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The next generation: tuberculosis vaccines that elicit protective CD8⁺ T cells

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Summary

Tuberculosis continues to cause considerable human morbidity and mortality across the world, particularly in people coinfecting with HIV. The emergence of multidrug resistance makes the medical treatment of tuberculosis even more difficult. Thus, the development of a tuberculosis vaccine is a global health priority. Here we review the data concerning the role of CD8⁺ T cells in immunity to tuberculosis and consider how CD8⁺ T cells can be elicited by vaccination. Many immunization strategies have the potential to elicit CD8⁺ T cells, and we critically review the data supporting a role for vaccine-induced CD8⁺ T cells in protective immunity. The synergy between CD4⁺ and CD8⁺ T cells suggests that a vaccine which elicits both T cell subsets has the best chance at preventing tuberculosis.

Keywords

Mycobacterium tuberculosis; BCG; vaccine; bacterial infection; CD4; CD8; T cell; animal models; microbial immunity

1. Introduction

In the 21st century, tuberculosis remains a plague in many parts of globe. In addition to the 8–10 million cases of tuberculosis that occur each year, nearly a third of the world's population is latently infected, which represents an enormous potential reservoir of disease. The development of multidrug resistant (MDR)¹ and extensive drug resistant (XDR) strains

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¹Abbreviations: Ad, adenovirus; APC, antigen presenting cells; β 2m, β 2 microglobulin; BCG, Bacille Calmette Guérin; DC, dendritic cell; ER, endoplasmic reticulum; HIV, human immunodeficiency virus; IFN γ , interferon- γ ; IV, intravenous; mAb, monoclonal antibody; MDR, multidrug resistant; MHC, major histocompatibility; TAP, transporter associated with antigen processing; TLR, Toll-like receptor; VV, vaccinia virus; XDR, extensive drug resistant;

of *Mycobacterium tuberculosis* make the task of treating tuberculosis even more daunting and highlight the need for strategies to prevent transmission, such as prophylactic vaccination of susceptible individuals. The only vaccine available against *M. tuberculosis* is the attenuated strain of *M. bovis* known as Bacille Calmette Guérin (BCG), which is used in many parts of the world. Unfortunately, data suggest that while BCG vaccination can reduce the incidence of severe infantile forms of tuberculosis such as meningoencephalitis and disseminated tuberculosis, BCG appears ineffective at preventing the most common adult form of tuberculosis, pulmonary tuberculosis. Therefore, there is wide spread consensus that a new vaccine against tuberculosis is needed – a vaccine that would be effective against pulmonary tuberculosis – the form of disease that is most contagious and represents the greatest threat to public health.

Abundant experimental and clinical data supports a critical role for T cells in immunity to *M. tuberculosis*. Consequently, eliciting immune T cells is proposed to be an essential property of any vaccine that is capable of providing protection against tuberculosis. Designing the best vaccine for clinical trials requires an understanding of the T cell subsets that contribute to protective immunity against tuberculosis. T cells come in a variety of flavors, each with unique properties and specialized functions. For example, CD4⁺ T cells recognize peptide antigens derived from proteins that enter the cell's endocytic system and are processed and presented by the class II MHC pathway. In contrast, CD8⁺ T cells recognize peptide antigens, derived from cytosolic proteins, such as those generated in virally infected cell, which are presented by the class I MHC pathway. In addition to MHC-restricted CD4⁺ and CD8⁺ T cells, other T cell subsets exist that are restricted by nonclassical MHC molecules. CD1-restricted T cells are one such well-characterized subset of T cells. CD1 is a family of non-polymorphic antigen presenting molecules that present lipid and glycolipid antigens to T cells. Group 1 CD1 (CD1a, CD1b, and CD1c), can present mycobacterial lipid antigens to human T cells, which are often CD4⁺ and CD8⁺ (so called "double negative" T cells). In contrast, group 2 CD1 (CD1d) is the restricting element for NKT cells, which are almost always CD4⁺ or CD4⁺8⁺. In designing a vaccine to prevent tuberculosis, it is important to consider which T cell subsets are desirable to activate. In this review, we will discuss the evidence that class I MHC-restricted CD8⁺ T cells are important in immunity to *M. tuberculosis*. An overview of vaccine strategies designed to elicit *M. tuberculosis*-specific CD8⁺ T cells will be presented. Finally, we will critically examine the how these strategies perform with respect to eliciting CD8⁺ T cells that recognize mycobacterial antigens and mediate protective immunity.

