, M.tb bacilli are engulfed by alveolar macrophages and later by parenchymal tissue macrophages, too. M.tb is protected by a robust cell wall, rich in waxes and other glycolipids [Kremer 2005], which is a main component for resistance against host defence. Thus, infected resting macrophages fail to destroy the bacteria without help and transport their cargo to the lung parenchyma and eventually to the draining lymphnodes. The infection triggers production of TNF-α, which then induces chemokine secretion and chemokine receptor expression. These chemokines will attract cells that have entered the lung to the sites of infected macrophages [Algood 2003]. Dendritic cells (DC) are also able to take up antigens from apoptotic vesicles, secreted from infected macrophages, and cross-present them to T cells [Winau 2006]. DC present in the lung can also be infected [Inaba 1993; Reljic 2005] because M.tb can mediate its entry into DC, for instance via DC-SIGN [Geijtenbeek 2003; Tailleux 2003]. Infected DC undergo maturation and migrate to the draining lymphnodes [Bhatt 2004; Humphreys 2006]. Despite the long-standing belief that macrophages represent the resident cells for M.tb, a recent publication identified DC as the largest population infected with M.tb early during infection [Wolf 2007]. These cells shuttle bacteria to the draining lymphnodes. Antigen is presented on MHC-I and MHC-II molecules to CD4 and CD8 T cells. Naïve CD4 or CD8 T cells which recognise the antigen become activated [Gonzalez-Juarrero 2001], start to proliferate and migrate to the site of infection, where they exert their effector functions [Saxena 2002]. IFN-γ and TNF-α, secreted by activated cells, synergise and are the major components responsible for activation of macrophages, recruitment of effector cells and the containment of M.tb inside the granuloma. TNF-α is a key cytokine that orchestrates granuloma formation. The importance of this cytokine became evident, as anti TNF-α therapy in arthritis lead to significant reactivation of TB [Brassard 2006]. One key effector

function of IFN-γ is activation of macrophages, thus enabling killing of the bacteria [Cooper 1993; Flesch 1990; Flynn 1993b]. Macrophages activated by T cell derived cytokines generate products with anti-mycobacterial activity, most importantly nitric oxide (NO) and reactive nitrogen intermediates (RNI). This was shown by *in vitro* experiments using murine macrophage cell lines infected with M.tb [Chan 1992; Denis 1991]. Anti-mycobacterial RNI is generated form $_L$-arginine by nitric oxide synthase 2 (NOS2). Whilst it is certain, that NO and RNI are major effector molecules in the control and containment of the infection in mice [Denis 1991; MacMicking 1997], their contribution is less clear in humans [Nathan 2002; Nathan 2006]. Expression of NO synthases by macrophages and giant cells inside granulomas was detected in surgically resected lungs from TB patients, but the contribution of NO and RNI to TB control in humans remains to be elucidated [Choi 2002]. The antimicrobial effector functions of activated macrophages also encompass phagolysosome fusion and generation of reactive oxygen intermediates (ROI) by oxidative burst.

This complex immune response ultimately results in formation of the granuloma around infected macrophages. The granuloma is composed of an aggregation of monocyte-derived macrophages surrounding the macrophages containing intracellular bacteria, followed by a ring of lymphocytes, mainly composed of CD4 and CD8 T cells, but γδ T cells and CD1-restricted αβ T cells as well as B cells are also found [Algood 2003; Ulrichs 2006]. Some of these T cells recognise TB-derived antigens and produce effector cytokines and chemokines that will sustain the proinflammatory response and attract further cells to the sites of infection. At later stages of infection, the granuloma will be surrounded by a fibrotic wall and lymphoid follicular structures, which might also help orchestrating the local immune response [Kahnert 2007; Ulrichs 2004]. The granuloma serves the immune system by containing the bacteria whilst limiting the extent of inflammation and tissue damage. However, even the most powerful immune response to TB only manages to control the infection but cannot eradicate the organism. This pathogen-host

interaction is well balanced and frequently leads to latent and symptomless infection albeit providing a niche for long-term survival for bacteria.
Even though the focus of this thesis is on the adaptive immune response elicited against TB, contributions of the innate immune signalling shall at least be briefly mentioned. Stimulation of toll-like receptor (TLR)-2 by lipoproteins of M.tb elicits a proinflammatory response, which can promote killing [Underhill 1999] and apoptosis of infected cells [Lopez 2003]. Experiments with knock-out mice revealed an important role for TLR-9 in maintaining the T_H1 granulomatous response [Ito 2007]. Signalling of lipoproteins via TLR-1 and TLR-6 also contributes to the proinflammatory response [Takeda 2002].

Ultimately, one important question remains: After having controlled the initial infection, why does the immune response stop short of eliminating the remaining bacteria? Anergy to PPD stimulation in TB infected and healthy controls has been associated with increased IL-10 and TGF-β1 levels and decreased IFN-γ production [Boussiotis 2000; Hirsch 1994]. An explanation for this might be the development of regulatory T cells, which have been detected in increased abundance in TB patients [Guyot-Revol 2006; Ribeiro-Rodrigues 2006]. Thus, prevention of tissue destruction becomes top priority to the expense of eradication of bacteria. Furthermore, bacteria have evolved a broad range of immune evasion and survival strategies making them extremely successful in persisting through millennia.

1.4 Bacterial factors modify the host immune response

As described, macrophages can kill mycobacteria via ROI and RNI. However, survival of bacteria inside macrophages suggests the existence of evasion or resistance mechanisms. Several genes of M.tb have been associated with evasion of RNI or ROI mediated cytotoxicity. The noxR1 and noxR3 genes have been identified as components involved in resistance to RNI, however the mechanisms are unknown [Ehrt 1997; Ruan 1999]. Another M.tb gene, ahpC encodes a peroxiredoxin which is part of an antioxidant complex that can neutralise RNI [Bryk 2002]. Also glbN, a gene which encodes for a

truncated haemoglobin, has been implicated in RNI metabolism [Ouellet 2002].

Another immune evasion strategy M.tb developed is to arrest the fusion of phagosomes with lysosomes, thus evading the antimicrobial activity of the lysosome and maintaining an intraphagosomal environment that favours its persistence. Also the iron-rich milieu of the early phagosome is beneficial for bacterial growth and survival [Schaible 2004]. One component of phagosome arrest is the phosphatidylinositol analogue lipoarabinomannan (LAM) of M.tb [Russell 2002; Vergne 2003]. Multiple factors from M.tb, including the ESX-1 secretion system [MacGurn 2007], are involved in modulating trafficking within the host. A strong association of ESAT-6 with cholesterol has been reported [de Jonge 2007]. Once released from its chaperone CFP-10 in acidic environments, ESAT-6 interacts with biomembranes in the phagosome and exhibits membrane-lysing activity. There are several interactions involved in arrest of phagosome maturation, i.e. aberrant retention of Rab5 and deficient recruitment of Rab7 [Clemens 2000; Via 1997] as well as retention of tryptophan-aspartate containing coat protein (TACO) [Ferrari 1999]. Recently, lipoamide dehydrogenase C (LpdC) was identified as a TACO binding protein. Binding of LpdC to TACO required cholesterol [Deghmane 2007]. Phagosome maturation is accompanied by a pH drop as to provide optimal conditions for the lysosomal acidic hydrolases [Hingley-Wilson 2003]. Retention of TACO by M.tb leads to exclusion of the vacuolar proton pump from the phagosomal membrane, thus arresting acidification [Ferrari 1999]. Moreover M.tb expresses urease which catalyses production of ammonia, thus neutralising the acidic pH in early phagosomes [Reyrat 1995]. In addition mycobacteria secrete a serine-threonine protein kinase G which also contributes to maturation arrest [Walburger 2004].

M.tb can also intercept antigen presentation. It has been reported that M.tb interferes with MHC-II dependent antigen processing and presentation [Singh 2006]. Lipoarabinomannan [Chan 1991], a 25-kDa M.tb glycolipoprotein [Wadee 1995] and a 19-kDa lipoprotein [Noss 2001] have been shown to attenuate the expression of MHC-II molecules and processing of soluble

antigens. Furthermore, interference with the generation of fully competent DC has been described as an escape mechanism utilized by M.tb [Mariotti 2002]. Immune evasion is not the only survival strategy of M.tb. Bacteria can also induce a dormancy programme in response to hostile conditions [Zhang 2004].

1.5 Bacterial factors involved in dormancy or persistence

The extraordinary ability of M.tb to survive has been shown more than 70 years ago. Researchers inoculated a culture bottle with M.tb and incubated it for 12 years before re-examination. Remarkably, they could identify viable micro-organisms in the sediment of the bottle [Corper 1933]. Dormant as well as persistent bacteria outlast in tissues despite a vigorous immune response or chemotherapy [Zhang 2004]. The term dormancy used in this thesis refers to bacteria being in a state of low to negligible metabolic activity and absence of growth and describes a reversible state of bacterial shutdown [Barer 1999]. Dormancy is not an exclusive phenomenon of M.tb, various bacterial species also form dormant bacteria i.e. streptococci, *Treponema palladium* and *Helicobacter pylori* under appropriate conditions such as aging, starvation, low temperature or low oxygen [McDermott 1958; Rhen 2003].

Most *in vitro* work on the dormancy response of M.tb was done in the so called Wayne model [Wayne 1994a]. Because M.tb is thought to persist in low oxygen environments *in vivo*, i.e. inside macrophages, granulomas or caseous lesions, Wayne established an *in vitro* model of dormancy. In this model cultures of M.tb are subjected to gradual oxygen depletion by incubation in sealed containers with controlled agitation [Wayne 1976; Wayne 1982]. This leads to two stages of non replicating persistence: First a microaerophilic state and later an anaerobic state where alterations in drug-susceptibility are observed [Wayne 1996]. Whilst in a stage of non-replicating persistence, organisms were sensitive to metronidazole, which is generally efficient against anaerobic bacteria. Reintroduction of oxygen lead to resumption of growth.

Dormancy is reversible and bacterial cultures can be resuscitated, i.e. by using spent culture supernatant [Sun 1999]. More recently a family of five secreted proteins has been identified which stimulate bacterial growth and have the ability to resuscitate dormant M.tb organisms. They have been termed resuscitation promoting factors (Rpf) [Biketov 2000; Mukamolova 2002]. Analysis of the structure of RpfB revealed homology to lysozymes and it has been speculated that the activation of dormant cells is facilitated by peptidoglycan lysis, which alters cell wall properties and might serve as a wake up call [Cohen-Gonsaud 2005; Keep 2006].

Several mycobacterial factors involved in dormancy or persistence have already been identified. It must be stressed, that some of these are putative and still need to be tested in adequate *in vivo* models of latent or persistent infection. Sigma factors SigF [DeMaio 1996], SigB [DeMaio 1997] and SigJ [Hu 2001] have been implicated in persistence as they are up-regulated in stationary phase cultures and under stress conditions, however none of these were up-regulated in a Wayne model microarray study [Sherman 2001]. This could indicate different responses under low oxygen conditions compared to other stress responses. Other genes connected to persistence or dormancy encode for enzymes needed for alternative energy metabolism, i.e. Isocitrate lyase (ICL) [Graham 1999] and Glycine dehydrogenase (GDH) [Wayne 1982]. In addition, cell wall strengthening seems to be important for dormancy, i.e. PcaA, CmaA1 and CmaA2 [Cole 1998] and the 16-kDa alpha crystalline (hspX) [Cunningham 1998] have been linked to this.

In vivo experiments showed that the number of bacilli in a lesion correlated well with the degree of oxygenation [Canetti 1955]. Dormancy is linked to hypoxic conditions inside the host [Wayne 2001]. Staining of human lung tissue samples indicated that areas of necrosis are likely to be hypoxic, due to the lack of endothelial cells and reduced vascularisation [Tsai 2006; Ulrichs 2005]. The initial hypoxic response of M.tb involves 47 induced genes and results in growth arrest [Sherman 2001]. Among the genes induced was the two-component response regulator Rv3133c. Long-term hypoxic survival of BCG also required Rv3133c, which was named dormancy survival regulator (DosR) [Boon 2002]. By targeted disruption of the DosR locus and

transcriptome analysis the same 47 genes were identified as powerfully regulated by hypoxia and requirement of DosR for their induction [Park 2003]. These authors showed that this protein is needed to induce nearly all M.tb genes that respond to a hypoxic signal. Inhibition of respiration by NO induced the same set of genes, indicating that the dormancy programme of M.tb can also be initiated by NO [Voskuil 2003]. These genes are not distributed randomly across the chromosome but appear to be clustered in nine discrete modules that form the so-called dormancy regulon. Recently it has been shown that Rv3132c (DosS) and Rv2027c (DosT) encode sensor kinases which can phosphorylate DosR *in vitro*, thus initiating the dormancy response [Roberts 2004]. There is evidence that the sensor for oxygen and NO concentration is a heme-containing protein [Voskuil 2003]. The two-component regulatory system DosR and DosS is expressed in M.tb [Park 2003; Sherman 2001], BCG [Boon 2002] and also in *M.smegmatis* [O'Toole 2003] but not in other mycobacterial strains, linking the dormancy response to virulence. However, validation that this DosR regulon is required for dormancy in latent human infection has not been achieved, yet [Boshoff 2005].

It is important to mention that there is still no evidence linking physiological states of M.tb *in vitro* with latent human infection. Latent infection simply indicates the host is infected, but has not developed symptoms. However, the term latency does not describe the metabolic or growth state of the bacteria inside the host. Most likely, the bacterial population will be heterogeneous with regard to their metabolic state, some populations will die, while others survive and remain viable and others might even be actively replicating.

1.6 Of mice and man: The mouse as a model for *Mycobacterium tuberculosis* infection

The most frequently used experimental animal species to evaluate new vaccines against pulmonary TB is the mouse. In contrast to humans, who usually develop a latent infection with very low bacterial burden, the infection course in mice differs. Even though mice are quite resistant to infection with

M.tb, and usually survive for more than 200 days, they develop a persistent infection in the lung and other organs, mainly spleen and liver, with very high bacterial titres [Orme 2003]. Administration of BCG will usually reduce bacterial burden approximately 10-fold in the lung and it is generally expected that subunit vaccines like naked DNA encoding one single TB antigen at best will result in five times less bacteria present in the lung and/or spleen compared to non-vaccinated controls [Orme 2006a].

Whilst it seems to be certain, that the few remaining bacilli in human granulomas are in a state of non-replicating persistence, referred to as dormancy, [Zhang 2004] it is unlikely that this also holds true for mice during persistent infection. It is possible that dormancy-associated antigens are expressed during persistent infection in mice, because *in vitro* and *in vivo* experiments indicate that some of the DosR-regulated genes are already up-regulated during the transition from exponential growth to non-replicating persistence phase and also in response to NO which is produced by activated macrophages [Shi 2003; Voskuil 2003]. Furthermore during persistent infection the bacterial population inside the host is likely heterogeneous, with regard to their metabolic state. Thus besides a large proportion of actively replicating microorganisms a fraction of dormant mycobacteria could exist as well.

Several models to study latency and reactivation have been described in the literature. Basically they can be divided into drug-induced models which are all variations of the Cornell model [McCune 1956] and low dose chronic infection models [Orme 1988]. While antibiotics are used in the Cornell model to artificially reduce bacterial burdens, infection is solely controlled by the host's immune response in the low dose chronic infection model. Reactivation occurs naturally after a prolonged latency period (up to 15 months) or has to be induced by immunosuppressive agents in the Cornell model. Each model has certain limitations and various parameters like challenge dose, route of infection, length of rest period, duration of drug-treatment, and mouse strain greatly influence the course of infection and reactivation ranging from imminent spontaneous reactivation to sterile eradication of bacteria. If those models represent "true" latency as observed in humans remains debatable.

Taken together, standardised models for vaccination in latent disease as well as for safety and toxicology are missing. But these are crucial for clinical development of new vaccines [Orme 2006a]

1.7 Vaccination against tuberculosis – where are we now?

The most effective and cost effective intervention for disease control will be the prevention of infection through vaccination. BCG was first used as a human vaccine in 1921 and is one of the most widely used and safest vaccines in the world. BCG vaccination prevents tuberculous meningitis and miliary TB in young children [Trunz 2006]. However, despite the widespread global administration of BCG, evidence of protection in other forms of TB in adolescent and adult populations is less convincing. A meta-analysis revealed efficacies ranging form 0-80% in adult populations [Andersen 2005; Colditz 1994]. Even re-vaccination with BCG did not provide substantial additional protection against pulmonary TB [Rodrigues 2005].

The first preclinical data for new TB vaccines was published more than ten years ago [Huygen 1996; Tascon 1996]. Since then, hundreds of reports have been published demonstrating some level of protection with new TB vaccine candidates in mouse models of TB infection [Fletcher 2007] but only a few of those candidates have progressed into phase I clinical trials [Skeiky 2006]. The Global plan to Stop TB supports the development of new anti-TB drugs and improved vaccines with a 50 billion dollar programme [Stop TB partnership and WHO 2006]. But, given the fact that only 5-10% of people infected with TB develop disease, and to complicate testing further, active disease may occur decades after the initial infection, results from phase II/III clinical trials are not expected in the near future. Thus, vaccination is expected to make a major contribution to the goal of eliminating TB worldwide only by 2050 [Young 2006]. New vaccine strategies against TB include recombinant BCG strains, attenuated M.tb strains, subunit vaccine approaches as well as non-replicating viral vector based delivery systems used alone or in prime-boost regimes [Skeiky 2006]. Generally subunit vaccines aim at boosting the immune response induced by a BCG prime, whereas improved, recombinant BCG should replace the current BCG vaccine strain [Kaufmann 2006].

A recombinant BCG strain expressing Ag85B [Horwitz 2003] has already been tested in a phase I clinical study and showed no serious adverse effects. Re-introduction of the region of difference-1 (RD-1) genes, which are expressed in M.tb but absent in BCG [Brosch 2000], into BCG has also been reported [Pym 2003]. Our lab engineered a recombinant ureaseC-deficient BCG strain expressing membrane-perforating listeriolysin (Hly) of *Listeria monocytogenes* named Δ*ureC hly+* rBCG (recBCG) and showed that it induced much better protection against aerogenic challenge with M.tb than the parental strain. Especially at late stages of infection recBCG was much more effective [Grode 2005]. A phase I clinical trial is expected to start in 2008. Live vaccines based on attenuated M.tb are also under investigation, e.g. auxotrophic mutants with targeted deletions in PanC and PanD, incapable of pantothenic acid synthesis and thus not replicating *in vivo* [Sambandamurthy 2005]. An M.tb PhoP mutant, where the virulence associated gene phoP has been deleted, is also scheduled for a phase I clinical trial in 2008 [Asensio 2008].
Several subunit vaccines, mostly fusion proteins in adjuvants, are also under investigation at the moment. These encompass Hybrid-1 (Ag85B-ESAT-6 fusion) [Weinrich 2001] and HyVac-4 (Ag85B-TB10.4) [Dietrich 2005] as well as Mtb72F (Mtb39-Mtb32) which has passed phase I clinical trials [Skeiky 2004]. A modified vaccinia virus Ankara expressing Ag85A has also passed phase I clinical trials [McShane 2004].

By far the most extensive testing of new vaccine candidates has been done by administration of DNA vaccines encoding the protein of interest. A substantial amount of research has been done on the Ag85 family. Ag85A and Ag85B have been shown to confer protection against aerosol infection in mice, whereas Ag85C has not [Lozes 1997; Montgomery 1997]. Also priming with DNA vaccines encoding for Apa, hsp60 or hsp70 and boosting with BCG conferred increased protection against subsequent intravenous (i.v.) challenge in mice [Ferraz 2004]. A plasmid DNA vaccine encoding ESAT-6, even though highly immunogenic, did not result in protection [Kamath 1999]. The PstS family of lipoproteins of M.tb has also been tested as DNA vaccines. Whilst all three members were immunogenic, only PstS-3 was protective in an

i.v. challenge model [Tanghe 1999]. The resuscitation-associated conserved hypothetical protein Rv3407 has also been successfully used in a BCG prime, DNA boost regime [Mollenkopf 2004]. As plasmid DNA vaccines only showed limited protective capacity compared to BCG, they were also tested in pools. A DNA vaccine cocktail consisting of plasmid DNA encoding Ag85B, ESAT-6, KatG, MPT8.4, MPT12, MPT63, MPT64 and MPT83 resulted in reduced bacterial burden in the lungs even in mice lacking CD4 T cells [Derrick 2004a]. Several other DNA vaccine cocktails have been evaluated and shown to confer modest protection but significantly prolonged survival of vaccinated mice [Delogu 2002; Morris 2000]. However these candidates were not protective in a post-exposure model [Repique 2002].

Almost all of these new vaccines have been tested in pre-exposure experimental settings in mice. But none of these vaccine candidates was efficient when given therapeutically to an already infected host [Turner 2000a]. An explanation might be that a potent host response against these antigens has already been established during the course of infection and additional vaccination cannot act synergistically. Only one report described spectacular protection in pre- and post-exposure experiments by administration of a plasmid DNA vaccine encoding hsp65 from *M.leprae* [Lowrie 1999]. However, these results could not be reproduced by other groups. On the contrary, vaccination seemed to induce severe pathology rather than conferring protection [Taylor 2005; Turner 2000b].
All antigens encoded in plasmid DNA vaccines and reported to show some efficacy against M.tb infection are abundantly expressed by actively replicating bacteria, whereas dormancy-associated antigens have not been tested so far.

1.8 The principles of DNA vaccination

More than 15 years ago, it was shown that plasmid DNA injection could elicit an immune response in animals [Tang 1992]. Soon after this report, plasmid DNA vaccines were shown to confer protection against viral challenge in mice and chickens [Robinson 1993; Ulmer 1993]. DNA vaccines have many

advantages compared to protein based or live vaccines, especially safety in immunocompromised hosts and the lack of vector immunity induction [Huygen 2005]. The production is easy and similar between different plasmids, which will be important for up-scaling processes. DNA is also very stable at higher temperatures, which will be an important for countries where a cold chain cannot be guaranteed. Different antigens can be encoded in the same vector, enabling the expression of multiple antigenic proteins in one construct. Also, DNA vaccines are often highly immunogenic and elicit a broad range of T_H1, T_H2 as well as cytotoxic T cell responses and antibodies [Bivas-Benita 2004]. The main safety concern is the possibility of chromosomal integration into the host genome that could activate oncogenes or disrupt tumour suppressor genes. However, it has been calculated that the possibility of integration was three orders of magnitude lower than the spontaneous mutation frequency [Nichols 1995].

Plasmid vectors designed for vaccination have to have certain essential elements: (I) A bacterial backbone with an origin of replication (ORI), usually from *E.coli*, which enables amplification of DNA for purification. This should not be active in mammalian cells, in order to prevent plasmid integration. (II) A prokaryotic marker gene, e.g. ampicillin resistance, to facilitate selection of organisms carrying the plasmid. (III) A strong eukaryotic promoter to drive expression in the host, usually from cytomegalovirus (CMV). (IV) A transcription terminator, e.g. the polyA signal from bovine growth hormone (BGH). (V) Last but not least, the DNA sequence encoding the antigen of interest [Donnelly 1999; Doria-Rose 2003]. Plasmids encoding a secreted form of the protein by fusion of the protein gene and the signal sequence gene of human tissue plasminogen activator (tPA) are generally more immunogenic [Baldwin 1999; Li 1999].

The concept of DNA vaccination is simple. DNA is introduced into host cells where expression, processing and presentation of the encoded bacterial protein takes place, very similar to what happens after infection with an intracellular pathogen. Plasmid DNA vaccines stimulate both the exogenous (MHC-II restricted) as well as endogenous (MHC-I restricted) antigen

presentation pathway and thus elicit strong CD8 as well as CD4 T cell responses [Huygen 2005]. Priming of T cells after DNA vaccination can be achieved in different ways [Bins 2007]. Cellular targets of intramuscularly (i.m.) administered plasmid DNA vaccines are first of all myocytes. Although myocytes have been shown to take up most of the DNA and produce more protein than other cells types, they lack the molecules needed for co-stimulation and thus direct priming by somatic cells does not elicit a strong immune response. However a recent publication showed *in vitro* and *in vivo* that plasmid transfection improved the ability of muscle cells to act as antigen presenting cells (APC), thus some contribution of myocytes cannot be ruled out [Shirota 2007].

Presentation by professional APC that were directly transfected by DNA elicits a strong immune response. These APC rapidly migrate to the draining lymphnodes and initiate an immune response [Condon 1996]. Bacterial DNA can enter professional APC by endocytosis into acidic vesicles for transport into the nucleus, enabling T cell priming via antigen presentation on MHC-I molecules [Tonkinson 1994]. It has been shown that cross-presentation of plasmid DNA encoded antigens is a major mechanism for T cell induction after i.m. vaccination [Corr 1999; Doe 1996]. Cross-presentation is a phenomenon in which a professional APC presents antigens which have been acquired from other cell types, a transfected somatic cell or an APC, to T cells [Bevan 1976]. APC acquire these antigens either by uptake of secreted proteins, processed peptides or by phagocytosis of apoptotic or necrotic bodies [Albert 1998]. Bacterial DNA, comprising so-called CpG motifs, has also adjuvant properties and triggers co-stimulatory cytokine secretion, as it can directly interact with TLR-9 on the surface of the early endosome in APC [Hemmi 2000]. This results in IL-12, IFN-γ, TNF-α and IL-6 induction, thus directing the immune response towards a T_H1 phenotype [Halpern 1996].

2 Aims of this thesis

As described in the introduction, a major problem in TB control is the high number of individuals already latently infected with M.tb. It is estimated, that an effective pre-exposure vaccine would initially prevent only approximately 20% of TB cases, whereas a successful post-exposure vaccine would prevent 30-40% of cases [Barry 2007; Ziv 2004]. The ultimate strategy for long-term TB control would thus be a combination of improved pre- and post-exposure vaccination. Although CD4 T cells play a key role in protection against TB, CD8 T cells are also important, especially to prevent reactivation of latent infection [van Pinxteren 2000]. Induction of a strong CD8 as well as CD4 T cell response is the main characteristic of plasmid DNA vaccines, which can only be induced in comparable strength by live vaccines but not by protein/adjuvant formulations [Huygen 2005]. In the last years, hundreds of new vaccines have been tested in pre-exposure experimental settings in mice. However, none of these vaccine candidates was efficient when given therapeutically to an already infected host. All antigens encoded in plasmid DNA vaccines and reported to show some efficacy against M.tb infection are abundantly expressed by actively replicating bacteria, whereas dormancy-associated antigens have not been tested, so far.

One objective of this thesis was to evaluate the protective potential of plasmid DNA vaccines encoding dormancy-associated antigens to prevent infection or reactivation of M.tb. The four selected candidate antigens are part of the dormancy regulon: TB31.7, hspX, Rv1733c and Rv2628. HspX seems to be involved in cell wall strengthening during dormancy [Cunningham 1998], the functions of the other three proteins are still unknown. HspX, TB31.7 and Rv2628 were among the genes most powerfully induced by hypoxia [Park 2003]. More importantly, all four candidate antigens were recognised by latently infected individuals [Leyten 2006]. TB31.7 contains a so-called universal stress protein domain (Usp) [Florczyk 2001]. Usp are a family of proteins that are produced by bacteria in response to a range of environmental stressors [Kvint 2003]. The DosR regulon encompasses five proteins containing Usp domains, making them interesting vaccine

candidates. Specific aims of the first part of this thesis were: (I) Evaluation of the immunogenicity of these plasmid DNA vaccines encoding dormancy-associated antigens. (II) Determination of the protective capacities of these plasmid DNA vaccines against aerosol infection with M.tb in pre- and post-exposure vaccination regimes. (III) Establishment of a short-duration drug-induced model for latent TB infection in mice. (IV) Evaluation of the protective capacity of plasmid DNA vaccines to prevent reactivation in the established murine latency model.

The identification of immune surrogate markers of protection, often called “biomarkers” or “correlates of protection” was another objective of this thesis. This will help facilitate the rational vaccine design for the prevention of M.tb infection or clearance of latent TB infection. Immune correlates of protection could be used as early endpoints in TB vaccine efficacy trials and thus reduce the duration of vaccine field trials as well as enable testing of larger numbers of vaccine candidates. In the last five years, clinical studies focussing on recently infected, healthy contacts of TB patients were initiated in order to define correlates of protection [Fletcher 2007]. Nevertheless, despite extensive research investigating the immunity to TB, the fundamental components of the protective memory immune response have yet to be elucidated. In the context of BCG vaccination, antigen-specific memory CD4 T cells are difficult to detect, due to the lack of an immunodominant antigen. Currently the most widely used correlate for protection is the increase in the frequency of CD4 T cells producing IFN-γ. However in recent years this strategy has been questioned, as in several newly developed vaccines increased production of IFN-γ did not result in equivalent or improved protection compared to BCG [Goldsack 2007]. Immune correlates of protection should be identified by comparison of the immune responses elicited after vaccination with BCG or the superior vaccine recBCG [Grode 2005]. Specific aims were: (I) Analysis of the vaccine-induced memory response against PPD and Ag85A prior to aerosol infection with M.tb. (II) In-depth analysis of the cell mediated immune response, against PPD and Ag85A, 90 days after aerosol infection with M.tb in BCG or recBCG vaccinated mice.

