Mycobacterial Diseases
ISSN: 2161-1068

Garcia Contreras et al., J Mycobac Dis 2012, S:1
DOI: 10.4172/2161-1068.S1-002

Review Article Open Access

Inhaled Vaccines for the Prevention of Tuberculosis

Garcia Contreras L[1], Awashthi S[1], Hanif SNM[1] and Hickey AJ[2,3]*

[1]Department of Pharmaceutical Sciences, The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73126-0901, USA
[2]Division of Molecular Pharmaceutics, University of North Carolina at Chapel Hill, NC 27599-7571, USA
[3]RTI International, 3040 Cornwallis Road, P.O. Box 12194, Research Triangle Park, NC 27709-2194, USA

Abstract

Tuberculosis a major challenge to global health exacerbated by emerging multi drug resistant (MDR) and extensively drug resistant (X-DR) strains of M. tuberculosis and co-infection with HIV. BCG, the only approved vaccine, has variable protection ranging between 0-80%. Compared to the large number of new vaccine candidates a modest effort has been expended to investigate other routes of administration, such as pulmonary and intranasal. Vaccination by these routes is relevant since TB infection is mainly acquired by inhalation of a few aerosol droplets containing as little as 3-5 viable bacilli. The lungs have many attractive immunological features including bronchoalveolar lymphoid tissue (BALT) and local antigen presenting cells (APCs) sampling airborne pathogens. Aerosol vaccination is a noninvasive method of antigen delivery that may facilitate mass vaccination campaigns. Administration by non-medical personnel and the ability to eliminate transmission of blood borne diseases arising from poor practice in injected procedures in remote areas is beneficial. The current dogma does not include mucosal immunity for protection against TB but its contribution may not be ruled out. The selection of the appropriate antigen, aerosol formulation and inhaler will determine the success of this approach. Dry powder formulations of antigen/adjuvant combinations have efficiency of delivery, stability and sterility advantages over liquid formulations. Dry powder vaccines can be manufactured as micro particles and nanoparticles prepared with different materials including polymers, sugars and amino acids. Examples of these novel vaccine formulations and their evaluation in animal models are discussed in the present review. The proposed pharmaceutical and clinical advantages of inhaled dry powder vaccines justify further evaluation.

Keywords: Dry Powder; Formulation; Inhaled Vaccines; Tuberculosis

Introduction

Mycobacterium tuberculosis (MTB) is one of the most prominent human pathogens infecting one-third of the world's population [1] and mutates into multidrug-resistant (MDR) and extensively-drug resistant (XDR) strains. Infection with and mutation of MTB, the co-infection with the human immunodeficiency virus (HIV), the limitation in current therapies and the lack of patient compliance for tuberculosis (TB) treatment have all contributed to the significant threat to the global control of TB.

Effective vaccination strategies to prevent the disease would be the best intervention for the global control of TB. The current vaccine against TB, bacilli Calmette-Guérin (BCG) was developed by the French scientists Calmette and Guérin after *Mycobacterium bovis* was isolated from a cow with tuberculous mastitis in 1908 [2]. Small children in many countries worldwide have been routinely vaccinated with BCG for over a century. BCG is considered safe, has minor side effects and it is inexpensive to produce large quantities. Unfortunately, protection conferred by BCG during childhood wanes after 10 or 15 years and has variable degree of protection to pulmonary tuberculosis in adults [3]. Large scale randomized control trials and case-control studies demonstrated a 0% protective efficacy in Chingleput, India and 80% protection in Haiti and Canada [4,5]. In addition to different BCG strains used for these trials (Montreal, Danish, Phillips, Tice, Glaxo, Madras and Birkhaug) [5], the efficacy of BCG appeared to be dependent upon temperature and location of the geographical area in which the vaccine was evaluated. Good protection appeared to occur mainly in temperate regions, whereas tropical regions of the globe appeared to show poor protection. Several factors have been implicated in the variability of BCG protection, including nutritional differences in the population, the use of different BCG strains, and the variability in the exposure of different populations to environmental mycobacteria and parasites [4,6].

A major research effort has been expended to develop new TB vaccines that provide the same effective protection despite of the population or the region of the world. Hundreds of TB vaccine candidates have been reported after the publication of the MTB genome in 1998 [7]. These include recombinant BCG or other vectors expressing mycobacterial antigens, attenuated or recombinant strains of mycobacteria, DNA vaccines, protein or peptide vaccines in adjuvants and non-peptide vaccines [8]. Compared to the number of new vaccine candidates produced, little research has gone into investigating other routes of administration, such as the pulmonary, intranasal and oral routes. This is surprising since TB infection is mainly acquired by inhalation of a few aerosol droplets containing as little as 3-5 viable bacilli. Inhaled vaccines are potentially powerful tools to provide immunity since the pulmonary route of immunization follows the natural route of infection, thus closely mimicking the induction of immunity in the respiratory tract by inhaled pathogens. From the perspective of vaccine development, the lungs have many attractive immunological features including organized lymphoid follicles, known as the bronchoalveolar lymphoid tissue (BALT) and local antigen presenting cells (APCs) located ideally to sample antigens entering the airways [9]. In addition, the pulmonary epithelium has a crucial role in host defense against inhaled pathogens as it possesses

***Corresponding author:** Anthony J. Hickey, Division of Molecular Pharmaceutics, Research Triangle Institute, 3040 Cornwallis Road, Research Triangle Park, NC 27709, USA, Tel: 919-541-6771; Fax: 919-541-6936; E-mail: ahickey@rti.org

Received April 25, 2012; **Accepted** May 23, 2012; **Published** May 25, 2012

Citation: Garcia Contreras L, Awashthi S, Hanif SNM, Hickey AJ (2012) Inhaled Vaccines for the Prevention of Tuberculosis. J Mycobac Dis S1:002. doi:10.4172/2161-1068.S1-002

natural defense mechanisms including the mucociliary escalator and secretion of antimicrobial agents, chemokines and cytokines into the mucous layer covering the airway epithelium to prevent colonization of microorganisms [10,11]. Aerosol vaccination is a noninvasive method of antigen delivery that may facilitate mass vaccination campaigns, the administration by non-medical personnel and the ability to eliminate transmission of blood borne diseases arising from poor practice in injected procedures in remote areas [12]. Finally, this route of immunization has the potential to be successful in children, for whom the persistence of maternal antibodies does not appear to interfere with mucosal immunization but does interfere with subcutaneous immunization [13].

The first recorded use of a mass aerosol vaccination can be traced to 1951 when Johnson and Gross immunized a flock of chicken against Newcastle disease by atomization of the B1 strain of the virus [14]. The success of this approach led to the use of aerosol vaccination to immunize other small farm animals against common diseases [15,16]. In 1968, pioneering studies by Rosenthal et al. compared the immunization with aerosolized BCG in guinea pigs and in a few human subjects [17]. However, perhaps the most successful example of a human mass aerosol vaccination has been the immunization of pre-school and school aged children against measles by the inhaled aerosol method undertaken in Mexico between 1988 and 1990 [18]. Fewer side-effects and a larger percentage of seroconversion (52-64%) were observed after aerosol immunization than after injection vaccine (4-23%) [19]. The World Health Organization (WHO) has recognized the potential of immunization using alternative routes of vaccine delivery, such as small-particle aerosol and large-droplet intranasal administration [20] and has considered the use of different devices to administer these vaccines [13]. The pulmonary route of immunization employed to deliver small-particle aerosol is currently preferred, as it has been shown to induce strong and long-lived systemic and mucosal immune responses [21-23].

This review presents the factors that can influence the successful delivery and effectiveness of an inhaled vaccine for tuberculosis including the type of vaccine or antigen used, the relationship between the route of immunization and the nature of the immune response elicited, the formulation and the device employed to deliver the vaccine and the animal model in which the vaccine is tested. Examples of inhaled vaccines for TB will be also discussed.

The Immune Response

Success of a vaccine depends on an efficient presentation of vaccine-antigens by antigen-presenting cells followed by generation of antigen-specific effect or immunity and persistence of immunological memory. As long as the memory response persists, a vaccinated individual can clear the infectious organisms upon contact through a recall immune response. This immunity has to be initiated quickly before the infectious organisms evade the immune mechanisms and get an opportunity to establish pathology. Consequently, delivery of the vaccine-antigens to the lungs should be more effective for prevention of respiratory infectious diseases, such as TB and other microbial infections that are initiated at the pulmonary mucosa [24,25].

The cellular and biochemical milieu in the lung, including surfactant, mucin, proteins, peptides, glycoproteins, glycolipids, will naturally affect the antigen-uptake and presentation by antigen-presenting cells (APCs) [26]. Among different APCs, dendritic cells (DCs) are the most effective because of their flexible morphology, variant phenotypes and unique ability to physically move within the tissue [27,28]. Upon taking up the antigens, the DCs are activated and develop unique features. These activated DCs extend many long dendrites which can cover a considerable zone of cell-cell contacts at targeted site by crossing these natural barriers [29,30]. Therefore, a small number of DCs are required to activate antigen-specific adaptive immune response. Other typical APCs, epithelial cells and macrophages, are also critical even if they are not as flexible and robust in mounting immune response, cross-talk between macrophages and DCs through cross-antigen-presentation, secretion of cytokines, chemokines and trans-signaling can significantly affect this process. The mechanism of phagocytosis and antigen-processing of TB antigens by DCs is temporally different from that in macrophages. DCs first take up the TB antigens directly or by cross-priming. Then, vesicles carrying TB antigens are released from infected macrophages that are taken up by the bystander non-infected APCs, including DCs (Figure 1) [31-33]. Phagocytosis of TB antigens results into maturation and migration of DCs through activation of cytokines, chemokines, release of reactive oxygen intermediates, and increased expression of antigen presentation (MHC class II, CD1) and T cell co-stimulatory molecules (CD40, CD80, CD86) [32].

Migration of mature DCs is mediated by upregulation of receptors such as CCR7. The processed antigen, expressed on the cell-surface MHC molecules as antigenic peptides, is presented to the naïve T cells, NK cells or other immune cells. Dendritic cells show versatility in expressing the antigens on MHC class I or MHC class II molecules, depending on the nature of the epitope, thereby inducing CD8 cytotoxic T cell or CD4 helper T cell response, respectively (Figure. 2). The $CD4^+$ T cells have shown to mount protective immune response against TB, while contribution of $CD8^+$ T cells is not confirmed [34].

Depending on the density of the TB antigens presented, types of co-stimulatory molecules, and cytokines secreted by the DCs, naïve T cells differentiate into either Th1 or Th2 cells and B cells produce antibody. A relatively new paradigm suggests that even non-lymphoid organs, such as lung, also have lymphoid structures in their stroma that are efficient in inducing local immune responses [35,36]. This set up will make it more convenient for the local DCs to produce effector immunity and memory in lung against TB antigens. Experimental evidence suggests that pulmonary vaccination is capable of inducing systemic immunity [37,38].