2. CD8⁺ T cells mediate host defense against *M. tuberculosis*

Although extensive experimental data supports the contention that T cells play a critical role in mediating immunity to *M. tuberculosis*, the data that support an essential role for T cells in people is not as abundant. One exception concerns the role of CD4⁺ T cells. Here, abundant clinical data supports the observation that people infected with human immunodeficiency virus 1 (HIV-1) have a greater risk of developing both primary and reactivation tuberculosis. Since one of the central immunological defects following HIV infection is a loss of CD4⁺ T cells and their function, the association is commonly interpreted as evidence that CD4⁺ T cells mediate immunity to tuberculosis in people. In contrast, clinical data supporting a role for CD8⁺ T cells is not available; however, it is clear from a variety of experimental systems that CD8⁺ T cells are an important component of protective immunity to *M. tuberculosis*.

The transfer of cells or serum from immune donors into naïve recipients can passively transfer immunity. When done experimentally, such adoptive transfer experiments were classically used to distinguish between cell-mediated versus humoral immunity. For

immunity to tuberculosis, Lefford found that splenocytes but not serum from BCG vaccinated mice could protect irradiated recipient mice against BCG or *M. tuberculosis* [1]. The capacity of splenocytes from BCG vaccinated mice to transfer protection was significantly impaired if T cells were depleted from the splenocytes prior to transfer. As better T cell purification techniques became available, Orme and his colleagues confirmed that T cells from BCG vaccinated mice could provide protection to T cell deficient recipient mice challenged with virulent *M. tuberculosis* [2]. Orme also determined the differential ability of CD4⁺ and CD8⁺ T cells to provide protection to mice challenged with *M. tuberculosis* [3]. Splenic CD4⁺ and CD8⁺ T cells from mice infected intravenously (IV) with *M. tuberculosis* were transferred into uninfected irradiated mice, which were then challenged with 10⁵ CFU of *M. tuberculosis* by the IV route and protection was assessed 10 days later by determining the bacterial burden in the lungs. Both CD4⁺ and CD8⁺ splenic T cells mediated protection; however, CD4⁺ T cells provided greater protection and acquired the capacity to transfer protection earlier than CD8⁺ T cells. These kinetics may reflect a dependence of CD8⁺ T cell function on CD4⁺ T cell helper function and reflect a requirement for CD4⁺ T cell licensing of CD8⁺ T cell effector function. Using the same model, Orme showed that memory T cells also provide protection to naïve irradiated mice challenged with *M. tuberculosis*; however, in the case of memory T cells, only CD4⁺ and not CD8⁺ T cells were effective [4]. Similarly designed studies have shown that T cells mediate protection against challenge with *M. bovis* BCG [5,6]. Britton's lab established an adoptive transfer model using RAG-1 ^{-/-} mice, which lack B and T cells, as immunodeficient hosts to study T cell mediated protection against mycobacterial infection. They find that protection against aerosolized *M. bovis* BCG can be mediated by the adoptive transfer of both CD4⁺ and CD8⁺ T cells. In all of these studies, CD8⁺ T cells provide less protection than an equivalent number of CD4⁺ T cells [6]. Nevertheless, these adoptive transfer experiments show that CD8⁺ T cells, even in the absence of CD4⁺ T cells, can mediate protection against tuberculosis.

Administration of monoclonal antibodies (mAb) to CD4 and CD8 is used to deplete T cells subsets in mice, and this experimental approach has been used to determine whether CD4⁺ and/or CD8⁺ T cells are required for immunity to tuberculosis. Antibody mediated depletion has a more robust effect in thymectomized mice, since they lack the capacity to regenerate new CD4⁺ and CD8⁺ T cells. Muller et al treated thymectomized C57BL/6 mice with monoclonal antibodies (mAb) specific for CD4 or CD8 and then infected the mice with *M. tuberculosis* by the IV route. Depletion of either CD4⁺ or CD8⁺ T cells impaired control of bacterial replication in the spleen when measured 3 wks after infection [7]. Pedrazzini et al examined the effect of depleting CD4⁺ or CD8⁺ T cells on the growth of *M. bovis* BCG in mice. The growth of BCG in the spleen and lung was enhanced in anti-CD4 mAb treated mice, reaching a maximum by day 45 (30-fold increase), but subsequently returned to normal levels by day 100 post-infection. In contrast, treatment with anti-CD8 had no effect on bacterial levels [5]. Similar experiments were done by Cox et al using BCG to infect both resistant (C57BL/6) and susceptible (CBA/Ca) mouse strains. Anti-CD4 treatment resulted in a 0.5–0.6 log increase in splenic CFU in both mouse strains. The effect of anti-CD8 mAb treatment was strain dependent: CD8⁺ T cell depletion had no effect in CBA/Ca mice, while there was a modest exacerbation (0.3–0.4 log) in C57BL/6 mice [8]. Overall, these results suggest that CD8⁺ T cells have a role in mediating immunity to mycobacterial infection, and this effect is more important in resistance to virulent bacterial strains.

