CHAPTER 6.3

INHALABLE VACCINES FOR TB

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Vaccine formulation is one of the most important determinants of the success or failure of a vaccine candidate.

‘I saw light in the windows and youth singing and without intention, I was dreaming.’

Monologue
Silvio Rodriguez
INTRODUCTION

Since TB infection is first established in the lungs, it is of interest to generate a protective immune response to the bacillus in the airway and lung mucosa. TB vaccine delivery to the lungs and airways faces an additional challenge in that the largest recipient group comprises neonates and infants. The respiratory system in the first few years of life is very different from the adult version, and requires attention to low-pressure, rapid-rate, nose-only breathing, the composition of mucus and lung surfactant, and a variety of anatomical (1) and physiological considerations (2). However, the widespread use of inhalations as intervention in childhood dyspnea (3) and recent translational efforts in the area of measles (4) and influenza (5) vaccines for pediatric populations indicate that TB vaccination through this route may certainly be feasible (6–7). The first section summarizes formulation approaches and delivery methods for inhaled vaccines. The following sections will address immunological issues with inhaled vaccination as well as the different cell types involved and the impact they have on vaccine-induced immunity.

METHODS OF AIRWAY AND PULMONARY VACCINATION

Formulations

The diversity of forms in which a TB vaccine may be presented precludes the possibility of outlining general principles of formulation. Live, attenuated, or killed whole-cell (or virus) preparations; single or multiple glycosylated or unglycosylated proteins and polypeptides possessing requisite secondary and tertiary structure; and DNA vaccines—all present specific problems that can be addressed with varying degrees of success by formulation approaches. In respect of vaccine delivery through the respiratory tract, however, the overarching concern is obviously that the formulation should be inhalable. Inhalable formulations require materials (in addition to the adjuvant, if any) and additional processing steps. In general, there is
an emerging consensus that vaccines formulated in dry powder form enjoy several benefits, including reproducibility of formulation performance, enhanced storage stability, even at room temperature, and in many cases, enhanced efficacy (7–8). Spray-drying and freeze drying are therefore emerging as methods of choice for preparing inhaled vaccines.

Dosimetry, or more specifically, biodistribution of inhaled dose is another concern in inhaled vaccine delivery. Deciding the dose of an inhaled vaccine might require preclinical studies to establish not only the total amount of antigen (Ag) administered, but also what proportions reach the respiratory mucosa-associated lymphoid tissue (MALT) and not buccal or gastro-intestinal secondary lymphoid organ components. This objective might require sophisticated equipment and facilities (9), or the use of alternative techniques for deep lung delivery, such as the PennCentury Microsprayer (7) to establish responses to accurately measured doses. Specifically in TB vaccination, the primary objective would remain deep lung delivery.

Thus, the goals of successful formulation of inhalable TB vaccines can be listed as follows, in no particular order of priority:

1. Preservation of viability (of live vaccines),
2. Preservation of secondary/tertiary structure,
3. Ability to be dispensed as aerosols, without compromising 1 or 2 above,
4. Ability to deposit preferentially in deep lung rather than nasopharyngeal, oropharyngeal or upper airway mucosa,
5. Facilitation of Ag presentation on HLA class I
6. Facilitation of preferential induction of T helper (Th)-1 response, and
7. Storage stability.

Preservation of viability of spray-dried BCG was elegantly demonstrated by Wong et al. (10), who used leucine as a spray-drying excipient in the preparation of an inhalable vaccine. It was also demonstrated that minimization of osmotic stress to the drying bacteria, by avoiding the use of salts and anti-freeze agents like glycerol in the bacterial suspension to be spray-dried, improves viability and storage stability in the dry state. Viruses, such as influenza (11) and measles (10) remain viable on spray-drying, and appropriate live viral delivery vehicles may be employed for TB vaccines, particularly DNA vaccines. Preservation of higher-order protein/peptide structure during formulation has traditionally been achieved through the use of cryoprotectants such as polyols (12), and in at least one report, through the use of an innocuous ‘sacrificing’ polypeptide in excess of the Ag of interest (13).
Garcia-Contreras et al. (14) also employed leucine as a spray-drying additive for live BCG, which not only imparted good aerosol and flow properties to the preparation, but also enhanced lung deposition of the vaccine formulation. The improvement in flow and aerosolization properties was attributable to small (nanometer-sized) particles of leucine that coated the rod-shaped bacilli, promoting lubricated slippage past each other. Enhancement of pulmonary delivery was on account of retention of the morphology of the bacilli that have evolved to invade their host through the respiratory route.