Considering the utility of the pulmonary delivery, a sophisticated and efficient vehicle is needed to carry the vaccine antigen. Viral and some of the non-viral vectors (for example, lipid-based vectors) may

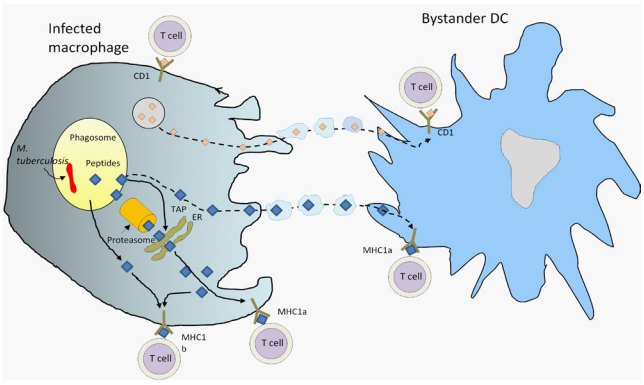


Figure 1: Cross priming mechanism for stimulation of major histocompatibility complex (MHC) I and CD1 restricted T cells. (DC = dendritic cell; ER = endoplasmic reticulum; TAP = transporter associated with antigen presentation) (Adapted from (60).

cause severe toxic responses including inflammation and mortality as reported in few lung disease-specific clinical trials [39,40]. Thus, unique non-viral vectors are required that can carry the vaccine antigens and target the lungs. For the development of an efficient immune response, stable but not necessarily persistent expression of vaccine antigens is desired [41]. In the lungs, DCs are usually present in a quiescent phase and in a very low number; therefore, adjuvants can be very important in vaccine formulations as a stimulus to the immune cells.

New Vaccine Candidates

Live bacterial vaccines are thought to induce the greatest protection because they carry multiple antigens and they could live in tissues for sufficient time to ensure efficient immunological memory is induced and maintained [42]. However, these assumptions may not apply to all mycobacterial vaccines. Since the earliest use of BCG, there has been debate about the benefits or disadvantages of using this vaccine. BCG may be capable of switching on multiple immune mechanisms in response to compounds in the bacterial cell wall, secreted proteins and naturally occurring adjuvants (innate, CD4/CD8, and gamma cell responses, respectively). In addition, it has a long track record of safety, protects repeatedly and uniformly against severe childhood forms of TB and leprosy and it is very inexpensive [8]. However after the failure of BCG to protect large populations particularly in the developing world, hundreds of vaccine candidates have been developed to address one or more limitations of BCG. Over the last decades, several types of vaccines have been evaluated in animal models for protection against TB infection including, recombinant BCG, BCG over-expressing native proteins, attenuated MTB, subunit and DNA vaccines. Except for attenuated MTB, none of these vaccines can provide the same or better level of protection as the universal BCG in animal models [43].

In order to develop improved vaccines against TB, BCG has been employed as homologous vector to over express mycobacterial antigens. BCG can supply a number of relevant mycobacterial antigens, recombinant antigens and it also had the capability to express MTB proteins in native form and release them in a manner that makes them being processed similarly to MTB proteins (Table 1). The work of Tullius et al. [44] is noteworthy because recombinant BCG strains were developed specifically to vaccinate immunocompromised persons, such as HIV patients. These vaccines can replicate *in vitro*, in media containing specific supplements and in cultured macrophages but their replication in animal models is highly restricted; thus avoiding serious disseminated disease in these patients. Other forms of BCG have been developed to express immunomodulatory cytokines (Table 1) or genetically modified to enhance antigen presentation [43]. Two approaches have been used to improve antigen presentation either by enhancing MHC I or MHC II antigen presentation. Grode et al. modified BCG to secrete listeriolysine to promote perforation of the phagosomal membrane and with deleted urease gene to reduce the pH in the phagosome to be optimum for the action of listeriolysine [45].

Another strategy, employing whole bacterium, has been to attenuate MTB by deleting one or more virulence factor genes [46]. In theory, the use of modified MTB is better than BCG because MTB contains over 120 specific genes that are not found in BCG, thus being likely more effective for immune activation. Some of these vaccine candidates are shown in table 1. In addition to public perception and acceptance of these vaccine candidates, the main concerns are the danger of reversion from attenuated to virulent state and the potential release of antibiotic gene markers [47].

Non-viable subunit vaccines have been considered valuable for safety reasons mainly with respect to immunocompromised patients or those who have been previously vaccinated with BCG. Subunit vaccines include one or more immunodominant MTB antigens and can be administered with an adjuvant or a viral vector to increase its potency [46]. Examples of these vaccine candidates are given in table 2.

Potential vaccines for TB have been developed from antigens such as Ag85 and ESAT-6 and their fusion proteins, which have been shown to confer some degree of protection in animal models [48]. More frequently, subunit vaccines have been employed in prime-boost vaccine strategies in animals that are primed with BCG, named heterologous prime-boost immunization strategies. These vaccine strategies can evoke powerful T cell immune responses and may be useful to develop an improved vaccine regimen [1].

The use of viral vectors with MTB antigens, either as prime or boost, also shows great potential to produce a new vaccine. One of the most promising candidates in this category of vaccines is the recombinant strain of the modified vaccinia Ankara virus expressing Ag85A from MTB (MVA85A). Initial clinical studies with MVA85A performed in 2002 were designed to demonstrate its safety in BCG-naïve, tuberculin test negative adults [6]. From 2002 to 2008, 258 subjects were vaccinated without signs of adverse events, but in a recent study antigen-specific IL-17A-producing T cells were detected in the systemic circulation of healthy individuals after vaccination with MVA85A [49]. A newer strategy employing MVA expressing IL-15 plus the ESAT-6/Ag85 fusion protein has improved this approach in the mouse model [50].

It is encouraging that several of the vaccine candidates shown in tables 1 and 2 are actually in different stages of clinical trials, making more tangible the promise of a new vaccine against TB.

Animal Models for Vaccine Testing

The use of an animal model relevant to human disease is fundamental to new vaccine development. Overall, there is no ideal animal model of TB, but each of the available models can represent certain aspects of the human disease. However, when analyzing the outcome of a study, it is important to consider the similarities and differences of the animal model selected with respect to those of humans, and their susceptibility to infection with a determined bacterial strain. It is also important to understand that complete protection imparted by a vaccine in terms of complete sterilization of the lungs of immunized animals or reduction in bacterial burden cannot be obtained because the animal model may have a limited "window of protection" [51]. For example, in the mouse this "window of protection" is quite small, with only 1.0-1.2 log protection usually achievable. In the guinea pig, in which the control inoculum grows to higher values because of the inherent susceptibility of this animal, a larger range of 2.0-3.0 logs can be attained [8,52]. Thus, it is of paramount importance to know the advantages and disadvantages of each animal model for vaccine testing.

Among TB animal models, the most frequently used model is the inbred mouse [53] because of the detailed knowledge of its immune system and the large variety of reagents available [51]. In addition, the mouse genome has been completely sequenced and the genetic manipulation of this species is highly advanced so that various strains of knockout mice are accessible [51]. However, the most attractive advantages of the mouse are its cost (for animal purchasing, husbandry and housing) and smaller space requirements. Thus, large numbers of vaccine candidates can be tested in this model, and the most promising candidates can be tested in other more relevant species at a later time. Similarities between the basic immune response of mice and men

Vaccine	Description	Results (compared to BCG)	Reference
Recombinant BCG overexpressing native MTB proteins			
rBCG30	BCG over expressing the 30kDa protein (Ag85B)	The first vaccine demonstrating more potency than BCG against TB	[114]
rBCG/Ag85A	BCG over expressing Ag85B	Fewer CFU in lung and spleen in vaccinated guinea pigs; fewer CFU in lung but not in spleen in cynomolgus monkeys	[115,116]
rBCG/Ag85C	BCG over expressing Ag85C	In guinea pigs, reduced CFU in lung and spleen; reduced pathology in lung, spleen and liver; reduced pulmonary fibrosis	[117]
rBCG/ESAT-6 (±CFP10)	BCG secretes both ESAT-6 and CFP10	Tested in mice and guinea pigs; same protection in lung but better in spleen; not good in immunocompromised mice	[118]
rBCG/38 kDa protein	BCG over expressing 38kDa glycoprotein	Immunized mice survived longer	[119]
Recombinant BCG expressing MTB fusion proteins			
rBCG/72f	BCG secreting a hybrid of proteins Mtb39 +Mtb32 (named 72f)	In cynomolgus monkey induced immune and protective responses but not different than BCG controls	[120]
rBCG/Ag85B-ESAT-6	BCG secreting fusion proteins of Ag85B and ESAT-6	Protective responses similar to BCG controls in mice	[121]
rBCG/Ag85B-Mpt64$_{190-198}$-Mtb8.4	BCG expressing a fusion protein of Ag85B with immunodominant peptide Mpt64 and Mpt8.4	Comparable or slightly better efficacy than BCG in mice	[61]
Recombinant BCG overexpressing native proteins and additionally attenuated for safety in HIV-positive persons			
rBCG(mbtB)30	Rendered sideropore dependent by deletion of the gene mbtB	Safer than BCG in SCID mice and more potent than BCG in the guinea pig model of TB	[44]
rBCG(panCD)30	Rendered pantotenate dependent by deletion of the panCD genes	Can multiply in vitro, but multiplication is limited in vivo; safer than BCG in SCID mice in protection comparable to BCG in the guinea pig model of TB	[44]
Recombinant BCG expressing immunomodulatory cytokines			
rBCG/GM-CSF	BCG secreting murine granulocyte macrophage colony stimulating factor	Immunized mice had higher numbers of antigen presenting cells; IFN-gamma secreting cells, and ~1 log fewer CFU in spleen after virulent challenge	[122]
rBCG/IL-2	BCG secreting murine IL-2 to counter a Type 2 immune response	Immunized mice exhibited greater splenocyte proliferation and IFN-gamma production in response to PPD, comparable protection to BCG	[123]
rBCG/IL-15	BCG secreting a fusion protein of Ag85B and murine IL-15	Immunized mice had greater absolute numbers of CD4+ and CD8+ T cells in lung and spleen; greater numbers of IFN-gamma secreting CD4+ cells and lower CFU in the lung but not in the spleen	[124]
Recombinant BCG overexpressing native proteins and escaping the phagosome			
rBCG-Aeras403	BCG over expressing Ag85B and TB10.4 with endosome escape	Safer than BCG in SCID mice; induced stronger responses in mice and guinea pigs compared to BCG; mice survived longer than with BCG	[125]
rBCG ΔureC hly+	BCG that expresses membrane perforating listeriolysine and is devoid of urease	Induced superior protection in mice than BCG. Proven to be safe and immunogenic in phase I clinical trial	[126]
Modified or attenuated MTB			
MTB phoP mutant SO2	SO2 strain engineered by a disruption in the *phoP* gene of MTB	Impaired multiplication *in vitro* in mouse macrophages and *in vivo* in infected mice; Enhanced ability to bind human macrophages	[127]
MTB ΔRD1 ΔpanCD	MTBH37Rv with deletion of the primary attenuating mutation of BCG (DeltaRD1) and two genes for the synthesis of pantothenate (DeltapanCD).	long-lived protective immune responses and longer survival in wild type mice, and CD4-deficient mice against an aerosol challenge with virulent MTB. Safe in guinea pigs and SCID mice	[128]
MTB Δ leuD Δpan	MTB with two independent attenuating auxotrophic mutations in leucine	Long-term protection and survival in guinea pigs against challenge with virulent MTB similar to BCG. No vaccine-associated adverse effects (clinical, hematological and bacteriological) in SIV-positive or SIV-negative Rhesus macaques	[129]