The most convincing data that CD8⁺ T cells are required for host immunity after *M. tuberculosis* infection comes from experiments using “knockout” mouse strains. Knockout mouse strains that lack protein expression of β 2 microglobulin (β 2m), transporter associated with antigen presentation-1 (TAP-1), and the class I MHC heavy chains (K^bD^b), all have impaired class I MHC antigen presentation, either because they lack class I MHC structural

proteins ($\beta 2m$ and K^bD^b) or because they lack proteins (TAP-1) that transport peptides from the cytosol into the ER. Since expression of class I MHC/peptide complexes in the thymus is required for positive T cell selection during T cell ontogeny, all three of these knockout mouse strains lack conventional MHC-restricted $CD8^+$ T cells. Following infection with virulent *M. tuberculosis* by the IV route, all three of these knockout mouse strains are more susceptible than appropriate genetically intact control mice [9–12]. Although caveats exist concerning the interpretation of these experiments (discussed in reference [13]), these data support the hypothesis that $CD8^+$ T cells are required for optimum immunity to tuberculosis.

Many of the studies described above that demonstrate a role for $CD8^+$ T cells in the control of *M. tuberculosis* infection were performed in mice infected with *M. tuberculosis* by the IV route. Robert North addressed the relative importance of $CD4^+$ and $CD8^+$ T cells following infection by the aerosol route [14]. After aerosol infection with virulent *M. tuberculosis*, class II MHC knockout mice (which lack conventional $CD4^+$ T cells) are much more susceptible than $\beta 2m$ knockout mice (which lack conventional $CD8^+$ T cells), as measured by survival. Similarly, C57BL/6 mice treated with anti- $CD4$ mAb succumbed earlier than mice treated with anti- $CD8$ mAb. Nevertheless, mice without $CD8^+$ T cells were always more susceptible than immunocompetent “wild-type” control mice. Mice treated with the combination of anti- $CD4$ plus anti- $CD8$ mAb were more susceptible than mice treated with anti- $CD4$ alone, suggesting that $CD8^+$ T cells have a unique non-redundant role.

Most studies that have compared the contribution of $CD8^+$ and $CD4^+$ T cells to anti-mycobacterial immunity have found that the protective effect of $CD8^+$ T cells is less than that described for $CD4^+$ T cells. This comparison, however, is an oversimplification. First, the mouse may not accurately model all stages of the natural history of *M. tuberculosis* infection in people. For example, latency, which is arguably one of the most important but least understood phases of infection, is not easily studied in animal models. Using a modified version of the Cornell latency model, in which small numbers of bacteria persist despite antibiotic therapy, van Pinxteren et al showed that although $CD4^+$ T cells were critical during acute infection, $CD8^+$ T cells were of greater importance during latency [15]. Thus, the effector functions of different T cell subsets may be particularly well suited to controlling bacterial replication at different phases of disease.

A second caveat is that certain features of an optimum $CD8^+$ T cell response are dependent on $CD4^+$ T cells for licensing or “help”, and mice lacking $CD4^+$ T cells have suboptimal $CD8^+$ T cell effector function and memory responses [16]. These principles are derived from a variety of model systems but are likely to be important for adaptive immunity following *M. tuberculosis* infection. For example, the cytotoxic activity of CTL elicited following *M. tuberculosis* infection is defective in $CD4$ knockout mice, perhaps because of a need for IL-2 and IL-15 [17]. Such findings are taking on even more importance since we now appreciate that antigen-specific $CD8^+$ T cells elicited following infection have cytotoxic activity in vivo [18,19]. Through the use of vaccination studies, Hill’s group has shown that an protection correlates with an optimum $CD8^+$ T cell response to Ag85A, which is critically dependant upon $CD4^+$ T cell help [20]. Thus, hosts lacking class II MHC-restricted $CD4^+$ T cells may not be able to mount an optimal $CD8^+$ T cell response to *M. tuberculosis*, and consequently studies that deplete $CD4^+$ T cells, whether genetically or by treatment with mAb, are likely to overestimate the contribution of $CD4^+$ T cells relative to $CD8^+$ T cells. After reviewing the literature, we believe that immunological synergy exists between the $CD4^+$ and $CD8^+$ T cell response to *M. tuberculosis* and that the best chance for inducing protective immunity is to elicit antigen-specific T cells from both T cell subsets.