Preferential presentation of TB vaccine-derived peptides in the context of HLA class I may possibly be achieved by two strategies. First, the use of delivery systems that are made from ‘fusogenic’ material, capable of integrating with the membrane of antigen-presenting cells (APCs) and delivering the contents into the cytosol. Makidon et al. reported nasal sprays of several Ags formulated as nanoemulsions (15) and Ansari et al. recently demonstrated improved efficacy of a candidate TB vaccine incorporated in liposomes prepared using, among other materials, lipids isolated from the cell wall of Archaebacteria (16). More traditional fusogenic liposomes have been well-characterized for applicability to Ag delivery for presentation on MHC class I. Recent developments indicate the possibility of engineering pH-sensitivity and enhanced storage stability in lipid vesicles (17). The feasibility of formulating protein-containing liposomes as dry powders for inhalation has been demonstrated by several workers, including Lu and Hickey (18).

A second approach to cytosolic delivery is suggested by molecules that mediate escape from phagosomes in APCs, such as listeriolysin O (19), which uses transient changes in cytosolic ion concentrations to affect pore formation and phagosome membrane rupture (20).

**Administration of inhaled vaccines**

*Instillation*

Nose drops could be the simplest way to administer vaccine Ags to the respiratory tract. Nasopharyngeal tonsils (adenoids) are important components of the airway mucosa-associated lymphoid tissue (21) and are potentially capable of linking up the process of Ag presentation down to deep lung. Although induction of deep airway allergy following uptake of an allergen from the nasal mucosa is known (22), recent reports of robust immune responses to Ag instilled in the nares of mice further indicate the possibility of using nose drops for vaccination (23–24). Nose drops are fairly simple to formulate, requiring isotonicity and sterility as key
formulation characteristics, and could represent a particularly easy way to achieve immunization.

However, there are reasons to expect that the nature of responses elicited when the Ag is administered to the nasal mucosa alone may differ from those generated on upper airway and pulmonary vaccine delivery. Thus, Minne et al. reported marked gradations of mucosal responses when influenza viral vaccines were delivered to the nares, upper airways, central airways and deep lung of mice. Equally important, they observed that the greater the depth of Ag delivery, the greater was the magnitude of the Th1 component of the response (25). It would thus appear that inhalations might be more efficacious for TB vaccines as well.

As stated earlier, modalities of administration of inhaled TB vaccines differ in respect of children (who breathe exclusively through the nose) and adults (who use both mouth and nose). In the first case, the use of nebulizers may be unavoidable.

**Nebulizers**

The design evolution of nebulizers has addressed many of the limitations of the technique in respect of deep lung delivery, but dose delivery and lung deposition patterns resulting from nebulization are still significantly different from other methods of pulmonary delivery. The fact remains, however, that from the vaccine recipient’s perspective, nebulizers are especially welcome because they are the simplest and easiest to use among pulmonary delivery devices (26). It is convenient to classify nebulizers, according to the technique used for aerosolization of the formulation, into jet nebulizers, ultrasonic and piezoelectric nebulizers, and vibrating mesh nebulizers. Additional refinements have resulted in the development of ‘breath-actuated,’ ‘breath-enhanced’ and ‘adaptive’ aerosol delivery versions.