Table 1: Summary of new vaccine candidates against TB by modification of the whole bacterium (modified from [43,46]).

include toll-like receptors, NK and NKT cells, and production of antimicrobial molecules such as nitric oxide. However, there are several differences in the response to TB infection and the progression of the disease between mice and man. Besides size, anatomy, vasculature and lymphatic system, the most important differences are in the pathology of the granuloma and the cell mediate immunity. Mice lack CD1b and CD1c cells, there are no visible lesions in the mouse lungs after 10 days of infection, and after 20 days only perivascular cuffing of mononuclear cells with central aggregation of lymphocytes is visible with no evident necrosis [53]. This may be one of the main factors contributing to the concern that vaccines being effective in the mouse may not be effective in humans.

The guinea pig is the smallest animal model that most closely resembles TB infection in humans. Guinea pigs are highly susceptible to TB infection with only a few droplets containing 3-5 bacilli and the progression of the disease is similar to that in humans [52]. Guinea pigs are outbred and in that respect their population is reflective of human populations. Studies in this species have been instrumental to understand the relationship between mycobacteria and their mammalian hosts as well as key elements of their immune response. Discrete lesions can be observed in the guinea pig lungs after 10 days of infection with extra-pulmonary dissemination to the spleen and other organs occurring 10-14 days after infection humans [52]. The "classic" granuloma can be observed 21 days after infection with mineralization of the central core occurring during the chronic phase of the disease

Vaccine	Description	Results	Reference
Fusion proteins from MTB antigens as vaccines			
Ag85B-TB10.4/IC31	Ag85B-TB10.4 delivered in IC31 adjuvant	Vaccination resulted in high numbers of polyfunctional CD4 T cells co-expressing IL-2, IFN-gamma and TNF-alpha. This correlated with protection against virulent challenge in the TB mouse model	[131]
Mtb72F	a 72-kDa polyprotein genetically linked in tandem in the linear order Mtb32(C)-Mtb39-Mtb32(N)	Immunization of guinea pigs resulted in more than 1 year survival after virulent challenge when compared to BCG.	[132]
Mtb72F + AS02	Mtb72F in the GSK proprietary adjuvant system AS02	Vaccine was immunogenic and caused no adverse reactions in cynomolgus monkeys. Superior protection afforded with BCG prime- Mtb72F in AS02A boost, compared to that with BCG alone. Monkeys also survived longer and showed reversal of disease progression	[133]
ID83	fusion protein (ID83), which contains the three Mtb proteins Rv1813, Rv3620 and Rv2608	ID83 + synthetic TLR4 or TLR9 agonists generated a T helper-1 immune response and protected mice against Mtb challenge with regardless of route.	[134]
Viral-vectored vaccines			
MVA85A/ Aeras-485	Attenuated vaccinia Ankara virus expressing Ag85A	Induced high levels of antigen-specific IFN-gamma-secreting T cells when used alone in BCG-naive healthy volunteers. In BCG vaccinated volunteers, substantially higher levels of antigen-specific IFN-gamma-secreting T cells were induced, and at 24 weeks after vaccination these levels were 5-30 times greater than in those receiving a single BCG dose.	[135]
Crucell Ad35/ Aeras 402	Adenovirus 35 expressing 3 antigens	Confers protection against MTB after intranasal or IM immunization in the mouse model. Histological evaluation revealed differences in immune response between two mouse strains that were elucidated by epitope mapping analysis. a dominant CD8 T-cell response was detected in BALB/c (H-2(d)) mice whereas a more balanced CD4/CD8 T-cell responses were observed in C57BL/6 (H-2(b)) mice	[136]
AdAg85A	Adenovirus 5 expressing only Ag85A	A single intranasal but not IM immunization was able to induce potent protection from MTB challenge in a mouse model Also used to boost mice primed with SC BCG, showing enhanced protection that was correlated with the numbers of IFN-gamma CD4+ and CD8+ in airway lumen	[137]

Table 2: Summary of new subunit vaccine candidates against TB (modified from [49,130]).

[54]. As lung tissue damage progresses, guinea pigs, like humans, lose weight and depending of the severity of the disease can die in less than six months [55]. Guinea pigs can develop an intense, indurate delayed hypersensitivity reaction 24 hours after injection of a low dose purified protein derivative (PPD), which makes them the "gold standard" for potency testing and standardization of PPD for use in the human skin test [52]. It is also for this reason that they are the preferred animal model that is used to test vaccines when the final point does not require the sacrifice of the animal. Perhaps the main disadvantage of this animal model is the scarcity of immunologic reagents specific to this species, but this may change in the near future as various investigators are currently working specifically toward the goal of developing and acquiring novel reagents to study the immune response of the guinea pig to infection [56].

The rabbit is the species lowest on the phylogenetic scale in which cavitary tuberculosis can be studied [57]. After infection with virulent strains of Mycobacterium bovis, rabbits can develop cavitary tuberculosis similar to that observed in adult immunecompetent patients. Susceptible rabbit families can also develop tuberculosis similar to that observed in babies and immune-compromised patients by hematogenous reseeding [58]. However, since inbred rabbits are difficult to obtain significant variations in resistance to infection are observed [59]. Disadvantages of the rabbit model of tuberculosis include their cost, space required and the limited number of reagents available to study their immune response compared to other models [60]. Dannenberg classified rabbits by their susceptibility to infection as "susceptible" and "resistant" [61]. In "susceptible" rabbits virulent bovine bacteria initially multiplied to a greater extent than in "resistant" animals where macrophages rapidly and effectively engulfed the bacteria and acquired cellular resistance was developed. In "resistant" rabbits the caseous center of tubercles is surrounded by activated macrophages that can engulf the bacteria that escape from the caseous center. Mycobacterial strains that affect humans are less virulent for rabbits than the bovine strains. It has been reported that when the H37Rv strain is aerosolized to infect rabbits, it has as much as 50% chance of being inactivated by macrophages. In addition, when rabbits were challenged with aerosolized bacteria that infects humans after standard vaccination with BCG, the number of primary tubercles observed in "resistant" rabbits was decreased by 80% whereas in "susceptible rabbits it decreased by 15% only. Therefore, it is important to interpret the results of this study on the context of the model, as this study would indicate that BCG vaccination would increase the protection of BCG vaccination in "resistant" rabbits by 5-fold, but the protection in "susceptible" rabbits would be only 1.2-fold [62].

The pathology and immune response to TB infection in non-human primates is also very similar to humans, which is a big advantage of this model because most human reagents cross-react with non-human primates [63]. The confirmation of infection and progression of the disease can be tracked by a variety of serological methods, cytokine levels, chest X-rays and weight loss. This model is also unique in that TB co-infection with the human immunodeficiency virus (HIV) can be studied in this species, since they are susceptible to the simian immunodeficiency virus [64]. The disease progression in non-human primates can be classified in three categories [65]: 1) rapid infection/ rapid progression, where advanced disease can be observed by 3 months post-infection; 2) active/chronic infection, where the signs of disease can be observed but the progression is slow; 3) latent infection, where no clinical signs of the disease can be observed. Based on this classification, Flynn et al. were the first researchers to develop a tractable model of latent tuberculosis infection [66,67]. In addition, this model has been successfully employed to evaluate long-term protection of experimental vaccines [68]. It is considered that studies in this model should be restricted to final experiments prior to clinical

trials with drugs and vaccines in humans, not only due to the high cost of each animal and its housing but also for ethical reasons.

Lastly, the bovine model of tuberculosis is infrequently employed for the evaluation of experimental vaccines for humans. This model is more commonly used to develop diagnostic reagents to differentiate between vaccinated and infected individuals [69-71]. An advantage of this model is that large volumes of blood can be collected, thus providing the opportunity to measure a large number of immunological parameters [72]. This can be explored to identify immune correlates of protection for TB that are still not defined [73]. Notably, this model has been employed to explore one of the causes attributed to the low efficacy of the current BCG vaccine: exposure to environmental bacteria. In this study [74], a group of calves were vaccinated within 8 h of birth to mimic vaccination policies in African countries. A second group was vaccinated at 6 weeks of age, and a third group was primed at 8 h after birth and boosted at 6 weeks. Non-vaccinated calves were employed as controls. All animals were challenged at 4 months with *Mycobacterium bovis* and sacrificed 4 months after challenge. The study revealed that calves became responsive to environmental bacteria at 6 weeks and that the responsiveness was lost at 15 weeks of age, which did not appear to hamper the protection provided by BCG. Surprisingly, animals in the prime-boost regimen had significantly less protection than those only vaccinated at birth. The results of this study indicate that the bovine model of tuberculosis may be of use to study novel TB vaccines.

Selection of the animal model employed to evaluate vaccines delivered to the lungs depends upon the characteristic of the aerosol being assessed which includes: efficiency of aerosol delivery; immunogenicity; protection or; toxicity [75]. The anatomy and physiology of species differ significantly and in general the ease of delivery of aerosols increases as the size of the animal increases. Since the method of delivery of the particles to the lungs dictates the efficiency and reproducibility this must be considered in parallel to selection of the animal model [75].

Pharmaceutical Forms and Delivery Devices

Successful stimulation of the common mucosal immune system in the airways is a challenge that inhaled vaccines may face at the present stage of development. This is also needed to make the effectiveness of the inhaled vaccines against respiratory pathogens superior to injected conventional formulations. The selection of the appropriate aerosol formulation and the right inhaler device for delivery in concert with the appropriate antigen may address this challenge.

Several methods have been employed to vaccinate different animal models, individual patients or populations. For animal models, these range from direct administration methods such as intra-tracheal instillation, spray instillation and insufflation, to passive inhalation methods such as exposure chambers (whole body or nose only) [75]. For humans, three different inhaler devices can be employed, depending on the vaccine formulation: pressurized metered dose inhalers (pMDIs), nebulizers and dry powder inhalers (DPIs) [76].