3 Material

Addresses and contact information for suppliers are listed in the appendix. Standard laboratory chemicals used to prepare buffers, staining solutions and the like, were purchased from Sigma, Merck or Roth in per analysis quality.

3.1 Enzymes

Restriction endonucleases	Fermentas
Shrimp alkaline phosphatase	
T4 DNA ligase	
Restriction endonucleases	New England Biolabs
Collagenase D	Roche
Expand High Fidelity DNA Polymerase	
Collagenase VIII	Sigma

3.2 Equipment

Spectrophotometer Ultrospec 2100 pro	Amersham Biosciences
Flow cytometer FACSCanto™II and LSRII	Becton Dickinson
CO_2-Incubator	Binder
Electrophoresis power supply PowerPAC300	Bio-Rad
Electrophresis chamber Bio-Rad Mini Sub™ DNA Cell	
Bio-Plex™ System	
Gene Pulser® II Electroporation System	
Cell counting chamber Neubauer improved	Brand
Mastercycler gradient	Eppendorf
Thermoblock	
Microcentrifuge Biofuge fresco	Heraeus
Biological Safety Cabinets Class II	
Megafuge 1.0R (sepatech#2704 rotor)	
Multifuge 4KR (LH4000 rotor)	
Stir- and heating plate RCT basic	IKA-Labortechnik
Vortex MS1 Minishaker	
Electronic pipetter Pipetboy acu	Integra Biosciences
pH-meter 761 calimatic	Knick
Lab-Shaker	Lab-Therm
Microscopes	Leica
Paraffin Embedding Centre	MICROM International GmbH
Paraffin Rotation Microtome	
MultiScreen Filtration System	Millipore
Water purifier	
ELISA-reader SpectraMAX190	Molecular Devices
Scales	Sartorius
Glas-Col Inhalation Exposure System	Schuett-Biotec
Shandon Citadell 1000 Tissue Processor	Shandon

3.3 Other material

Rompun	Bayer
PARAFILM®	Brand
Serum seperator tubes Microtainer® SST™	Becton Dickinson
Cell strainer (40µm)	
1ml Sub-Q syringes	
Syringes	B.Braun
Whirl-Pak® sterile sample bags	Carl Roth
Spin-X® centrifuge tube filters	Corning
Brefeldin A	Invitrogen
Streptavidin-PE (1mg/ml)	
Filter plates for Bio-Plex MultiScreen®-BV	Millipore
ELISA-plates Immuno Maxi-Sorp	Nunc
Ketavet	Pfizer
Felix 500 base pair ladder	Qbiogene
Jules™ molecular weight marker 100bp ladder	
Metal sieves Paco-roundfilter FFO290	Regenerier Service Wolfgang Franz
Sterile filters and membranes	Schleicher & Schüll
Plasticware	Corning, Sarstedt or TPP
Tuberculin PPD Batch RT50	SSI

3.4 Kits

Bio-Plex Mouse Cytokine T_H1/T_H2 panel	Bio-Rad
Bio-Plex Mouse IL-6 Assay	
Bio-Plex Mouse IL-17 Assay	
Cytokine reagent kit	
TOPO-TA cloning	Invitrogen
QIAquick® Gel Extraction Kit	Qiagen
QIAprep® miniprep Kit	
Endo Free® Plasmid Giga Kit	

3.5 Proteins and peptides

Recombinant hspX was a gift from Dr P. Andersen, Statens Serum Institute Copenhagen, Denmark. Recombinant proteins TB31.7, Rv1733c and Rv2628 were kindly provided by Prof. Dr T.H.M. Ottenhoff, Leiden University Medical Centre, Netherlands. Recombinant Ag85A was a gift from Prof. Dr A.S.V. Hill, the University Of Oxford, United Kingdom. All proteins received were expressed in, and endotoxin-free purified from, *Escherichia coli*.
All peptides used for *in vitro* re-stimulation (IVR) were purchased from JPT Peptide Technologies GmbH in crude purity (>70%). Aliquots of 10^{-2}M stock solutions were stored at -20°C until use. Peptides were either known CD4 or CD8 T cell epitopes [Denis 1998] or putative CD8 T cell epitopes identified with the web-based MHC-I antigenic peptide processing prediction (MAPPP) program: A combination of the proteasomal processing software FRAGPREDICT and PAProC with the MHC binding ligand predictions

SYFPEITHI and BIMAS in the expert mode of MAPPP were used [Hakenberg 2003]. Parameters for FRAGPREDICT were set at 0.6 (minimal residue cleavage probability and minimal fragment cleavage probability) and at 0.6 in PAProC. Binding score for BIMAS was set at 0.3 and 0.5 for SYFPEITHI. The murine haplotypes H2Kd, H2Dd and H2Ld and a fragment length of 8 to 10 amino acids were used. The threshold was set at an overall score of 0.9.

name	aa	restricted	sequence	reference
Rv1733c p38	38-48	H2-K^d CD8	DRLEAVVMLL	MAPPP
Rv1733c p45	45-55	H2-K^d CD8	MLLAVTVSLL	MAPPP
hspX p1	1-20	I-E^d CD4	MATTLPVQRHPRSLFPEFSE	Roupie
hspX p111	111-130	I-A^d CD4	DDIKATYDKGILTVSVAVSE	Roupie
hspX p9	9-18	H2-L^d CD8	HPRSLFPEF	MAPPP
hspX p31	31-40	H2-L^d CD8	RPTFDTRLM	MAPPP
hspX p93	93-102	H2-K^d CD8	AYGSFVRTV	MAPPP
TB31.7 p43	43-52	H2-L^d CD8	VSPEVATWL	MAPPP
TB31.7 p119	119-128	H2-L^d CD8	GSGRWPGRL	MAPPP
Rv2628 p2	11-30	I-A^d CD4	IRAVGPYAWAGRCGRIGRWG	Roupie
Ag85A p15	99-118	I-E^d CD4	TFLTSELPGWLQANRHVKPT	Denis
Ag85A p70	70-78	H2-L^d CD8	MPVGGQSSF	Denis
Ag85A p145	145-152	H2-K^d CD8	YAGAMSGL	Denis

3.6 Primer

Sequences of synthetic oligonucleotides (primer) used for amplification of M.tb genes. Primers were obtained from MWG, diluted to 10µM stock solutions and stored at -20°C until needed. Specific primers were designed using the web-based software Primer3. BamHI restriction sites were added in order to excise and re-ligate the amplified sequences.

primer-name	5'-3' sequence	annealing temperature
Rv1733c_for	GGATCCATGATCGCCACAACCCGCGAT	62°C
Rv1733c_rev	GGATCCTCACCGCTGCGTGCAGAACAG	62°C
hspX_for	GGATCCATGGCCACCACCCTTCCCGTT	65°C
hspX_rev	GGATCCTCAGTTGGTGGACCGGATCTG	65°C
Rv2623_for	GGATCCATGTCATCGGGCAATTCATCT	57°C
Rv2623_rev	GGATCCAGTGTTCGGTGCTTGTCGTT	57°C
Rv2628_for	GGATCCATGTCCACGCAACGACCGAGG	64°C
Rv2628_rev	GGATCCGTTAGGTGGTGCTTAGACCGC	64°C

3.7 Antibodies

An overview of the monoclonal antibodies used is given in the table below. Monoclonal antibodies (mAb) marked with a plus (+) were purified from hybridoma supernatants by protein-G sepharose. The mAb used for flow cytometry were conjugated with the fluorescent dyes FITC or PerCP, for *in vitro* re-stimulation (IVR) or Fc-receptor blocking (block) mAbs were only purified. For ELISA antibodies were obtained coupled to alkaline phosphatase (AP) instead of fluorochromes.

Specificity	Clone	Application	fluorescent dye	source
CD4	RM4.5	FACS	Pacific blue	eBioscience
CD8α	YTS169	FACS	PerCP	ATCC+
IL-2	JES6-5H4	FACS	APC	eBioscience
IL-17	TC11-18H10	FACS	PE	BD
IFN-γ	XMG1.2	FACS	PE-Cy7	eBioscience
TNF-α	XT22	FACS	FITC	ATCC+
CD3	145-2C11	IVR	None	ATCC+
CD28	37.51	IVR	None	ATCC+
CD16/CD32	2.4G2	Block	None	ATCC+
mouse IgG1	X56	ELISA	AP	BD
mouse IgG2a	R19-15	ELISA	AP	BD
rat Ig		Block	None	purified rat serum

3.8 Buffers and solutions

Solutions were made up in H_2O prepared with a Millipore water purifier, unless stated otherwise. Where indicated (*), solutions were sterilised by autoclaving for 25min at 121°C, or filter-sterilised through a 0.2µm membrane.

3.8.1 Cell culture media

RPMI 1640 medium, FCS, penicillin/streptomycin solution, L-glutamine and Percoll were obtained from Biochrom.

RPMI medium*:
10% (v/v) FCS
0.2mM L-glutamine
10U/ml penicillin and streptomycin
50µM 2-mercaptoethanol

Collagenase digestion medium*:
10% (v/v) FCS
0.2mM L-glutamine
10U/ml penicillin and streptomycin
50µM 2-mercaptoethanol
0.075mg/ml collagenase D
0.175mg/ml collagenase VIII

Red blood cell (RBC) lysis buffer*:	155mM NH_4Cl 10mM $KHCO_3$ 0.1mM EDTA

3.8.2 Bacterial culture media

Luria Bertani (LB) medium*:	1% (w/v) Bacto-Tryptone 0.5% (w/v) yeast extract 85.5mM NaCl titrated to pH7.5 with NaOH

50mg/l ampicillin was added as indicated in the methods section. 50g/l stock solutions of ampicillin were stored at –20°C. For LB-agar plates 1.5% (w/v) agar was added prior to autoclaving.

S.O.C. medium*:	2% (w/v) Bacto-Tryptone 0.5% (w/v) yeast extract 10mM NaCl 2.5mM KCl 10mM $MgCl_2$ 10mM $MgSO_4$ titrated to pH7.0 with NaOH added after autoclaving: 20mM glucose
Middlebrook 7H9 broth*:	4.7g Difco™ Middlebrook 7H9 powder 2ml glycerol 900ml water added after autoclaving: 100ml BBL™ Middlebrook ADC enrichment 0.05% (v/v) Tween80
Dubos broth*:	6.5g Difco™ Dubos broth base 50ml glycerol 850ml water added after autoclaving: 100ml Difco™ Dubos medium albumin
7H11-agar plates*:	21g Difco™ Mycobacteria 7H11 agar 5ml glycerol 900ml water added after autoclaving: 100ml BBL™ Middlebrook OADC enrichment

50mg/l ampicillin and 100mg/l cycloheximide were added after cooling. 50g/l stock solutions of ampicillin were stored at –20°C. 10g/l stock solutions of cycloheximide were stored at 4°C.

PBS/Tween (PBST)*:	0.05% (v/v) Tween80 in PBS

3.8.3 Buffers used in molecular biology

Chloroform/Methanol:	3:1 ratio

Chloroform saturated with water: Chloroform and water are mixed at a 1:1 ratio, left to settle and the lower phase is then mixed with $^1/_3$ methanol.

GTC solution:	80ml of 5M Guadinium thiocyanate 10ml 1M Tris/HCl pH7.5 2.5ml 20%(v/v) N-Lauroylsarcosine 6.5ml water added directly before use 1ml 2-Mercaptoethanol
TE buffer:	10mM Tris-HCl (pH7.6) 1mM EDTA (pH8.0)
TAE running buffer:	36mM Tris-HCl 30mM Na_2HPO_4 30mM NaH_2PO_4
DNA loading buffer:	50% (v/v) glycerol 1mM EDTA 0.4% (w/v) bromophenol blue 0.4% (w/v) xylene cyanol

3.8.4 Buffers needed for ELISA, Bio-Plex and flow cytometry

Phosphate buffered saline (PBS):	8g NaCl 0.2g KCl 0.2g KH_3PO_4 1.3g Na_2HPO_4
ELISA coating buffer:	100mM Na_2CO_3 100mM $NaHCO_3$ titrated to pH9.6
ELISA wash buffer:	0.1% (w/v) BSA 0.05% (v/v) Tween 20 in PBS
ELISA blocking buffer:	5% (w/v) BSA in PBS
ELISA serum dilution buffer:	1% (w/v) BSA in PBS
ELISA stop solution:	3M NaOH
Bio-Plex assay buffer A:	1% (w/v) BSA 0.05% (v/v) Tween 20 in PBS
Bio-Plex wash buffer A:	0.5% (w/v) BSA 0.05% (v/v) Tween 20 in PBS

FACS buffer (PBS/B)*:	0.2%(w/v) BSA in PBS
FACS blocking buffer:	1µg/ml mAb CD16/CD32 and rat Ig diluted in PBS/B
Fixation buffer:	1%(v/v), 2%(v/v) or 4%(v/v) formalin in PBS
Permeabilisation buffer*:	0.1% (w/v) BSA 0.5% (w/v) saponin 1µg/ml mAb CD16/CD32 and rat Ig diluted in PBS

3.9 Software

Tables, calculations, statistic and graphics	
GraphPad Prism 4.0	GraphPad Software
Excel	Microsoft
Photoshop	Adobe Systems
Flow cytometric analysis	
FACSDiva v5.0.2	BD
FACS data Analyser (v0.9.8)	Christian Köberle
Bio-Plex analysis	
Bio-Plex Manager 4.1.1	Bio-Rad
DNA sequence analysis	
Clone manager 5.0	Scientific&Educational Software
Chromas	Copyright Conner McArthy
Text	
Word	Microsoft
References	
Reference Manager 10	ISI ResearchSoft

3.10 Web resources

DNA sequence identification:
http://www.ncbi.nlm.nih.gov/BLAST/
DNA sequence comparison:
http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi
MHC-I antigenic peptide processing prediction:
http://www.mpiib-berlin.mpg.de/MAPPP/
Oligonucleotide-primer design:
http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
Sequence information on M.tb genes:
http://genolist.pasteur.fr/TubercuList/
Information on cytokines and chemokines:
http://www.copewithcytokines.de/cope.cgi

4 Methods

4.1 Molecular biological methods

The general molecular biological methods like *E.coli* cultivation, agarose gel preparation, DNA electrophoresis, DNA precipitation, DNA digestion and determination of DNA concentration were performed according to Molecular cloning: A laboratory manual [Sambrook 2001]. DNA sequencing was performed by AGOWA GmbH in Berlin. Sequences were verified using Chromas and the web-based program blast 2 sequences. DNA restriction digests were planned with the Clone manager program.

4.1.1 Culture of mycobacteria

The M.tb strain H37Rv was originally obtained from J.K. Seydel, Forschungsinstitut Borstel, Germany. The BCG Pasteur 1173P2 strain was kindly provided by B. Gicquel, Institut Pasteur, Paris, France. M.tb was grown in Middlebrook 7H9-broth and BCG was cultured in Dubos broth, both at 37°C with shaking until bacterial growth reached an OD_{600}=0.7, corresponding to a cell density of approx. 10^8cells/ml. These mid-logarithmic cultures were harvested by centrifugation, washed with PBS, re-suspended in 10% glycerol, aliquoted and stored at -80°C until use. All mycobacteria stocks were titrated prior to use by plating serial dilutions onto 7H11-agar plates and counting the colony forming units (cfu) after three weeks. Aliquots were homogenised prior to use by repeated transfer through a syringe with a 26G needle.

4.1.2 Purification of genomic DNA from *M.tuberculosis*

One ml of a mid-logarithmic M.tb culture was harvested by 10min centrifugation at 13000rpm (microcentrifuge). The bacterial pellet was re-suspended in 130µl chloroform/methanol and vortexed to lyse the cells. When lysis was complete, 130µl phenol was added, the mixture again vortexed, 200µl GTC solution was added and mixed well. In order to separate phases, the mixture was centrifuged for 15min at 13000rpm. Then 250-300µl of the upper phase were removed and carefully transferred into a new reaction tube, which was taken out of the BSL3 facility. Next, 300µl 2-propanol was added to

precipitate the DNA, followed by 30min centrifugation at 13000rpm. The DNA pellet was washed with 1ml 70% ethanol, air-dried for 30min at RT, re-suspended in 100µl TE-buffer, and used as template for PCR amplification of dormancy-associated antigens.

4.1.3 PCR

Selective amplification of DNA sequences was performed as described [Mullis 1986].
Standard PCR mix:

Expand buffer (with $MgCl_2$)10x	5µl
DNA template (20-200ng)	1µl
Forward and reverse primer	1µl
dNTPs (10nmol/µl)	1µl
Expand high fidelity polymerase (3.5U)	1µl
Water	40µl

Standard PCR protocol:

30cycles of		
denaturation	1min	94°C
annealing	45sec	55-66°C
extension	1min	68°C

4.1.4 Preparation of competent *E.coli* DH5α for electroporation

A single *E.coli* DH5α colony was grown to an overnight culture in 5ml LB-medium at 37°C. 3ml of this culture were used to inoculate 300ml LB-medium and grown to an OD_{600}=0.6 corresponding to a concentration of approximately $6x10^8$ bacteria/ml. The culture was centrifuged at 4°C for 20min at 2500rpm (Megafuge 1.0R). Pelleted bacteria were re-suspended in 50ml ice-cold water and centrifuged at 4°C for 20min at 2500rpm. This washing step was repeated two more times. Bacteria were finally re-suspended in 2.5ml ice-cold 10% (v/v) glycerol, 0.1ml aliquots were shock-frozen in liquid nitrogen and kept at -80°C until use.

4.1.5 Transformation of plasmid DNA into competent *E.coli*

1-2µl plasmid DNA (approximately 0.1pmol) was pipetted into a 0.2cm cuvette placed on ice. Competent cells were thawed on ice and added onto the DNA drop. Electroporation was carried out with 25µFD, 400Ω, 2.5kV and an average time constant of 9msec. After electroporation, 1ml of S.O.C. medium

was added and cells incubated for 1h at 37°C. Aliquots were then plated on selective, ampicillin-containing, LB-agar plates.

4.1.6 Isolation and purification of plasmid DNA

For all preparations, anionic ion exchange columns were purchased from Qiagen together with necessary buffers. Small-scale preparations (miniprep) for analytical purposes were carried out starting with pelleting the cells from 2.5ml culture and plasmid DNA was eluted from miniprep columns with 50µl water. Large scale preparations (gigaprep) for DNA vaccinations were carried out starting with pelleting the cells from 2.5L culture and endotoxin-free plasmid DNA was precipitated with 2-propanol, air-dried for 15min and re-dissolved in 1ml endotoxin-free water. The protocols for miniprep and maxiprep were performed according to the manufacturer`s instructions.

4.1.7 Covalent linking of DNA fragments using T4 DNA ligase

DNA preparations were ligated using a 3x molar excess of insert. Insert and vector backbone were precipitated with ethanol. DNA fragments were re-dissolved in 4µl water, 0.5µl T4 DNA ligase (1U) and 0.5µl 10x ligase buffer were added. The reactions were carried out overnight at 16°C and 1µl of the ligation solution was used for electroporation into competent *E.coli*.

4.1.8 Generation of DNA vaccines encoding dormancy-associated antigens

Sequences of dormancy-associated genes from M.tb were obtained from the TubercuList and specific primers for PCR were designed with the web-based primer3 software. BamHI restriction sites were added in order to excise and re-ligate the amplified sequences. A standard PCR was performed to amplify the M.tb genes, 10µl of the PCR mixture were run on a 1% agarose gel. Bands were excised, purified with a Qiagen Gel extraction Kit, and the isolated fragments finally cloned into the sequencing vector pCR2.1 using the TOPO-TA Cloning Kit. Plasmids were introduced into *E.coli* by electroporation and bacteria plated onto LB-agar plates containing ampicillin as a selection agent. Plasmids were isolated from single colonies and sent to AGOWA for sequencing. Obtained sequences were analysed using the web-based

program blast two sequences. Plasmids containing correct inserts were digested with BamHI and run on a 1% agarose gel. DNA fragments encoding the dormancy-associated genes were excised and purified with a Qiagen Gel extraction Kit. The target vector pCMVtPA was a kind gift from Dr J. Ulmer, Chiron-Behring, Emeryville, USA. The vector was also digested with BamHI and purified from 1% agarose gel followed by 1h de-phosphorylation with 1U of shrimp alkaline phosphatase directly added to the restriction digest mixture. Insert and linearised vector were covalently linked overnight and then introduced into *E.coli* by electroporation. Bacteria were grown over night on LB-agar plates containing ampicillin as selection agent, single colonies were picked, grown to 2.5ml cultures, and plasmid DNA was isolated using a miniprep kit. In order to identify the correct orientation of the inserts in the vector, restriction digests were performed with enzymes chosen by analysis of the constructs with the Clone manager programme. Aliquots derived from a single colony carrying sequence-verified inserts in correct orientation were stored in 15% glycerol at -20°C. For vaccination studies, a small amount of bacteria, taken with an inoculation loop directly of the frozen stock, was plated onto an LB-agar plate containing ampicillin and incubated over night. Next day a single colony was picked and inoculated into 5ml of LB-medium also containing the antibiotic. This starter culture was used for inoculation of 2.5L of LB-ampicillin medium and plasmids were isolated with an Endo Free® Plasmid Giga Kit according to the manufacturer's instructions. The DNA concentration was determined using a UV/Visible spectrophotometer. Isolated plasmid DNA vaccines were diluted in endotoxin-free PBS to 100µg/50µl.

4.1.9 Preparation of Chitosan-DNA nanoparticles

Chitosan-DNA particles were prepared as described [Mao 2001]. Briefly, low molecular weight Chitosan (Sigma) was dissolved at 55°C in 1% acetic acid and the pH adjusted to 5.5 with NaOH. The Chitosan solution was diluted to a final concentration of 0.02%(w/v) and 5mM acetate, the pH controlled again and passed through a 0.2µm filter. DNA was diluted to 200µg/ml with 50mM Na_2SO_4. Chitosan and DNA in a ratio of 1:1 were heated separately to 55°C, rapidly united and then vigorously vortexed for 1min. The maximum absorbing

capacity of Chitosan particles was 200µg/ml of DNA. As Chitosan-DNA nanoparticles were administered via the intranasal (i.n.) route the volume deployed, and therefore the amount of DNA, was limited to 40µl. This corresponded to 4µg of complexed DNA per mouse and vaccination.

4.2 Animal procedures

Female BALB/c mice were bred at the Bundesinstitut für Risikobewertung (BfR), Berlin. Mice were six to eight weeks of age at the beginning of the experiments and kept under specific pathogen-free (SPF) conditions in filter bonnet cages with food and water ad libitum in our BSL3 mouse facility at the Max Planck Institute for Infection Biology in Berlin. The experiments were conducted according to the German animal protection law.

4.2.1 Vaccination

Intramuscular (i.m.) vaccinations were given into the right lower leg muscle of restrained mice with a total volume of 50µl containing 100µg of plasmid DNA, diluted in PBS if necessary. Subcutaneous (s.c.) vaccinations with BCG were carried out in close proximity to the tail base with 200µl injection volume per mouse. Injections were done with 1ml Sub-Q syringes. Before i.n. vaccination, mice were anaesthetised according to animal protection law with a mixture of 10µl (0.2mg) Rompun, 10µl (1.2mg) Ketavet and 30µl PBS per mouse injected i.m.. 20µl of the Chitosan-DNA mixture was given as droplets onto the nostrils. Mice were rested for 3-5min followed by application of another 20µl.

4.2.2 Aerosol infection

Infection of mice was performed using a Glas-Col inhalation exposure system. An aliquot of frozen M.tb was thawed and diluted (as determined in titration experiments performed for every infection stock) with water. Aerosol infection deposited approximately 100-200cfu of the bacteria in the lung of each mouse. 24h after aerosol exposure five mice were killed, the lungs removed, homogenised and plated onto 7H11-agar to verify the initial challenge dose.

4.2.3 Determination of bacterial burden in lung and spleen

Mice were sacrificed at distinct time points after infection by cervical dislocation. Lungs and spleens were transferred into sterile sample bags containing 1ml PBST and either processed immediately or stored at -80°C. Organs were homogenised in the sample bags and after serial dilution in PBST plated on 7H11-agar plates which were sealed with PARAFILM® and wrapped in aluminium foil. After three weeks of incubation at 37°C cfu were counted.

4.3 Immunological methods

4.3.1 Histology

Tissue was fixed in 4% formalin for 24h at RT, transferred into water-containing 15ml tubes and stored at 4°C. Lung sections were then dehydrated and embedded in paraffin overnight; 5µm sections were cut, re-hydrated and stained with Hematoxylin and Eosin Y (H&E). Re-hydration was performed by 2x10min xylene, 2x5min 95% ethanol, 2x5min 80% ethanol, 5min 70% ethanol and finally 5min deionised H_2O. For H&E staining slides were incubated for 10min with Hematoxylin, rinsed with water for 10min, dipped in acid ethanol, rinsed with water, followed by Eosin Y staining for 1min. Slides were subsequently rinsed with water and dehydrated for 3min in 95% ethanol followed by 2min in xylene. Samples were mounted using coverslip slides and permount and photographed with 50 times magnification.

4.3.2 ELISA

Blood was collected into serum separator tubes and allowed to clot for 1h. In order to separate serum and red blood cells, the tubes were centrifuged for 1min at 13000rpm (microcentrifuge) and sera were immediately frozen and stored at -20°C until use. If mice had been infected with M.tb, sera were passed through 0.2µm Spin-X® centrifugal filter tubes (5min at 6500rpm) in order to remove bacteria before transferring samples out of the BSL3 facility.
Immuno Maxi-Sorp ELISA plates were coated with 100µl/well of 1µg/ml protein or PPD in ELISA coating buffer and incubated overnight at 4°C. Plates were washed 5x using ELISA wash buffer and blocked with 200µl/well ELISA

blocking buffer for 1h at 37°C. After repeating the washing step, sera were diluted 1:25 or 1:2500 in ELISA serum dilution buffer and 100µl of diluted sera were added to each well and incubated for 2h at 37°C. Plates were again washed 5 times prior to the addition of 100µl/well of alkaline phosphatase labelled antibodies, specific for murine IgG1 or IgG2a, diluted 1:2000 in ELISA serum dilution buffer. After 1h incubation, plates were washed 5x followed by the addition of 50µl SIGMA*FAST*™ p-Nitrophenyl phosphate substrate. The reaction was allowed to proceed for 20min in the dark before being stopped by addition of 50µl 3M NaOH. The plate was read within 24h in a SpectraMAX190 ELISA reader at 405nm.

4.3.3 Single cell isolation from the spleen

Mice were killed by cervical dislocation and spleens aseptically removed. If mice were infected with M.tb, spleens were cut in half and one half used for cfu determination and the other for IVR. In case of non-infected mice, whole spleens were processed for IVR. Experimental groups consisted of five mice, 2x two spleens were pooled and one processed individually, resulting in three samples of splenocytes per group of five mice for subsequent IVR. Spleens were mashed through a metal sieve with the plunger of a 5ml syringe in 20ml PBS/B and the cell suspension centrifuged for 5min at 1500rpm (Megafuge1.0R or Multifuge4KR in the BSL3 facility) and 4°C. The cell pellet was re-suspended in 2ml RBC lysis buffer at RT to lyse red blood cells. Lysis was stopped after 1min by addition of 18ml PBS/B and the cell suspension centrifuged for 5min at 1500rpm and 4°C. Isolated splenocytes were re-suspended in 5ml RPMI, passed through a 40µm cell strainer to remove debris that might clog the flow cytometer and viable cells counted by trypan blue exclusion. Cells were kept on ice during the whole isolation process until IVR was started.

4.3.4 Single cell isolation from the lung

Lymphocytes from the lungs were isolated as described [Kursar 2007]. Briefly, mice were killed by cervical dislocation, lungs were perfused with 10ml PBS through the heart, in order to flush out blood-circulating lymphocytes, and then aseptically removed. If mice were infected with M.tb, $^1/_5$ of the lung (lower left

lung lobe) was used for cfu determination and the remaining lobes for IVR, in case of non-infected mice, whole lungs were processed. Here too, lung tissue was pooled as described in section 4.3.3.. Lung tissue was transferred into a sterile Petri dish, carefully minced into small pieces and 10ml of collagenase digestion medium added. Lung tissue was digested for 30min at 37°C and then mashed through a metal sieve with the plunger of a 5ml syringe in 10ml PBS/B. The cell suspension was centrifuged for 5min at 1500rpm and 4°C (Megafuge1.0R or Multifuge4KR in the BSL3 facility) and the pellet thoroughly re-suspended in 15ml of 40% (v/v) Percoll in RPMI without additives. 5ml of this suspension was carefully layered onto 3ml of 70% (v/v) Percoll in RPMI without additives in 15ml tubes and centrifuged for 25min at 1800rpm and RT. The resulting interphase containing the lymphocytes was transferred into 50ml tubes already containing 30ml ice cold PBS/B and centrifuged for 5min at 1500rpm and 4°C. The cell pellet was re-suspended in 2ml RBC lysis buffer at RT to lyse red blood cells. Lysis was stopped after 1min by addition of 18ml PBS/B and the cell suspension was centrifuged for 5min at 1500rpm and 4°C. Cells were re-suspended in 2ml RPMI medium, passed through a 40µm cell strainer and viable cells counted by trypan blue exclusion. Cells were kept on ice during the whole isolation process until IVR was started.