Jet nebulizers are designed on Bernoulli’s principle of ‘entrainment’ of particles or droplets in an air stream, based typically on a Venturi throat and a jet of compressed air (Figure 6.3.1A). This design uses a source of compressed air to aerosolize a liquid formulation in an ‘atomizer’. The resulting mist of droplets emerges from an orifice interfaced with a face mask to be placed over the nose (and mouth) of the user. Rudimentary versions of jet nebulizers generate a mist that a user has to inhale over periods ranging from a few seconds to a few minutes, depending on the dose to be delivered. Current technologies are able to adjust dose delivery in proportion to the rate of inspiration, and even guide the vaccine recipient and care provider through the process of inhalation for optimization of dose delivery (27).
Electronic nebulizers employ piezoelectric devices to actuate ultrasonic crystals (28) or vibrating perforates (29) to aerosolize formulations (Figure 6.3.1B). This kind of equipment is based on ultrasonic energy to produce high-amplitude oscillations or even cavitation of an inert fluid, which then carries the medicament through an outlet in the form of a mist. The construction is usually robust. Ultrasonic and vibrating mesh nebulizers are portable, and with an adequate supply of disposable patient interface masks, are adaptable for use in the field for administering vaccines.

Figure 6.3.1 Schematic representation of jet (A) and ultrasonic (B) nebulizers.

*Note:* Jet nebulizers utilize a stream of compressed air that entrains droplets of medicament during its passage through a Venturi throat, to emerge as a mist from the outlet. Portable, electronic nebulizers employ piezoelectric activation of ultrasonic crystals to create turbulence in the carrier fluid. Fan and baffle arrangements may also be used to achieve aerosolization. The medicament is usually placed in a separate reservoir, and is entrained in the mist generated through ultrasonic vibrations.

**Inhalers**

Pressurized, metered-dose inhalers (MDI, pMDI) and dry powder inhalers (DPI) possess some advantages in vaccine delivery. Despite the major drawback of being difficult to use, especially for the pediatric populations, they are more efficient at deep-lung delivery, can store the vaccine in a dry state to enhance stability, and can be filled aseptically to maintain sterility. While pMDI require formulation with a propellant, DPI rely solely on the indrawn breath of the recipient to deliver their payload. The aerodynamic behavior of particles to be delivered to the deep lung by inhalers is a crucial factor with regard to the success of deep lung delivery. Thus, particles must acquire sufficient moment of inertia to traverse the upper airways without depositing on the walls, yet retain sufficient density to permit
deposition in the alveoli. Particles that are too large to navigate the oropharynx and upper airways deposit in distal portions of the respiratory tract. Below a certain optimal aerodynamic size, particles are likely to remain suspended in inspired air and be emitted during exhalation. Apart from particle engineering to obtain desired aerodynamic characteristics, inhalers are therefore designed to achieve aerosolization of the formulation. Propellants do this by merely entraining solids as they escape from a release valve, whereas DPI inhalers are designed in a variety of geometries, using baffles, fans, and fluid dynamics to aerosolize the powder.

Figure 6.3.2 illustrates DPI devices used for administering a live attenuated measles virus vaccine to rhesus macaques (4). This study also demonstrated that aerosol immunization through the oropharynx delivered relatively larger amounts of the vaccine in comparison to aerosol delivery through the nose alone. Similar apparatus can be fabricated and validated for drug delivery to mice and guinea pigs by almost any research group (30).

![Figure 6.3.2 DPI devices used for administering a live attenuated measles virus vaccine to rhesus macaques](image)

**Note:** Basic designs of dry powder inhalers are usually dependent solely on the indrawn breath of the user to generate an aerosol, but modifications such as the PuffHaler (A) or Solovent (B) may be used for prior generation of an aerosol, which is confined within a spacer. The user receives the aerosol through a mask or a nose-only adapter.

(Redrawn, with permission, from reference 4).

Finally, a novel technique of pulmonary delivery of vaccine candidates to rodents is offered by the PennCentury series of equipment (7, 31). This equipment is not, per se, an inhalation device, but possesses the undeniable advantage of being able to deliver precisely quantitated doses to the deep lung.
**Examples**

Pulmonary delivery of TB vaccines has been proposed for a long time (7). An early report of immunization of guinea pigs with BCG via the aerosol route demonstrated superiority over intradermal immunization at similar doses (32). Immunization through the airway route was accomplished by insufflations, and $10^5$ CFU administered thus elicited better protective responses than intradermally-administered bacilli.