Even though most inhaled drugs are delivered by pMDIs, only a few vaccines have been delivered by pMDIs. The main reason is that the formulation ingredients in pMDIs are not compatible with most vaccines, whole attenuated organisms or subunit vaccines. The propellants used to aerosolize pMDI contents as well as the surfactants or co-solvents needed to solubilize the active ingredients are detrimental for most antigens and adjuvants. Brown et al. determined that *Streptococcus* suis bacteria lost 50-70% of its antigenicity after being delivered with a propellant- driven device similar to a pMDI to the respiratory tract of a swine [77].

Most studies with inhaled vaccines have employed nebulizers and liquid vaccine formulations. However, delivery of therapeutic compounds with typical air-jet nebulizers can be cumbersome, inefficient and expensive. They require the use of compressed air, large volumes of the vaccine solution, long times to inhale the aerosol and it is estimated that approximately 10% of the dose placed in the device will be deposited in the periphery of the lung [78]. More effective nebulizers have been developed in the last few years, including the Pari eFlow, the Activaero AKITA and APIXNEB system co-developed by Pari and Activaero appears to deliver more than 80% of the drug loaded into the system [79]. One issue that is not resolved even with these newer technologies is that the sheer force generated by the nebulizer may decrease vaccine potency [80]. This was experienced by Coates et al. when they observed a 71% decrease in the potency of a measles vaccine after 20 minutes of continuous operation of the nebulizer [81]. They also determined that one-third of the vaccine potency was lost for each cycle of 30 seconds on/10 seconds off.

Liquid vaccine aerosols are frequently unstable on storage or during delivery. Manufacture of liquid vaccine formulations requires very stringent controls and the use of refrigeration throughout the process, the shipping and storage. Liquid vaccine formulations are also more prone to contamination when compared to dry formulations. Therefore vaccines such as BCG are freeze dried (lyophilized) to preserve their potency and sterility. Interestingly, the methods used to produce the injectable BCG vaccine used in the present day are quite similar as those described by Calmette many years ago. The few modifications that have been made in different laboratories are largely based on individual laboratory observations, personal preference, and convenience [82]. However, in the case of measles vaccine, the reconstituted form of the lyophilizate lost potency rapidly when exposed to temperatures of 25 and 37°C.

Perhaps the best example of inhaled vaccines for the prevention of TB is the pioneer work by Rosenthal et al. in 1968 [83]. Their work was based on the assumption that the sensitization to tuberculin, though not synonymous with immunity, was indicative of the immune state of an animal model. In this study, guinea pigs, children and medical students were exposed to BCG aerosols generated by nebulization of aqueous suspensions of cells. The indirect measure for immunization in this study was the rate and size of the tuberculin reaction following exposure to BCG aerosols. Thus, the results of these early studies were inconclusive due to the measure of effectiveness used and the variability in the nebulizer output [83]. Similar studies conducted by Lagranderie et al. [84] reported the effects of bacterial strain and dose in guinea pigs receiving intradermal or aerosol immunizations with BCG. In comparison with the intradermal route of vaccination, aerogenic vaccination with 10^5 CFU induced higher local cellular immune responses and substantially improved protective effect. As in a study conducted by the authors and presented in the last section of this review, the aerosol vaccination did not result in pathological alterations to the lung tissue at the doses studied [84].

Delivery of vaccines in a dry powder form by means of dry powder inhalers (DPIs) has several advantages over pressurized metered dose inhalers and nebulizers. DPIs are portable, inexpensive, and give a better control on the dose delivered. There are single dose devices (dose in a capsule or single reservoir) and multi-dose devices (doses in blister packs). The dose deposited in the lung among common devices ranges from 12-40% for passive DPIs, but is higher for active (battery driven)

DPIs [85]. Dry powder formulations of drugs or vaccines have greater stability than liquid formulations and do not require reconstitution or refrigeration to preserve their potency. They are usually packed in a manner that protects the powder from light and humidity, thus enhancing their stability. However, one of the most desirable properties of dry powder vaccines for inhalation is that APCs in the lung (macrophages and DCs) tend to uptake dry particles. This event elicits an additional immune response and can make particles act as adjuvants, depending on their composition. In general, microparticulate systems are known to enhance the immune response greatly in comparison to soluble antigens because they act as adjuvants and stimulate the immune response to help Th1 induction [86]. Antigens alone or with other adjuvants have been associated or encapsulated in micro- or nano-particles made of lipids, polymers, sugars, aminoacids or viral vectors. In addition to providing adjuvancy, the encapsulation of antigens into particles has other advantages for pulmonary delivery such as protecting the integrity of antigens, improving the aerosol dispersion of the particles and lung deposition, and in cases even control the time and amount of antigen released from these particles.

A group in Switzerland, led by Bachman developed novel virus-like particles (VLPs) to induce potent B cell responses in the absence of adjuvants [87]. The authors attributed this property to the highly repetitive and organized array of epitopes on viral surfaces efficiently cross-links B cell receptors constituting a strong activation signal that may even overcome B cell tolerance. A novel vaccine for tuberculosis was prepared from Influenza A VLPs were produced displaying a 20 amino acid sequence from MTB ESAT-6 [88]. Immunization of mice with this vaccine resulted in high serum antibody titers against ESAT-6, indicating that VLPs can be an efficient platform for epitope presentation.

Several experimental vaccines against TB have been formulated in liposomes and showed enhanced protection compared to the vaccine alone. Dimethyl dioctadecyl ammonium bromide (DDA) is a cationic, micelle-forming surfactant that has been used as adjuvant in few experimental TB vaccine, live or subunit, because of its Th1-stimulating effect and elicited protective immunity in mice [89-91]. Higher levels of protection have been obtained in this animal model when immunomodulators have been tested in combination with DDA. Some of these include trehalose 6,6 dibenenate (TDB), monophosphoryl lipid A (MPL), muramyl dipeptide (MDP), saponin, calcitrol, beta-glucan and n-hexadecane. The highest adjuvancy was obtained after vaccination of C57BL/6 mice with the DDA-MPL and the DDA-TDB combinations when the early secretory antigenic target 6 protein (ESAT-6) was used as the antigen [90]. All these experimental liposomal vaccines were administered by parenteral route. To the best of our knowledge, no liposomal vaccine has been administered by inhalation yet. However, it is possible to generate aerosols of liposomal vaccines from liquid or dry-powder forms. The latter is deemed to be more stable since the shear produced during nebulization can compromise the integrity of liposomes [92] and the antigen as described before. Dry powder liposomal vaccines can be prepared by spray drying the liquid liposomal formulation [93], or by freeze drying it followed by milling the resulting cake to obtain particles in the respirable size [94].

Polymeric micro- or nano- particles are the most used vehicles to encapsulate antigens for tuberculosis vaccines. Bivas-Benita et al. [95] prepared a chitosan-DNA nanoparticle vaccine encoding eight known HLA-A*0201-restricted T-cell epitopes derived from *M. tuberculosis* antigens: 19 kD, Ag85B (2 epitopes), Ag85A, PstA1, ThyA & RpoB and ESAT-6. The levels of IFN-gamma after spray instillation of a suspension of these nanoparticles were much higher in a transgenic mouse model compared to those after spray instillation of the nanoparticles alone of the plasmid by the intramuscular route. In another study, Zhu et al. produced chitosan microspheres containing an Ag85-MPT64-Mtb8.4 fusion protein made from MTB genes. Similarly, to the Bivas-Benita study, higher IFN-gamma levels were obtained after subcutaneous administration of the chitosan microspheres containing the plasmid, compared to administration of the plasmid in solution. These two studies suggest that chitosan particles can be effective carriers for potential tuberculosis vaccines. However, this should be confirmed by studies challenging immunized animals with viral strains of MTB. Polyethyleneimine (PEI) has been used in the same way as chitosan to produce nanoparticle subunit vaccines [96,97]. However, studies by Yu et al. showed a modest efficacy with Fe(3)O(4)-Glu-PEI in the MTB-infected mouse model [97].

Perhaps the polymers that have been most frequently used to encapsulate vaccines to be delivered by different routes are the derivates of the poly-lactic and poly-glycolic acids and their co-polymers. They are popular because they are biodegradable, biocompatible and their toxicity, if any, is minimal [9]. The poly-lactic-co-glycolic acid copolymer is probably the most used from this family of polymers. Micro- or nano particles can be produced by different techniques using these polymers and their efficiency to encapsulate compounds depends on the molecular weight of the compound, its physicochemical properties and the intended size of the particle. Some of the vaccine candidates against TB that have been formulated into PLGA particles include: MTB 38KDa protein [98], MTB 71KDa protein [99], DNA encoding mycobacterial hsp65 protein +TDM [100], Ag85 DNA [101], DNA encoding Ag85B+MPT-64+MPT83+DDA [102], Ag85+TDB [103], antigen TB10.4-Ag85B fusion protein [104], and rHsp65 protein+ artificial antimicrobial peptide KLK [105]. Invariable, the protection conferred by the vaccines encapsulated into particles is superior to that of the vaccine in its liquid form. As for other formulations, only one of these studies has delivered the particles in dry form by the pulmonary route [103].

As previously described (section 3), despite the many efforts to develop an effective vaccine, BCG still performs well, apparently better than most new TB vaccines, including some recombinant forms of BCG, modified forms of MTB or subunit vaccines. Several of these whole cell vaccines have shown to have advantages over subunit vaccines. The preparation of these whole cell vaccines into dry powders for pulmonary delivery requires a totally different formulation approach from the ones described for subunit vaccines. To our knowledge, only one group has prepared dry powders for inhalation containing a whole cell vaccine. Wong et al. [106] reported an alternative method to prepare BCG with leucin, by spray drying to provide a dry, flowable powder that is more viable and more stable over time at room temperature conditions than conventionally lyophilized BCG. It was demonstrated that by limiting osmotic stress on bacterial membranes during drying, i.e., by removing extracellular salts and cryoprotectants from suspensions of bacteria, bacterial viability on drying can be significantly improved relative to standard spray drying (with salts and cryoprotectants) and relative to lyophilization. BCG dried by this method also exhibited improved room-temperature shelf-life stability than with the lyophilized formulation. The size and form of the resulting particles (a mix of rods and small spheres) was thought to have a role not only in aerosol deposition but in extent of adjuvant effect achieved: the rod –shaped coated bacterium is associated with leucine spheres. The size of these particles (MMAD=2.1-3.7 μm) makes them ideal candidates for phagocytosis by APCs.