4.3.5 *In vitro* re-stimulation

IVR protocol for determination of cytokine secretion in cell culture supernatants using the Bio-Plex bead-based assay system: All IVR were set up in 96-well plates in a total volume of 200µl using $2*10^6$ splenocytes or $2*10^5$ lymphocytes isolated from the lung. For specific IVR, cells were stimulated with either 10µg/well recombinant protein or PPD or 10^{-5}M of each of the respective peptides as listed in section 3.5. Non-specific background activation was determined by culturing cells only in RPMI. As positive control, lymphocytes were stimulated with 5µg/well of purified anti-mouse CD3 and anti-mouse CD28 mAb. Plates were incubated at 37°C for 72h if DNA vaccines were analysed (section 5.1.2) or for 18h if vaccinated with BCG (sections 5.4.2 and 5.4.3). After the incubation period, 150µl of cell culture supernatant was transferred into a fresh 96-well plate and stored at -20°C. If mice were infected with M.tb, cell culture supernatants were passed

through 0.2µm Spin-X® centrifugal filter tubes (2min centrifugation at 6500rpm) in order to remove any residual bacteria before transferring samples out of the BSL3 facility.

IVR protocol for the evaluation of cytokine secretion on a single cell level using intracellular cytokine staining (ICCS): Apart from the addition of brefeldin A to the cell cultures, the IVR setup for ICCS is identical to the one described for the Bio-Plex assay. If DNA vaccines were analysed (section 5.1.2), brefeldin A was added after 4h of stimulation and incubation continued for another 16h, whereas brefeldin A was added directly during the setup and incubation stopped after 6h in case mice were vaccinated with BCG (sections 5.4.2 and 5.4.3). In both cases, staining for ICCS, as described in section 4.3.7, immediately succeeded the incubation period.

4.3.6 Bio-Plex bead-based assay

The Bio-Plex Mouse Cytokine T_H1/T_H2 panel, Mouse IL-6 Assay and Mouse IL-17 Assay were performed according to the manufacturer's instructions with some slight modifications. Only 0.5µl beads in 50µl Assay buffer A, 0.15µl (50x concentrated) or 0.075µl (100x concentrated) of the detection antibodies in 25µl Assay buffer A and 0.2µl Streptavidin-PE (1mg/ml) in 50µl Assay buffer A per well were used. Beads as well as detection antibodies from all three kits were mixed together in order to analyse all cytokines in parallel within the same sample.

4.3.7 Flow cytometry

After 6 or 20h IVR, the 96-well plates were centrifuged for 3min at 1200rpm and 4°C (Megafuge1.0R or Multifuge4KR in the BSL3 facility), supernatants were discarded, cells re-suspended in 50µl FACS blocking buffer and incubated for 10min. Diluted CD4-Pacific blue and CD8-PerCP in 50µl PBS/B was added and cells incubated for 20min. 100µl PBS was added, plates were centrifuged for 3min at 1200rpm and 4°C, washed with 200µl PBS and finally fixed with 2% fixation buffer for 20min at RT. Plates were centrifuged for 3min at 1200rpm and 4°C, washed with 200µl PBS/B and fixed cells re-suspended in 100µl permeabilisation buffer. After 15min incubation, 10µl of a mixture of IL-2-APC, IL-17-PE, TNF-α-FITC and IFN-γ-PE-Cy7 antibodies, diluted in

permeabilisation buffer, was added and incubation continued for 30min. 100µl PBS was added, plates were centrifuged for 3min at 1200rpm and 4°C, cells were washed with 200µl PBS and fixed with 200µl 1% fixation buffer for 24h at 4°C. The 96-well plates were kept on ice during the whole procedure and covered with aluminium foil to prevent bleaching of the fluorochromes. Purchased antibodies were diluted as suggested in the respective data sheets; every batch of antibodies purified from cell culture supernatants was titrated before use. Prior to flow cytometric analysis, plates were centrifuged for 3min at 1200rpm, 4°C and stained cells were re-suspended in 200µl PBS/B. Cells isolated from non-infected mice were measured on an LSRII, whereas cells isolated from infected mice were analysed on a FACSCanto™II in our BSL3 mouse facility. In both cases acquisition was stopped, when 20000 CD8 T cells had been recorded. Flow cytometric data was analysed with the FACSDiva software and statistics calculated and complied using the FACS data Analyser.

4.4 Statistical analysis

Statistical significance of the results was determined by Mann-Whitney test with two tailed p values for non-parametric data using GraphPad Prism 4.0.

5 Results

5.1 DNA vaccines encoding dormancy-associated antigens are immunogenic

Almost 20 years after the first report that injection of naked DNA into muscle cells resulted in expression of the encoded protein [Wolff 1990], DNA vaccines have been shown to confer protection in various animal models of parasitic, viral and bacterial infections [Gurunathan 2000]. They induce robust CD8 and CD4 T cell responses and are comparatively easy to produce in large quantities whilst showing minimal side effects. Vaccination with plasmid DNA encoding prominent antigens of actively replicating M.tb has already been attempted by several groups [Lowrie 2006]. However, during the course of infection, mycobacteria can progress from an actively replicating metabolic state to a dormant one, eventually leading to the expression of different antigens rendering a vaccine directed against actively replicating bacteria ineffective. One objective of this thesis was to investigate the potential of plasmid DNA vaccines encoding dormancy-associated antigens, instead of antigens expressed by metabolically active bacteria, to induce protection against M.tb infection. This was assessed by the experiments described below.

5.1.1 Generation of plasmid DNA vaccines encoding dormancy-associated antigens

The dormancy-associated M.tb genes Rv1733c, hspX (Rv2031c), TB31.7 (Rv2623) and Rv2628 [Leyten 2006] were amplified by PCR from purified genomic DNA of M.tb with gene-specific oligonucleotide primers containing the additional sequence GGATCC, thus introducing BamHI restriction sites at both ends. PCR products were run on a 1% agarose gel to assess amplification and subcloned into pCR®2.1-TOPO®. After transformation, single colonies were picked, plasmids were purified and sequenced by a commercial company (AGOWA).

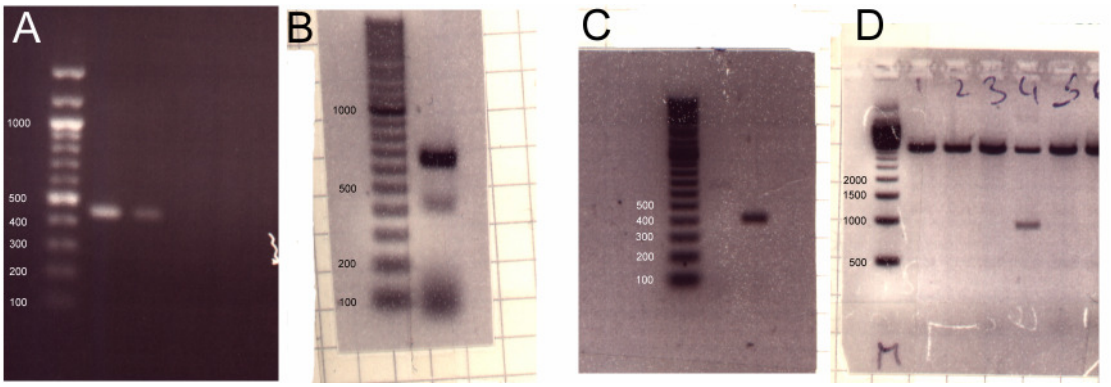

Figure 1: Amplification of open reading frames by PCR
A-C: 10µl PCR products or 100bp marker run on a 1% agarose gel to verify amplification. A: HspX size 447bp. B: Rv1733c size 635bp. C: Rv2628 size 387bp. D: PCR product already subcloned into pCR®2.1-TOPO®. Digestion with BamHI revealed 925bp TB31.7 insert in colony 4 only, a 500bp marker was used.

The sequences of amplified Rv1733c, TB31.7 and Rv2628 were found to be identical to the ones published (http://genolist.pasteur.fr/TubercuList/). Amplification of hspX introduced two point mutations which did not result in amino acid exchanges, one at position 351 (A replaced by G) and one at position 385 (T replaced by A). The amplified open reading frames were excised from the pCR®2.1-TOPO vector by restriction digest with BamHI and subsequently cloned into pCMVtPA also digested with BamHI. Correct orientation of the inserts was checked by restriction digests of purified plasmids from picked colonies.

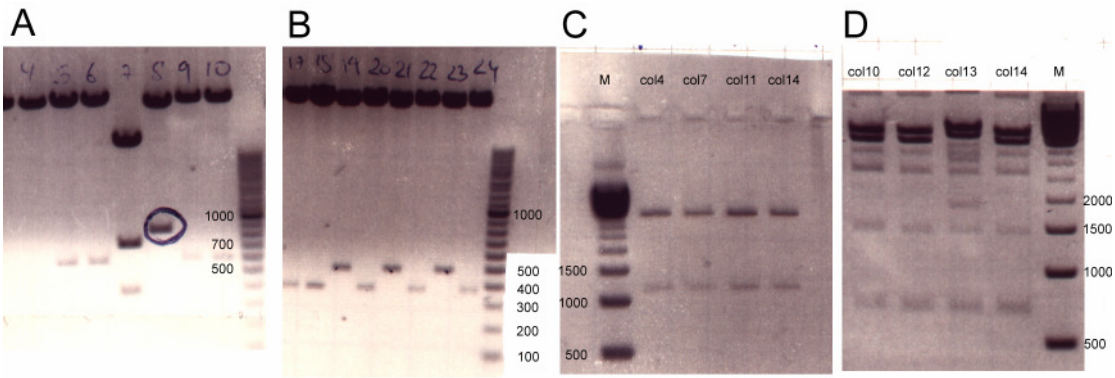

Figure 2: Orientation of cloned open reading frames in pCMVtPA
A: Restriction digest of pCMVtPA-Rv1733c with PvuII. Fragment size in correct orientation 835bp and 574bp if reversed B: Restriction digest of pCMVtPA-hspX with EcoRI. Fragment size in correct orientation 483bp and 365bp if reversed. C: Restriction digest of pCMVtPA-TB31.7 with PvuII. Fragment size in correct orientation 1159bp and 464bp if reversed. D: Restriction digest of pCMVtPA-Rv2628 with FseI and DraI. Fragment size in correct orientation 1442bp and 1187bp if reversed and an additional 692bp fragment in both cases.

Each of the cloning experiments yielded at least one *E.coli* DH5α colony-containing pCMVtPA with one of the four inserts hspX, Rv1733c, TB31.7 or Rv2628 in correct orientation. Aliquots of transformed bacteria were stored at -80°C and DNA for vaccine studies was purified using EndoFree Plasmid Giga Kits from Qiagen as described in the methods section.

5.1.2 Immune response evoked by plasmid DNA vaccines encoding dormancy-associated antigens

In a first set of experiments, the quality and magnitude of the immune response elicited by injection of naked DNA was assessed. Intracellular cytokine staining (ICCS) was chosen to detect frequencies of antigen-specific T cells because this is a very sensitive technique, which, in contrast to ELIspot, also enables distinction between responding CD4 and CD8 T cells.

Preliminary experiments indicated that at least 16 hours incubation with peptides were needed before cytokine secretion could be detected by ICCS. Therefore, isolated splenocytes were stimulated for 4 hours with the respective peptides followed by addition of brefeldin A and incubation continued for another 16 hours. The cells were then stained for surface expression of CD4 and CD8 as well as intracellular presence of IL-2, IL-17, IFN-γ and TNF-α (Figure 3).

The strongest CD4 as well as CD8 T cell responses were detected after vaccination with pCMVtPA-Rv2628. CD4 T cells secreted all four cytokines in response to stimulation, 0.6% of CD4 T cells secreted IL-17 and 0.27% IFN-γ, IL-2 and TNF-α were also detected above background levels but in lower amounts (both 0.13%). About 1.23% of CD8 T cells stained positive for IL-17. IFN-γ and TNF-α were also produced (0.32% and 0.4%) in response to Rv2628 peptide. Interestingly only 0.02% of CD8 T cells made IL-2.

Vaccination with any of the three vectors pCMVtPA-Rv1733c, pCMVtPA-hspX or pCMVtPA-TB31.7 resulted in ten times less T cells producing any of the four cytokines analysed as compared to pCMVtPA-Rv2628. However, cytokine secretion above background levels, as measured by 20h culture in medium only, was detected. After vaccination with pCMVtPA-TB31.7 CD4 and

CD8 T cells secreting IFN-γ (0.11% of both T cell populations after peptide stimulation; 0.04% medium control) and IL-17 (0.07% peptide stimulation; 0.03% medium control) were detected. Vaccination with pCMVtPA-hspX induced an even weaker immune response; the proportion of CD4 T cells secreting one of the four cytokines in response to specific stimulation was only twice as high as if cells were cultured in RPMI. Only 0.02% of CD8 T cells produced IL-17 or TNF-α after stimulation with peptides, IL-2 and IFN-γ were not detectable above background levels. The weakest immune response was evoked against Rv1733c: Four weeks after the last vaccination no antigen-specific CD8 T cells were detected. CD4 T cells secreting IFN-γ (0.06%), IL-17 and TNF-α (both 0.02%) were present above background levels (0.04%, 0.01% and 0.004% medium control, respective).
The same 20 hour stimulation protocol as described for splenocytes was applied to cells isolated from lungs or inguinal lymphnodes but no cytokine secretion in response to peptide stimuli was observed (data not shown).

For a more detailed characterisation of antigen-specific T cells, lymphocytes isolated from spleens, lungs or inguinal lymphnodes were re-stimulated for 72h with the respective peptides and culture supernatants were assayed for secretion of cytokines using a Bio-Plex bead-based assay (Figure 4). This assay enables the detection of various cytokines within one sample with a detection limit as low as 1pg/ml. Beads covering a broad range of well known T_H1/T_H2 cytokines were chosen for analysis: IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, GM-CSF, IFN-γ and TNF-α.
No cytokine secretion by cells isolated from the lungs or draining lymphnodes of vaccinated mice was detected after 72h re-stimulation with peptides in this assay.

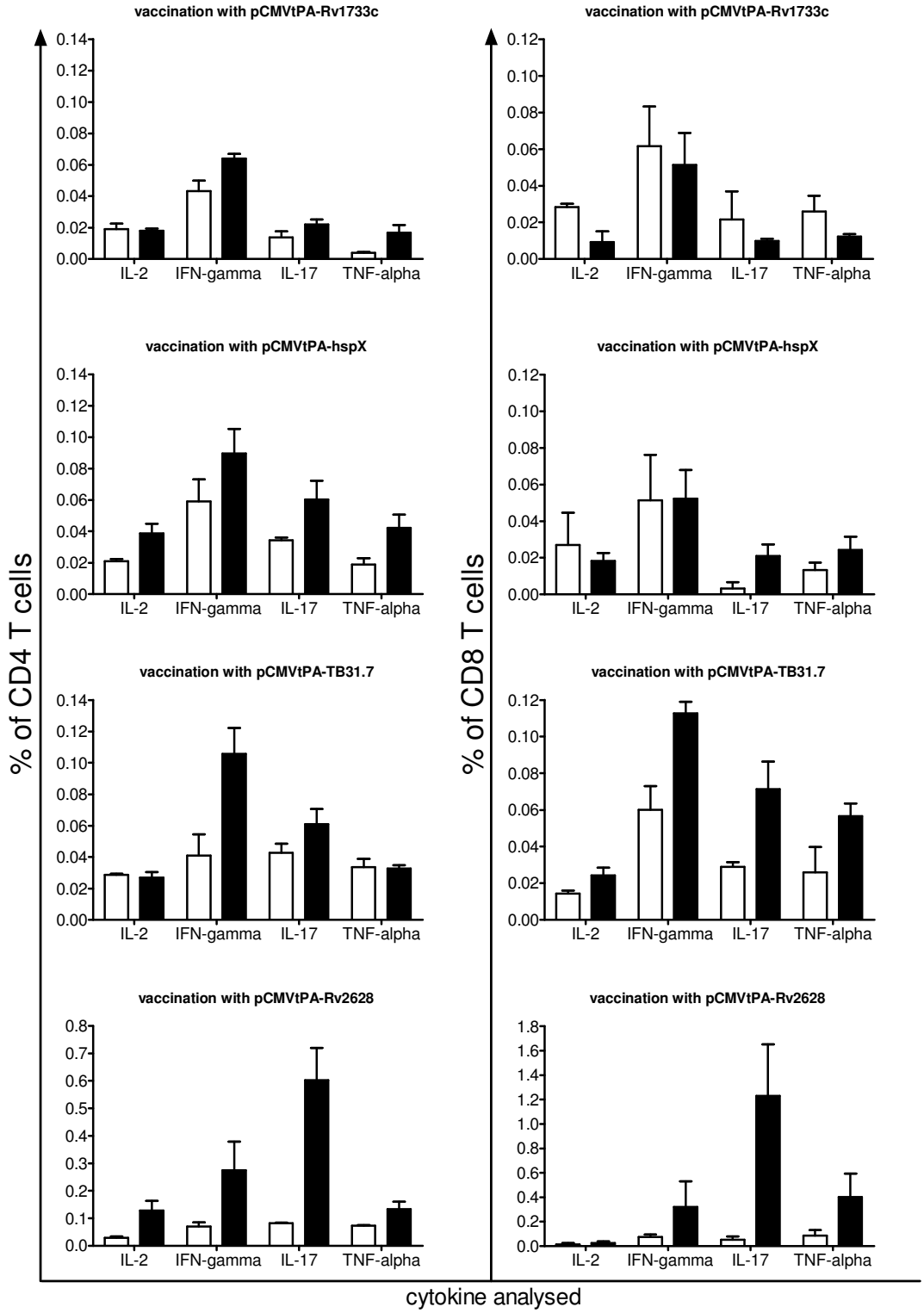

Figure 3: ICCS of splenocytes after DNA vaccination
BALB/c mice were vaccinated i.m. with 100µg of purified plasmid DNA three times in 14-day intervals and splenocytes isolated four weeks after the third vaccination. Cells were re-stimulated for 20h with either RPMI (white bars) or the respective peptides (black bars). The left panel shows percentage of CD4 T cells responding to stimulation and the right percentage of CD8 T cells. All four cytokines were measured at the same time within the same sample. Bars represent the means of three samples; each sample contained cells pooled from two mice, error bars depict SEM. One of two experiments is shown.

Secretion of TNF-α, IL-2 (both not shown) and IL-4 did not exceed background levels after three days stimulation with any of the peptides. As already observed by ICCS, vaccination with pCMVtPA-Rv1733c did not result in cytokine secretion of antigen-specific T cells after stimulation with peptides. Vaccination with pCMVtPA-hspX and pCMVtPA-TB31.7 and subsequent stimulation with the corresponding peptides induced secretion of IL-5, IL-6, IL-10, IL-12p70, IL-17, GM-CSF and IFN-γ with cytokine concentrations always being higher in cells from pCMVtPA-TB31.7 vaccinated mice.

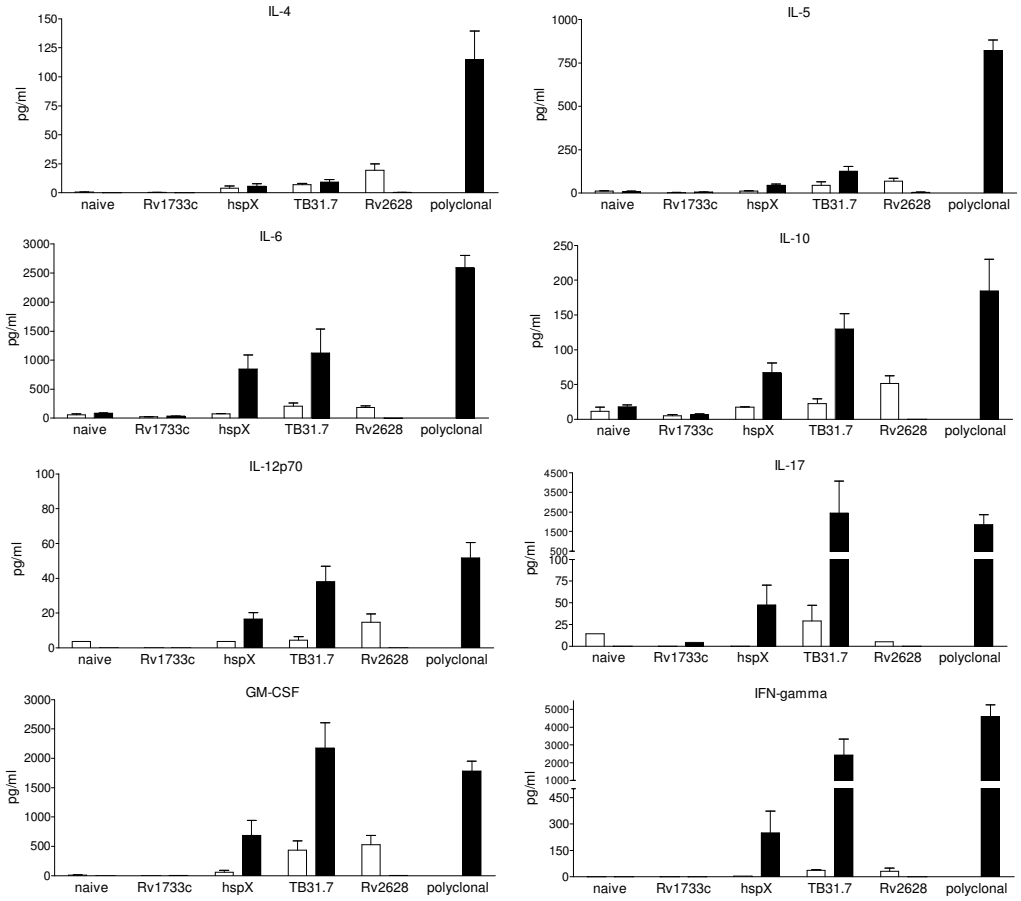

Figure 4: Cytokine secretion measured by Bio-Plex
BALB/c mice were vaccinated i.m. with 100µg of purified plasmid DNA three times in 14-day intervals and splenocytes isolated four weeks after the third vaccination. $2x10^6$ cells were re-stimulated for 72h with either RPMI (white bars) or the respective peptides (black bars). Cells stimulated with purified anti-CD3 and anti-CD28 antibodies (25µg/ml each) were used as positive control. Cytokines were measured in supernatants by Bio-Plex bead-based assay. Bars represent the means of three samples where each sample contained cells pooled from two mice, error bars depict SEM.

Of note is that massive secretion of IL-17, IFN-γ, GM-CSF and IL-6 (ranging from about 3500pg/ml of IL-17 to 1500pg/ml of IL-6) was detected after

vaccination with pCMVtPA-TB31.7 and those concentrations being in the same ranges as the polyclonal controls, again confirming results from ICCS where mostly IFN-γ and IL-17 were detected. No cytokine secretion was observed after application of pCMVtPA-Rv2328, which contradicts the results from ICCS where vaccination with this plasmid induced the highest percentages of antigen-specific T cells.

Plasmid DNA vaccination also induced production of antigen-specific antibodies. Interestingly only after vaccination with pCMVtPA-hspX high titres of IgG1 and IgG2a antibodies were detected. Vaccination with pCMVtPA-Rv1733c also resulted in production of specific antibodies but to a very low degree, whereas antigen-specific antibodies were not detected after vaccination with pCMVtPA-TB31.7 or pCMVtPA-Rv2628.

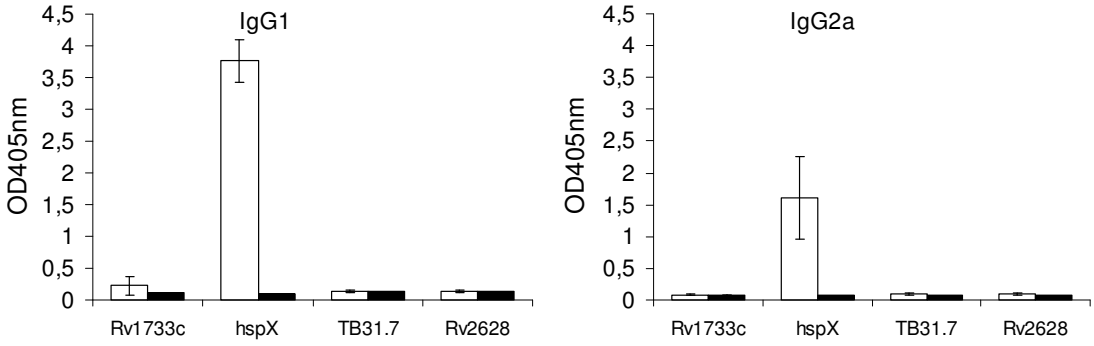

Figure 5: Antigen-specific antibodies after DNA vaccination
Detection of antigen-specific antibodies in sera from vaccinated (white bars) or in naïve animals (black bars). Bars represent mean OD_{405} values of serum, diluted 1:20, of six (vaccinated) or two (naïve controls) mice. Error bars show SD.

In summary, T cells specific for all four dormancy-associated antigens were detected in the spleen after application of plasmid DNA, however the frequencies of those cells were very low. The strongest T cell response, measured by ICCS, was evoked after vaccination with pCMVtPA-Rv2628 whereas the strongest response measured by Bio-Plex was detected after vaccination with pCMVtPA-TB31.7. T cell responses were pre-dominantly T_H1/T_H17. Specific antibody production was only detected after vaccination with pCMVtPA-hspX. As vaccination with any of the four plasmids gave rise to antigen-specific cells, they were assessed in therapeutic and prophylactic vaccination experiments against M.tb.

5.2 Vaccination with plasmid DNA encoding dormancy-associated antigens during persistent infection with *Mycobacterium tuberculosis*

Due to the lack of an adequate murine model for latent pulmonary TB, the plasmids encoding dormancy-associated antigens were tested either as post-exposure vaccines during persistent infection with M.tb or using the classical approach of prophylactic vaccination and subsequent aerosol challenge. The gold standard vaccine against TB is BCG, but even this only confers limited protection against establishment of persistent pulmonary TB infection. Administration of BCG will usually reduce bacterial burden approximately 10-fold in the lung [Orme 2006a] and it is generally expected that subunit vaccines, i.e. naked DNA encoding one single TB antigen, at best will result in 5-fold reduction of bacteria in the lung and/or spleen compared to non-vaccinated controls.

5.2.1 Intramuscular vaccination with plasmid DNA after aerosol infection with *Mycobacterium tuberculosis*

One third of the world's population is thought to be latently infected with M.tb. Thus, a vaccine which could help eradicate an already established infection would be desirable. Regardless of the difference in infection course in mice, the plasmid DNA vaccines encoding dormancy-associated antigens were tested in a post-exposure vaccination regime (Figure 6). Also taking into account, that the majority of individuals are vaccinated with BCG after birth, mice were primed with BCG, infected via aerosol with M.tb and boosted with plasmid DNA after development of a persistent infection. In order to minimise the number of mice used, three of the plasmids were mixed: DosR Mix is composed of 33.3µg pCMVtPA-Rv1733c, 33.3µg pCMVtPA-TB31.7 and 33.3µg pCMVtPA-Rv2628.