Bivas-Benita et al have, since 1994, contributed a series of articles demonstrating pulmonary delivery of a variety of TB vaccine candidates. Employing nanoparticles of chitosan (33), a cationic (fusogenic) nano-emulsion (34) and composite particles prepared from poly(lactide-co-glycolide) and poly(ethyleneimine) (35), the authors demonstrated elegant strategies for delivering DNA vaccine candidates. They also found that the DNA vaccine is taken up and expressed in airway epithelial cells, and that the delivery systems are able to induce maturation of dendritic cells (DCs) to express CD80 and CD86 costimulatory molecules. Vaccine delivery to epithelial cells is an interesting observation, since it may be expected to present antigen in the context of MHC class I, a desirable outcome of immunization against TB. The same group recently recommended that an immunization strategy based on priming with a DNA vaccine and boosting with the cognate protein antigen might provide additional benefits (35). Another long-term interest of this group is in employing excipients capable of transducing APC-activating signals through the Toll-like receptor (TLR) family (36–38). It has been demonstrated that appropriately-modified chitosan can engage TLR2 and activate THP-1-derived macrophages. The approach, however, requires caution to ensure that promiscuous signaling through TLRs does not engender hypersensitivity to booster doses.

Bhaskar et al. have explored pulmonary immunization with a heat-killed or live attenuated vaccine strain earlier referred to as *M. w.* (39–40) and now formally named *M. indicuspranii*. In a detailed study of protective efficacy, the authors report significantly higher protective efficacy through the aerosol route of immunization, and equally significant advantages over the classical vaccine strain BCG.

Hickey’s group has investigated defined antigens, Ag85B (41) and TB10.4-Ag85B (42–43). These authors used poly(lactide-co-glycolide) microparticles and incorporated muramyl dipeptide as an adjuvant in the particles used for pulmonary immunization. The response, in terms of antigen presentation by THP-1 cells to a T cell hybridoma *in vitro*, is extremely encouraging.
The candidate vaccine AERAS-402/Crucell Ad35 viral vaccine showed promise upon intramuscular immunization of BCG-primed adults (44), and has been formulated as a dry powder inhalation (45). Further developments are eagerly awaited.

Inhaled vaccines and the induction of an immune response

The immune status of the lung is a delicate balancing act due to its critical function of distributing oxygen throughout the body. The body can, to a point, tolerate a reduction in this capacity. However, there is a limit to this tolerance and, obviously, a cessation of function quickly leads to death. With regards to protection from pathogenic organisms, this is a double-edged sword: infections need to be prevented, if possible, but otherwise quickly controlled and cleared. However, the bystander damage resulting from a potent immune response can be just as damaging as the infection itself. The lung also regularly encounters nonpathogenic organisms and potentially immunogenic particulates from the environment against which a strong cytotoxic immune response would cause unnecessary damage. The understanding of the unique mechanisms employed by the lung to deal with this dichotomy is necessary to design a strategy for harnessing lung immunity with vaccination.

Immune compartmentalization

Unlike traditional vaccinations, aerosol vaccines have the unique ability to target and prime cells in the mucosal immune system rather than the systemic immune system (7,46). The concept of a separate mucosal immune system is not new (47–48). The mucosal immune system, also referred to as mucosa-associated lymphoid tissues (MALT), is highly compartmentalized and is recognized as being separate from “normal” systemic immunity (49–50). Vaccine studies in mice by Hodge et al. demonstrated that intranasal immunization, but not parenteral immunization, elicited antibody responses in the lung and nasal passages as well as cellular infiltration in the lungs. Interestingly, intranasal immunizations were also able to generate antibody responses in the genital tract (51). We are now beginning to understand the molecular basis for the compartmentalization of immune responses. Picker proposed that T cells generated in lung-associated lymph nodes (LALNs) may express homing receptors that direct them back to the lungs (52). Subsequently, studies have demonstrated that APCs, particularly DCs, are responsible for the imprinting of antigen-specific T cells such that they return to the tissue where the
dendritic cell resided. Thus far, gut immunity has been the focus for identifying the molecular basis for immune imprinting. For example, intestinal DCs isolated from mesenteric lymph nodes (MLNs) were shown to upregulate the expression of α4β7 integrin in T cells, regardless of the site from which T cells were isolated (53). Similarly, Mora et al. demonstrated that Peyer’s patch dendritic cells also stimulated expression of this gut-homing integrin (54). Retinoic acid was later identified to play a role in the ability of MLN-derived DCs to promote the upregulation of α4β7 on antigen-specific T cells (55). Together, these studies suggest that there also exists similar imprinting properties in pulmonary DCs. Utilization of the mucosal immune system and, in particular, the imprinting capabilities of lung-resident dendritic cells can be a valuable tool for the elicitation of lung-homing antigen-specific memory T cells. By priming the mucosal immune system, aerosol vaccines can trigger a more rapid, robust, and localized immune response to pathogenic infection in the lungs (56). More importantly, aerosol vaccines can elicit lung-resident memory responses that may be able to respond to infection more rapidly than memory cells in other locations.