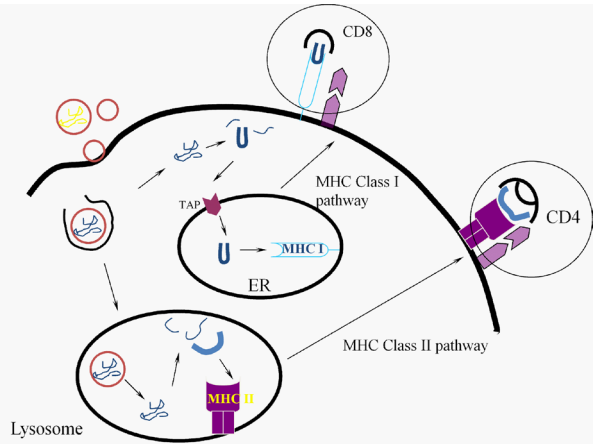
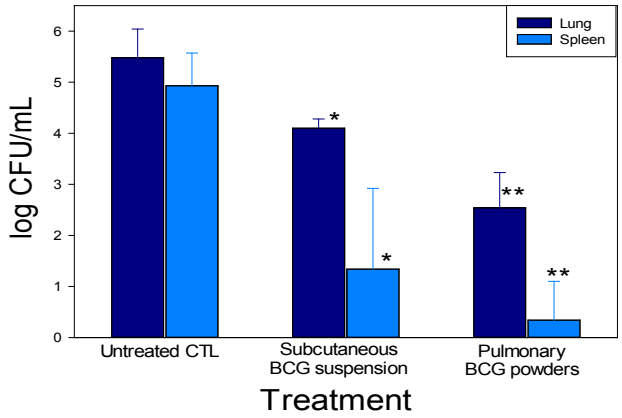


Figure 2: Subunit antigen conformation versus antigenicity in antigen presenting cells. (MHC = major histocompatibility complex; ER = endoplasmic reticulum; TAP = transporter associated with antigen presentation) (70)



* Significantly smaller than untreated controls, ** significantly smaller than BCG SQ and untreated control

Figure 3: Number of viable bacteria (CFU/mL) at necropsy in lung and spleen tissues after bacterial challenge of guinea pigs immunized with different BCG formulations by different routes (average ± std deviation, n=6).

More recently the same approach was employed to manufacture a powder vaccine intended for aerosol delivery by spray drying the Ad35-vectored tuberculosis (TB) AERAS-402 vaccine with mannitol-based stabilizers. Physicochemical properties of the powder vaccine were determinate as well as the integrity of the virus during spray drying and storage [107]. The resulting powder vaccine formulated with a combination of mannitol-cyclodextrin-trehalose-dextran was deemed appropriate for pulmonary vaccination of humans and stable when stored at different temperatures.

Evaluation of Inhaled Vaccines against Tuberculosis

Despite the success shown with the inhaled measles vaccine in human population, immunization by the pulmonary route in the prophylaxis and therapy of infectious diseases is still in its early stages [12]. Besides the influenza vaccine, a few other studies have achieved successful immunization by the nasal route, including papilloma virus and cytomegalovirus [108]. Given the large number of new vaccine candidates against tuberculosis and the variety of novel formulations and aerosol delivery systems, there are only a few studies formulating these new vaccine candidates for inhalation, and fewer evaluating their efficacy in animal models.

Our laboratory evaluated the BCG powder vaccine, manufactured by Wong et al. [106], administered by the pulmonary route to prevent tuberculosis in the guinea pig model [109]. Animals were divided in different groups immunized by different routes (pulmonary, intradermal and subcutaneous routes) with commercial BCG solution, BCG powders in suspension BCG dry powders. Ten weeks after immunization and 4 weeks after aerosol infectious challenge with MTB H37Rv, the bacterial burden in the lungs of all immunized animals was, as expected, significantly lower than that of unimmunized controls, regardless of the BCG dose or the route of immunization (Figure 3). Most notably, the bacterial burden of lungs of animals immunized by the pulmonary route with BCG particles was significantly lower than that of animals immunized by the parenteral route (with either solution or particles). Histopathological analysis mirrored the bacteriology results, showing less lung tissue damage (in the form of granulomas) in animals vaccinated by parenteral routes whereas in animals immunized by the pulmonary route, lung tissue appeared almost normal (Figure 4). Studies that may give an insight into the way that BCG replicates and disseminates when delivered by the parenteral or pulmonary routes and possible differences in the nature of the immune response have been completed by our group and the data is being analyzed. At this point we postulate that delivering BCG in dry particle form rather than in aqueous bacterial suspension may have increased the residence time in the lungs, thus increasing the possibility of uptake by APCs. Lagranderie et al. have shown that activation of alveolar macrophages occurs more readily after inhalation than after intradermal vaccination [84]. Dannenberg suggested that at the local site, products of bacilli and those from sensitized lymphocytes are at higher concentration than occur systemically, and suggested that macrophages in the local lesion achieve much higher antimicrobial resistance that that observed for systemic macrophages [62]. It is possible that delivery of the vaccine to the lungs would favorably influence the mucosal response in the lungs and improve the local immunity. A decade ago, Kauffman suggested that a vaccine that could generate neutralizing antibodies in the respiratory tract, such that it will kill rather than just phagocytize MTB before macrophage uptake would solve the problems of the present vaccines [51,108]. For years the dogma has been that the cellular

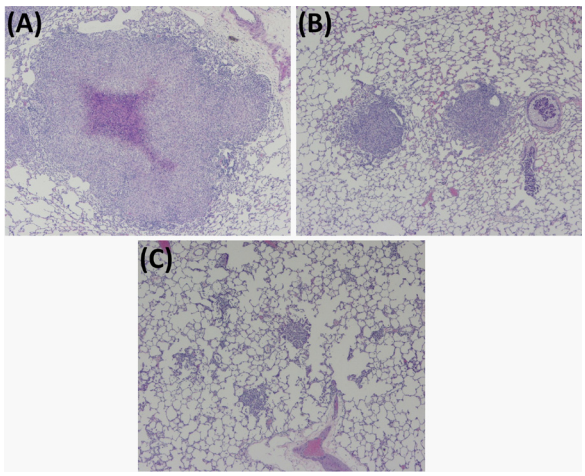


Figure 4: Lung histopathology after bacterial challenge of guinea pigs immunized with different BCG formulations by different routes: (A) Untreated controls; (B) animals immunized with BCG powder suspension by the subcutaneous route; and (C) animals immunized with BCG dry powders by the pulmonary route.

immune response is the only defense against MTB, but the importance of the mucosal immune response has not been established. However, is interesting to question how the absence of a mucosal response can be postulated when mucosal routes have not been used for immunization.

Microspheres delivered as aerosols to the lungs in the size range 1-5 mm can reside at the site of deposition for extended periods of time prior to uptake by APCs [26,58]. Microspheres are mainly taken up by macrophages, which are mobile and number almost a billion in the periphery of the lungs [110]. Given the efficient targeting of microspheres to APCs, the particles have the ability to elicit strong immune responses even with small amounts of antigen [86]. Therefore, role of alveolar macrophages as initial host cells for MTB was employed by our group in a second vaccine strategy with a subunit antigen. Poly (lactide-co-glycolide) (PLGA) microparticles were manufactured in respirable sizes as carriers for recombinant Ag85B (rAg85B) to be delivered by pulmonary route as a novel vaccine against TB [103]. Continuous release of antigens from PLGA has been shown to provide a prolonged immunological response in animals and avoids the need for multiple boosting [111,112]. Recombinant Ag85B was expressed from two *Escherichia coli* strains and encapsulated it by spray-drying in PLGA microspheres (rAg85-PLGA) with/without adjuvants. The ability of these rAg85-PLGA microspheres to deliver antigen to macrophages for subsequent processing and presentation to the specific CD4 was assessed in the T-hybridoma cells DB-1. These cells recognize the Ag85B97-112 epitope presented in the context of MHC class II and secrete IL-2 as the cytokine marker. The rAg85-PLGA microspheres (3.4 - 4.3 μm) were suitable for aerosol delivery to the lungs and targeting alveolar macrophages. THP-1 macrophage-like cells exposed with PLGArAg85B microspheres induced the DB-1 cells to produce IL-2 at a level that was two orders of magnitude larger than the response elicited by soluble rAg85B. These results demonstrated that rAg85-PLGA microspheres in respirable sizes were effective in delivering rAg85B in an immunologically relevant manner to macrophages [103]. They also demonstrated that dry powder delivery of rAg85B using a microsphere formulation has potential as a vaccine strategy for preventing TB or to be employed as a promising boosting vaccine.

It is important to recognize that adult populations in many developing countries have been immunized previously with BCG or exposed to MTB, which constitutes a priming exposure, and a boost approach is more appropriate for these populations. Therefore, there is a need to develop an effective boosting immunization that could enhance and prolong the protective immunity based on BCG, or prior MTB, initiated immunity.

Based on the results obtained with rAg85B-PLGA, our group designed and performed studies to evaluate the protection afforded in the guinea pig model against virulent MTB challenge by combinations of prime-boost strategies with BCG delivered by the pulmonary and intradermal routes [113]. PLGA microparticles containing rAg85B in sizes suitable for delivery by inhalation were prepared as described above and delivered as dry powders to the lungs of guinea pigs in single or multiple doses of homologous and heterologous immunization strategies. BCG was delivered subcutaneously as the positive control and as part of heterologous immunization strategies. Immunized animals were challenged with a low-dose aerosol of *MTB* H37Rv to assess the extent of protection measured as reduction in bacterial burden (CFU) in the lungs and spleens of guinea pigs. Histopathological examination and morphometric analysis of those tissues was also performed. The heterologous strategy of BCG prime-P-rAg85B aerosol boosts appeared to enhance protection from bacterial infection, as indicated by a

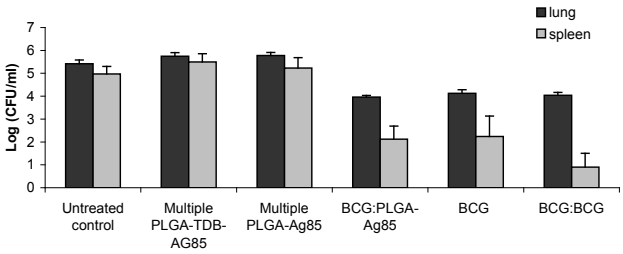


Figure 5: Number of viable bacteria (CFU/mL) at necropsy in lung and spleen tissues after bacterial challenge of guinea pigs immunized with different rAg85 formulations by different routes: PLGA-TDB-AG85 = microparticles containing rAg85B and trehalose dibehenate as adjuvant; PLGA-AG85 = microparticles containing rAg85B; BCG = BCG solution. Microparticles were administered by the pulmonary route and BCG was administered subcutaneously. (average ± std deviation, n=6).

reduction in CFU in both the lungs and spleens compared with untreated controls (Figure 5). Although the CFU data were not statistically different from the BCG and BCG–BCG groups, the histopathological and morphometric analyses indicated the positive effect of BCG-P-rAg85B in terms of differences in area of tissue affected and number and size of granulomas observed in tissues. Therefore, it was suggested that direct pulmonary immunization using these particles enhanced the protection afforded by primary immunization with BCG against TB infection in guinea pigs. Since a large proportion of the population of the world has been immunized with BCG as infants, an aerosol boost would be a promising strategy to enhance and prolong the protective immunity based on BCG-initiated immunity [113].