3. Eliciting CD8⁺ T cells and measuring protection

Bacteria and extracellular particulate antigens are internalized by antigen presenting cells (APC) via phagocytosis, receptor-mediated endocytosis or macropinocytosis. An array of diverse hydrolytic enzymes resides in the endocytic compartment and can catabolize even intact bacteria. Peptides generated by proteolysis can bind class II MHC and the resulting complex is transported to the cell surface for recognition by CD4⁺ T cells. The preferential entry of exogenous antigens into the class II MHC pathway makes the job of eliciting CD4⁺ T cells by vaccination relatively straightforward. Both inactivated pathogens (e.g., influenza A) and purified components (e.g., tetanus toxoid), can stimulate antigen-specific CD4⁺ T cells. The development of newer adjuvants approved for human use promises to make this process even more effective. In contrast, inducing antigen-specific CD8⁺ T cells is more complicated. Antigens that are recognized by CD8⁺ T cells are usually derived from cytosolic proteins, such as viral proteins that are found in the cytosol during virus assembly. Cytosolic antigens are cleaved by the proteasome, a large enzymatic structure which normally functions to catabolize proteins that the cell has targeted for degradation [21]. The resulting peptides are transported into the endoplasmic reticulum (ER) by transport proteins including TAP-1 and tapasin. Once in the ER, peptides compete for binding to “empty” class I MHC and β_2m heterodimer [22]. After a trimeric complex is formed, it is transported to the cell surface where it can be recognized by CD8⁺ T cells. Therefore, the trick to inducing antigen-specific CD8⁺ T cells is to target the antigen of interest to the cytosol of an APC.

Several innovative methods have been developed to target proteins to the cytosol for the purpose of eliciting CD8⁺ T cell responses. These approaches fall into three main categories. One strategy is to alter physicochemical properties of the APC using techniques such as osmotic shock, which can be used in vitro to deliver extracellular proteins to the cytosol. A second approach takes advantage of defined physiological properties of APC. For example, if the minimal epitope has been defined for a particular antigen, the corresponding synthetic peptide can be cultured with the APC in vitro. If the peptide binds to class I MHC with high affinity, it can bind directly to cell surface class I MHC proteins, bypassing the requirement for cytosolic processing. DC are the APC of choice, since they uniquely prime naïve cells when injected back into the host. Cells also have a propensity to take up DNA under certain conditions, and this has led to the concept of DNA vaccination. DNA encoding the antigen of interest is administered by the parenteral route and several delivery methods have been developed including intramuscular or intradermal injection or epidermal delivery via a gene gun. The DNA is taken up by local cells and gene expression leads to antigen production, some of which is acquired by local DC, which can migrate to the regional LN and induce an immune response. DNA vaccination is tremendously versatile and the gene encoding the antigen can be engineered to target the protein to either the class I or class II MHC pathway by changing the signal peptide. In addition, adjuvant-like signals can be provided by co-vaccination with plasmids encoding cytokines or using immunostimulatory DNA sequences that activate toll-like receptors (TLR). The third, and perhaps most successful vaccine approach, is to hijack the properties of pathogens that naturally target or enter the cell's cytosol. The CD8⁺ T cell response is an important component of adaptive immunity to most viruses and many intracellular bacteria. Attenuated versions of several viruses and bacteria have been developed as successful vaccines. For example, *M. bovis* BCG, an attenuated form of *M. bovis* that is related to *M. tuberculosis*, is the current vaccine for tuberculosis and elicits antigen-specific CD8⁺ T cells in both experimentally infected animals [23] and in BCG vaccinated people [24]. Similarly, vaccinia is a pox virus that successfully protects people against smallpox infection. Using recombinant DNA methodology, both of these vectors can be engineered to express antigens of interest for the purpose of eliciting a strong immune response.