**Induction of cellular immunity**

In terms of a TB vaccine, the most important vaccine targets in the lung mucosa are generally resident macrophages and dendritic cells, as these members of the innate immune system are the first line of defense against a TB infection and play the important roles in the presentation of antigens to prime the adaptive immune system (7). However, another cell type that should not be overlooked as a vaccine target is respiratory epithelial cells (ECs). The following sections will address both the cell targets and how they can lead to the induction of an immune responses as well as the potential role of the different facets of the immune response in the generation of lung immunity.

**Antigen presenting cells**

Antigen presenting cells are critical targets for lung immunization, particularly DCs, whether those cells are targeted directly or through designing vaccines specifically to facilitate the delivery of antigens to resident APCs along with the requisite activation signals. APCs are uniquely capable of bridging innate and adaptive immune responses through both secretion of cytokines and chemokines at the site of antigen exposure to recruit innate effectors as well as the processing and presentation of antigens for the stimulation of the adaptive response in tissue-draining lymph nodes (57–60).
Dendritic cells are recognized as the preeminent professional APC, fully capable of acquiring, processing, and presenting antigen with the full capacity to elicit responses from naïve T cells (57, 60). DCs are therefore an attractive target for vaccination in the lungs. Dendritic cells in the lungs share many characteristics with their counterparts in other tissues. DCs in the lung possess an array of receptors which allows them to sample the microenvironment and acquire antigens, including Fc and mannose receptors (61), and have the capacity to acquire many antigenic forms, such as soluble (62–63) and particulate antigen (61). Importantly, DCs serve as sentinels and immune messengers, displaying a broad array of pattern recognition receptors, most notably TLRs, which together serve to instruct the DCs on the appropriate response to stimulate both the innate and adaptive immune systems (64–66). Dendritic cells in the lung reside below a layer of macrophages and epithelial cells. However, they extend projections into the lung lumen such that they are able to sample the antigenic milieu, a process referred to as “snorkeling” (67). Once appropriate signals are received, DCs undergo maturation and traffic to draining lymph nodes where they can stimulate naïve antigen-specific T cells through the interaction of MHC/peptide with the T cell receptor (signal 1), providing costimulation (signal 2), and relaying critical direction as to which way the cells should respond through a combination of cytokines and possibly through other cell surface receptors (signal 3) (58, 65). Ultimately, any vaccination approach in the lung will, by necessity, trigger responses by lung dendritic cells, as they are uniquely capable of stimulating naïve T cells. The pattern recognition receptors, including TLRs, expressed by dendritic cells make them prime targets for elicitation of immune responses. A broad array of microbial vectors as well as adjuvanted particulate and soluble antigens can easily trigger DC maturation. However, this also raises the concern of inflammation. In tissues such as the skin, the ability of DCs to induce very high levels of inflammation can be beneficial in eliminating potential microbial threats. However, the lung represents a unique environment where massive inflammation can not only be damaging, but potentially deadly to the individual. It is clear that other cells, particularly alveolar macrophages, may be responsible for limiting the inflammatory activity of DCs.