An alternative prime-boost strategy was proposed by Bivas-Benita et al. [96]. They evaluated the immunogenicity of a DNA vaccine encoding the MTB latency antigen Rv1733c and to evaluate the effect of pulmonary delivery and co-formulation with PLGA–polyethyleneimine (PEI) nanoparticles on host immunity in mice. Characterization of the PLGA–PEI nanoparticles indicated they were positively charged and that their nanometer size was preserved after concentration. The nanoparticles were able to mature human dendritic cells and stimulated them to secrete IL-12 and TNF-α comparable to levels observed after lipopolysaccharide (LPS) stimulation. MTB latency antigen Rv1733c DNA prime combined with Rv1733c protein boost enhanced T cell proliferation and IFN-γ secretion in mice in response to Rv1733c and MTB hypoxic lysate. Rv1733c DNA adsorbed to PLGA–PEI nanoparticles and applied to the lungs increased T cell proliferation and IFN-γ production more potently compared to the same vaccinations given intramuscularly. The strongest immunogenicity was obtained by pulmonary priming with nanoparticles-adsorbed Rv1733c DNA followed by boosting with Rv1733c protein. Their results confirmed that PLGA–PEI nanoparticles are an efficient DNA vaccine delivery system to enhance T cell responses through pulmonary delivery in a DNA prime/protein boost vaccine regimen and the immunogenicity of DNA vaccines can be strongly enhanced in case of pulmonary delivery by formulating the DNA with PLGA–PEI nanoparticles, followed by protein boosting.

In summary, the lungs could be a suitable route of administration for novel dry powder vaccines that may offer not only clinical advantages but also the pharmaceutical advantages of absence of needles in a mass immunization program and absence of a cold chain for delivery globally and also will address the issue of the need to increase efficacy that confers better local mucosal as well as systemic immunity, the need

to demonstrate safety during administration by reducing the risk of contamination by sharps and needles; the need to eliminate powder reconstitution, thus increasing stability during transport, storage, administration; and the need to improve cost effectiveness.

References

1. Martin C (2006) Tuberculosis vaccines: past, present and future. Curr Opin Pulm Med 12: 186-191.
2. Huebner RE (1996) Bacillus of Calmette and Guerin p. 893-904. In W. N. Room and S. Garay (ed.), Tuberculosis. Little, Brown and Co., new York, NY.
3. Sterne JA, Rodrigues LC, Guedes IN (1998) Does the efficacy of BCG decline with time since vaccination? Int J Tuberc Lung Dis 2: 200-207.
4. Horwitz MA, Harth G, Dillon BJ, Maslesa-Galić S (2009) Commonly administered BCG strains including an evolutionarily early strain and evolutionarily late strains of disparate genealogy induce comparable protective immunity against tuberculosis. Vaccine 27: 441-445.
5. Roche PW, Triccas JA, Winter N (1995) BCG vaccination against tuberculosis: past disappointments and future hopes. Trends Microbiol 3: 397-401.
6. McShane H (2009) Vaccine strategies against tuberculosis. Swiss Med Wkly 139: 156-160.
7. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, et al. (1998) Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 393: 537-544.
8. McMurray DN (2003) Recent progress in the development and testing of vaccines against human tuberculosis. Int J Parasitol 33: 547-554.
9. Bivas-Benita M, Ottenhoff TH, Junginger HE, Borchard G (2005) Pulmonary DNA vaccination: concepts, possibilities and perspectives. J Control Release 107: 1-29.
10. Diamond G, Legarda D, Ryan LK (2000) The innate immune response of the respiratory epithelium. Immunol Rev 173: 27-38.
11. Zhang P, Summer WR, Bagby GJ, Nelson S (2000) Innate immunity and pulmonary host defense. Immunol Rev 173: 39-51.
12. Lu D, Hickey AJ (2007) Pulmonary vaccine delivery. Expert Rev Vaccines 6: 213-226.
13. Laube BL (2005) The expanding role of aerosols in systemic drug delivery, gene therapy, and vaccination. Respir Care 50: 1161-1176.
14. Johnson EP, Gross WB (1951) Vaccination against pneumoencephalitis (Newcastle disease) by atomization or nebulization with the B1 virus. Vet Med 46: 55-59.
15. Deuter A, Southee DJ, Mockett AP (1991) Fowlpox virus: pathogenicity and vaccination of day-old chickens via the aerosol route. Res Vet Sci 50: 362-364.
16. Murphy D, Van Alstine WG, Clark LK, Albregts S, Knox K (1993) Aerosol vaccination of pigs against Mycoplasma hyopneumoniae infection. Am J Vet Res 54: 1874-1880.
17. Rosenthal SR, McEnery JT, Raisys N (1968) Aerogenic BCG vaccination against tuberculosis in animal and human subjects. J Asthma Res 5: 309-323.
18. Fernández-de Castro J, Kumate-Rodríguez J, Sepúlveda J, Ramírez-Isunza JM, Valdespino-Gómez JL (1997) [Measles vaccination by the aerosol method in Mexico]. Salud Publica Mex 39: 53-60.
19. Bennett JV, Fernandez de Castro J, Valdespino-Gomez JL, Garcia-Garcia Mde L, Islas-Romero R, et al. (2002) Aerosolized measles and measles-rubella vaccines induce better measles antibody booster responses than injected vaccines: randomized trials in Mexican schoolchildren. Bull World Health Organ 80: 806-812.
20. Cutts FT, Clements CJ, Bennett JV (1997) Alternative routes of measles immunization: a review. Biologicals 25: 323-338.
21. Bellanti JA, Zeligs BJ, Mendez-Inocencio J, García-Garcia ML, Islas-Romero R, et al. (2004) Immunologic studies of specific mucosal and systemic immune responses in Mexican school children after booster aerosol or subcutaneous immunization with measles vaccine. Vaccine 22: 1214-1220.
22. Dilraj A, Cutts FT, de Castro JF, Wheeler JG, Brown D, et al. (2000) Response to different measles vaccine strains given by aerosol and subcutaneous routes to schoolchildren: a randomised trial. Lancet 355: 798-803.
23. Dilraj A, Sukhoo R, Cutts FT, Bennett JV (2007) Aerosol and subcutaneous measles vaccine: measles antibody responses 6 years after re-vaccination. Vaccine 25: 4170-4174.
24. Blank F, Stumbles P, von Garnier C (2011) Opportunities and challenges of the pulmonary route for vaccination. Expert Opin Drug Deliv 8: 547-563.
25. Torrado E, Robinson RT, Cooper AM (2011) Cellular response to mycobacteria: balancing protection and pathology. Trends Immunol 32: 66-72.
26. Gehr P, Geiser M, Im Hof V, Schürch S, Waber U, et al. (1993) Surfactant and inhaled particles in the conducting airways: structural, stereological, and biophysical aspects. Microsc Res Tech 26: 423-436.
27. Palucka K, Banchereau J, Mellman I (2010) Designing vaccines based on biology of human dendritic cell subsets. Immunity 33: 464-478.
28. Steinman RM (1989) Dendritic cells: clinical aspects. Res Immunol 140: 911-918.
29. Miller MJ, Wei SH, Parker I, Cahalan MD (2002) Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. Science 296: 1869-1873.
30. Westermann J, Bode U, Sahle A, Speck U, Karin N, et al. (2005) Naive, effector, and memory T lymphocytes efficiently scan dendritic cells in vivo: contact frequency in T cell zones of secondary lymphoid organs does not depend on LFA-1 expression and facilitates survival of effector T cells. J Immunol 174: 2517-2524.
31. Behar SM, Martin CJ, Nunes-Alves C, Divangahi M, Remold HG (2011) Lipids, apoptosis, and cross-presentation: links in the chain of host defense against Mycobacterium tuberculosis. Microbes Infect 13: 749-756.
32. Deretic V (2011) Autophagy in immunity and cell-autonomous defense against intracellular microbes. Immunol Rev 240: 92-104.
33. Russell DG (2011) Mycobacterium tuberculosis and the intimate discourse of a chronic infection. Immunol Rev 240: 252-268.
34. Winslow GM, Cooper A, Reiley W, Chatterjee M, Woodland DL (2008) Early T-cell responses in tuberculosis immunity. Immunol Rev 225: 284-299.
35. Randall TD (2010) Bronchus-associated lymphoid tissue (BALT) structure and function. Adv Immunol 107: 187-241.
36. Randall TD (2010) Pulmonary dendritic cells: thinking globally, acting locally. J Exp Med 207: 451-454.
37. Lau YF, Wright AR, Subbarao K (2012) The contribution of systemic and pulmonary immune effectors to vaccine-induced protection from H5N1 influenza virus infection. J Virol 86: 5089-5098.
38. Song K, Bolton DL, Wei CJ, Wilson RL, Camp JV, et al. (2010) Genetic immunization in the lung induces potent local and systemic immune responses. Proc Natl Acad Sci U S A 107: 22213-22218.
39. Ruiz FE, Clancy JP, Perricone MA, Bebok Z, Hong JS, et al. (2001) A clinical inflammatory syndrome attributable to aerosolized lipid-DNA administration in cystic fibrosis. Hum Gene Ther 12: 751-761.
40. Vandenberghe LH, Wilson JM (2007) AAV as an immunogen. Curr Gene Ther 7: 325-333.
41. Mingozzi F, High KA (2007) Immune responses to AAV in clinical trials. Curr Gene Ther 7: 316-324.
42. Andersen P (2001) TB vaccines: progress and problems. Trends Immunol 22: 160-168.
43. Tullius, M. V., and M. A. Horwitz. (2011) New generation BCG vaccines, p. 119-169. In C. M. Philip R. Dormitzer, Rino Rappuoli (ed.), Replicating Vaccines: A New Generation. Springer Basel AG, Basel Switzerland.
44. Tullius MV, Harth G, Maslesa-Galic S, Dillon BJ, Horwitz MA (2008) A Replication-Limited Recombinant Mycobacterium bovis BCG vaccine against tuberculosis designed for human immunodeficiency virus-positive persons is safer and more efficacious than BCG. Infect Immun 76: 5200-5214.
45. Grode L, Seiler P, Baumann S, Hess J, Brinkmann V, et al. (2005) Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guérin mutants that secrete listeriolysin. J Clin Invest 115: 2472-2479.
46. Checkley AM, McShane H (2011) Tuberculosis vaccines: progress and challenges. Trends Pharmacol Sci 32: 601-606.
47. Skeiky YA, Sadoff JC (2006) Advances in tuberculosis vaccine strategies. Nat Rev Microbiol 4: 469-476.