The “prime-boost” strategy is increasingly being considered in the design of vaccines. An attenuated pathogen, such as BCG, can be used to “prime” an immune response, which is then boosted by a second immunization using a distinct vaccine that shares only a subset of antigens with the first vaccine, such as adenovirus or vaccinia virus expressing recombinant mycobacterial proteins. Boosting repeatedly with the same vaccine can lead to a suboptimal T cell response because neutralizing antibodies ultimately limit the functional availability of the administered antigens to restimulate T cells. In contrast, by using a different vector for the boost, a primary immune response is generated to the “vector”, but a secondary (and hence more effective) response to the recombinantly expressed protein antigens. Although this approach shares some of the disadvantages of subunit vaccines such as the problems inherent in vaccinating an immunogenetically heterogeneous population with a single antigen, it is nevertheless a powerful way to elicit a robust adaptive immune response to defined antigens.

One strategy would be to prime the immune system with one vaccine, such as a DNA vaccine, and boost with a second vaccine, such as a recombinant virus. In this case both the DNA vaccine and the recombinant virus express the same mycobacterial antigen. In practice, this strategy would be most important for eliciting a potent immune to *M. tuberculosis* antigens not present in BCG. However, most of the world’s population, particularly in tuberculosis endemic regions, has been vaccinated with BCG. The protective effect of BCG against childhood disseminated TB ensures its continued use over the foreseeable future. In the BCG vaccinated population, the role of any novel vaccine would be to boost an immune system previously primed with BCG. Thus, many of the new vaccines against tuberculosis are designed around such a BCG prime-boost strategy.

Ultimately, vaccination is designed to protect the host against infectious disease. Because tuberculosis is a chronic infection, it is not trivial to determine the protective efficacy of vaccines. For animal studies, vaccinated subjects are challenged with *M. tuberculosis* and then analyzed to determine whether or not protective immunity has been induced. Although there is considerable variability in how protection is determined, there is an ongoing effort to standardize these studies [25–27]. First, challenge with *M. tuberculosis* is generally not performed until at least 4 weeks after the last immunization because of concern that non-specific activation by the vaccine, particularly of the innate immune system, may lead to an appearance of protection. Although many different routes of infection are used for animal models of tuberculosis, the IV and aerosol routes are the most commonly used. The aerosol route is increasingly preferred because of the use of low numbers of bacteria (e.g., 100–300 CFU/mouse) and similarity to the route of infection in people. It is imperative that virulent bacteria are used in all of these studies. Growth of less virulent bacteria is more easily inhibited by the innate immune system, whereas greater synergy between innate and adaptive immune pathways is required for the control of more virulent bacteria. How one determines whether vaccinated subjects are protected by the vaccine can be divided into “microbial” and “host” endpoints. Most commonly, the bacterial burden is measured, generally 4 weeks after infection which is near the peak of the adaptive immune response, as well as a later time point, usually 2–4 months after infection, which is during the plateau or chronic phase of infection. When possible, the bacterial burden in both the lung and the spleen should be measured. This is particularly important following aerosol infection because the immune mechanisms that limit bacterial growth in the lung may be different from how the immune system prevents systemic dissemination and controls the infection in other tissues. Many vaccines appear to be more effective at controlling systemic infection than pulmonary infection. Unfortunately, short-term control of the bacterial burden does not always correlate with long-term survival [28]. In part, this is because the pulmonary pathology caused by *M. tuberculosis* arises not only from direct toxic effects of the bacteria, but also as a consequence of immune-mediated tissue injury. Survival studies are an

important way to measure the long-term benefit of vaccination [29,30], and integrate both the beneficial effect of improved bacterial control and whether improved microbial control is at the cost of greater immune-mediated inflammation that paradoxically results in more tissue damage. In practice BCG has been the standard for comparison, regardless of the measure of protection used. This both reflects the desire to match, if not improve upon, the current *M. tuberculosis* vaccine, as well as allow for the comparison of different vaccine strategies.