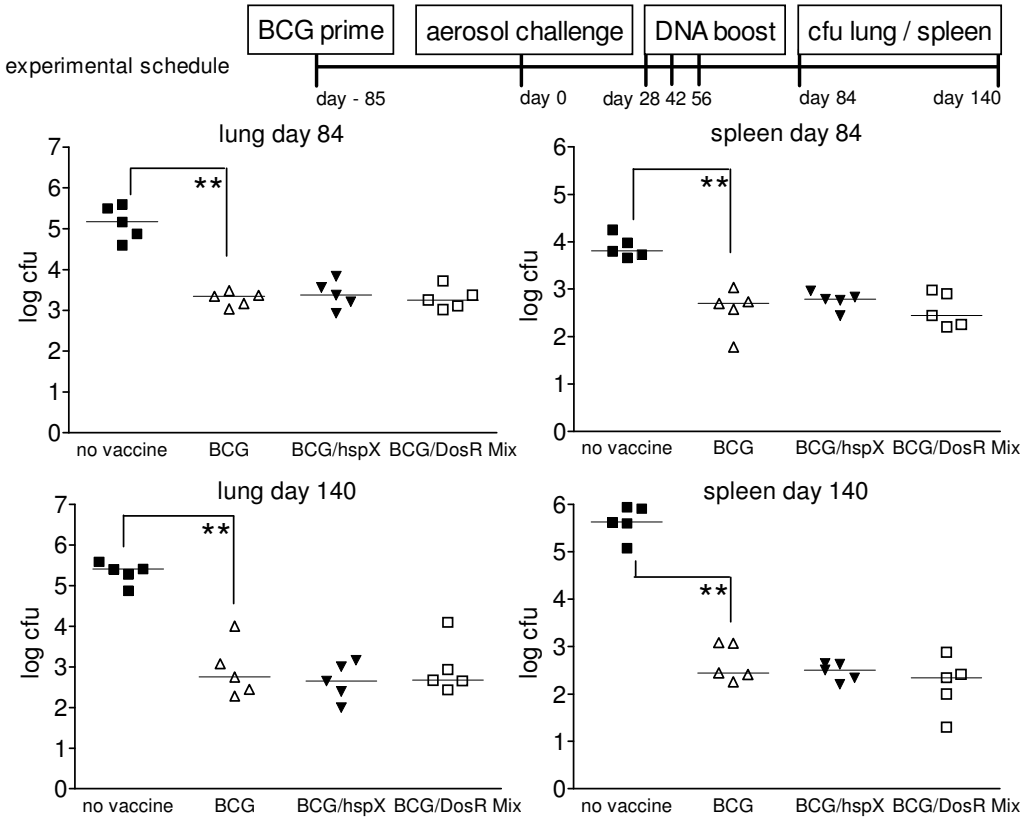

Figure 6: Bacterial burden after therapeutic boost with plasmid DNA
Mice were vaccinated with 10^6 cfu BCG s.c. at day -90, infected by aerosol with approximately 40cfu of M.tb at day 0, boosted with 100µg plasmid DNA i.m. at days 28, 42 and 56 post infection (p.i.). Bacterial burden in lungs and spleens were determined 4 and 12 weeks after the last boost. Experiment was done once. The asterisk indicates significance (** represents P=0.01-0.001) as determined by Mann-Whitney test with two tailed P values.

The plasmid encoding hspX was not included in the mixture, as there was the possibility of it inducing autoimmunity due to homology with murine heat shock proteins. Vaccination with BCG resulted in highly significant reduction of bacterial loads in lungs and spleens (P=0.0079 for all groups primed with BCG as compared to non-vaccinated control). However, neither boosting with pCMVtPA-hspX nor with a mixture of pCMVtPA-Rv1733c, pCMVtPA-tB31.7 and pCMVtPA-Rv2628 (33.3µg each) achieved an additional decrease in cfu counts in lung or spleen.

5.2.2 Intramuscular vaccination with plasmid DNA prior to aerosol infection with *Mycobacterium tuberculosis*

In a first experiment, the protective capacity of DNA vaccines administered intramuscularly was compared to that of BCG given subcutaneously (Figure 7 A-D). Significant and sustained protection in lung and spleen (p value 0.0159 for A-C and 0.0357 for D) were achieved by BCG s.c. vaccination but no protection with DNA vaccines was seen. Figure 7 E-F depict results from a different experiment, where mice were primed with BCG s.c. followed by three i.m. vaccinations with plasmid DNA prior to aerosol infection.

In this experiment, vaccination with BCG conferred highly significant protection with a 100-fold reduction of bacterial burden in the lung (p value 0.0079 for E) but no additional reduction of cfu counts after DNA boost in lung or spleen was observed.

In summary, none of the pre-exposure DNA vaccine regimes induced protection against subsequent challenge with M.tb in the lung or spleen.

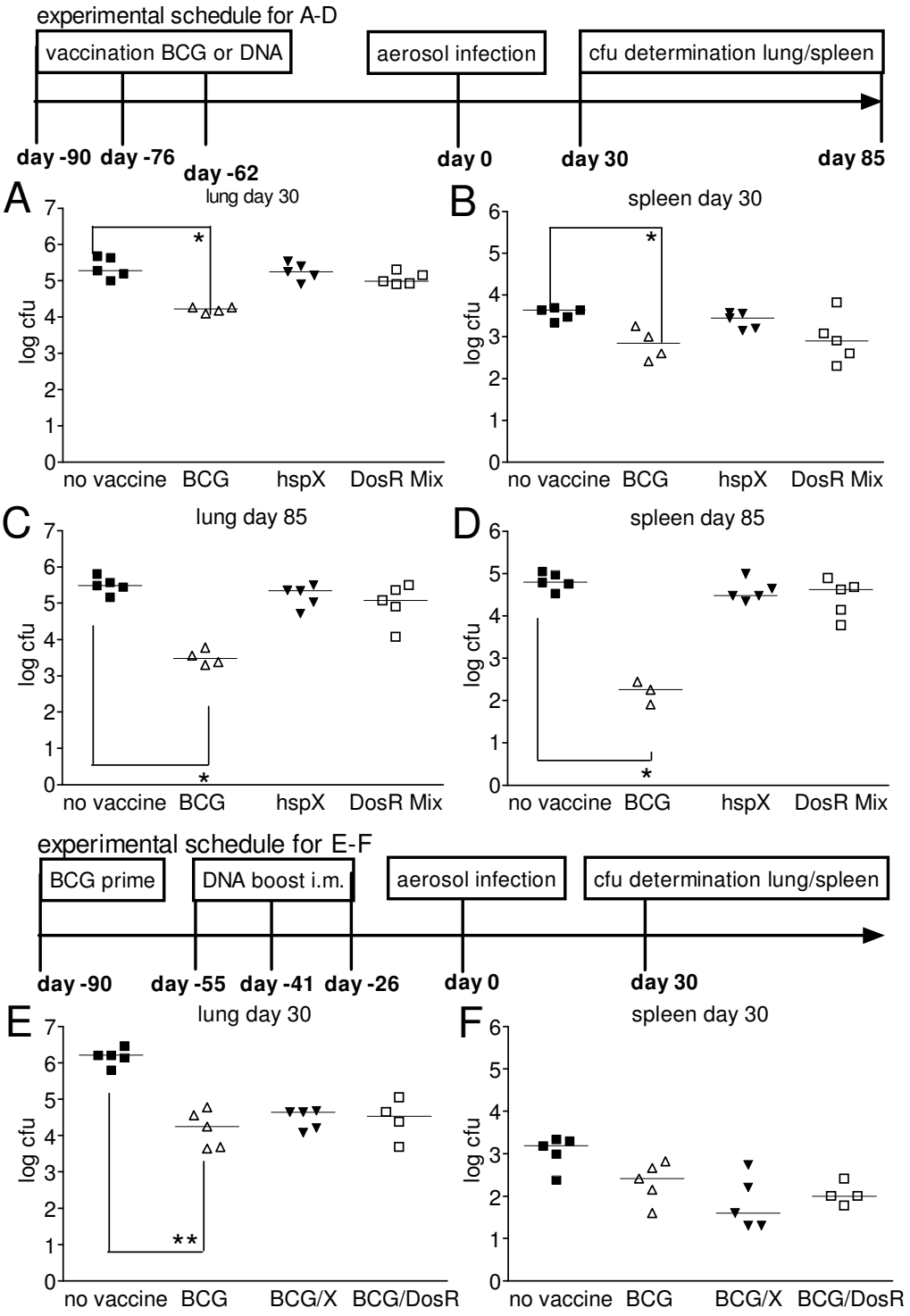

Figure 7: Bacterial burden in lungs and spleens after prophylactic DNA vaccination
Protection induced by BCG or DNA vaccines (A-D). Mice were vaccinated with 10^6cfu BCG s.c. at day -90 or 100µg plasmid DNA i.m. at days -90, -76 and -62; aerosol infection with 85cfu M.tb at day 0. Cfu determination at day 30 (A-B) and day 85 (C-D) p. i.. Booster effect of DNA vaccines (E-F). Mice were vaccinated with 10^6cfu BCG s.c. at day -90, boosted with 100µg plasmid DNA (X=pCMVtPA-hspX, DosR=mixture of pCMVtPA-Rv1733c, pCMVtPA-TB31.7 and pCMVtPA-Rv2628) i.m. at days -55, -41 and -26; aerosol infection with 220cfu M.tb at day 0. Cfu determination at day 30 p. i.. Experiments were done once. The asterisk indicates significance (* represents P=0.05-0.01 and ** represents P=0.01-0.001), determined by Mann-Whitney test with two tailed P values.

5.2.3 Intranasal vaccination with plasmid DNA before and after aerosol infection with *Mycobacterium tuberculosis*

One possible explanation for the failure of DNA vaccines to protect against pulmonary TB could be an inadequate application route. Intramuscular vaccination might induce antigen-specific T cells predominantly residing in the spleen, rather than cells homing to the lung. Therefore, intranasal application of DNA was tested next (Figure 8). The mucosal surface is a very strong barrier, which greatly reduces the uptake of DNA via the intranasal route. To overcome this barrier, DNA vaccines will have to be complexed onto carrier molecules. Chitosan has already been explored as a carrier for DNA because of its mucoadhesive properties and ability to open tight junctions [Bivas-Benita 2004; van der Lubben 2001]. Adsorption of DNA onto chitosan also has the advantage of reducing the amount of DNA needed for efficient induction of an immune response.

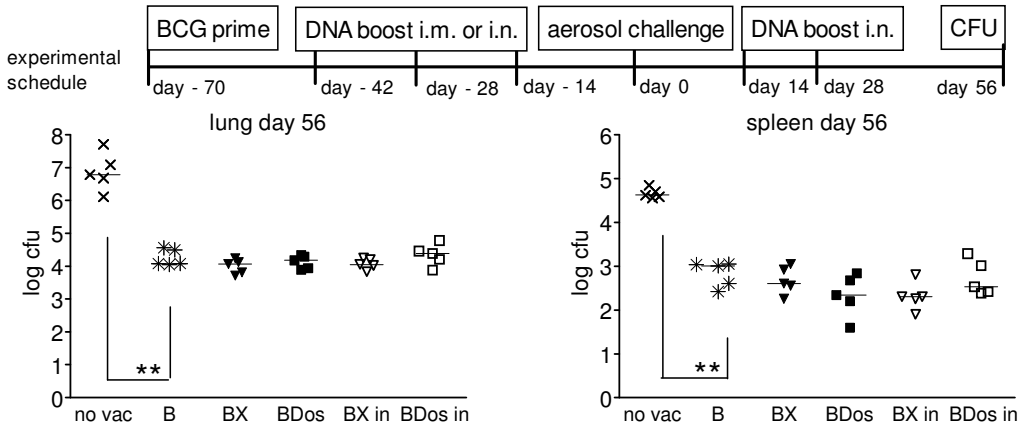

Figure 8: Intranasal application of plasmid DNA
Mice were vaccinated with 10^6cfu BCG s.c. at day -70, boosted with DNA i.m. (closed symbols) or i.n. (open symbols) at days -42, -28 and -14, infected by aerosol with 110cfu of M.tb at day 0 and boosted with plasmid DNA intranasal at days 14 and 28 p.i.. Bacterial burden in lungs and spleens was determined four weeks after the last boost. Abbreviations used: no vac=infected only control, B=10^6 cfu BCG; BX=10^6 cfu BCG + 100µg pCMVtPA-hspX i.m.; BDos: 10^6 cfu BCG + mixture of pCMVtPA-Rv1733c, pCMVtPA-Rv2623 and pCMVtPA-Rv2628 (50µg each) i.m.; in=pre-challenge boost given i.n.. I.n. vaccination with either 4µg pCMVtPA-hspX or 1.5µg of each plasmid in the mix. Experiment was done once. The asterisk indicates significance (* represents P=0.05-0.01 and ** represents P=0.01-0.001) as determined by Mann-Whitney test with two tailed P values.

Control experiments confirmed the maximum capacity of DNA adsorption onto chitosan as published (data not shown). Mice were first vaccinated with BCG, followed by i.m. application of naked plasmid DNA or i.n. administration of

DNA complexed onto chitosan (either hspX or a mixture of the other three plasmids for both). After aerosol infection, mice received two additional i.n. vaccinations with complexed DNA.

Vaccination with BCG resulted in highly significant reduction of bacterial loads in lungs and spleens (P=0.0079 for all groups primed with BCG as compared to non-vaccinated control). However, neither i.m. nor i.n. boosting prior to aerosol infection, followed by two additional i.n. vaccinations with pCMVtPA-hspX or a mixture of the other three plasmids post challenge, further diminished cfu counts in the lung or spleen.

Taken together, none of the plasmid DNA vaccine regimes tested so far could prevent or revert the development of persistent M.tb infection in the lung or spleen. A prerequisite for testing the efficacy of DNA vaccines encoding dormancy-associated antigens seems to be the development of a murine model for latent TB infection.

5.3 Plasmid DNA encoding dormancy-associated antigens in murine drug-induced latency models of *Mycobacterium tuberculosis* infection

Aerosol infection of mice with M.tb results in exponentially replicating bacteria, mainly in the lung, for the first four weeks after challenge until the adaptive immune response is fully developed. This will then lead to replication control and establishment of persistent infection with high bacterial titres. As shown in the previous experiments, DNA vaccines encoding dormancy-associated antigens did not confer protection in such challenge experiments. Therefore, a murine model of latent TB is needed in order to test vaccines potentially conferring protection against latent TB.

For screening of newly developed vaccines directed against dormant bacteria, a short-duration latency model would be desirable without the need for immunosuppressive agents or experiments lasting for over 18 months. Therefore a modified Cornell model [Botha 2002] was tested here, but instead of C57BL/6 mice the slightly more susceptible BALB/c strain was used, aiming

for a phase of latent infection followed by spontaneous reactivation after approximately three months.

5.3.1 Short-duration drug-induced latency model in BALB/c mice

The reactivation kinetics after low dose aerosol infection with M.tb and four-week treatment with rifampicin and isoniazid in the lungs of BALB/c mice were analysed (Figure 9A). Four-week treatment with antibiotics was sufficient to eradicate almost all bacteria from the lungs (average of three colonies per mouse detected) but only four weeks after completion of drug-therapy, reactivation had occurred with cfu numbers being in the same range as before treatment. Interestingly, later during the course of infection bacterial burden in mice treated with antibiotics remained approximately 10-fold lower than in control animals. Histology revealed granuloma formation in untreated control mice by week six post infection (p.i.) which increased over time. In drug-treated mice, no granulomas were visible after four-week therapy. Even though bacteria immediately reactivated, as measured by increased cfu counts, granuloma formation could still not be detected in those mice seven weeks after cessation of antibiotic treatment. In addition to four-week treatment with antibiotics mice were also vaccinated with plasmid DNA in a repeat experiment (Figure 9E). Here, too, reactivation occurred within four weeks after cessation of drug-therapy even though bacterial burdens in treated groups were still significantly lower as compared to those of the untreated control group ($P=0.0159$ for antibiotics only and $P=0.0079$ for antibiotics and DNA vaccines at day 70 p.i., $P=0.0159$ for all three at day 98 p.i.). Post-exposure vaccination with DNA encoding hspX or the plasmid mixture encoding the other three dormancy-associated antigens did not delay reactivation in the lung. Vaccination with the DosR mix seemed to delay reactivation in the spleen because only in two out of five spleens bacteria were detected at day 70 p.i.. Yet, the observed reduction in cfu was not significant ($P=0.0635$) compared to treatment with antibiotics alone and as reactivation in the lung progresses bacteria also reappear in the spleen.

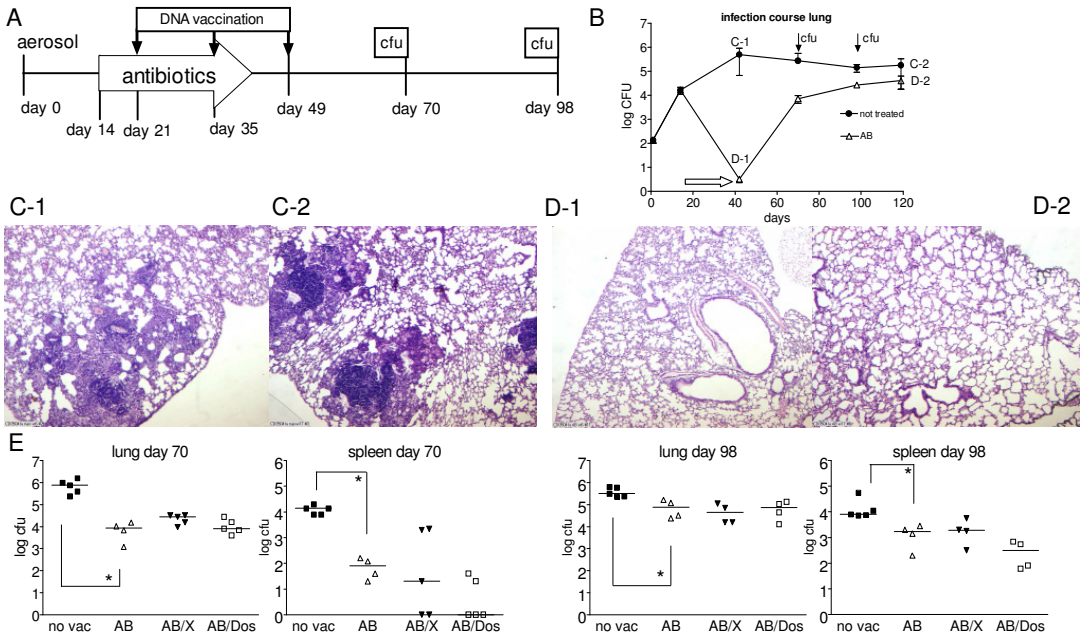

Figure 9: Rapid reactivation after four-week drug-treatment in BALB/c mice
A: Experimental setup. B: Infection course in the lung. BALB/c mice were infected via aerosol with 140cfu of M.tb, two weeks after challenge four-week drug-treatment (0.1g/L rifampicin and 0.1g/L isoniazid in drinking water) was initiated. Cfu determination in the lungs at indicated time points. Arrow depicts drug-treatment. One of three similar experiments is shown. C: H&E staining on paraffin sections of the lung week 6 (C-1) and week 17 (C-2) p.i.. Magnification: 50-fold. D: H&E staining on paraffin sections of the lung week 6 (D-1) and week 17 (D-2) p.i., mice received 4-week drug treatment. Magnification: 50-fold. E: BALB/c mice were infected via aerosol with 100cfu of M.tb, two weeks after challenge four-week drug-treatment (AB) and three weeks after challenge vaccination with plasmid DNA i.m. (X=100µg pCMVtPA-hspX or Dos=mixture of 33.3µg of each of the other three plasmids) was initiated. Experiment was done once. The asterisk indicates significance (* represents P=0.05-0.01 and ** represents P=0.01-0.001) as determined by Mann-Whitney test with two tailed P values.

In another four-week short course drug-treatment regime mice were again vaccinated with plasmid DNA in parallel but, as vaccination with the DosR mix indicated protection in the spleen, the plasmids pCMVtPA-Rv1733c, pCMVtPA-TB31.7 and pCMVtPA-Rv2628 were tested individually and compared to the mixture (Figure 10). Again, reactivation in the lung occurred immediately after cessation of drug-therapy and none of the vaccines tested prevented this. In contrast to the previous experiment, in which bacteria also reappeared in the spleen four weeks after antibiotics had been withdrawn, this was not the case in this repeat. Only in some of the plated samples bacteria were detectable, thus making it impossible to draw any conclusion about the efficacy of the DNA vaccines tested. It should be noted, that cfu levels in the lungs at day 70 p.i. were approximately 10-fold lower as compared to the first experiment, indicating some degree of variability in the reactivation kinetics

making it difficult to choose the appropriate time for determination of bacterial load after vaccination.

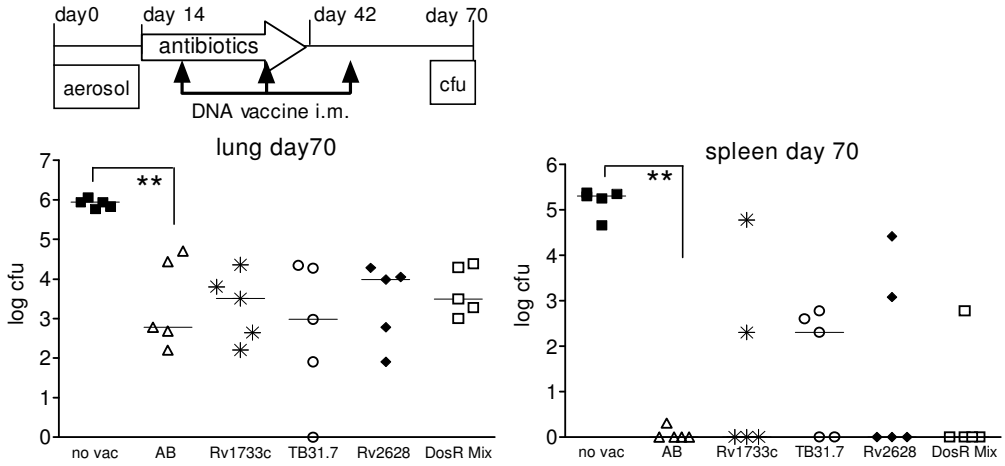

Figure 10: Plasmid DNA vaccination during four-week drug-treatment in BALB/c mice
Bacterial burden in lungs and spleens 4 weeks after withdrawal of antibiotics. BALB/c mice were infected by aerosol with 155cfu of M.tb, four-week drug-treatment was started after two weeks, followed by i.m. vaccination with plasmid DNA 3, 5 and 7 weeks after challenge. Abbreviations used: no vac=mice infected only, AB=4 weeks antibiotics only; Rv1733c, TB31.7, Rv2628 or DosR Mix=4 weeks antibiotics + three times 100µg (3x33.3µg in the mix) plasmid DNA. Experiment was done once. The asterisk indicates significance (* represents P=0.0159 and ** represents P=0.0079) as determined by Mann-Whitney test with two tailed P values.

Taken together these results indicate that four-week treatment with rifampicin and isoniazid is insufficient to induce latent infection in BALB/c mice after aerosol challenge with a low dose of M.tb. Therefore, post-exposure vaccination with DNA encoding dormancy-associated antigens did not prevent immediate reactivation of bacteria in the lung. Delayed, albeit not statistically significant, re-emergence of bacteria was observed in the spleen in one experiment.

5.3.2 Prolonged drug-treatment to induce latent infection in mice

As four-week therapy with rifampicin and isoniazid did not prevent immediate re-growth of M.tb, antibiotics were administered for eight weeks in the following experiments. Vaccination with plasmid DNA, given i.m. as well as i.n., was started in parallel. Even an increased period of eight-week treatment with antibiotics did not prevent immediate re-emergence of actively replicating bacteria in lungs of BALB/c mice infected with a low dose of M.tb and neither

i.m. nor i.n. application of plasmid DNA vaccines could delay this. Bacteria also reappeared in the spleen but with slightly delayed kinetics. Therefore any protection conferred by i.n. vaccination could not be detected four weeks after cessation of drug-therapy. However, cfu data from spleens of i.m. vaccinated mice were not significantly lower than those of drug-treated controls making it highly unlikely that i.n. vaccination would have been effective.

Several other variations of the Cornell model were assessed in addition to those already presented here, which shall only be briefly mentioned without showing any more data, as the results are all comparable. Even vaccination with BCG prior to challenge with M.tb and successive eight-week drug-therapy did not result in a long lasting period of latent infection in BALB/c mice. Four weeks after completion of antibiotic treatment bacterial burden in the lungs of mice vaccinated with BCG prior to infection were again comparable to those of mice, which had not received any antibiotics. Another approach was based on infecting F1 generation mice from BALB/c (susceptible to spontaneous reactivation) x C57BL/6 (very late or no spontaneous reactivation) mice, with a low dose aerosol challenge followed by six-week treatment with antibiotics. Yet again, vaccination with plasmid DNA encoding dormancy-associated antigens did not prevent reactivation in the lung and here too, reactivation kinetics in the spleens were unpredictable.

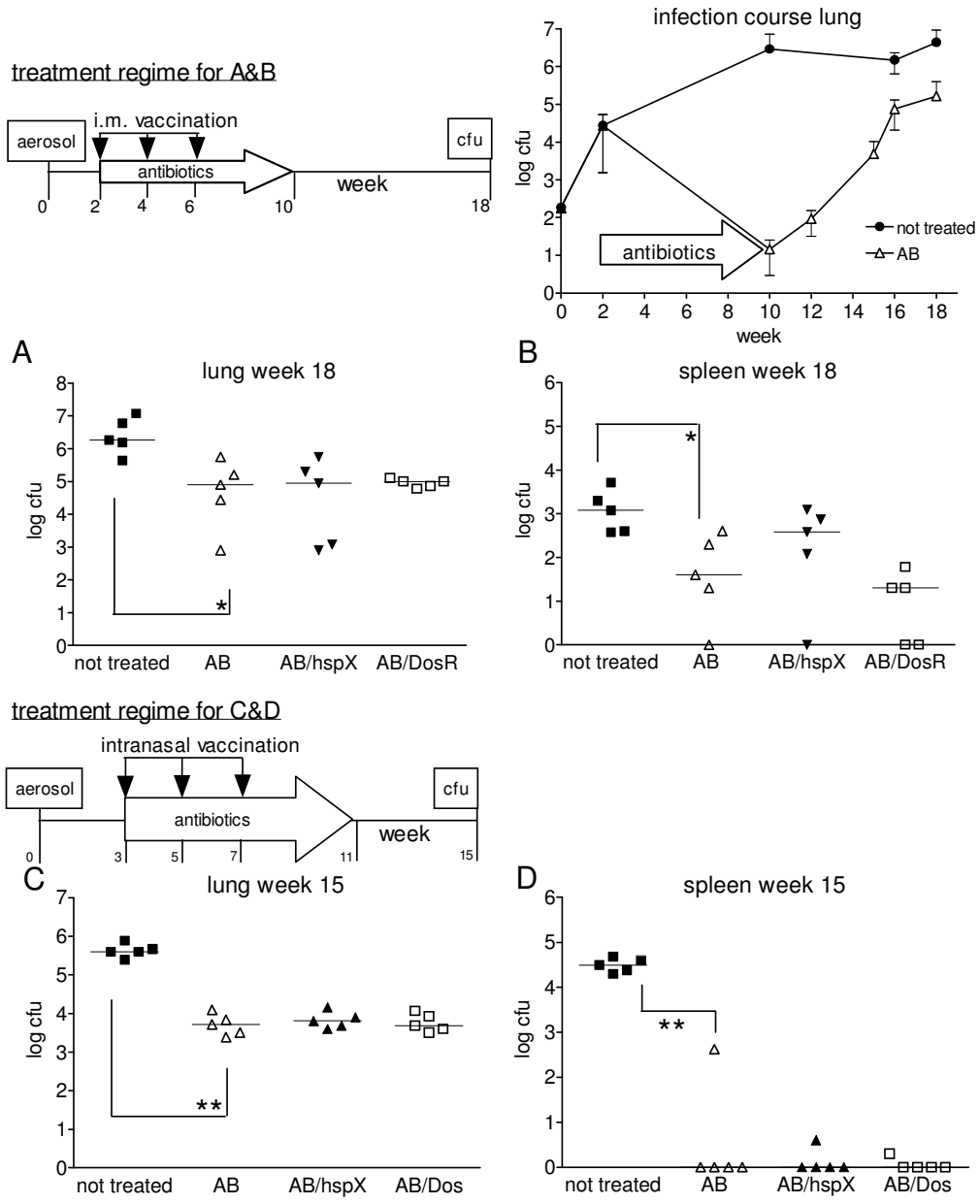

Figure 11: DNA vaccination during eight-week drug-treatment in BALB/c mice
Infection course in the lung following eight-week treatment with rifampicin and isoniazid and bacterial burden in lungs and spleens eight or four weeks after completion of therapy. A+B: I.m. DNA vaccination during eight-week treatment with rifampicin and isoniazid. Aerosol challenge dose was 180cfu of M.tb, drug-treatment was started after two weeks, accompanied by i.m. vaccination with plasmid DNA 2, 5 and 7 weeks after challenge. Abbreviations used: AB=8 weeks antibiotics only; AB/hspX or AB/Dos=8 weeks antibiotics + 3 times 100µg (3x33.3µg in the mix) plasmid DNA. C+D: I.n. DNA vaccination during eight weeks of treatment with antibiotics. Challenge dose was 155cfu. Drug-therapy started three weeks p.i. and vaccines were given on weeks 3, 5 and 7 p.i.. Experiments were done once. The asterisk indicates significance (* represents P=0.0159 and ** represents P=0.0079) as determined by Mann-Whitney test with two tailed P values.

Taken together it is still unclear, whether DNA vaccines encoding the dormancy-associated antigens Rv1733, hspX, TB31.7 or Rv2628 are capable

of conferring protection against reactivation of dormant bacteria, as it was not possible to test the vaccines in a robust mouse model of latent M.tb infection.