**Alveolar macrophages**

In the healthy lung, analysis has shown the composition of cells recovered from bronchoalveolar lavage to be primarily alveolar macrophages (75-80%), the remainder of which is composed of lymphocytes (20%), neutrophils (1%) and eosinophils (< 1%) (68). Because of their number and location, alveolar macrophages (AMs) are the first cells of the immune system to encounter inhaled
particles and pathogens. However, similar to (and in part because of) respiratory ECs, AMs have a noninflammatory phenotype known as alternative activation (alternatively activated AMs, aaAMs). This noninflammatory phenotype is designed to protect the lung from excessive inflammation as well as promote tissue repair (69). These cells can be detected by high levels of expression of arginase (which prevents the generation of nitrogen radicals (70)) and fibronectin (essential during tissue repair (71)). In contrast to the classical IFN-γ-mediated activation pathway, alternative activation is associated with, among others, priming by IL-4 and IL-13 (72). Alternative activation has also been linked to the constitutive expression of TGF-β from bronchial epithelial cells (73). This activation pathway leads to the secretion of IL-10 and IL-1R antagonist, inhibiting the proliferation of T cells and limiting the inflammatory response in neighboring DCs. In addition, in contrast to classically activated macrophages, aaAMs do not produce nitric oxide (NO) and are limited in their ability to undergo autophagy (72). This primes AMs for attack by intracellular pathogens such as MTB, which have evolved to hijack AMs, within which they reside and replicate. Vaccines delivered to the lung will need to take into consideration the modulatory function of AMs, either bypassing or overriding their suppressive activity or utilizing AMs to minimize damage from an otherwise harmful inflammatory response.

**Epithelial cells**

Respiratory epithelial cells line the pulmonary lumen. The lung epithelium, broadly divided into bronchial and alveolar epithelia, is composed of a variety of specialized cell types. Cells of the bronchial epithelium include basal cells, mucous goblet cells, ciliated epithelial cells and Clara cells. The alveolar epithelium is comprised mainly of type I and type II (TI and TII) cells, specializing in gas exchange and surfactant production, respectively.

Apart from their dual roles in acting as a physical barrier between host and environment and supporting oxygen exchange within the lung, many of these cells have innate immune effector functions. Mucous (produced by goblet cells) and ciliated epithelial cells function together to trap foreign particles and organisms and remove them from the lung airways via mucociliary transport, a formidable obstacle in the development of aerosol vaccines. In the lower airways, TII cells produce a variety of phospholipoprotein complexes known as surfactants. Surfactant proteins B and C function primarily to lower the surface tension within the lung, preventing alveolar collapse. However, surfactant proteins A and D have carbohydrate recognition domains that allow them to coat viruses and bacteria, promoting phagocytosis by alveolar macrophages.
Respiratory epithelial cells secrete anti-microbial peptides such as cathelicidins (LL-37 being the only cathelicidin identified in humans) and β-defensins (BDs), both of which are cationic peptides with broad anti-viral and anti-bacterial properties. BD-1 is constitutively expressed (74), while the expression of LL-37, BD-2 and BD-3 can be induced by mechanisms such as ER stress, TNF, IL-1β and TLR engagement (75–78). In addition to targeting microbes for phagocytosis, these peptides can differentially recruit and modulate the functions of monocytes, macrophages, mast cells, neutrophils and T cells (79).

Though epithelial cells are generally not an efficient target for TB, they can be a target of aerosolized vaccines depending on the vaccine vector. In particular, viral vaccine vectors can efficiently target the respiratory epithelium (80–81). Previously, studies have shown that respiratory epithelial cells can play a role in stimulating the innate immune system (82–83). Mechanisms such as mucociliary transport, surfactants and anti-microbial peptides allow the lung to deal with invading microorganisms in a manner that does not require the induction of a proinflammatory adaptive immune response. Epithelial (and other) cells also mediate a noninflammatory environment in the lung by secreting immune mediators to temper the immune response (e.g. TGF-β and IL-10 (73, 84)) or redirect it toward a less proinflammatory (e.g. Th2) response, including TSLP, IL-25 and IL-33 (85–86). These mediators also contribute to the alternative activation state of alveolar macrophages (discussed above).