48. Langermans JA, Doherty TM, Vervenne RA, van der Laan T, Lyashchenko K, et al. (2005) Protection of macaques against Mycobacterium tuberculosis infection by a subunit vaccine based on a fusion protein of antigen 85B and ESAT-6. Vaccine 23: 2740-2750.

49. Orme IM (2011) Development of new vaccines and drugs for TB: limitations and potential strategic errors. Future Microbiol 6: 161-177.

50. Kolibab K, Yang A, Derrick SC, Waldmann TA, Perera LP, et al. (2010) Highly persistent and effective prime/boost regimens against tuberculosis that use a multivalent modified vaccine virus Ankara-based tuberculosis vaccine with interleukin-15 as a molecular adjuvant. Clin Vaccine Immunol 17: 793-801.

51. Kaufmann SH (2001) How can immunology contribute to the control of tuberculosis? Nat Rev Immunol 1: 20-30.

52. McMurray DN (1994) Guinea pig model of tuberculosis, p. 135-148. In Bloom BR (ed.), Tuberculosis: Pathogenesis, Protection and Control. ASM press, Washington, DC.

53. Orme IM (2003) The mouse as a useful model of tuberculosis. Tuberculosis (Edinb) 83: 112-115.

54. McMurray DN (2003) Hematogenous reseeding of the lung in low-dose, aerosol-infected guinea pigs: unique features of the host-pathogen interface in secondary tubercles. Tuberculosis (Edinb) 83: 131-134.

55. Reed SG, Alderson MR, Dalemans W, Lobet Y, Skeiky YA (2003) Prospects for a better vaccine against tuberculosis. Tuberculosis (Edinb) 83: 213-219.

56. Padilla-Carlin DJ, McMurray DN, Hickey AJ (2008) The guinea pig as a model of infectious diseases. Comp Med 58: 324-340.

57. Dharmadhikari AS, Nardell EA (2008) What animal models teach humans about tuberculosis. Am J Respir Cell Mol Biol 39: 503-508.

58. Gupta UD, Katoch VM (2009) Animal models of tuberculosis for vaccine development. Indian J Med Res 129: 11-18.

59. Brennan MJ, Morris SL, Sizemore CF (2004) Tuberculosis vaccine development: research, regulatory and clinical strategies. Expert Opin Biol Ther 4: 1493-1504.

60. Reed S, Lobet Y (2005) Tuberculosis vaccine development; from mouse to man. Microbes Infect 7: 922-931.

61. Dannenberg AM (1994) Rabbit model of tuberculosis. In B. R. Bloom (ed.), Tuberculosis. Pathogenesis, protection and control. ASM Press, Washington, D.C.

62. Dannenberg AM Jr (2010) Perspectives on clinical and preclinical testing of new tuberculosis vaccines. Clin Microbiol Rev 23: 781-794.

63. Kaufmann SH (2003) Immune response to tuberculosis: experimental animal models. Tuberculosis (Edinb) 83: 107-111.

64. Mattila JT, Diedrich CR, Lin PL, Phuah J, Flynn JL (2011) Simian immunodeficiency virus-induced changes in T cell cytokine responses in cynomolgus macaques with latent Mycobacterium tuberculosis infection are associated with timing of reactivation. J Immunol 186: 3527-3537.

65. Flynn JL, Capuano SV, Croix D, Pawar S, Myers A, et al. (2003) Non-human primates: a model for tuberculosis research. Tuberculosis (Edinb). 83:116-118.

66. Capuano SV 3rd, Croix DA, Pawar S, Zinovik A, Myers A, et al. (2003) Experimental Mycobacterium tuberculosis infection of cynomolgus macaques closely resembles the various manifestations of human M. tuberculosis infection. Infect Immun 71: 5831-5844.

67. Tufariello JM, Chan J, Flynn JL (2003) Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection. Lancet Infect Dis 3: 578-590.

68. Reed SG, Coler RN, Dalemans W, Tan EV, DeLa Cruz EC, et al. (2009) Defined tuberculosis vaccine, Mtb72F/AS02A, evidence of protection in cynomolgus monkeys. Proc Natl Acad Sci U S A 106: 2301-2306.

69. Harboe M, Oettinger T, Wiker HG, Rosenkrands I, Andersen P (1996) Evidence for occurrence of the ESAT-6 protein in Mycobacterium tuberculosis and virulent Mycobacterium bovis and for its absence in Mycobacterium bovis BCG. Infect Immun 64: 16-22.

70. Pollock JM, Andersen P (1997) The potential of the ESAT-6 antigen secreted by virulent mycobacteria for specific diagnosis of tuberculosis. J Infect Dis 175: 1251-1254.

71. Vordermeier HM, Whelan A, Cockle PJ, Farrant L, Palmer N, et al. (2001) Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. Clin Diagn Lab Immunol 8: 571-578.

72. Hewinson RG, Vordermeier HM, Buddle BM (2003) Use of the bovine model of tuberculosis for the development of improved vaccines and diagnostics. Tuberculosis (Edinb) 83: 119-130.

73. McMurray DN (2001) A coordinated strategy for evaluating new vaccines for human and animal tuberculosis. Tuberculosis (Edinb) 81: 141-146.

74. Buddle BM, Skinner MA, Wedlock DN, de Lisle GW, Vordermeier HM, et al. (2005) Cattle as a model for development of vaccines against human tuberculosis. Tuberculosis (Edinb) 85: 19-24.

75. Cryan SA, Sivadas N, Garcia-Contreras L (2007) In vivo animal models for drug delivery across the lung mucosal barrier. Adv Drug Deliv Rev 59: 1133-1151.

76. Hickey AJ (2004) Summary of common approaches to pharmaceutical aerosol administration, p. 385-421. In A. J. Hickey (ed.), Pharmaceutical inhalation Aerosol Technology. Marcel Dekker, Inc., New York, NY.

77. Brown AR, George DW, Matteson DK (1997) Vaccinator device for delivering propellant-driven aerosols of Streptococcus suis bacterin into the respiratory tracts of swine. Vaccine 15: 1165-1173.

78. Dalby RN, Tiano SL, Hickey AJ (2007) Medical devices for the delivery of therapeutic aerosols to the lungs, p. 417-444. In A. J. Hickey (ed.), Inhalation Aerosols: Physical and Biological Basis for Therapy. Informa Health Care USA, Inc., New York, NY.

79. Garcia-Contreras L (2012) Inhaled antibiotics, p. In press. In M. Hindle (ed.), Pharmaceutical Aerosol Drug Delivery in the 21st Century. John Wiley & Sons, Inc., Hobroken, NJ.

80. Schwarz LA, Johnson JL, Black M, Cheng SH, Hogan ME, et al. (1996) Delivery of DNA-cationic liposome complexes by small-particle aerosol. Hum Gene Ther 7: 731-741.

81. Coates AL, Tipples G, Leung K, Gray M, Louca E; WHO Product Development Group for Measles Aerosol Vaccine (2006) How many infective viral particles are necessary for successful mass measles immunization by aerosol? Vaccine 24: 1578-1585.

82. WHO (1985) Varations in the preparation of BCG vaccine, WHO individual monograph. World health Organization, Geneva Switzerland.

83. Rosenthal SR, McEnery JT, Raisys N (1968) Aerogenic BCG vaccination against tuberculosis in animal and human subjects. J Asthma Res 5: 309-323.

84. Lagranderie M, Ravisse P, Marchal G, Gheorghiu M, Balasubramanian V, et al. (1993) BCG-induced protection in guinea pigs vaccinated and challenged via the respiratory route. Tuber Lung Dis 74: 38-46.

85. Hickey AJ, Crowder TM (2007) Next generation of dry powder inhalation delivery systems, p. 445-460. In A. J. Hickey (ed.), Inhalation Aerosols: Physical and biological basis for therapy. Informa Healthcare USA, Inc, New York, NY.

86. Raychaudhuri S, Rock KL (1998) Fully mobilizing host defense: building better vaccines. Nat Biotechnol 16: 1025-1031.

87. Jegerlehner A, Tissot A, Lechner F, Sebbel P, Erdmann I, et al. (2002) A molecular assembly system that renders antigens of choice highly repetitive for induction of protective B cell responses. Vaccine 20: 3104-3112.

88. Krammer F, Schinko T, Messner P, Palmberger D, Ferko B, et al. (2010) Influenza virus-like particles as an antigen-carrier platform for the ESAT-6 epitope of Mycobacterium tuberculosis. J Virol Methods 167: 17-22.

89. Derrick SC, Dao D, Yang A, Kolibab K, Jacobs WR, et al. (2012) Formulation of a mmaA4 Gene Deletion Mutant of Mycobacterium bovis BCG in Cationic Liposomes Significantly Enhances Protection against Tuberculosis. PLoS One 7: e32959.

90. Holten-Andersen L, Doherty TM, Korsholm KS, Andersen P (2004) Combination of the cationinc surfactant Dimethyl Dioctadecyl Ammonium Bromide and Synthetic Mycobacterial Cord Factor as an Efficient Adjuvant for Tuberculosis Subunit Vaccines. Infect Immun 72: 1608-1617.

91. Lindblad EB, Elhay MJ, Silva R, Appelberg R, Andersen P (1997) Adjuvant modulation of immune responses to tuberculosis subunit vaccines. Infect Immun 65: 623-629.

92. Niven RW, Schreier H (1990) Nebulization of liposomes. I. Effects of lipid composition. Pharm Res 7: 1127-1133.

93. Skalko-Basnet N, Pavelic Z, Becirevic-Lacan M (2000) Liposomes containing drug

and cyclodextrin prepared by the one-step spray-drying method. Drug Dev Ind Pharm 26: 1279-1284.

94. Lu D, Hickey AJ (2005) Liposomal dry powders as aerosols for pulmonary delivery of proteins. AAPS PharmSciTech 6: E641-648.

95. Bivas-Benita M, van Meijgaarden KE, Franken KL, Junginger HE, Borchard G, et al. (2004) Pulmonary delivery of chitosan-DNA nanoparticles enhances the immunogenicity of a DNA vaccine encoding HLA-A*0201-restricted T-cell epitopes of Mycobacterium tuberculosis. Vaccine 22: 1609-1615.

96. Bivas-Benita M, Lin MY, Bal SM, van Meijgaarden KE, Franken KL, et al. (2009) Pulmonary delivery of DNA encoding Mycobacterium tuberculosis latency antigen Rv1733c associated to PLGA-PEI nanoparticles enhances T cell responses in a DNA prime/protein boost vaccination regimen in mice. Vaccine 27: 4010-4017.