5.4 Analysis of the vaccine-induced immune response involved in protection against infection with *Mycobacterium tuberculosis*

Understanding the mechanisms involved in the development of a protective immune response against pulmonary TB will be crucial for the development of improved vaccines. Usually new vaccine candidates are monitored by measuring cytokine secretion, most commonly only IFN-γ, after vaccination and re-stimulation. Undoubtedly IFN-γ does play a crucial role in the defence against mycobacteria [Pearl 2001] but production of this cytokine alone cannot be considered as a reliable marker of a protective immune response. More likely a whole range of different cytokines will be involved in orchestrating a very complex immune response which ultimately leads to containment of infection. The second part of this thesis is dedicated to identifying correlates of protection against pulmonary TB after vaccination. This will provide valuable information for future vaccine development, especially for designing follow up experiments of individuals' immune responses after vaccination in clinical phase I/II trials. The only available vaccine against TB is BCG, one of the safest viable vaccines known. However, its protective efficacy against pulmonary TB in adults is debatable and protection wanes with time. Members of our lab engineered a recombinant ureaseC-deficient BCG strain expressing membrane perforating listeriolysin (Hly) of *Listeria monocytogenes* named Δ*ureC hly+* rBCG (recBCG) and showed that it induced much better protection against aerogenic challenge with M.tb than the parental strain. Especially at late stages of infection recBCG was much more effective [Grode 2005]. Comparing these two vaccines against each other and against untreated controls might lead to the identification of factors indicative of the development of a protective immune response against pulmonary TB. Vectors of immunity shall be identified through a detailed analysis of the immune response elicited after vaccination with recBCG or the parental strain one week prior to aerosol

challenge and 90 days p.i., when the superior protective effect of recBCG has become visible.

5.4.1 Increased vaccine efficacy of recombinant BCG expressing listeriolysin against infection with virulent *Mycobacterium tuberculosis*

In the published vaccine studies showing superior protection of recBCG, mice were vaccinated via the i.v. route. However, human vaccines are generally not administered i.v.. Therefore, in a first experiment the protective capacity of recBCG after s.c. injection was examined and found to be identical to i.v. vaccination. Bacterial burden in spleens and lungs were determined 90 days after infection with M.tb.

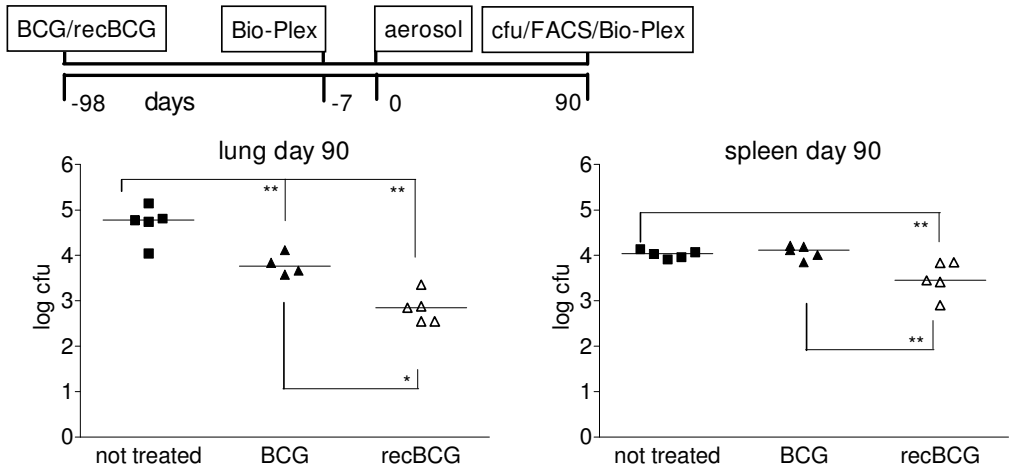

Figure 12: Bacterial burden in lung and spleen 90 days p.i.
Vaccination schedule and bacterial burdens in lungs and spleens 90 days p.i. with 200cfu of M.tb. Mice were vaccinated with $5x10^5$cfu of BCG or recBCG s.c., infected only controls were included. The asterisk indicates significance (* represents P=0.0159 and ** represents P=0.0079) as determined by Mann-Whitney test with two tailed P values. Experiment was done once.

Vaccination with parental BCG reduced bacteria in the lung significantly by approximately 10-fold. Cfu counts could be further decreased in the lung by application of recBCG. Roughly, 100 times less bacteria than in untreated controls were detected in the lung. This difference was highly significant and mice were also significantly better protected by vaccination with recBCG than by parental BCG. Vaccination with recBCG also conferred significant

protection against M.tb in the spleen, whereas application of parental BCG was not effective.

5.4.2 Characterisation of the memory response elicited by subcutaneous vaccination with BCG or recBCG

In order to identify vectors of immunity against infection with M.tb, the memory response elicited after vaccination with recBCG or the parental strain was analysed 90 days after vaccination. Cells were isolated from the lung or spleen and stimulated for 18h with PPD, Ag85A peptides or Ag85A protein. Cell culture supernatants were collected and analysed by Bio-Plex assay. Stimulation with Ag85A was included as it is known to be a major antigen of M.tb and detailed epitope mapping has already been conducted [Denis 1998]. Using the Bio-Plex bead-based assay a very robust response to PPD was detected in the spleen 13 weeks after vaccination (Figure 13). Almost all cytokines (GM-CSF, IFN-γ, IL-2, IL-6, IL-10, IL-12p70 and IL-17) analysed with this assay were detectable after stimulation with PPD whilst only minimal amounts of IL-4, IL-5 and TNF-α were produced. Splenocytes secreted mainly IFN-γ, IL-6, IL-17, IL-2 and GM-CSF (listed from highest to lower concentrations detected) in response to stimulation with PPD and there was no difference in the amount of IFN-γ, IL-2 and GM-CSF detectable after vaccination with recBCG or the parental strain. However elevated levels of IL-17, IL-6, IL-10 and IL-12p70 (listed from largest to smallest difference) where detectable after vaccination with recBCG compared to parental BCG. IL-2 and IL-6 secretion in response to stimulation with Ag85A peptides (CD4 and CD8 epitopes) or purified recombinant protein was also detected after vaccination with BCG, albeit to a much lesser extent than in response to PPD. No Ag85A specific T cell response was detected in the recBCG vaccinated group; note high background for IL-6 secretion.

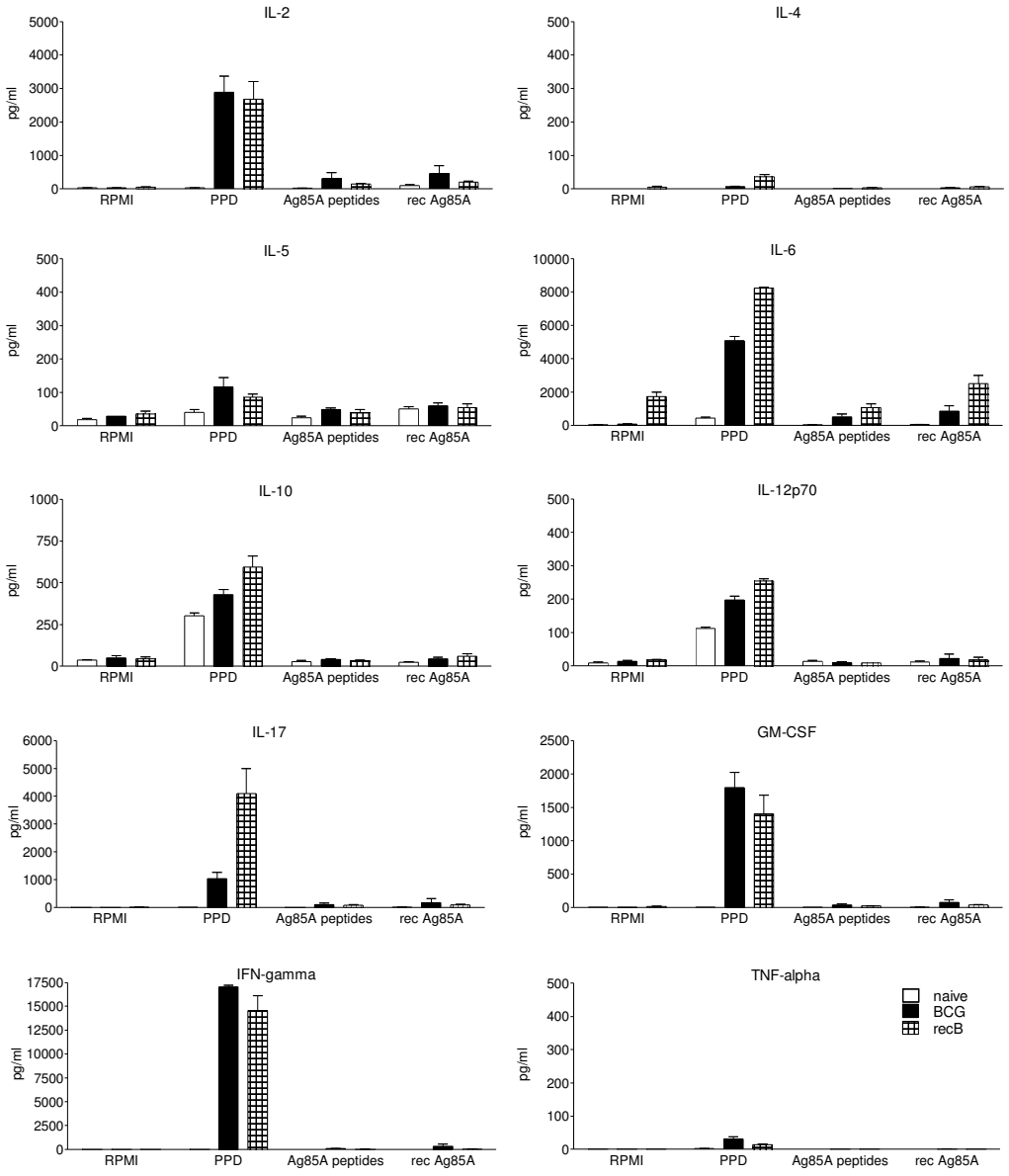

Figure 13: Cytokine secretion of splenocytes measured by Bio-Plex
Recall response in the spleen 13 weeks after vaccination. Mice were vaccinated with $5x10^5$cfu of BCG (black bars) or recBCG (chequered bars) s.c.; naïve controls (white bars). $2x10^6$ cells isolated from spleens were re-stimulated for 18h with either RPMI, PPD, Ag85A peptides or purified recombinant Ag85A protein. Cytokines were measured in supernatants by Bio-Plex bead assay. Bars represent the means of three samples where two samples contained cells pooled from two mice and one sample contained cells from one mouse only, error bars depict SEM. Experiment was done once.

The immune response elicited in the lung after vaccination with parental BCG or recBCG was also assessed (Figure 14). Cells isolated from the lung were stimulated with PPD, peptides or recombinant protein and cell culture supernatants tested in the Bio-Plex assay.

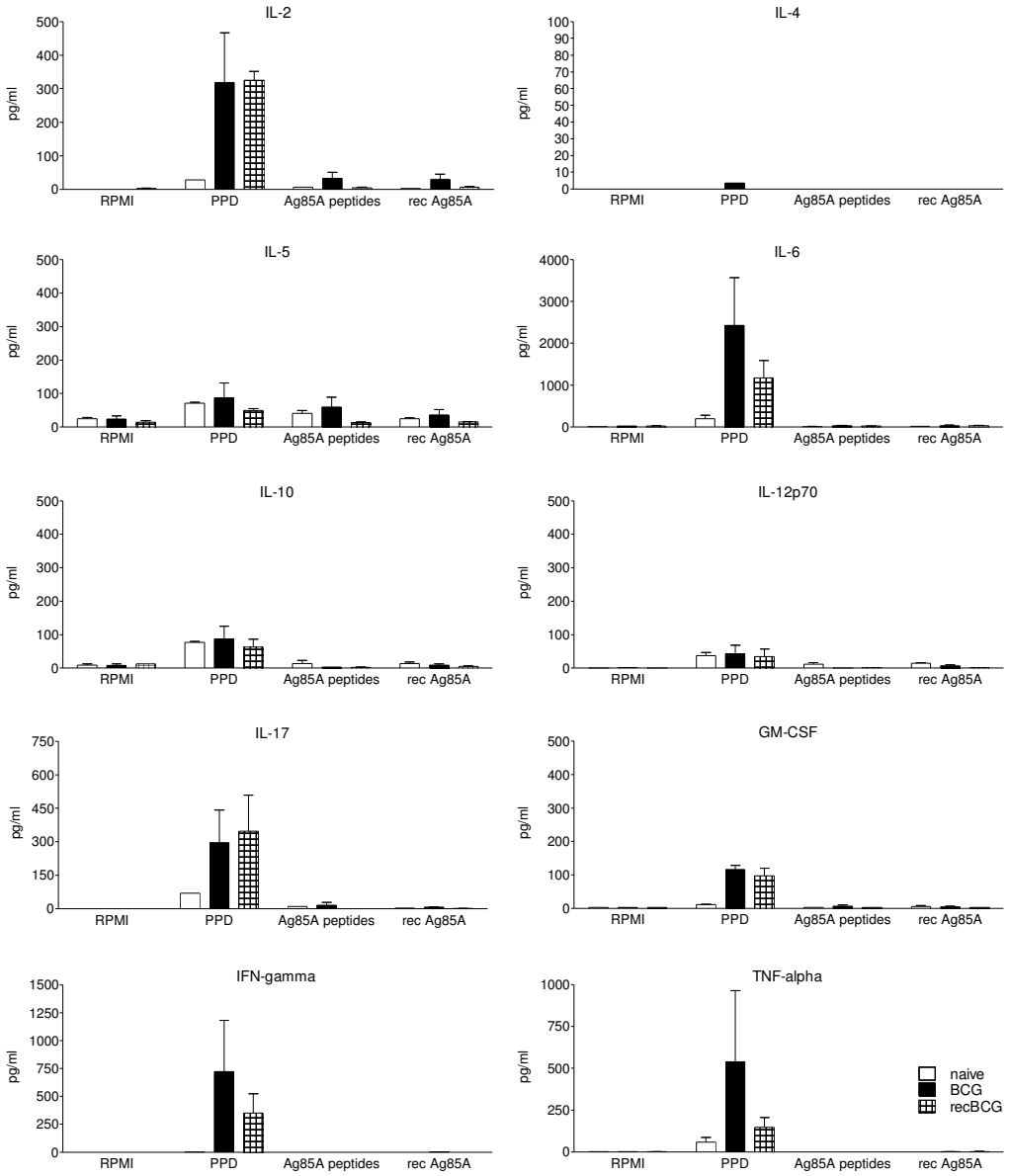

Figure 14: Cytokine secretion of lymphocytes isolated from the lung measured by Bio-Plex

Recall response in the lung 13 weeks after vaccination. Mice were vaccinated with $5x10^5$cfu of BCG (black bars) or recBCG (chequered bars) s.c.; naïve controls (white bars). $2x10^5$ cells isolated from lungs were re-stimulated for 18h with either RPMI, PPD, Ag85A peptides or purified recombinant Ag85A protein. Cytokines were measured in supernatants by Bio-Plex bead assay. Bars represent the means of three samples where two samples contained cells pooled from two mice and one sample contained cells from one mouse only, error bars depict SEM. Experiment was done once.

Apart from minimal levels of IL-2 after vaccination with parental BCG, no cytokine secretion in response to stimulation with Ag85A peptides or protein was detected in any of the experimental groups. However, lymphocytes isolated from the lungs of vaccinated mice did respond to stimulation with PPD by secretion of IL-2, IL-6, IL-17, GM-CSF, IFN-γ and TNF-α but to a

much lesser extend than splenocytes and no differences between BCG and recBCG were detected.

5.4.3 Potential correlates of protection 90 days after infection with *Mycobacterium tuberculosis*

The immune response directed against M.tb 90 days after infection, when recBCG vaccinated mice were significantly better protected than mice immunised with parental BCG, was assessed in greater detail in order to identify potential correlates of protection.

Two different assays were employed to analyse the immune response elicited after vaccination with parental or recBCG, namely intracellular cytokine staining (ICCS) for four T_H1/T_H17 polarising cytokines considered to be of vital importance in the defence against M.tb [Jung 2002; Khader 2007] as well as a Bio-Plex bead-based assay covering a broad range of well known T_H1/T_H2 cytokines: IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, GM-CSF, IFN-γ and TNF-α. Both assays were done in parallel with the same pools of isolated cells from lung or spleen.

Stimulation of lymphocytes isolated from the lungs 90 days p.i. revealed marked differences in the cytokine secretion profile depending on the vaccine that was given. Analysis of cell culture supernatants by Bio-Plex showed that lymphocytes isolated from the lung reacted strongly against PPD but only weakly against Ag85A peptides or protein. The concentrations of most cytokines were at least twice as high when mice had been vaccinated with recBCG compared to parental BCG. Most prominent was a 5-fold up-regulation of IL-2, observed in supernatants from the recBCG group. Vaccination with recBCG induced cells secreting high amounts of IL-6 (14,000pg/ml), IL-2 (11,000pg/ml), GM-CSF (3,500pg/ml) and TNF-α (1,500pg/ml) in response to stimulation with PPD and such high concentrations of cytokines in supernatants were not observed when parental BCG was administered. A response against Ag85A protein, namely IL-2, GM-CSF, IFN-γ and IL-6 secretion, was also detected when recBCG was used,

whereas IFN-γ and IL-17 were detected when parental BCG was administered.

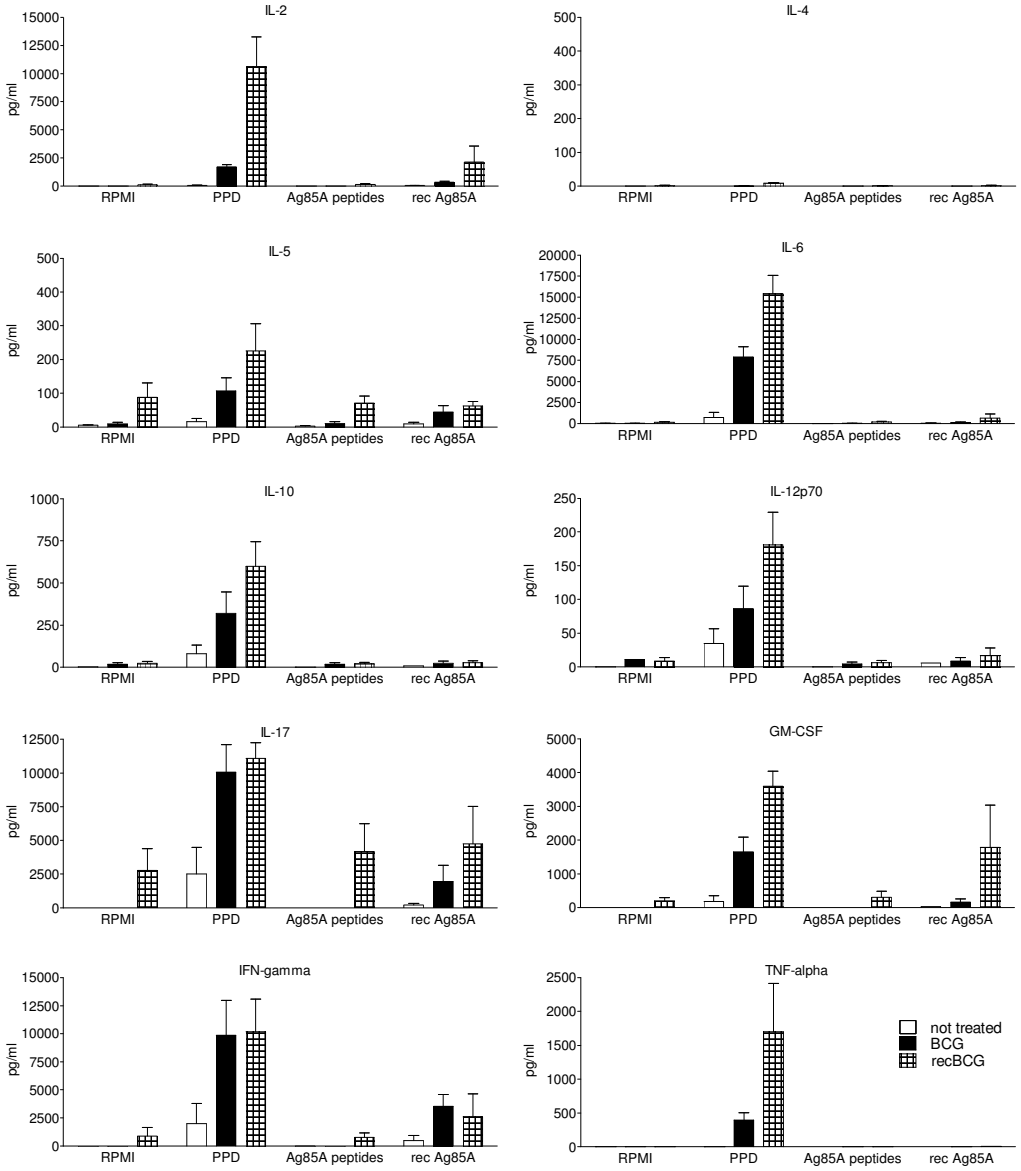

Figure 15: Cytokine secretion of lymphocytes isolated from the lung measured by Bio-Plex 90 days p.i.
Recall response 90 days p.i. in the lung. Mice were vaccinated with $5x10^5$cfu of BCG (black bars) or recBCG (chequered bars) s.c.; non-vaccinated controls (white bars). 13 weeks after vaccination mice were challenged with 200cfu M.tb via aerosol. 90 days p.i. mice were sacrificed and $5x10^5$ cells isolated from lungs were re-stimulated for 18h with either RPMI, PPD, Ag85A peptides or purified recombinant Ag85A protein. Supernatants were passed through 0.2µm centrifugal filters before transfer out of the BSL3 facility. Cytokines were measured in supernatants by Bio-Plex bead assay. Bars represent the means of three samples where two samples contained cells pooled from two mice and one sample contained cells from one mouse only, error bars depict SEM. Experiment was done once.

Only after vaccination with recBCG and stimulation with Ag85A peptides lymphocytes produced IFN-γ. No differences in the amounts of IFN-γ, IL-17

and IL-5 were found, if vaccination with recBCG and parental BCG, followed by stimulation with PPD, were compared. Of note is that in the recBCG-vaccinated group, cells readily secreting IFN-γ and especially IL-17 without specific stimulation (the RPMI control) were detected. If mice were only infected, but not vaccinated, the response to PPD stimulation was weak. IL-17 and IFN-γ were detected in the supernatants, albeit only a quarter of what was measured in supernatants from vaccinated groups.

Even though the analysis of cytokines in cell culture supernatants provided valuable insights into the immune response needed to control persistent TB infection this assay does not allow differentiation between responding CD4 and CD8 T cells. Therefore, IL-2, IFN-γ, IL-17 and TNF-α secretion was also measured by ICCS after 6h incubation with PPD or Ag85A peptides in the presence of brefeldin A. The data generated by Bio-Plex and by ICCS complement each other. While roughly 5 times more IL-2 was detected, if mice were vaccinated with recBCG, this was reflected in the percentage of CD4 T cells (2% in recBCG versus 1% in BCG) secreting IL-2 in response to stimulation with PPD. And as cells isolated from both BCG and recBCG primed mice released the same amount of IL-17 and IFN-γ into the culture medium, so were the percentages after stimulation of CD4 T cells with PPD also comparable. Interestingly the percentages of CD4 T cells secreting TNF-α in response to PPD stimulation were comparable between the BCG and recBCG groups even though in the cell culture supernatants of recBCG vaccinated mice 3 times more TNF-α was detected. This can be explained if the mean fluorescence intensities (mfi) after staining with FITC conjugated anti-murine TNF-α antibodies of these two groups are compared, the brighter the staining, the more cytokine present: The mfi of the recBCG group is increased by approximately 50% compared to the group vaccinated with parental BCG (from 850 to 1250, data not shown), indicating that the same proportion of antigen-specific CD4 T cells secrete more TNF-α per cell.

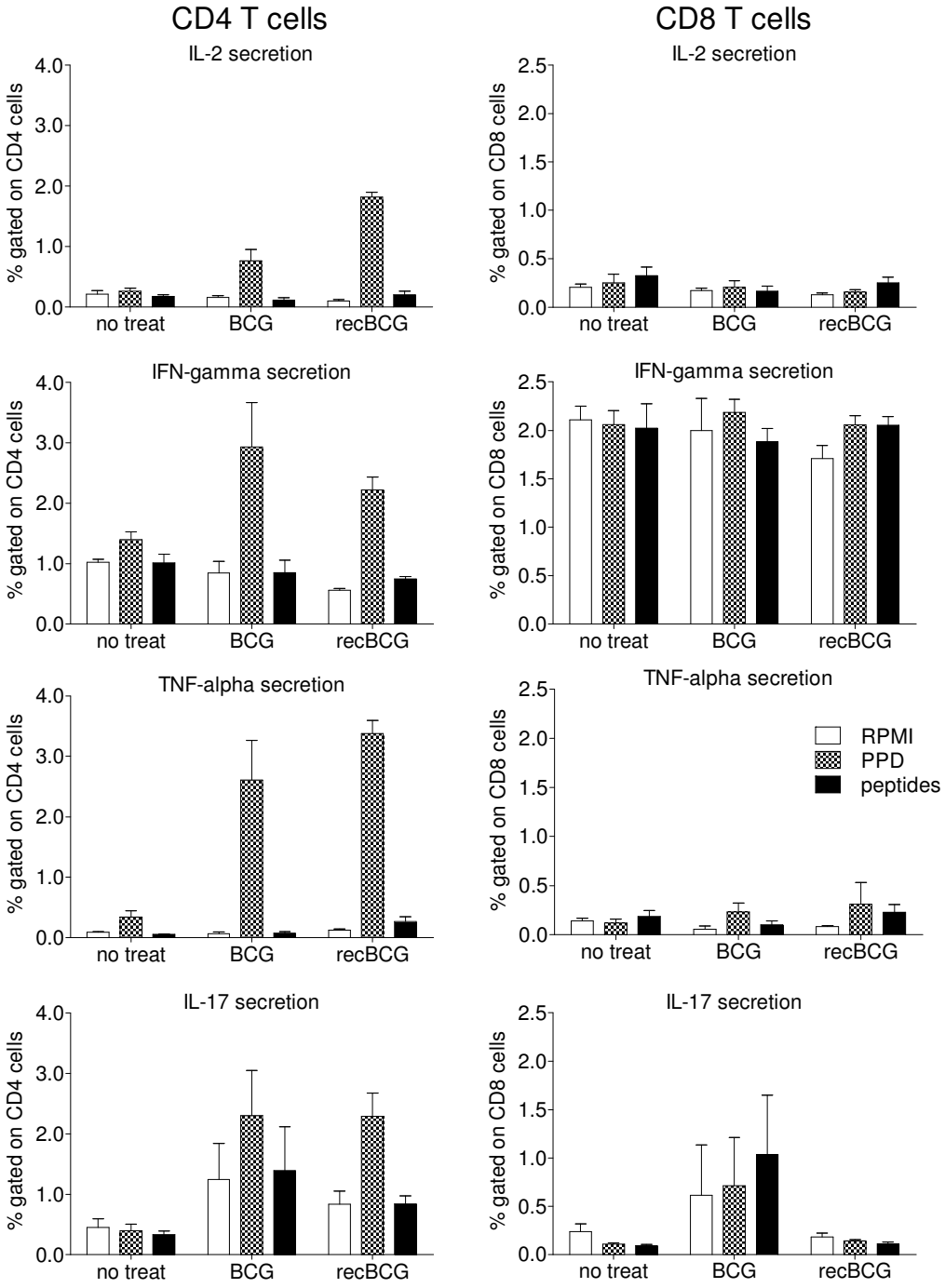

Figure 16: ICCS of lymphocytes isolated from the lung 90 days p.i.
Immune response in the lung 90 days p.i.. Mice were vaccinated with $5x10^5$cfu of BCG or recBCG s.c., non-vaccinated controls were included. 13 weeks after vaccination mice were challenged with 200cfu M.tb via aerosol. 90 days p.i. mice were sacrificed and $5x10^5$ cells isolated from lungs were re-stimulated for 6h with either RPMI, PPD or Ag85A peptides in the presence of brefeldin A. Cytokine secretion was measured by 6-colour flow cytometry. The x-axis labels indicate treatment of mice and stimulation is represented by differently filled bars: RPMI only (white bars), PPD (chequered bars) or Ag85A peptides (black bars). Panels on the left show responding CD4, panels on the right CD8 T cells. All four cytokines were measured at the same time within the same sample. Bars represent the means of three samples where two samples contained cells pooled from two mice and one sample contained cells from one mouse only, error bars depict SEM. Experiment was done once.

After vaccination with BCG or recBCG CD4 T cells secreting IL-2, IL-17, TNF-α and IFN-γ in response to stimulation with PPD were present in the lungs 90 days p.i. whereas only CD4 T cells secreting IFN-γ and TNF-α were detectable in the lungs of untreated controls. BCG and recBCG vaccination did not seem to induce large numbers of antigen-specific CD8 T cells. Some CD8 T cells responded to stimulation with Ag85A peptides or PPD by secretion of IL-2 and TNF-α, after vaccination with recBCG, but percentages were just barely above background levels.