While the combination or threshold of signals required to overcome this immune inhibition is not entirely defined, the lung is entirely capable of priming and recalling adaptive immune responses. Epithelial cells have the ability to secrete inflammatory mediators such as tumor necrosis factor (TNF), interleukin-1, and a variety of chemokines (87). The importance of recognizing and differentiating infection within the lung is highlighted by the fact that respiratory ECs express transcripts for all 10 human TLRs. Following recognition of pathogens by pattern recognition receptors (PRRs), including TLRs, ECs are capable of secreting proinflammatory cytokines and chemokines including TNF, IL-1β, IL-6, IL-8 and GM-CSF. IL-8, a potent neutrophil chemokine, is induced by various PRRs. Similar to gastrointestinal epithelium, the cellular localization of these receptors differs from that normally seen in APCs. For example, TLR4 and TLR5 are typically found on the cell surface of APCs. In respiratory epithelium, these receptors are found intracellularly and on the basolateral surface, respectively. However, migration to the apical membrane can be induced by RSV infection (TLR4, (88)) or flagella (TLR5, (89)). In contrast, TLR9 typically resides within endosomes, whereas apical surface expression can be seen on primary respiratory ECs (90). Together the data show that the lung epithelial cells are uniquely specialized to detect and differentiate
pathogens. Furthermore, the secreted cytokines in response to infections, and by design vaccinations, have a significant impact on resulting immunity.

**Vaccine-induced lung immunity**

Although the requisite components for TB immunity remain a mystery, for years the general dogma of TB vaccine research has pointed to T cells as critical components of the anti-mycobacterial immune response. Indeed, research has suggested a potent role for both CD4+ and CD8+ T cells (91–92). Operating under this dogmatic assumption, however, has led to a near complete disinterest of the role of B cells in mediating TB immunity. Several studies have reversed this trend by suggesting that B cells may play a role in granuloma formation (93–94), and there is renewed interest in how both T and B cells may mediate TB immunity. Furthermore, one of the most prominent researchers in the TB vaccine field, Stefan Kaufmann, has previously postulated that the “dream scenario” for TB immunity would be the establishment of TB-neutralizing antibodies in the lungs (95).

**T cell immunity**

T cells are known to play an important role in controlling TB. Perhaps the greatest evidence for this in human populations is with HIV/TB coinfection. It is well-known that HIV infection greatly increases the risk of TB reactivation and infection. Prevalence of TB is directly correlated with the loss of CD4+ T cells in HIV-infected patients (96). Very recent studies describe the development of an animal model to mimic HIV/TB coinfection. In this model, cynomolgus macaques latently infected with TB are challenged with the highly pathogenic SIVmac251. TB reactivation in this model was shown to be correlated with peripheral T cell loss (97). In addition to CD4+ T cells, CD8+ T cells also play a critical role in the control of TB. Chen et al. demonstrated that depletion of CD8+ T cells in BCG-vaccinated macaques resulted in the loss of control of TB infection (98). Together the data clearly indicate that T cells are required for controlling infection and suggest that T cell immunity may also play an important role in preventing disease. Therefore, aerosol vaccines should be designed to elicit T cell responses, preferably in both the CD4+ and CD8+ compartments. However, T cell responses can be very diverse functionally and the exact type of T cell response required is less clear.

Advances in the understanding of T cell immunity have greatly expanded over the past several years and it is clear that T cell function is very complex. The ever expanding T cell functional phenotypes include (but are not limited to) Th0, Th1, Th2, Th3, Th9, Th17, Th22, Tfh, and Treg (99–102). Each of these subsets can be
further divided by the pattern of cytokines secreted which in turn can be affected by the activation state of the T cell (i.e. effector, effector memory, central memory, transitional memory, etc), adding to its complexity. Currently, correlates of protection have not been defined for TB. However, there are some clues that will help in the development of an aerosol vaccine for TB. Th1 responses appear to be very important for controlling infection, particularly IFN-γ and TNF (reviewed by Redford et al. (103)). However, it is also clear that other facets of the immune response play a role in TB immunity. For example, IL-17 appears to play an important role in Th1 infection as well as vaccine-mediated protection in animal models (104–105), in particular the formation of granulomas (105). Regulatory T cells are also key players in lung immunity to TB as they secrete IL-10 and moderate inflammation to protect delicate lung tissues (103). Understanding the different T cell subsets, the cytokines they produce, and the positive and negative regulators of immunity in the lung and how to elicit the perfect protective balance will be vital in the development of an effective aerosol vaccine for TB.