97. Yu F, Wang J, Dou J, Yang H, He X,et al. (2012) Nanoparticle-based adjuvant for enhanced protective efficacy of DNA vaccine Ag85A-ESAT-6-IL-21 against Mycobacterium tuberculosis infection. Nanomedicine [Epub ahead of print].

98. Vordermeier HM, Coombes AG, Jenkins P, McGee JP, O'Hagan DT, et al. (1995) Synthetic delivery system for tuberculosis vaccines: immunological evaluation of the M. tuberculosis 38 kDa protein entrapped in biodegradable PLG microparticles. Vaccine 13: 1576-1582.

99. Dhiman N, Khuller GK (1998) Protective efficacy of mycobacterial 71-kDa cell wall associated protein using poly (DL-lactide-co-glycolide) microparticles as carrier vehicles. FEMS Immunol Med Microbiol 21: 19-28.

100. Lima KM, Santos SA, Lima VM, Coelho-Castelo AA, Rodrigues JM Jr (2003) Single dose of a vaccine based on DNA encoding mycobacterial hsp65 protein plus TDM-loaded PLGA microspheres protects mice against a virulent strain of Mycobacterium tuberculosis. Gene Therapy 10: 678-685.

101. Mollenkopf HJ, Dietrich G, Fensterle J, Grode L, Diehl KD, et al. (2004) Enhanced protective efficacy of a tuberculosis DNA vaccine by adsorption onto cationic PLG microparticles. Vaccine 22: 2690-2695.

102. Cai H, Hu XD, Yu DH, Li SX, Tian X, et al. (2005) Combined DNA vaccine encapsulated in microspheres enhanced protection efficacy against Mycobacterium tuberculosis infection of mice. Vaccine 23: 4167-4174.

103. Lu D, Garcia-Contreras L, Xu D, Kurtz SL, Liu J, et al. (2007) Poly (lactide-co-glycolide) microspheres in respirable sizes enhance an in vitro T cell response to recombinant Mycobacterium tuberculosis antigen 85B. Pharm Res 24: 1834-1843.

104. Shi S, Hickey AJ. (2010) PLGA microparticles in respirable sizes enhance an in vitro T cell response to recombinant Mycobacterium tuberculosis antigen TB10.4-Ag85B. Pharm Res 27: 350-360.

105. dos Santos SA, Zárate-Bladés CR, de Sá Galetti FC, Brandão IT, Masson AP, et al. (2010) A subunit vaccine based on biodegradable microspheres carrying rHsp65 protein and KLK protects BALB/c mice against tuberculosis infection. Hum Vaccin 6: 1047-1053.

106. Wong YL, Sampson S, Germishuizen WA, Goonesekera S, Caponetti G, et al. (2007) Drying a tuberculosis vaccine without freezing. Proc Natl Acad Sci U S A 104: 2591-2595.

107. Jin TH, Tsao E, Goudsmit J, Dheenadhayalan V, Sadoff J (2010) Stabilizing formulations for inhalable powders of an adenovirus 35-vectored tuberculosis (TB) vaccine (AERAS-402). Vaccine 28: 4369-4375.

108. Hokey DA, Misra A (2011) Aerosol vaccines for tuberculosis: a fine line between protection and pathology. Tuberculosis (Edinb) 91: 82-85.

109. Garcia-Contreras L, Wong YL, Muttil P, Padilla D, Sadoff J, et al. (2008) Immunization by a bacterial aerosol. Proc Natl Acad Sci U S A 105: 4656-4660.

110. Bezdicek P, Crystal RG (1997) Pulmonary macrophages, p. 859-875. In R. G. Crystal, J. B. West, and P. Weibel (ed.), The Lung: Scientific Foundations. Lippincott, Philadelphia, PA.

111. Men Y, Gander B, Merkle HP, Corradin G (1996) Induction of sustained and elevated immune responses to weakly immunogenic synthetic malarial peptides by encapsulation in biodegradable polymer microspheres. Vaccine 14: 1442-1450.

112. Shahin R, Leef M, Eldridge J, Hudson M, Gilley R (1995) Adjuvanticity and protective immunity elicited by Bordetella pertussis antigens encapsulated in poly(DL-lactide-co-glycolide) microspheres. Infect Immun 63: 1195-1200.

113. Lu D, Garcia-Contreras L, Muttil P, Padilla D, Xu D, et al. (2010) Pulmonary immunization using antigen 85-B polymeric microparticles to boost tuberculosis immunity. AAPS J 12: 338-347.

114. Horwitz MA, Harth G (2003) A new vaccine against tuberculosis affords greater survival after challenge than the current vaccine in the guinea pig model of pulmonary tuberculosis. Infect Immun 71: 1672-1679.

115. Sugawara I, Li Z, Sun L, Udagawa T, Taniyama T (2007) Recombinant BCG Tokyo (Ag85A) protects cynomolgus monkeys (Macaca fascicularis) infected with H37Rv Mycobacterium tuberculosis. Tuberculosis (Edinb) 87: 518-525.

116. Sun R, Skeiky YA, Izzo A, Dheenadhayalan V, Imam Z, et al. (2009) Novel recombinant BCG expressing perfringolysin O and the over-expression of key immunodominant antigens; pre-clinical characterization, safety and protection against challenge with Mycobacterium tuberculosis. Vaccine 27: 4412-4423.

117. Jain R, Dey B, Dhar N, Rao V, Singh R, et al. (2008) Enhanced and enduring protection against tuberculosis by recombinant BCG-Ag85C and its association with modulation of cytokine profile in lung. PLoS One 3: e3869.

118. Pym AS, Brodin P, Majlessi L, Brosch R, Demangel C, et al. (2003) Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. Nat Med 9: 533-539.

119. Castañon-Arreola M, López-Vidal Y, Espitia-Pinzón C, Hernández-Pando R (2005) A new vaccine against tuberculosis shows greater protection in a mouse model with progressive pulmonary tuberculosis. Tuberculosis (Edinb) 85: 115-126.

120. Kita Y, Tanaka T, Yoshida S, Ohara N, Kaneda Y (2005) Novel recombinant BCG and DNA-vaccination against tuberculosis in a cynomolgus monkey model. Vaccine 23: 2132-2135.

121. Shi C, Wang X, Zhang H, Xu Z, Li Y, et al. (2007) Immune responses and protective efficacy induced by 85B antigen and early secreted antigenic target-6 kDa antigen fusion protein secreted by recombinant bacille Calmette-Guérin. Acta Biochim Biophys Sin (Shanghai) 39: 290-296.

122. Ryan AA, Wozniak TM, Shklovskaya E, O'Donnell MA, Fazekas de St Groth B, et al. (2007) Improved protection against disseminated tuberculosis by Mycobacterium bovis bacillus Calmette-Guerin secreting murine GM-CSF is associated with expansion and activation of APCs. J Immunol 179: 8418-8424.

123. Young SL, O'Donnell MA, Buchan GS (2002) IL-2-secreting recombinant bacillus Calmette Guerin can overcome a Type 2 immune response and corticosteroid-induced immunosuppression to elicit a Type 1 immune response. Int Immunol 14: 793-800.

124. Tang C, Yamada H, Shibata K, Maeda N, Yoshida S, et al. (2008) Efficacy of recombinant bacille Calmette-Guérin vaccine secreting interleukin-15/antigen 85B fusion protein in providing protection against Mycobacterium tuberculosis. J Infect Dis. 197: 1263-1274.

125. Sun R, Skeiky YA, Izzo A, Dheenadhayalan V, Imam Z, et al. (2009) Novel recombinant BCG expressing perfringolysin O and the over-expression of key immunodominant antigens; pre-clinical characterization, safety and protection against challenge with Mycobacterium tuberculosis. Vaccine 27: 4412-4423.

126. Desel C, Dorhoi A, Bandermann S, Grode L, Eisele B, et al. (2011) Recombinant BCG ΔureC hly+ induces superior protection over parental BCG by stimulating a balanced combination of type 1 and type 17 cytokine responses. J Infect Dis 204: 1573-1584.

127. Ferrer NL, Gomez AB, Neyrolles O, Gicquel B, Martin C (2010) Interactions of attenuated Mycobacterium tuberculosis phoP mutant with human macrophages. PLoS One. 5: e12978.

128. Sambandamurthy VK, Derrick SC, Hsu T, Chen B, Larsen MH (2006) Mycobacterium tuberculosis DeltaRD1 DeltapanCD: a safe and limited replicating mutant strain that protects immunocompetent and immunocompromised mice against experimental tuberculosis. Vaccine 24: 6309-6320.

129. Sampson SL, Mansfield KG, Carville A, Magee DM, Quitugua T, et al. (2011) Extended safety and efficacy studies of a live attenuated double leucine and pantothenate auxotroph of Mycobacterium tuberculosis as a vaccine candidate. Vaccine 29: 4839-4847.

130. Kaufmann SH (2011) Fact and fiction in tuberculosis vaccine research: 10 years later. Lancet Infect Dis 11: 633-640.

131. Aagaard C, Hoang TT, Izzo A, Billeskov R, Troudt J, et al. (2009) Protection and polyfunctional T cells induced by Ag85B-TB10.4/IC31 against Mycobacterium tuberculosis is highly dependent on the antigen dose. PLoS One 4: e5930.

132. Skeiky YA, Alderson MR, Ovendale PJ, Guderian JA, Brandt L, et al. (2004) Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or recombinant protein. J Immunol 172: 7618-7628.

133. Reed SG, Coler RN, Dalemans W, Tan EV, DeLa Cruz EC, et al. (2009) Defined tuberculosis vaccine, Mtb72F/AS02A, evidence of protection in cynomolgus monkeys. Proc Natl Acad Sci U S A 106: 2301-2306.

134. Baldwin SL, Bertholet S, Kahn M, Zharkikh I, Ireton GC, et al. (2009) Intradermal immunization improves protective efficacy of a novel TB vaccine candidate. Vaccine 27: 3063-3071.

135. McShane H, Pathan AA, Sander CR, Keating SM, Gilbert SC, et al. (2004) Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. Nat Med 10: 1240-1244.

136. Radosevic K, Wieland CW, Rodriguez A, Weverling GJ, Mintardjo R, et al. (2007) Protective immune responses to a recombinant adenovirus type 35 tuberculosis vaccine in two mouse strains: CD4 and CD8 T-cell epitope mapping and role of gamma interferon. Infect Immun 75: 4105-4115.

137. Santosuosso M, McCormick S, Zhang X, Zganiacz A, Xing Z (2006) Intranasal boosting with an adenovirus-vectored vaccine markedly enhances protection by parenteral Mycobacterium bovis BCG immunization against pulmonary tuberculosis. Infect Immun 74: 4634-4643.

This article was originally published in a special issue, **Mycobacterial Diseases** handled by Editor(s). Dr. Mario Alberto Flores-Valdez, UUniversidad Nacional Autónoma de México, Mexico; Dr. David A. Hokey, University of Pennsylvania, USA.