About 2% of CD8 T cells secreted IFN-γ after 6h incubation regardless of vaccination regime or stimulation used indicating some level of non-specific activation within the CD8 T cell population in the lung 90 days p.i. and in addition high background secretion of IL-17 was detected in the BCG vaccinated group. This was also observed for IFN-γ and IL-17 in cell culture supernatants, but only when mice were vaccinated with recBCG. Interestingly no IL-2, IL-17 or IFN-γ secretion was measurable after 6h incubation with Ag85A peptides by ICCS, whereas this was the case if cell culture supernatants were analysed.

Recently T cells producing multiple cytokines, i.e. IL-2, IFN-γ and TNF-α, so called poly-functional T cells, gained substantial interest in the field of vaccinology as their presence seems to be a valuable marker for the development of a protective T cell response [Darrah 2007]. Figure 17 depicts poly-functional CD4 and CD8 T cells in the lung 90 days after infection. Within the CD8 T cell compartment there were hardly any poly-functional secreting cells detectable after 6h re-stimulation with PPD, whereas cells secreting any combination of two or three of the four cytokines analysed represented approximately 2% or 1%, respectively, of the CD4 T cells when BCG or recBCG was used for vaccination. An in-depth analysis of the poly-functional CD4 T cells revealed that in untreated mice, the few detectable cells secreting two cytokines were predominantly producing IFN-γ and TNF-α. This population was reduced in BCG vaccinated animals, and an even stronger decrease was observed in the recBCG-vaccinated group. In mice vaccinated with BCG the population of CD4 T cells secreting IL-2 and IFN-γ was increased. In addition, a minute proportion of CD4 T cells producing TNF-α

and IL-2 was detected. The percentages of CD4 T cells secreting IFN-γ and IL-17 or IL-17 and TNF-α were comparable to those in the non-vaccinated group.

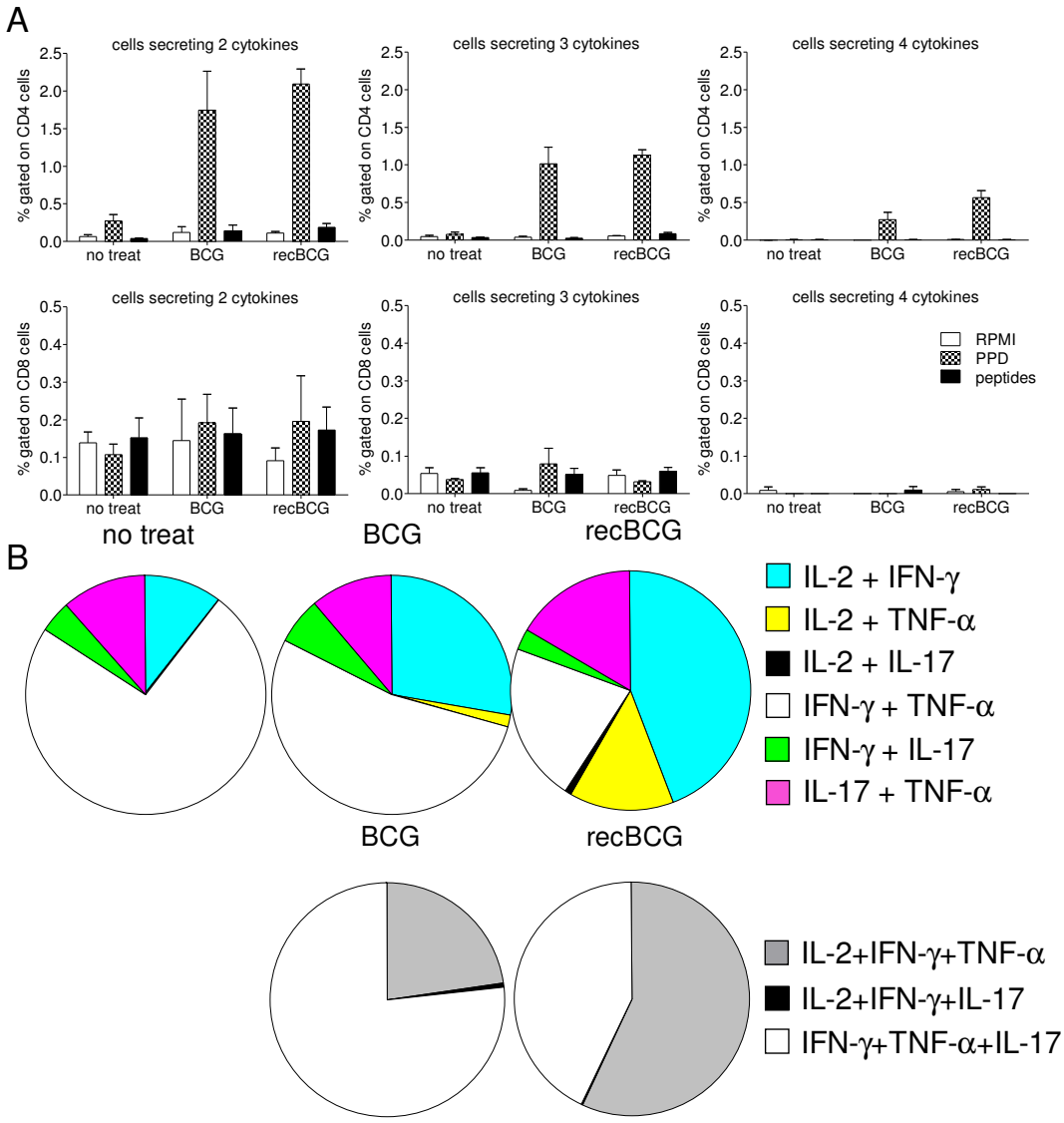

Figure 17: Poly-functional lymphocytes in the lung 90 days p.i.
$5x10^5$ cells isolated from lungs were re-stimulated for 6hwith either RPMI, PPD or Ag85A peptides in the presence of brefeldin A. Cytokine secretion was measured by 6-colour flow cytometry. A: Summary of poly-functional cells. The x-axis labels indicate treatment of mice. Stimulation is represented by differently filled bars: RPMI only (white bars), PPD (chequered bars) or Ag85A peptides (black bars). Graphs depict cells secreting two or three (any possible combination of IL-2, IL-17, IFN-γ and TNF-α) or all four cytokines analysed. Upper panels show responding CD4, lower panels CD8 T cells. Bars represent the means of three samples where two samples contained cells pooled from two mice and one sample contained cells from one mouse only, error bars depict SEM. Experiment was done once. B: In-depth analysis of poly-functional CD4 T cells. Pie charts depict the means of all detected cytokine combinations after stimulation with PPD. As there were hardly any cells secreting three cytokines in non-vaccinated mice, no pie chart is shown.

Even though the percentages of CD4 T cells secreting two cytokines were comparable between BCG and recBCG vaccinated mice, the cytokine combinations differed. The amount of CD4 T cells producing IL-2 and IFN-γ was increased further if recBCG was administered. In addition a larger proportion of cells stained positive for IL-2 and TNF-α or IL-17 and TNF-α. In non-vaccinated mice, hardly any CD4 T cells secreting three cytokines were detected. Three quarters of triple positive cells from BCG vaccinated mice produced IFN-γ, TNF-α and IL-17. In contrast to this, more than half of all triple positive CD4 T cells from mice vaccinated with recBCG secreted IL-2, IFN-γ and TNF-α. The amount of cells producing IL-2, IFN-γ and IL-17 was negligible in both. Finally, the amount of CD4 T cells secreting all four cytokines in response to stimulation with PPD was twice as high in the lungs of mice vaccinated with recBCG compared to parental BCG.

The immune response in the spleen 90 days p.i. was also analysed. Whilst bacterial burden in the lungs of mice vaccinated with parental BCG were significantly reduced in comparison to untreated controls this protective effect was not observed in the spleen. Here only vaccination with recBCG was effective.

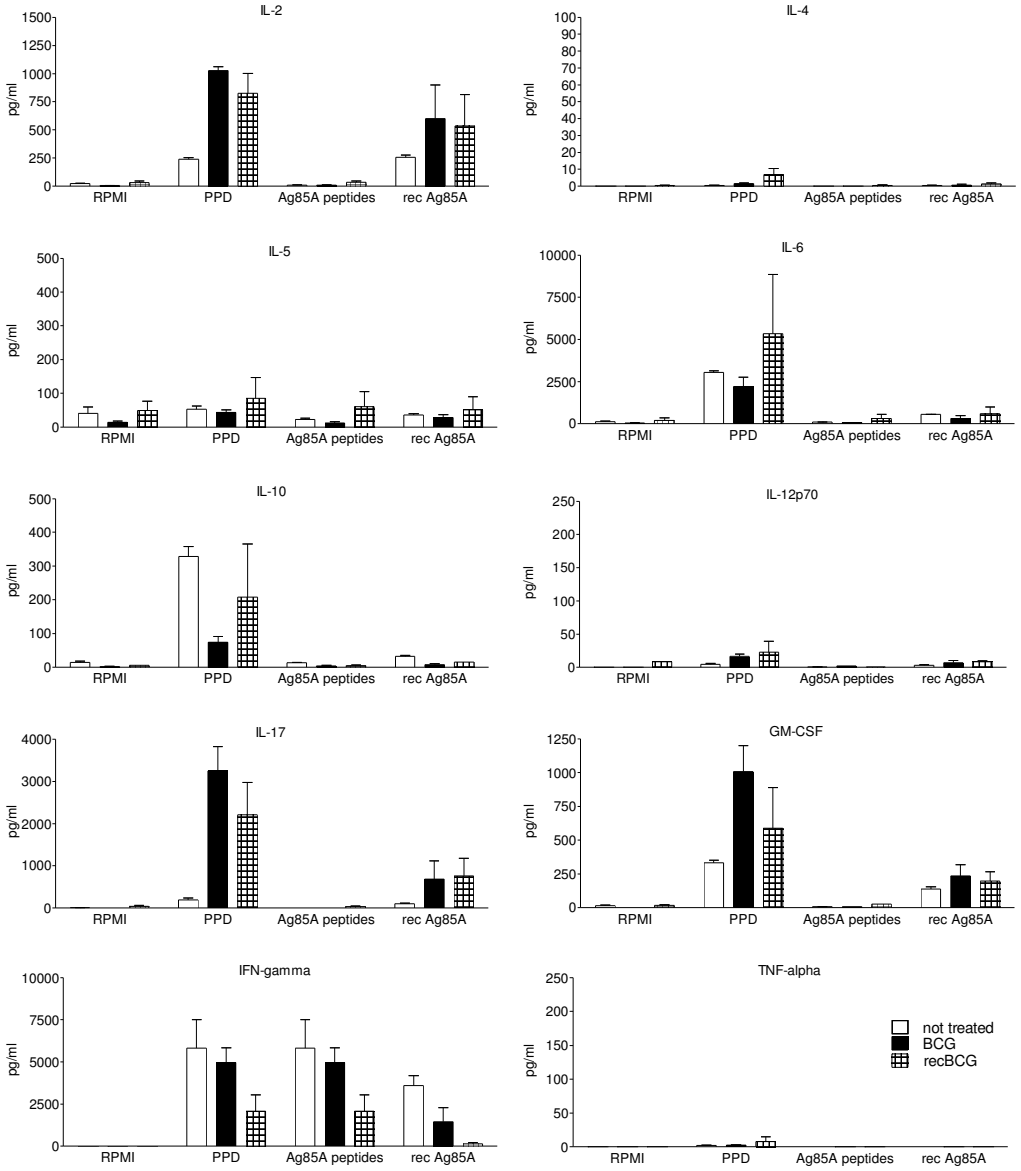

Figure 18: Cytokine secretion of splenocytes measured by Bio-Plex 90 days p.i.
Recall response 90 days p.i. in the spleen. Mice were vaccinated with $5x10^5$cfu of BCG (black bars) or recBCG (chequered bars) s.c.; non-vaccinated controls (white bars). 13 weeks after vaccination mice were challenged with 200cfu M.tb via aerosol. 90 days p.i. mice were sacrificed and $2x10^6$ cells isolated from the spleen were re-stimulated for 18h with either RPMI, PPD, Ag85A peptides or purified recombinant Ag85A protein. Supernatants were passed through 0.2µm centrifugal filters before transfer out of the BSL3 facility. Cytokines were measured in supernatants by Bio-Plex bead assay. Bars represent the means of three samples where two samples contained cells pooled from two mice and one sample contained cells from one mouse only, error bars depict SEM. Experiment was done once.

In order to get a good overview about the ongoing immune response in the spleen 90 days p.i., cell culture supernatants were analysed by Bio-Plex assay.

Even though 4 times more cells isolated from the spleens were stimulated with PPD, Ag85A peptides or protein, cells isolated from the lungs produced approximately 10-fold more cytokines. Whereas elevated cytokine secretion was measurable in the lungs post infection, splenocytes produced less cytokines after aerogenic challenge compared to concentrations measured before infection. After 18h stimulation, hardly any cells secreting IL-4, IL-5 or TNF-α were detected. The most prominent finding was that IFN-γ secretion was lowest in recBCG-protected mice and highest in non-vaccinated controls regardless if PPD, peptides (in fact, only IFN-γ secretion was detectable after stimulation with peptides) or whole protein was used for stimulation. Isolated cells also responded to Ag85A protein stimulation. Regardless if vaccinated or not, cells produced IL-2, GM-CSF and IL-6 whereas IL-17 production was only observed in vaccinated groups. The strongest response however was measured if PPD was used for re-stimulation. The highest IL-10 secretion was detected in the untreated control group. IL-6 secretion was detected in all groups to the same extent. Clearly, vaccination with BCG or recBCG induced cells secreting most of the cytokines analysed here. Pronounced differences were mostly observed, if these groups were compared to non-vaccinated controls. Even though only recBCG conferred protection in the spleen, there were hardly any differences between both BCG strains with regard to IL-6, IL-17, IL-2 or GM-CSF secretion.

In the spleen too, the contribution of CD4 and CD8 T cells was determined by 6-colour flow cytometry after stimulation with PPD or Ag85A peptides in the presence of brefeldin A. The first observation was that the percentages of responding T cells isolated from the spleen were lower, if compared to cells isolated from the lung. After vaccination with recBCG, antigen-specific CD4 T cells secreted TNF-α, IL-2 (both large error bars), IFN-γ and IL-17 in response to PPD as well as TNF-α and IL-17 after stimulation with Ag85A peptides.

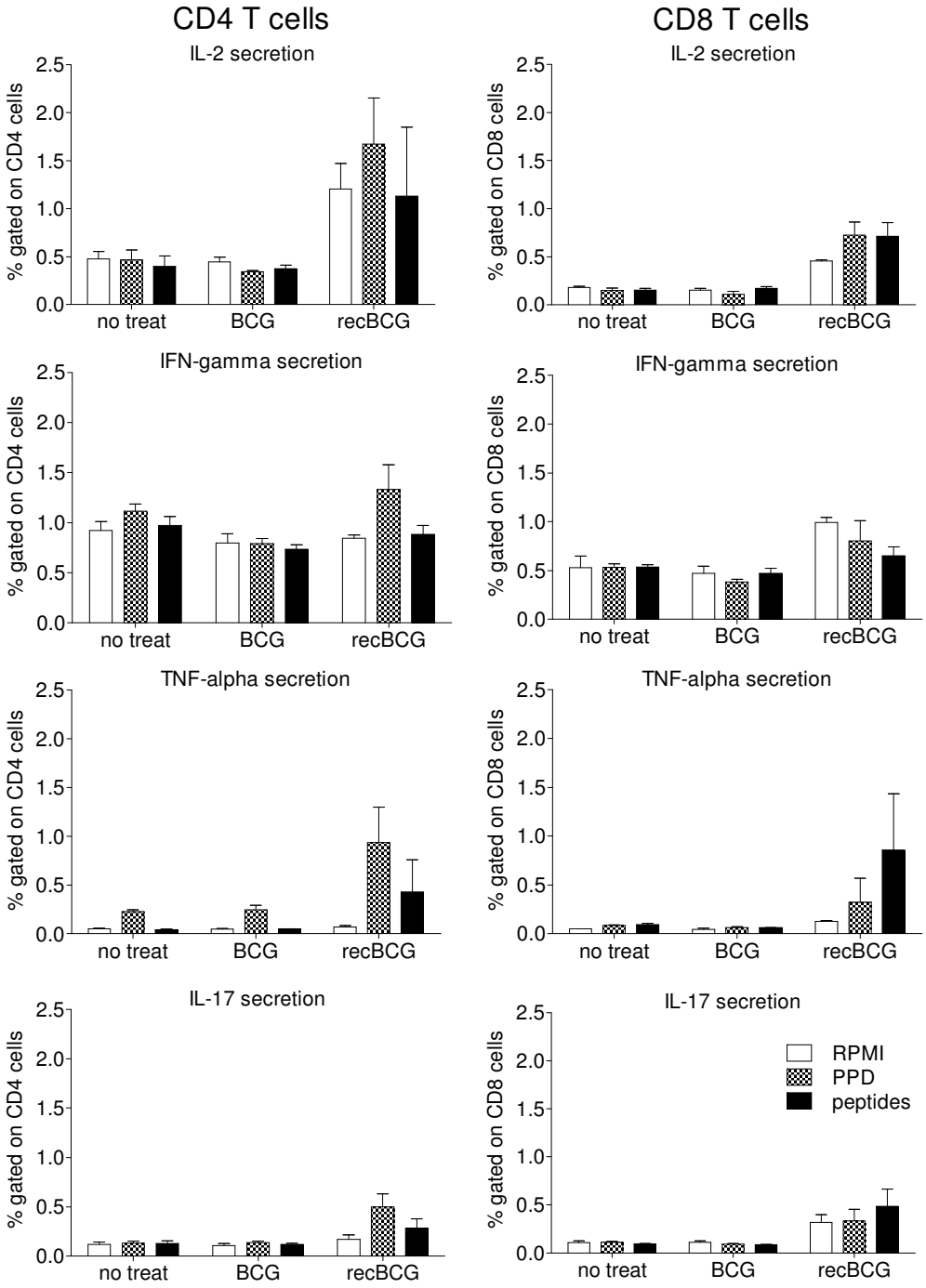

Figure 19: ICCS of lymphocytes isolated from the spleen 90 days p.i.
Immune response in the spleen 90 days p.i.. Mice were vaccinated with $5x10^5$cfu of BCG or recBCG s.c., non-vaccinated controls were included. 13 weeks after vaccination mice were challenged with 200cfu M.tb via aerosol. 90 days p.i. mice were sacrificed and $2x10^6$ cells isolated from the spleen were re-stimulated for 6h with either RPMI, PPD or Ag85A peptides in the presence of brefeldin A. Cytokine secretion was measured by 6-colour flow cytometry. The x-axis labels indicate treatment of mice and stimulation is represented by differently filled bars: RPMI only (white bars), PPD (chequered bars) or Ag85A peptides (black bars). All four cytokines were measured at the same time within the same sample. Bars represent the means of three samples where two samples contained cells pooled from two mice and one sample contained cells from one mouse only, error bars depict SEM. Experiment was done once.

Of note is that also increased background activation (RPMI control), with regard to IFN-γ and IL-2 secretion, was observed. CD4 T cells isolated from spleens produced TNF-α regardless if mice were vaccinated or not. 3-fold more of this cytokine was detected in the recBCG-vaccinated group as compared to parental BCG or untreated controls. As already observed in CD8 T cells isolated from the lungs, CD4 and CD8 T cells isolated from the spleens showed some degree of non-specific activation by secretion of IFN-γ no matter which stimuli were given.

Antigen-specific CD8 T cells were only detectable, if mice had been vaccinated with recBCG. The CD8 T cells secreted IL-2 and TNF-α in response to stimulation with PPD or Ag85A peptides. Percentages of IL-17 secreting cells were minute in all samples analysed.

Poly-functional T cells were also present in the spleen 90 days p.i.. Even in non-vaccinated controls and also after vaccination with parental BCG some CD4 T cells secreting two or three of the four cytokines (IL-2, IL-17, IFN-γ or TNF-α) measured were detected after stimulation with PPD. However, recBCG was a much more potent inducer of these poly-functional CD4 T cells, twice as many cells produced two and three times more cells secreted three cytokines. Regarding the combinations of secreted cytokines, in response to PPD stimulation, no differences in composition were observed in BCG and non-vaccinated mice. In both groups the highest proportion were CD4 T cells secreting IFN-γ and TNF-α followed by equal percentages of cells secreting either IL-2 and IFN-γ or IFN-γ and IL-17. A smaller fraction produced IL-2 and TNF-α or IL-2 and IL-17; the smallest contribution came from cells secreting IL-17 and TNF-α. If mice were vaccinated with recBCG, not only were more poly-functional CD4 T cells detectable, but also the combinations of secreted cytokines differed. As already observed in cells isolated from the lungs, almost 50% of cells producing two cytokines secreted IL-2 and IFN-γ in response to stimulation with PPD and also a higher proportion of cells produced IL-17 and TNF-α. Consequently, the fractions of cells secreting IFN-γ and IL-17 or IFN-γ and TNF-α were smaller. As for CD4 T cells secreting three cytokines there were also no differences in the distribution patterns between BCG and non-vaccinated.

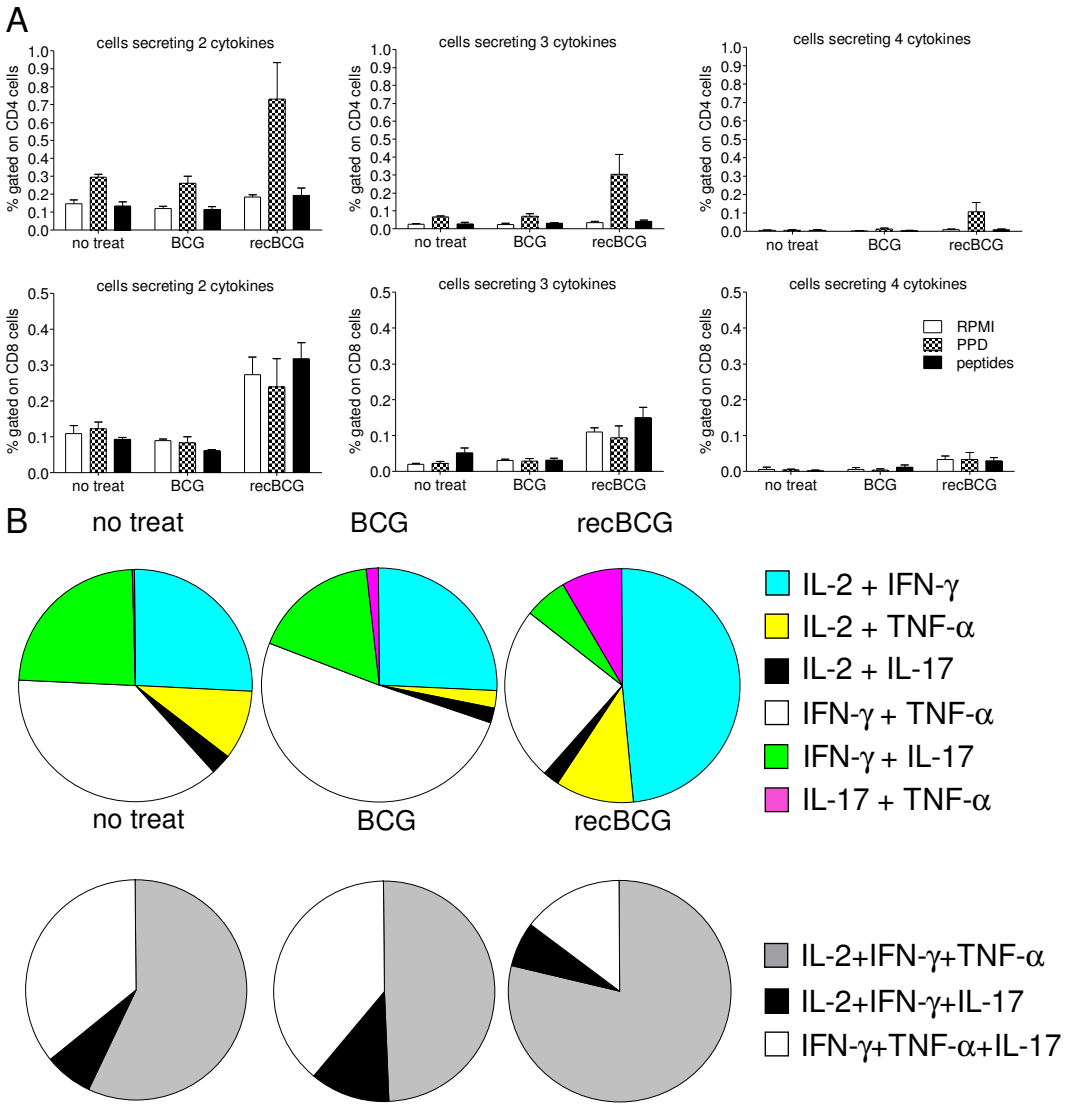

Figure 20: Poly-functional lymphocytes in the spleen 90 days p.i.
$2x10^6$ cells isolated from spleens were re-stimulated for 6h with either RPMI, PPD or Ag85A peptides in the presence of brefeldin A. Cytokine secretion was measured by 6-colour flow cytometry. A: Summary of poly-functional cells. The x-axis labels indicate treatment of mice and stimulation is represented by differently filled bars: RPMI only (white bars), PPD (chequered bars) or Ag85A peptides (black bars). Graphs depict cells secreting two or three (any possible combination of IL-2, IL-17, IFN-γ and TNF-α) or all four cytokines analysed. Upper panels show responding CD4, lower panels CD8 T cells. Bars represent the means of three samples where two samples contained cells pooled from two mice and one sample contained cells from one mouse only, error bars depict SEM. Experiment was done once. B: In-depth analysis of poly-functional CD4 T cells. Pie charts depict the means of all detected cytokine combinations after stimulation with PPD.

Generally, these cells accounted for only 0.1% of the total CD4 T cells in BCG vaccinated or non-vaccinated mice and 0.3% in recBCG-vaccinated mice. Approximately 50-60% of these cells secreted IL-2, IFN-γ and TNF-α. This fraction increased to 75% in recBCG. The smallest fractions in all three groups were CD4 T cells secreting IL-2, IL-17 and IFN-γ. Most importantly

only after vaccination with recBCG 0.1% of CD4 T cells stained positive for all four cytokines. Within the CD8 T cell compartment there were hardly any poly-functional secreting cells detectable after 6h of re-stimulation with PPD and some non-specific activation was detected after vaccination with recBCG.

The cytokine milieu also dictates isotype switching of antibodies, thus analysis of the humoral immune response provides additional information into the type of helper T cell response elicited by vaccination. IL-4 induces switching to IgG1 and inhibits IgG2a switching. IFN-γ by contrast induces switching to IgG2a whilst inhibiting IgG1 [Stavnezer 1996]. Antibodies of IgG1 isotype directed against PPD were detected after vaccination with recBCG but not parental BCG (Figure 21). In contrast, a small amount of IgG2a antibodies, but none of IgG1 isotype, directed against Ag85A were only measured after vaccination with parental BCG. 90 days after challenge, no Ag85A specific antibodies were detected. Antibodies binding proteins from PPD increased approximately 100-fold after infection with M.tb. Mice from all groups irrespective of treatment produced comparable amounts of PPD-specific antibodies of IgG1 isotype, with a tendency of increased levels after vaccination with parental BCG. Vaccination, in contrast, seemed to suppress the production of IgG2a antibodies, with recBCG exerting stronger effects than parental BCG.

Taken together, a very prominent immune response was induced in the spleen after s.c. vaccination. Cells strongly reacted to PPD, but only weakly to Ag85A peptides, by secretion of IFN-γ, IL-2 and GM-CSF regardless which BCG strain was administered. Only after vaccination with recBCG, cells produced high amounts of IL-17 and also approximately 20% more IL-6 in response to PPD stimulation. A recall response directed against PPD was also detected in the lungs, but no differences were found between the BCG strains.