**Mucosal-associated invariant T cells**

The immune system has clearly evolved to be as efficient as possible in the defense against microbes. As mentioned above, pattern recognition receptors, such as Toll-like receptors, recognize conserved microbial motifs and result in immune activation. In a similar fashion, a population of T cells has been identified that appears to help protect against mycobacteria. Mucosal-associated invariant T (MAIT) cells have been identified in both mice and humans and express a conserved T cell receptor α chain. MAIT cells are not restricted by classical HLA molecules, but rather are reactive to antigen presented by the highly conserved MHC class I-related MR1 molecule. Importantly, MAIT cells have been shown to be reactive to mycobacterial antigens and are recruited to the site of mycobacterial infection in humans (106). It may prove useful for an aerosol vaccine to stimulate and expand populations of lung-homing MAIT cells.

**Humoral immunity**

For researchers seeking to prime a B cell mediated immune response, aerosol vaccines are a powerful tool. Resident B cells are present throughout the lung (107), and could be readily accessed via aerosol. Though pulmonary B cells can be activated via Th2 CD4+ T cells, they can also be activated in a T cell independent manner, particularly by bacterial carbohydrates (108). This may prove to be an important consideration when designing an aerosol vaccine for TB.
Priming B cell immunity in the lungs can have a number of advantages. First, as mentioned previously, priming a mucosal immune response can initiate a more rapid response in the lungs than priming a systemic immune response via traditional intradermal vaccination. Theoretically, by using an aerosol vaccine, the anti-TB primed B cells would already be resident in the lungs, meaning that neutralizing antibodies could be present in sufficient quantities to prevent a pathogenic infection. Secondly, resident B cells in the pulmonary mucosa have the ability to generate both IgG and IgA serotypes of antibodies (107, 109). Generally, B cells in the systemic immune system primarily generate the IgG serotype, and generation of IgA (particularly, secretory IgA) antibodies has been shown as a key factor in immunity against infections of the mucosal tissues (110). Whether or not the IgA serotype is significant for TB immunity is still unknown, but the ability to generate a potent IgA response may be a unique advantage provided by aerosol vaccines.

One disadvantage of vaccines and B cells is the generation of anti-vaccine antibodies (111), and this too is the case for aerosol vaccines. Though vaccine researchers hope to generate neutralizing antibodies to TB, they have to be cautious about the production of antibodies to the vaccine vector. The presence of vaccine-neutralizing antibodies could mean that no anti-TB immune response is primed, invalidating the usage of that vaccine.

Another disadvantage of resident B cells in the pulmonary mucosa is the immunopathology created by antibody induction in the lungs. At least two studies (51, 112) have shown that resident B cells in the lung can cause an IgE response similar to an allergic reaction, which leads to inflammation and tissue damage. In combination with the factors described above, the resident B cells in the lungs can be important effector cells for an aerosol vaccine, but also represent a significant challenge for aerosol vaccine safety.

**CHALLENGES AND OPPORTUNITIES**

There are clear challenges associated with lung immunization, the most pressing of which is the risk of inducing high levels of inflammation that lead to immunopathology. However, the regulatory environment in the lung may help to limit that threat. Despite the risks, the advantages of mucosal vaccination are substantial. The ability to elicit potentially protective lung-resident immunity where mycobacterial infection starts may significantly tip the scale in favor of the host. The results of vaccine studies in animal models have been promising. Ballester et al. recently published data demonstrating enhanced protective efficacy against
TB using mucosally-delivered vaccine adjuvanted with a TLR9 agonist, CpG. Interestingly, this immunization strategy enhanced the polyfunctionality of Th1 cells and stimulated production of IL-17, providing some clues to what types of immune responses may be protective (113). More research is required to evaluate different strategies for eliciting specific patterns of immune responses, such as through the use of Toll-like receptors, particularly in the setting of lung vaccination. However, these data suggest aerosol vaccines may prove valuable in the fight against TB.

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