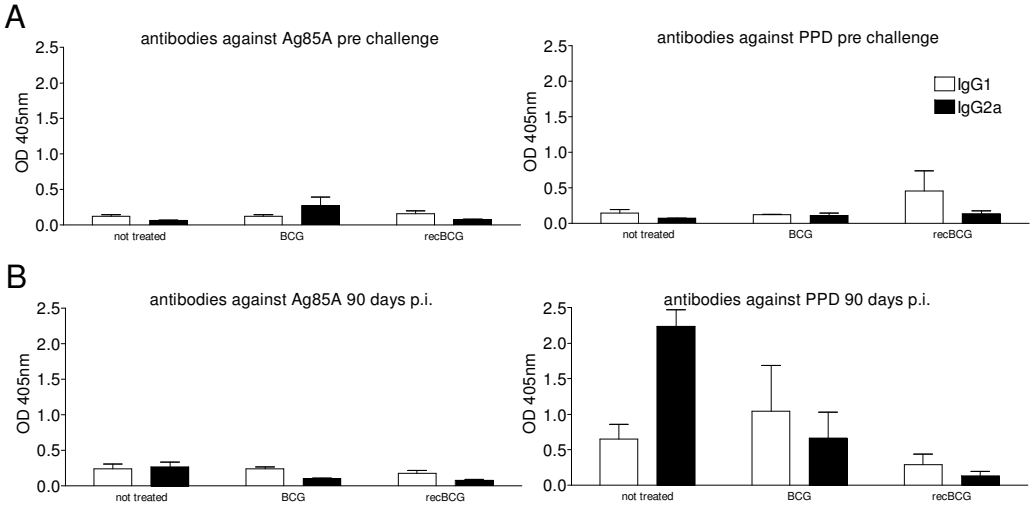

Figure 21: Antibody formation after vaccination with BCG or recBCG
Antibody formation one week prior to aerosol infection (A) and 90 days p.i. (B), measured by sandwich ELISA. PPD or Ag85A protein were used for coating and secondary antibodies labelled with alkaline phosphatase recognising either murine IgG1 (white bars) or IgG2a (black bars) antibodies for detection. Sera prior to aerosol infection and for Ag85A detection p.i. were diluted 1:25, sera for PPD detection p.i. were diluted 1:2500.

After infection with M.tb, marked differences between the immune response elicited in the lungs by s.c. vaccination with parental and recBCG were evident. Cells isolated from the lungs of mice immunised with recBCG secreted huge amounts of IL-6, IL-2, GM-CSF and TNF-α after stimulation with PPD. Flow cytometry revealed mostly CD4 T cells responding and more importantly a higher percentage of them being poly-functional after vaccination with recBCG. The differences between parental and recBCG were less prominent in the spleen 90 days p.i.. Secretion of IL-6, IL-17, IL-2 and GM-CSF were detected after stimulation with PPD regardless, which BCG was used even though only recBCG conferred protection. Production of IFN-γ, detected by Bio-Plex, was reduced in the recBCG group but only in this group intracellular presence of IL-2, IFN-γ, IL-17 and TNF-α (single and poly-cytokine producers) was detected in CD4 T cells after stimulation with PPD. Most interestingly also CD8 T cells responding to stimulation with Ag85A peptides by TNF-α secretion (and to a lesser extent also IL-17) were present after vaccination with recBCG.

6 Discussion

In this thesis novel vaccine candidates against pulmonary TB were assessed in models of persistent and latent infection. In the first part immunogenicity and protective capacity of plasmid DNA vaccines encoding the dormancy-associated antigens hspX, TB31.7, Rv1733c or Rv2628 was analysed in pre- and post-exposure vaccination experiments. In order to test the capability of these DNA vaccines to prevent reactivation in latent TB infection, short-duration drug-induced latency models had to be evaluated. In the second part the immune response elicited by vaccination with BCG or recBCG involved in the defence against infection with M.tb was analysed in great detail. Defining correlates of protection is of vital importance for the design of future vaccines against pulmonary TB.

6.1 Plasmid DNA vaccines encoding dormancy-associated antigens are immunogenic

The immune response elicited after vaccination is routinely assessed by isolation of lymphocytes, followed by IVR for three to six days and subsequent analysis of either cytokines present in cell culture supernatants, by ICCS, ELISpot or chromium release assays to analyse cytotoxicity. However, any assay requiring *in vitro* expansion of responding cells has its limitations, the most important one being the inability to quantify antigen-specific cells and the creation of false positive measures as such a strategy might vastly overestimate the frequencies of antigen-specific T cells due to bystander effects and expansion during this long period of stimulation [Tough 1996]. Therefore a direct ex-vivo ICCS protocol with only 20h stimulation was exploited here, as this technique has been shown in several studies to be more sensitive than measuring cytokines in cell culture supernatants after 72h stimulation [Asemissen 2001; Kabilan 1990] and also shown to correlate with cytotoxic activity of antigen-specific CD8 T cells [Horton 2004].

Only recently it has been shown, that DNA vaccination with plasmids encoding the dormancy-associated antigens Rv1733c, Rv2628 and hspX and

subsequent re-stimulation with recombinant proteins induced significant antigen-specific IFN-γ production, thus ruling out the possibility of an inherent inability of BALB/c mice to respond to these latency antigens [Lin 2007]. However, this group used a different vector, albeit also containing a tPA leader sequence, for vaccination and did not carry out any protection studies. Furthermore, they only analysed IFN-γ secretion after 72h IVR in cell culture supernatants. A second group also started investigating the immunogenic potential of DNA vaccines, which were identical to the ones applied by Lin et al., but they administered four instead of three doses and again no protection experiments were reported [Roupie 2007]. This group showed, by 72h (for IFN-γ) or 24h (for IL-2) IVR with whole recombinant protein, that a DNA vaccine encoding Rv1733c was only weakly immunogenic, which is in line with the results presented here. Vaccination with plasmids encoding hspX or Rv2628 induced profound IFN-γ responses, whereas IL-2 secretion was only observed after vaccination with hspX. In contrast, results presented here and gained by ICCS, showed that both IFN-γ and IL-2 were induced after vaccination with any of these two plasmids. Roupie et al. also reported B cell responses, by measuring total murine antigen-specific IgG content, after vaccination with plasmids encoding hspX or Rv1733c but not with Rv2628, which is also in line with the data shown here.

Another advantage of ICCS over cytokine determination in cell culture supernatants is the differentiation into responding CD4 and CD8 T cells. It is advisable for such an in-depth analysis to use peptides encoding CD4 or CD8 T cell epitopes instead of whole proteins for re-stimulation, because processing and presentation of whole protein requires time. The detailed analysis of antigen-specific T cells was complicated by the fact that, not for all dormancy-associated antigens tested here, CD8 or CD4 T cell epitopes are known. For hspX and Rv2628 some CD4 T cell epitopes have been identified, whereas none were found for Rv1733c [Roupie 2007]. MHC-I Antigenic Peptide Processing Prediction (MAPPP) [Hakenberg 2003] was used here to identify putative CD8 T cell epitopes for Rv1733c, hspX and TB31.7.

As shown here, vaccination with plasmid DNA encoding hspX induced only a weak CD8 T cell response, even though three putative MHC-I epitopes were

used for stimulation. Vaccination with pCMVtPA-hspX elicited a dominant CD4 T cell response: IL-17 and TNF-α and, to a lesser extent, IL-2 and IFN-γ were detected by ICCS and confirmed by 72h IVR followed by examination of cell culture supernatants. The CD4 T cell response was a mixed T_H1/T_H2 type, because also T_H2 polarising cytokines were detected by Bio-Plex and furthermore a strong B cell response, which was also mixed T_H1/T_H2, developed. It was already published, that vaccination with DNA encoding hspX induced a very robust antibody response with predominantly IgG1 antibodies [Morris 2000].

Neither CD4 nor CD8 T cell epitopes have been described for TB31.7, therefore peptides predicted to bind to MHC-I were used for re-stimulation and results indicate, that the predictions were correct. CD8 T cells produced IFN-γ, IL-17 and small amounts of TNF-α in response to 20h IVR. Interestingly, also CD4 T cells secreted IFN-γ after stimulation, even though 9-mer peptides are highly unlikely to bind to MHC-II [Rudensky 1991]. This can be explained by bystander activity during the 20h incubation period as the first 4h incubation were done without brefeldin A. The highest concentrations of cytokines after 72h stimulation were measured, if pCMVtPA-TB31.7 was used for vaccination. Here, too, vaccination induced a mixed T_H1/T_H2 response, with T_H1 polarising cytokines clearly being present in higher concentrations. No antigen-specific B cell response was elicited.

The strongest T cell response, measured by ICCS, was detected after vaccination with pCMVtPA-Rv2628. CD4 T cells produced all four cytokines in response to stimulation with the 19-mer peptide. Of note, CD8 T cells also responded strongly to stimulation with this peptide, even though MHC-I usually presents peptides of 8-10 amino acids [Hunt 1992]. But the 19-mer peptide used here contained three putative CD8 T cell epitopes: One H2-L^d and two H2-K^d restricted. It is possible that this peptide can either bind directly to MHC-I molecules because of very strong affinity or taken up by APC and presented to CD8 T cells via cross-presentation [Bevan 1976] during the first 4h incubation period without brefeldin A. The observed CD8 T cell response might as well be due to non-specific activation, as CD4 T cells will secrete cytokines into the culture medium within the first 4h incubation without

brefeldin A. It is well known that IL-2 is an un-specific T cell growth factor and stimulates the production of IFN-γ as well as TNF-α [Boothby 2001]. In contrast to the strong T cell response observed by ICCS, no cytokine secretion in cell culture supernatants was detected after 72h IVR. The observed discrepancy between a profound cytokine response detected by ICCS and the absence of cytokines in the cell culture supernatants, measured by Bio-Plex, might be explained by a phenomenon termed activation-induced cell death (AICD). Several studies have shown that activated T cells are prone to apoptosis upon antigen-specific re-stimulation [Nagata 1995]. There is evidence that CD8 T cells undergo apoptosis by endogenous Granzyme B upon CD3/TCR stimulation [Laforge 2006]. Also $CD4^+CD25^+$ T regulatory cells can induce contact-dependent activation-induced apoptosis via Granzyme B [Gondek 2005; Laforge 2006]. AICD is mainly associated with antigen-specific CD8 T cells and as the frequency of responding CD8 T cells was 10 times higher after vaccination with Rv2628 compared to vaccination with any of the other three vectors; it is possible that such a strong CD8 T cell recall response induced AICD, making it impossible to detect cytokines after 3 days IVR. Only recently it has been shown that IFN-γ can mediate death of T_H1 cells in a paracrine manner [Foulds 2008].

It is noteworthy to point out, that TNF-α was not measured in 72h IVR cultures but was readily detected by ICCS. TNF-α may be secreted only during the initiation of the memory response elicited by vaccination and consumed by neighbouring cells, which then secreted other cytokines in response i.e. IFN-γ.

In summary, it was shown that plasmid DNA vaccination encoding one of the dormancy-associated antigens Rv1733c, Rv2628, hspX or TB31.7 lead to the generation of antigen-specific T cells, thus proving that the encoded antigens were expressed *in vivo*. In order to visualise the true proportion of antigen-specific T cells generated after vaccination, the stimulation period was reduced which resulted in low, albeit measurable, frequencies of antigen-specific T cells detected. The ranking order, based on the frequencies of antigen-specific T cells generated after vaccination, is as follows: Rv2628 > TB31.7 > hspX > Rv1733c. Vaccination with DNA encoding hspX or Rv1733c

also induced B cell immune responses. All plasmid DNA vaccines elicited predominantly T_H1 responses but T_H2 cytokines were also detected.

6.2 Vaccination with plasmid DNA encoding dormancy-associated antigens during persistent infection with *Mycobacterium tuberculosis*

Plasmid DNA vaccines expressing a variety of M.tb antigens, in combination or administered individually, have been found to be partially protective against pulmonary TB in a range of animal models, predominantly in mice but also in guinea pigs and cattle [Huygen 2005]. But in most reported studies the induced immune responses did not lead to sterile eradication of bacteria, only reduction in bacterial burden was achieved. The majority of these DNA vaccines encoded immunodominant proteins released into the bacterial culture supernatant, mostly from the Ag85 family [Derrick 2004b; Romano 2006b], heat shock proteins of mycobacteria [Tascon 1996] or antigens expressed in M.tb but absent in BCG [Mollenkopf 2004]. All these antigens are considered to be abundantly expressed by actively replicating bacteria. No dormancy-associated DNA vaccine candidates have been tested in experimental infections so far.

Even though information about the dormancy response of M.tb is mostly derived from *in vitro* experiments [Park 2003; Voskuil 2003], there is some evidence, that dormancy-associated proteins are expressed during persistent infection *in vivo*. HspX protein was detected from week four p.i. onwards in the lungs of guinea pigs infected i.m. [Sharma 2006] and also four weeks p.i. by real-time RT-PCR in aerosol-infected mice [Timm 2003]. The expression pattern of hspX and TB31.7 during persistent M.tb infection was also analysed by real-time RT-PCR. Increased expression of both hspX and TB31.7 from day 15 p.i. onwards was observed whereas mRNA levels decreased again by day 50 p.i. [Shi 2003]. If and when the dormancy-associated antigens Rv1733c and Rv2628 are expressed *in vivo* during the course of pulmonary TB infection in mice, has not been analysed, yet. Moreover, it is not known,

whether these dormancy-associated proteins access the antigen processing machinery, thus becoming a target for the T cell system.

For the majority of *in vivo* vaccination experiments presented here, blood was collected when mice were sacrificed and the antibody response analysed. Antibodies, IgG1 as well as IgG2a, specific for hspX, Rv1733c, Rv2628 or TB31.7 were detected in most sera from non-vaccinated mice (data not shown) indicating that these latency antigens were at least transiently expressed and gained access to the MHC-II pathway, during the course of infection.

In the first experiment, the efficacy of plasmid DNA vaccines against an already established M.tb infection was assessed, as this scenario mirrors the majority of TB cases in humans best. Mice were vaccinated with BCG and 90 days later infected with virulent M.tb H37Rv by aerosol. Vaccination with DNA was commenced four weeks p.i., which coincided with the completed development of an M.tb specific adaptive immune response, resulting in control of infection with stable, albeit high, bacterial titres. Prophylactic vaccination with BCG conferred highly significant protection, as defined by reduction of bacterial burden in lungs and spleens, however additional therapeutic vaccination with DNA encoding hspX or a mixture of plasmids encoding Rv1733c, Rv2628 and TB31.7 did not further decrease bacterial load.

It has recently been shown that BCG, even though equipped with a DosR regulon sharing 97% homology to that of M.tb, induced only weak immune responses against dormancy-associated antigens in humans and mice [Lin 2007]. Therefore, it was tested, whether BCG induced protection could be improved by booster vaccination with plasmids encoding dormancy-associated antigens. However, the DNA vaccines failed to protect in a second experiment where they were administered prior to aerosol infection. Neither were they protective against primary TB infection, compared to BCG, nor did they increase BCG-induced protection, if given as a booster before aerogenic challenge.

If the observed lack of vaccine efficacy could at least partly be attributed to an inadequate route of administration, was also evaluated. It has been shown that the presence of antigen in the airways is needed for recruitment, priming

and maintenance of antigen-specific T cells in the airway lumen and that this is much better achieved by i.n. instead of parenteral vaccination [Santosuosso 2005]. In another experiment, the plasmid DNA was therefore administered i.n., pre- and post-infection, in mice primed with BCG. I.n. application of the vaccines did not protect against establishment of persistent pulmonary TB infection. In order to facilitate i.n. uptake of DNA vaccines, they were complexed with Chitosan to form nanoparticles. Due to the limited absorption capacity of Chitosan, only 4µg plasmid DNA were delivered per vaccination. However this was sufficient for eliciting an antigen-specific T cell response in the lung (analysed for pCMVtPA-Rv2628 vaccination by ICCS, data not shown), indicating that i.n. vaccination per se was successful, but the immune response elicited, failed to protect.

It could be argued that plasmid DNA vaccines, administered before or after aerosol challenge, were unsuccessful in reducing bacterial burden in infected mice, because the frequencies of antigen-specific CD4 and CD8 T cells generated were too low, or were elicited too late, to mediate protection. However there is evidence that low frequencies of antigen-specific CD8 T cells, ranging from 0.03% to 0.2% of total splenocytes, conferred protection against lethal challenge with lymphocytic choriomeningitis virus within one week after a single DNA vaccination [Hassett 2000]. Also influenza reactive T cells from healthy human subjects were found to range between 0.01% to 0.34% of CD8 T cells [Asemissen 2001]. If such low frequencies of antigen-specific T cells are sufficient for control of M.tb infection, remains to be examined.

The sequence of BCG and DNA vaccination may also play an important role in conferring protection against pulmonary TB. There are reports showing that plasmid DNA vaccines are only effective when given as the initial vaccine followed by BCG boost [Ferraz 2004; Romano 2006a], whereas other groups found that the sequence of vaccination did not impair the outcome of infection [Skinner 2005]. If the vaccine sequence also has an influence on the efficacy of the DNA vaccines encoding dormancy-associated antigens tested here, has not been evaluated yet.

DNA vaccines given in a post-exposure model also raised safety concerns, as it was observed by others that vaccination with a DNA vaccine encoding

hsp65, showing promising results when administered prior to infection, caused severe necrosis and pneumonia in the lungs, if given therapeutically [Taylor 2005; Turner 2000a]. It was suggested, that a massive immune activation would exacerbate lung pathology via TNF-α induced inflammation without reducing bacterial burden, a reaction resembling the so-called Koch phenomenon [Rook 1996]. Signs of a Koch reaction however, were not observed in any of the therapeutic vaccination experiments presented here.

The failure of the DNA vaccines to confer protection against persistent infection can be best explained by the nature of the mouse models used for these studies. During the initial phase of infection bacteria are actively replicating and will not express dormancy-associated antigens, thus vaccination cannot prevent exponential growth. The development of an adaptive immune response slows bacterial growth and a persistent infection with high bacterial titres is developed. A dynamic equilibrium between replication and killing of bacteria is maintained. Some bacteria might up-regulate genes from the DosR regulon in response to RNI secreted by activated macrophages or due to hypoxic conditions inside the granuloma, but the majority of bacteria will remain metabolically active.

Taken together: Given that bacteria do not express the antigens chosen for vaccination, antigen-specific CD4 and CD8 T cells do not have any target, thus rendering these vaccines ineffective. Whether DNA vaccines directed against hspX, Rv1733c, Rv2628 or TB31.7 are protective, can only be assessed in a “true” model of latent pulmonary TB.

6.3 Plasmid DNA encoding dormancy-associated antigens in murine drug-induced latency models of *Mycobacterium tuberculosis* infection

The development of vaccines directed against dormant bacteria is hampered by the lack of a standardised mouse model for latent pulmonary TB infection. Short-duration drug-induced latency models in BALB/c mice were evaluated here using a modification of the Cornell model [Botha 2002]. Taken together,

all treatment regimes tested failed to induce a stable phase of low bacterial burden in the lungs lasting for more than two weeks. Spontaneous reactivation occurred almost immediately after cessation of drug-therapy in all experiments. Reactivation kinetics in spleens however were difficult to predict and showed a substantial degree of variability. None of the experimental setups yielded a working model for latent infection in mice underlining the need for further optimisation. Not surprisingly, none of the vaccine regimes administering plasmid DNA encoding dormancy-associated antigens, conferred protection in the lung. In some of the experiments, delayed reactivation was observed in the spleen, if DNA vaccines were applied, however this effect was only transient and bacteria always reappeared in the spleen to the same extent as in mice only treated with antibiotics.

Several other drug-induced mouse models for latent infection were also tested, which will be only briefly mentioned. First, the more resistant C57BL/6 strain was tested. Here, drug-treatment induced a prolonged latency phase with low bacterial burden. Aminoguanidine has been reported to induce reactivation, thus making it possible to assess a vaccine without having to wait until natural reactivation occurs [Flynn 1998]. This did not work in our hands. If the published dose of Aminoguanidine was administered, the mice died within one week, albeit not from high bacterial burdens. A reduced dose of immuno-suppressant, given for four weeks, did not induce reactivation. The corticosteroid Dexamethasone has also been reported to induce reactivation [Scanga 1999]. This too, did not work in our hands. An explanation might be the use of different M.tb strains, inoculation doses and routes of infection.

Second, CB6F1 mice were tested. This model has been evaluated by Dr C. Vingsbo-Lundberg, Statens Serum Institute Copenhagen, Denmark and successful vaccination with a starvation-associated protein was presented at the TB-VAC Fourth Annual Meeting in 2007. In our hands this model yielded inconsistent results, vaccination with pCMVtPA-Rv2628 resulted in delayed reactivation in the spleen in one experiment out of two.

Two recent publications showed that in persistent pulmonary TB infection the mouse lung, in contrast to humans, is not hypoxic [Aly 2006; Tsai 2006]. Tsai et al. reported that murine tuberculous granulomatous tissues were relatively

aerobic. Aly et al. did not find any signs of hypoxia in mouse lungs even one year after TB infection and proved by direct oxygen measurement, that even though oxygen levels were reduced in infected mice, they did not reach severe hypoxia. Thus, mouse models of persistent infection are most likely not suitable for the evaluation of candidate antigens being predominantly expressed under hypoxic conditions. However, these studies by Aly et al. and Tsai et al. did not provide insight into the conditions bacteria might encounter inside host cells. It is tempting to speculate that the observed transient protection in the spleen is due to hypoxic conditions in this organ. Whether hypoxic areas develop in the spleen during infection with M.tb is currently under investigation.

Whether M.tb enters a truly dormant stage *in vivo*, or if persistence is maintained by a balance of slow growth and killing by the immune response is still one of the outstanding questions in TB research. In pursuit of an answer, several other strategies, apart from the mouse models presented here, are currently being explored. Currently other species, listed below, are under investigation by different groups in search for a feasible model for dormancy and reactivation of mycobacteria.

It was suggested to develop a drug-induced latency model in guinea pigs [Orme 2006b]. However this has not been explored, yet. The advantage of using guinea pigs is the susceptibility of these animals to TB and the development of caseating granuloma, whereas the drawback is the expense of keeping the animals for long time periods and large group sizes needed for statistical power [Lenaerts 2004].

If rabbits develop a paucibacillary latent TB infection, as seen in humans, was evaluated by another group [Manabe 2007]. One third of the rabbits developed caseous granuloma and corticosteroid immune suppression resulted in reactivation of the disease. If this really mirrors latent infection still needs to be investigated, however it is highly unlikely, that rabbits will be routinely used for screening of new vaccine candidates.

For the study of human viral and bacterial respiratory diseases, the American cotton rat (*sigmodon hispidus* genus of rodents) is attracting growing attention and has recently been introduced as a novel model for pulmonary TB. These

animals developed granulomatous disease with central necrosis, which is not observed in mice [Elwood 2007]. But in this preliminary report latent infection was not discussed. Furthermore, research with these animals is hampered by the lack of reagents, i.e. antibodies.

The use of a streptomycin-auxotrophic mutant, which does not grow but remains viable for at least six months, if not supplemented with exogenous streptomycin, has been reported [Kashino 2006]. To what extent auxotrophic mutants display characteristics of dormancy *in vivo* has not been investigated yet.

An intriguing approach is the *in vivo* adaptation of the Wayne model for latent TB [Woolhiser 2007]. This group transferred dormant bacteria, grown *in vitro* under oxygen depletion, into mice and observed a significant delay in growth compared to bacteria cultured under optimal conditions. Provided that the metabolic state of bacteria grown under Wayne conditions [Wayne 2001] resembles that of latent bacteria in humans, this model might represent a rapid test for latency vaccines if these are administered prior to infection.

For the time being Cynomolgus macaques might resemble human latent pulmonary TB infection best. Approximately 40% of monkeys infected via bronchoscopic instillation of a low dose of M.tb into the lungs had clinical characteristics of latent TB infection and did not progress to disease within 20 months studied [Capuano, III 2003]. For obvious reasons working with non-human primates does not pose an option for screening of new vaccine candidates but might be inevitable for testing of promising vaccines prior to clinical phase II/III trials.

In summary: It was not possible to induce protection against latent pulmonary TB by plasmid DNA vaccines encoding dormancy-associated antigens in any of the pre- or post-exposure experiments. Whether these vaccines are simply not inducing a strong protective immune response, and/or the immune response evoked by vaccination is irrelevant, because the bacteria are not dormant and thus not expressing these antigens, awaits further examination. It is most likely though, that in none of the murine models for pulmonary TB infection tested here, bacteria were in a “true” state of dormancy.

6.4 Analysis of the vaccine-induced immune response involved in protection against infection with *Mycobacterium tuberculosis*

Understanding the mechanisms involved in the formation of a protective immune response against pulmonary TB will be crucial for the development of improved vaccines. Therefore, the second part of this thesis was dedicated to finding correlates of protection against pulmonary TB after vaccination. Members of our department already published that a recombinant BCG expressing listeriolysin (recBCG) significantly better protected against TB than the parental strain [Grode 2005]. That this recBCG also conferred superior protection if administered subcutaneously was confirmed here. By comparing these two vaccines against each other and against untreated controls, factors indicative for the development of a protective immune response against pulmonary TB could be identified.

6.4.1 Characterisation of the memory response elicited by vaccination with BCG or recBCG

Cells isolated from the spleens of mice vaccinated with BCG or recBCG readily secreted IFN-γ, IL-6, IL-2, IL-17 and GM-CSF (listed from highest to lower concentrations detected in supernatants) in response to re-stimulation with PPD. Hardly any T_H2 polarising cytokines were detected. IL-12p70 and IL-10 were also detected in supernatants but to a lesser degree and also in non-vaccinated controls, most likely as a result of non-specific activation via TLR signalling through components present in the PPD preparation. Cells isolated from the lungs produced the same panel of cytokines, albeit at lower concentrations, and in addition TNF-α, in response to re-stimulation with PPD. Bearing in mind that 10 times less cells were used for re-stimulations of lung cells. However, even though vaccination with recBCG conferred superior protection over BCG, the concentrations of secreted cytokines were comparable between both vaccine strains.

The most prominent T_H1 cytokine is IFN-γ. It is well recognised that IFN-γ secretion is critical in the cell mediated immune response to TB [Flynn 1993a;

Orme 1993; Ottenhoff 1998]. Mostly activated CD4 and CD8 T cells secrete IFN-γ. It induces up-regulation of MHC-II and MHC-I molecules on APC and of the CD4 molecule on T cells. It also stimulates secretion of TNF-α from monocytes and macrophages and the formation of reactive oxygen intermediates (ROI) in macrophages. IFN-γ can induce antiviral and antiparasitic, but mostly immunomodulatory, functions. It can synergise with TNF-α, promotes growth of T cells and potentiates the response to other growth factors or to mitogens. TNF-α also plays a vital role in the defence against TB, most importantly it helps maintaining the integrity of granulomas [Flesch 1990; Flynn 1995; Tufariello 2003].

IL-2 is mainly produced by CD4 T cells upon activation but also from NK and lymphokine activated killer cells. The IL-2 receptor expression on monocytes is induced by IFN-γ. IL-2 is a growth factor for all subpopulations of T cells and induces antigen independent proliferation as well as secretion of TNF-α and IFN-γ [Taniguchi 1993].

GM-CSF is produced by T cells and macrophages upon activation. Other cell types like B cells, endothelial cells and fibroblasts can also secrete GM-CSF, if induced by TNF-α, TNF-β, IL-1, IL-2 or IFN-γ. Myeloid and endothelial, but not lymphoid cells, express the receptor for GM-CSF [Demetri 1991]. This cytokine is a strong chemo-attractant for neutrophils. It enhances the microbicidal and phagocytic activity as well as oxidative metabolism of neutrophils and macrophages and also improves the cytotoxicity of these cells [Caux 1992; Tazi 1993]. GM-CSF is known to increase the immunostimulatory capacity of APC by up-regulation of MHC-II molecules and enhanced antigen presentation [Fischer 1988]. A role for GM-CSF in pulmonary homeostasis has been observed [Dranoff 1994].

IL-6 is one of the major physiological mediators of the acute phase reaction. The main cellular sources for IL-6 are monocytes, fibroblasts and endothelial cells but also macrophages, T cells and B cells, granulocytes and eosinophils can produce IL-6 upon appropriate activation. IL-6 is induced by stimulation with IL-1 and TNF-α. IL-6 is also a potent T cell activator. In combination with IL-2 it induces differentiation into cytotoxic T cells. Apart from inducing T_H17 cells in collaboration with TGF-β, another function of IL-6 is to increase

expression of intercellular adhesion molecule-1 (ICAM-1), the ligand for leukocyte function associated antigen-1 (LFA-1) which is expressed on activated T cells. Thus secretion of IL-6 in the inflamed tissue facilitates transmigration of activated T cells [Chen 2006]. TNF-α and IFN-γ can induce up-regulation of adhesion molecules as well.

In summary after vaccination with BCG or recBCG memory T cells, predominantly in the spleens but also T cells resident in the lungs, readily secreted cytokines, which increase cytotoxicity, induce activation and proliferation of CD4 and CD8 T cells as well as macrophages and other APC. In addition chemo-attractants are produced which guide additional cells capable of eliminating or containing M.tb to the site of infection. These are mainly neutrophils, macrophages and activated T cells. Taken together, these cytokines induced a potent and protective T_H1 immune response against pulmonary TB upon infection. However, this does not explain improved protection of recBCG over BCG.

The only difference observed after vaccination with BCG versus recBCG was the generation of IL-17 producing cells in the spleen. Splenocytes from mice vaccinated with recBCG produced approximately 4 times more IL-17.

Recently it has been shown that an IL-17 producing CD4 T cell population was generated by APC activated by mycobacteria [Cruz 2006]. BCG induced secretion of TGF-β and IL-6 and activated APC secreted IL-23, which induced newly activated T cells to become IL-17 producers. TGF-β is a key cytokine responsible for the initiation of IL-17 secretion [Mangan 2006]. Costimulation with IL-6 modulates the action of TGF-β, so that IL-17 producing cells rather than regulatory T cells are induced [Bettelli 2006]. Unfortunately, neither IL-23 nor TGF-β secretion was analysed here. Nevertheless, a high concentration of IL-6 in cell culture supernatants was detected after vaccination with BCG or recBCG. IL-17 induces chemokines, growth factors, adhesion molecules and leads to neutrophil accumulation [Linden 2005]. Generally the development of IL-17 producing T cells is inhibited by IFN-γ, but mature IL-17 producing cells are resistant to these effects and can secrete IL-17 in T_H1 or T_H2 environments [Harrington 2005]. This indicates that IL-17 producing T cells in

the spleen have matured early after vaccination with recBCG, because 90 days after vaccination the immune response was predominantly of T_H1 type and nevertheless substantial amounts of IL-17 were detected. It has also been shown that IL-2 is a potent suppressor of T_H17 cell differentiation [Laurence 2007]. IL-1 can subvert this suppressive effect, another explanation why T_H17 cells can be found in a T_H1 environment [Kryczek 2007]. It has been shown that virtually all human IL-17 producing CD4 T cells express CCR6 [Singh 2008]. This receptor has been associated with trafficking of T cells, B cells and DC to epithelial sites. IL-17 driven inflammation is characterised by neutrophil infiltration and neutrophils produce CCL20, the only chemokine ligand for CCR6 [Scapini 2001]. IL-17 has been shown to strongly induce CCL20 [Chabaud 2001]. This could amplify the inflammatory response by the recruitment of IL-17 producing CD4 T cells to the site of infection.

Even though IL-17 secreting T cells are dispensable for the primary immune response against TB [Khader 2005], an important role in the development of a protective immune response after vaccination (with a peptide of the M.tb 6-kDa early secreted antigenic target protein (ESAT-6) and MPL/TDM/DDA adjuvant) was reported [Khader 2007]. These findings indicate that IL-17 producing memory T cells populate the lung and respond more rapidly upon challenge and recruit IFN-γ producing effector cells, which then orchestrate containment of the bacteria, leading to enhanced and earlier bacterial clearance and sustained T cell responses. It is tempting to speculate that the generation of a T_H17 memory T cell population is responsible for increased protection against TB after vaccination with recBCG. An accelerated immune response against M.tb could also be achieved by vaccination with recBCG, however the early memory response after aerosol infection was not analysed. Still, this does not explain why vaccination with recBCG is superior to BCG at later stages of infection, from approximately day 60 onwards (routinely observed in our lab).

One central question that remains to be answered is, whether newly activated, effector memory or central memory T cells responded to PPD re-stimulation 90 days after vaccination. The analysis of memory T cell responses elicited after vaccination with BCG is challenging due to potential antigen persistence.

Persistent antigen can result in continuous activation signals to T cells which could impair conversion from effector to memory T cells [Junqueira-Kipnis 2004]. Preliminary results from members of our department indicate that BCG persists for more than 90 days in spleens and lungs [Dr S. Seibert, personal communication]. It is likely that persistence of BCG has implications on the memory immune response generated. Whether and how the persistence of BCG and recBCG effects the development of a protective immune response against TB, is currently under investigation.

Responses in spleens and lungs after stimulation with Ag85A peptides or protein were low after vaccination with BCG and not detectable after vaccination with recBCG, thus indicating that BCG is a poor inducer of Ag85A specific T cells in BALB/c mice. The peptides used here for re-stimulation were based on a publication which identified Ag85A specific CD4 and CD8 T cell epitopes in TB infected or plasmid DNA vaccinated BALB/c mice, but not in BCG vaccinated animals [D'Souza 2003].

6.4.2 Potential correlates of protection 90 days after infection with *Mycobacterium tuberculosis*

Prior to aerosol infection, no differences, apart from IL-17, in cytokine secretion were observed after vaccination with BCG or recBCG. However, 90 days after aerosol challenge cells isolated from the lungs of mice vaccinated with recBCG secreted substantially higher amounts of IL-6, IL-2, GM-CSF and TNF-α in response to stimulation with PPD, compared to vaccination with BCG, whereas concentrations of IL-17 and IFN-γ were similar. Cells isolated from mice not vaccinated responded only weakly to stimulation with PPD by producing some IFN-γ and IL-17. As already described in detail in section 6.4.1, all these T_H1 polarising cytokines play vital roles in the defence against TB. These differences in cytokine responses cannot simply be explained by an increase in the total numbers of activated cells residing in the lungs, as isolated cells were counted and found to be within the same range, albeit increased compared to untreated controls (data not shown).

Members from our department published recently, that adoptively transferred regulatory CD4 T cells dampen the protective immune response against M.tb [Kursar 2007]. Therefore, the presence of regulatory T cells was also examined here (data not shown). The total number of regulatory CD4 T cells, defined by expression of FoxP3 with and without co-expression of CD25, in the spleen was similar in all groups. In the lungs, the total number of regulatory CD4 T cells was slightly higher in BCG or recBCG vaccinated mice compared to non-vaccinated controls, yet there was no difference between BCG and recBCG.

The detailed analysis of antigen-specific T cells by ICCS provided additional information. CD4 T cells reacted to stimulation with PPD, whilst an antigen-specific CD8 T cell response was not detectable. It has been suggested that recBCG confers superior protection against pulmonary TB because of an enhanced CD8 T cell response due to listeriolysin expressing mycobacteria escaping into the cytosol [Grode 2005]. No evidence for an increased CD8 T cell response after vaccination with recBCG was found in spleens or lungs. This might be explained by the choice of antigen used for re-stimulation. As already observed before aerosol challenge, and also 90 days p.i., vaccination with BCG or recBCG, or infection with M.tb, did not induce a strong Ag85A specific CD8 or CD4 T cell response. Peptides containing CD8 epitopes of Ag85A could be inapt for detecting CD8 T cell responses in BALB/c mice. Analysis of antigen-specific CD8 T cell responses after vaccination with recBCG are currently ongoing using peptides derived from dominant CD8 epitopes in C57BL/6 mice, i.e. TB10.4 [Billeskov 2007] and Mtb32 [Irwin 2005].

Data generated by ICCS are in line with Bio-Plex results: Antigen-specific CD4 T cells generated after vaccination with recBCG secreted more IL-2 and TNF-α whilst IL-17 and IFN-γ production was comparable to that from BCG immunised mice. Also equal proportions of CD4 T cells produced two or three cytokines after stimulation with PPD, whereas two times more cells produced all four cytokines after vaccination with recBCG.

These poly-functional antigen-specific CD4 T cells are likely to be important mediators for increased protection against TB infection. Not only increased numbers of poly-functional secreting cells were detected after vaccination with recBCG but also the combination of cytokines produced differed, compared to BCG and untreated controls. The small proportion of CD4 T cells secreting two cytokines, isolated from the lungs of untreated controls, mostly produced IFN-γ and TNF-α. This population was reduced in BCG and even more so in recBCG vaccinated mice. Instead, more cells appeared which secreted IL-2 and IFN-γ or IL-2 and TNF-α. Cells secreting three cytokines were not detected in unvaccinated controls. In BCG vaccinated mice CD4 T cells producing IFN-γ, TNF-α and IL-17 were dominant, whereas the largest fraction of three cytokine producing cells after recBCG made IL-2, IFN-γ and TNF-α.

Undoubtedly, IFN-γ is a key cytokine in the defence against TB. However there is growing evidence questioning the correlation between the frequency of IFN-γ producing T cells and the level of protection [Majlessi 2006; Mittrucker 2007]. Here too, IFN-γ secretion was not a correlate of protection. As well as regulating homing of activated CD4 T cells to the lung during TB infection, IFN-γ is also the main inducer of AICD and activated CD4 T cells are the main targets [Feuerer 2006]. In line with this it has been shown that IFN-γ induces apoptosis of activated CD4 T cells during mycobacterial infection [Dalton 2000]. The suppressive regulatory functions of IFN-γ might thus impair the establishment and, more importantly maintenance, of a protective T_H1 memory response. In a series of adoptive transfer experiments, it was also shown that only antigen-experienced T_H1 type CD4 T cells which did not produce IFN-γ persisted for a prolonged period of time and were able to secrete IFN-γ upon re-stimulation [Wu 2002]. It is tempting to speculate that this could explain why superior protection conferred by recBCG becomes apparent at later stages of infection.

Some studies in mice and non-human primates [Wille-Reece 2006] have already shown that CD4 and CD8 T cells [Joshi 2007; Saparov 1999] capable of co-producing IL-2, IFN-γ and TNF-α preferentially survive and provide greater protection against challenge as compared to cells producing only one

cytokine. It has been speculated that IL-2 is not only required for programming secondary memory responses in CD8 T cells but also in CD4 T cells, enabling long term survival of primed T cells [Dooms 2007]. Moreover these triple cytokine producing cells have recently been detected in healthy mycobacteria-exposed adults [Scriba 2008]. Thus, cells secreting both IFN-γ and IL-2 or both TNF-α and IL-2 might better support their own expansion and survival, because IL-2 is a potent growth factor which provides an autocrine signal [Yarilin 2004]. The relative increase in the proportion of cells secreting IFN-γ and IL-2, TNF-α and IL2, IL-17 and IL-2 and finally yet importantly all three cytokines, IFN-γ, TNF-α and IL-2, could reflect better maintenance of a stable effector T cell response and this will likely be an explanation for increased protective efficacy of recBCG.

Bio-Plex data revealed that splenocytes from mice vaccinated with BCG or recBCG secreted comparable amounts of IL-17, IL-6, IL-2 and GM-CSF in response to stimulation with PPD even though only recBCG conferred protection. Cells isolated from spleens of non-vaccinated mice also produced these cytokines, albeit at lower concentrations. Generally, cytokine secretion from splenocytes was much lower than from cells isolated from the lungs, even though 10 times more cells were used for re-stimulation. This could be explained either by lower bacterial burden in this organ or by presence of cells showing a central memory type rather than an activated effector phenotype. T cells of the central memory lineage respond with some delay to antigen-specific stimulation [Sallusto 1999]. ICCS from splenocytes did not correlate with Bio-Plex results. Thus, either 6h re-stimulation was not sufficient for detection of cytokine secretion by central memory T cells or cells other than CD4 or CD8 T cells were responsible for cytokine secretion p.i. in the spleen. ICCS revealed antigen-specific CD4 T cells producing IL-2, IFN-γ, TNF-α and IL-17 only after vaccination with recBCG, whereas CD4 T cells from untreated or BCG vaccinated mice only secreted TNF-α. In the spleen too, no antigen-specific CD8 T cell responses were detected. Interestingly, Bio-Plex data showed higher IFN-γ secretion in untreated or BCG vaccinated mice and reduced production of this cytokine in recBCG-immunised mice. This is in line

with a report from Wu et al. showing that transferred, antigen experienced CD4 T cells, which were able to produce IFN-γ upon re-stimulation, were rarely present in lymphoid organs but survived much longer in the lungs [Wu 2002]. Hardly any poly-functional CD4 T cells were detected in non-vaccinated or BCG vaccinated mice. As already discussed for the lung data p.i., protection in the spleen might be correlated with the appearance of poly-functional CD4 T cells, albeit at lower proportions than in the lung, and more importantly an increase of T cells producing IL-2 in combination with IFN-γ, TNF-α or IL-17.

The antibody response to Ag85A and PPD was analysed before and after aerosol infection. Hardly any Ag85A specific antibodies were detected, which is in line with Bio-Plex and ICCS data, confirming that the immune response against Ag85A is very weak in BALB/c mice. Comparable amounts of IgG1 antibodies to PPD were detected 90 days p.i. indicating the presence of IL-4, which drives isotype class switching to IgG1. The concentration of PPD-specific antibodies of the IgG2a class, which are induced by IFN-γ, most likely represented bacterial burden in the lungs, with the lowest OD 405nm measurable in recBCG-vaccinated mice. However, experiments with B cell knock out mice have shown that an antibody response, but not the presence of B cells, is dispensable for efficient TB control [Bosio 2000; Turner 2001].

In summary, it was shown that subcutaneous vaccination with recBCG conferred superior protection, compared to BCG, against airborne TB infection in the lungs and spleens. Analysis of the immune response elicited after vaccination revealed the development of a strong T_H1 polarised immune response regardless which strain of BCG was used for vaccination and one probable correlate of protection prior to aerosol infection: The amount of IL-17 secreted in response to PPD stimulation was 4 times higher in the spleens of mice vaccinated with recBCG. 90 days p.i. cells isolated from the lungs of mice vaccinated with recBCG produced substantially more IL-2, IL-6, GM-CSF and TNF-α, whereas IFN-γ and IL-17 secretion was comparable in BCG vaccinated mice. The most striking difference was the appearance of poly-

functional CD4 T cells and increased proportions of T cells producing IL-2 in combination with the other three cytokines (IL-17, IFN-γ, and TNF-α) analysed after vaccination with recBCG. Most likely these cells are indicative of a more sustained memory T cell response generated by vaccination with recBCG. This would also explain why the superior protection of recBCG is observed later during the course of M.tb infection: The protective T_H1 memory T cell response after vaccination with recBCG is maintained, whereas the memory response generated by vaccination with BCG wanes over time.

Some concluding remarks: The experiments presented here indicate, that the immune responses elicited after BCG or recBCG vaccination and subsequent exposure to TB are highly complex. It is therefore unlikely that focussing on one single cytokine will provide sufficient information to identify a protective vaccine against TB. The bead-based cytokine array technology seems a useful tool, as it is possible to analyse up to 90 cytokines and chemokines in parallel in one small sample volume. This assay in combination with ICCS, together with bioinformatical approaches based on cytokine secretion patterns, could provide vital clues in the hunt for correlates of protection.
Compared to BCG, vaccination with recBCG induced T cells secreting significantly more T_H1 cytokines upon re-stimulation with PPD. This correlated with a more vigorous and sustained immune response and translated to an additional 10-fold reduction in bacterial burden, more than conferred by any other vaccine tested. How much more cytokine producing cells are then needed for sterile eradication of bacteria? Will we ever be able to achieve complete elimination of bacteria through T_H1 polarised T cells or will we not rather tip the balance towards tissue destruction?

7 Summary

In this thesis, novel vaccine candidates against pulmonary tuberculosis (TB) were assessed in models of persistent and latent infection. In the first part, immunogenicity and protective capacity of plasmid DNA vaccines encoding the dormancy-associated antigens hspX, TB31.7, Rv1733c or Rv2628 was analysed in pre- and post-exposure vaccination experiments. In order to test the capability of these DNA vaccines to prevent reactivation of latent M.tb infection, short-duration drug-induced latency models were evaluated. However, none of the experimental setups yielded a working model for latent TB infection in mice. It was not possible to induce protection against pulmonary TB by plasmid DNA vaccines in any of the experiments. It is most likely that in none of the murine models for pulmonary TB infection tested here, bacteria were in a “true” state of dormancy. If the bacteria are not dormant and thus not expressing dormancy-associated antigens, plasmid DNA vaccines encoding these antigens cannot confer protection against TB infection.

In the second part, the immune response elicited by vaccination with BCG or recBCG, involved in the defence against infection with M.tb was analysed in detail. Vaccination with recBCG conferred superior protection against TB. After vaccination a strong T_H1 polarised immune response was detected, regardless which strain of BCG was used. Splenocytes of mice vaccinated with recBCG were capable of producing more IL-17 in response to PPD stimulation. Post infection, cells isolated from the lungs of mice vaccinated with recBCG produced substantially more IL-2, IL-6, GM-CSF and TNF-α. Flow cytometry revealed mostly CD4 T cells responding and a higher percentage of them were poly-functional (secreting any combination of IL-2, IL-17, IFN-γ and TNF-α) after vaccination with recBCG. Most likely these cells are indicative of a more sustained memory T cell response generated by vaccination with recBCG. Thus, the protective T_H1 memory T cell response after vaccination with recBCG is maintained, whereas the memory response generated by vaccination with BCG wanes over time.

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9 Appendix

9.1 Zusammenfassung

Ziel dieser Arbeit war es, experimentelle Impfstoffe gegen Tuberkulose (TB) in latenten und persistenten murinen Infektionsmodellen zu evaluieren. Im ersten Teil wurde die Immunogenität, sowie das protektive Potential, von Plasmid-DNA Vakzinen, die die Dormanz-assoziierten Antigene hspX, TB31.7, Rv1733c oder Rv2628 kodieren, in Tiermodellen untersucht. Um eine Schutzfunktion von Dormanz-assoziierten DNA Vakzinen gegen Reaktivierung latenter TB untersuchen zu können, wurden Kurzzeit Antibiotika-induzierte Latenzmodelle getestet. Es konnte jedoch mit keiner Behandlung ein zuverlässigens Tiermodell latenter TB etabliert werden. Da sich die Mykobakterien in keinem der untersuchten Tiermodelle in einem echten Dormanz-Zustand befanden und folglich auch keine Latenz-assoziierten Antigene exprimierten, konnte in keinem der Versuche eine Schutzfunktion von Plasmid-DNA nachgewiesen werden.

Im zweiten Teil dieser Arbeit sollte die schützende Immunantwort gegen TB nach Vakzinierung mit BCG oder rekombinantem BCG (rekBCG) untersucht werden. Vakzinierung mit rekBCG vermittelte einen wesentlich stärkeren Schutz als BCG. Nach Vakzinierung mit beiden BCG Stämmen konnte eine starke T_H1 Immmunantwort nachgewiesen werden. Milzzellen von Mäusen, die mit rekBCG vakziniert wurden, sekretierten wesentlich mehr IL-17 nach Stimulation mit PPD. 90 Tage nach Infektion sekretierten Lymphozyten, die aus den Lungen von rekBCG vakzinierten Mäusen isoliert wurden, deutlich mehr IL-2, IL-6, GM-CSF und TNF-α. Mit Hilfe der Durchflußzytometrie konnten CD4 T-Zellen als Hauptquelle dieser Zytokine identifiziert werden. Außerdem konnte ein erhöhter Anteil von polyfunktionalen T-Zellen, die Kombinationen von IL-2, IL-17, IFN-γ sowie TNF-α produzieren, nachgewiesen werden. Die hier durchgeführten Experimente lassen den Schluß zu, daß die durch rekBCG vermittelte, schützende T_H1 Immunantwort, besser erhalten wird, wogegen die durch BCG vermittelte Immunantwort im Verlauf der TB Infektion nachläßt.

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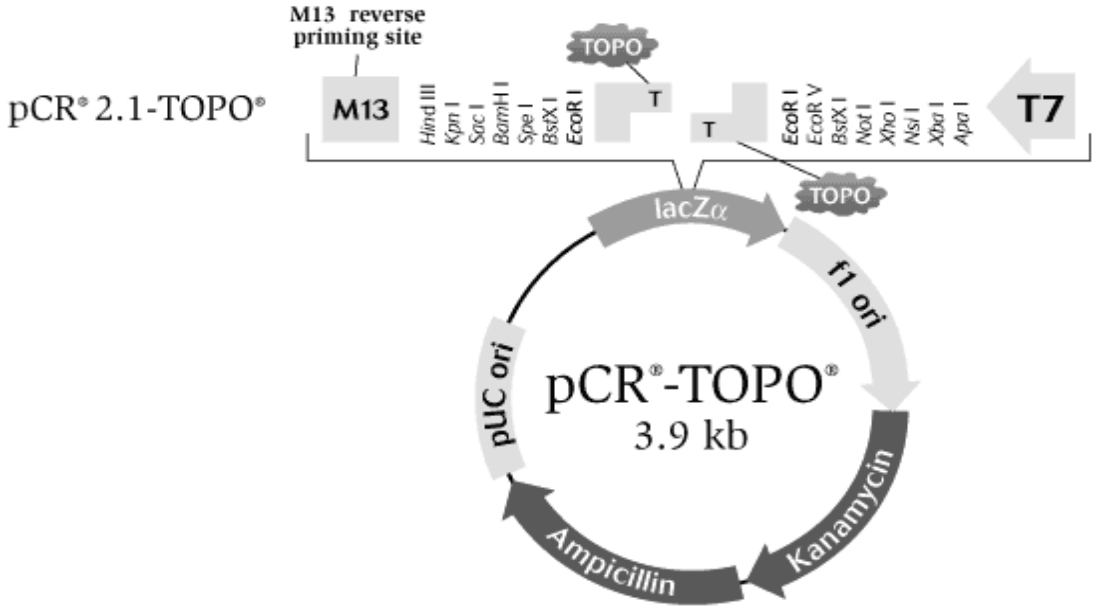
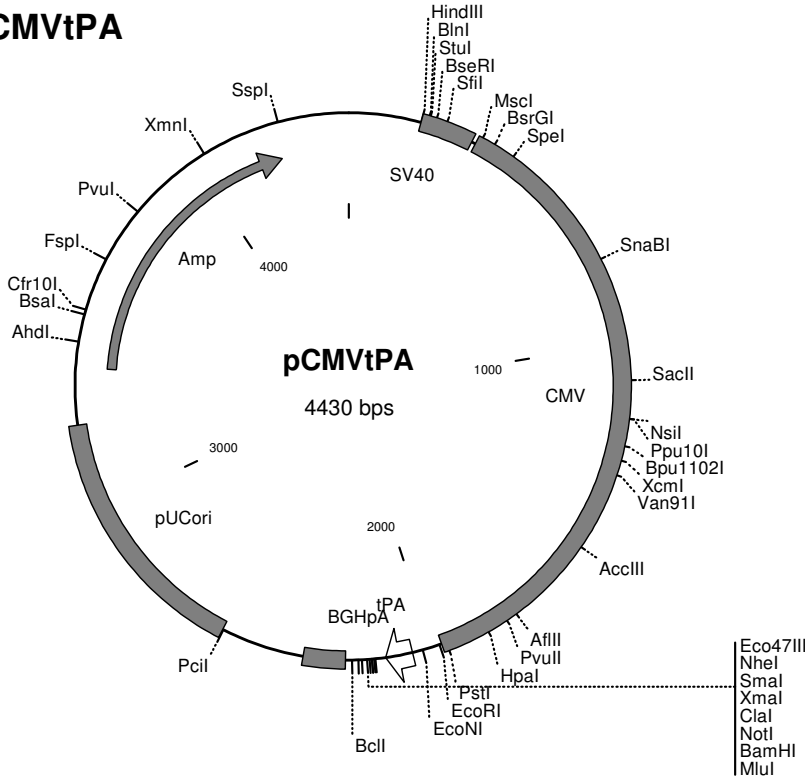
9.3 Plasmid maps

9.3.1 pCR®-TOPO®



Information on pCR®2.1-TOPO®
3931 nucleotides, *Lac*Zα fragment: bases 1-547, M13 reverse priming site: bases 205-221, Multiple cloning site: bases 234-357, T7 promoter/priming site: bases 364-383, M13 Forward (-20) priming site: bases 391-406, f1 origin: bases 548-985, Kanamycin resistance ORF: bases 1319-2113, Ampicillin resistance ORF: bases 2131-2991, pUC origin: bases 3136-3809

9.3.2 pCMVtPA



9.4 Supplier

Supplier	location	URL
Adobe Systems GmbH	Ohmstrasse 1 D-85716 Unterschleißheim	www.adobe.de
AGOWA GmbH	TGS Haus 8 Ostendstrasse 25 D-12459 Berlin	www.agowa.de
Amersham Biosciences	GE Healthcare Europe GmbH Oskar-Schlemmer Str. 11 D-80807 München	www1.gelifesciences.com
American Type Culture Collection (ATCC)	P.O.Box 1549 Manassas, VA 20108, USA	www.atcc.org
B.Braun Melsungen AG	Carl-Braun-Strasse 1 D-34212 Melsungen	www.bbraun.de
Becton Dickinson GmbH: BD Biosciences, BD Pharmingen, BD Difco™, BBL™ and BD Clontech	Tullastrasse 8-12 D-69126 Heidelberg	www.bdbiosciences.com
Binder	Im Mittleren Ösch 5 D-78532 Tuttlingen	www.binder-world.com
Biochrom Labs, Inc.	1719 South 13th Street, P.O. Box 996, Terre Haute, IN 47808, USA	www.biochrom.com
Bio-Rad Laboratories GmbH	Heidemannstrasse 164 D-80939 München	www.bio-rad.com
Brand	Otto-Schott-Strasse 25 D-97877 Wertheim	www.brand.de
Carl Roth GmbH & Co. KG	Schoemperlenstrasse 1-5 D-76185 Karlsruhe	www.carl-roth.de
Corning B.V. Life Sciences	Koolhovenlaan 12 NE-1119 Schiphol-Rijk	www.corning.com
e-Bioscience	6042 Cornerstone Court West San Diego, CA 92121, USA	www.ebioscience.com
Eppendorf	Barkhausenweg 1 D-22339 Hamburg	www.eppendorf.com
Fermentas GmbH	Opelstrasse 9 D-68789 St.Leon-Rot	www.fermentas.de
Graph Pad Software, Inc.	11452 El Camino Real, 215 San Diego, CA 92130, USA	www.graphpad.com
Heraeus Instruments: Kendro Laboratory Products GmbH	Robert-Bosch-Strasse 1 D-63505 Langenselbold	www.heraeusinstruments.de
ICN Biomedicals GmbH	Thüringer Strasse 15 D-37269 Eschwege	www.icnbiomed.com
IKA Labortechnik: IKA-Werke GmbH & Co.KG	Janke & Kunkel-Strasse 10 D-79219 Staufen	www.ika.net
INTEGRA Biosciences GmbH	Ruhberg 4 D-35463 Fernwald	www.integra-biosciences.de
Invitrogen GmbH: Invitrogen, NOVEX and Gibco	Technologiepark Karlsruhe Emmy-Noether-Strasse 10 D-76131 Karlsruhe	www.invitrogen.com
JPT Peptide Technologies GmbH	Volmerstrasse 5 (UTZ) D-12489 Berlin	www.jpt.com
Knick Elektronische Messgeraete GmbH & Co.	Beuckestrasse 22 D-14163 Berlin	www.knick.de

Supplier	location	URL
Lab therm: Adolf Kuehner AG	Dinkelbergstrasse 1 CH-4127 Birsfelden	www.kuhner.com
Leica Camera AG	Oskar-Barnack Strasse 11 D-35606 Solms	www.leica-camera.de
Merck	Frankfurter Strasse 250 D-64293 Darmstadt	www.merck.de
MICROM International GmbH	Robert-Bosch-Str. 49 D-69190 Walldorf	www.microm-online.com
Microsoft Deutschland GmbH	Katharina-Heinroth-Ufer 1 D-10787 Berlin	www.microsoft.de
Millipore GmbH	Am Kronberger Hang 5 D-65824 Schwalbach	www.millipore.de
Molecular Devices GmbH	Gutenbergstrasse 10 D-85737 Ismaning	www.moleculardevices.com
MWG-Biotech AG	Anzingerstrasse 7a D-85560 Ebersberg	www.mwg-biotech.com
New England Biolabs GmbH (NEB)	Bruningstrasse 50 D-65926 Frankfurt am Main	www.neb.com
Nunc GmbH und Co.KG	Hagenauer Strasse 21A D-65203 Wiesbaden	www.nuncbrand.com
Qbiogene Inc.: MP Biomedicals	Waldhofer Strasse 102 D-69123 Heidelberg	www.qbiogene.com
QIAGEN GmbH	QIAGEN Strasse 1 D-40724 Hilden	www.qiagen.com
Regenerier Service Wolfgang Franz	Balsaminenweg 42 D-12623 Berlin	
Roche Diagnostics GmbH: Roche Applied Science	Sandhofer Strasse 116 D-68305 Mannheim	www.roche-appliedscience.com
Sarstedt AG & Co.	Rommelsdorfer Strasse D-51582 Nümbrecht	www.sarstedt.com
Sartorius	Weender Landstrasse 94-108 D-37075 Goettingen	www.sartorius.de
Schleicher & Schuell BioScience GmbH MicroScience GmbH	Hahnestrasse 3 D-37586 Dassel/Relliehausen	www.schleicher-schuell.de
Schuett-Biotec GmbH	Rudolf-Wissell-Strasse 13 D-37079 Goettingen	www.schuett-biotec.de
Scientific Software Group	P.O. Box 708188, Sandy, Utah 84070, USA	www.scisoftware.com
SERVA Electrophoresis GmbH	Carl-Benz-Strasse 7 D-69115 Heidelberg	www.serva.de
Sigma-Aldrich Chemie GmbH	Eschenstrasse 5 D-82024 Taufkirchen	www.sigmaaldrich.com
Statens Serum Institut (SSI)	Artillerivej 5 2300 Copenhagen, DK	www.ssi.dk
Thermo Electron	Thermo Fisher Scientific, Inc. 81 Wyman Street Waltham, MA 02454, USA	www.thermo.com
Thermo Shandon, Inc.	171 industry Dr., Pittsburgh, PA 15275, USA	www.shandon.com
TPP AG	Zollstrasse 155 Ch-8219 Trasadingen	www.tpp.ch
Zapf Instruments	Emmy-Noether-Strasse 5 D-31157 Sarstedt	www.zapf-instruments.de