4. Do CD8⁺ T cells mediate vaccine-induced immunity?

To address whether eliciting mycobacterial antigen-specific CD8⁺ T cells should be a goal of vaccine development for tuberculosis, we will review studies that use different vaccination strategies to induce *M. tuberculosis*-specific CD8⁺ T cell responses. One difficulty in interpreting the literature is that many of these vaccines elicit both CD4⁺ and CD8⁺ T cell responses, and it is difficult to distinguish between the effect of CD4⁺ and CD8⁺ T cells in the standard *M. tuberculosis* challenge models. Conversely, because few mycobacterial epitopes that are recognized by CD8⁺ T cells have been defined, it is difficult to know whether the failure of vaccines designed to elicit CD8⁺ T cells to induce protective immunity is due to a failure to elicit antigen-specific CD8⁺ T cells, or because the CD8⁺ T cells elicited by vaccination do not recognize *M. tuberculosis* infected cells, or whether the *M. tuberculosis*-specific CD8⁺ T cells lack effector functions required to protect mice from tuberculosis.

What is acceptable evidence that CD8⁺ T cells elicited by vaccination protect mice against tuberculosis? Just as a variety of techniques have been used to show the role of CD8⁺ T cells in immunity to tuberculosis following infection, similar approaches have been used to prove that CD8⁺ T cells elicited by vaccination protect mice against tuberculosis in a challenge model. One way is to transfer CD8⁺ T cells from vaccinated mice to naïve recipients to determine whether the elicited CD8⁺ T cells provide passive immunity against challenge with virulent *M. tuberculosis*. Investigators have depleted CD4⁺ and CD8⁺ T cells in vivo after vaccination to show the role of different T cell subsets in resistance to tuberculosis. Sophisticated strategies include the use of vaccines that preferentially elicit CD8⁺ T cell responses in mice that lack CD4⁺ T cells, with the assumption that any protective effect can be ascribed to CD8⁺ T cells. As we will discuss, although these studies have a great deal of promise, there exist many caveats and the results need to be carefully interpreted.

DC vaccination

Dendritic cells (DC) are the ultimate APC [31]. More than any other APC, DC efficiently prime naïve T cells and this is their most important in vivo function. Their capacity to activate antigen-specific T cells and initiate the adaptive immune response is based on four important properties. First, they constitutively sample the extracellular milieu by macropinocytosis [32]. Second, they efficiently present antigen, in part because they do not rapidly degrade foreign proteins, which prolongs the half life of antigens and increases the probability that antigenic peptides will be presented to T cells [33]. Third, an array of costimulatory molecules is expressed by DC, particularly following their activation and maturation, which enhances their ability to activate T cells [34]. Finally, unlike macrophages, DC have the capacity to recirculate. Immature DC are found in all major tissue beds, and when they encounter pathogens or other danger signals, they undergo activation and maturation, which allows them to migrate to the regional LN. In the LN, DC can interact with naïve T cells until ones are encountered that are specific for the antigens presented by the DC. Instead of delivering protein and adjuvant into the skin and expecting that the vaccine is picked up by DC, a more efficient T cell vaccination strategy is to add specific antigens to DC in vitro, and then inject the DC into the host. The expectation is that

the DC will traffic back to the LN and trigger an adaptive immune response to the specific antigens it carries.

In principle, DC vaccination can protect mice from virulent tuberculosis. Administration of BCG-infected DC induces anti-mycobacterial immunity and provides some protection to mice challenged with aerosolized *M. tuberculosis* [35]. The power of DC vaccination is based on its ability to focus the immune response on a select group of protective antigens. Conversely, one can screen candidate proteins using DC vaccination in order to identify ones that are protective. Loading the DC with antigen can be as simple as culturing the cells with the intact protein antigen. For the purposes of generating CD8⁺ T cell responses, this strategy has the added benefit since the cells can be subjected to osmotic shock to load the protein into the cytosol, or if the minimal epitopes have been defined, synthetic peptides can be used to exogenously load the cell surface class I MHC. Another way to load antigens is to infect DC with a virus expressing a mycobacterial protein. This approach has been used to identify mycobacterial antigens recognized by human CD8⁺ T cells. Lewinsohn et al infected human DC with recombinant adenovirus expressing the protein Mtb39 [36]. CD8⁺ T cells from individuals with latent tuberculosis recognized autologous DC infected with rAd.Mtb39, demonstrating that Mtb39-specific CD8⁺ T cells are elicited in people after *M. tuberculosis* infection. Vaccinating with DC infected with recombinant adenovirus could be even more efficient than vaccinating with the virus alone, since in vivo, many non-APC would be infected, which would be unable to directly prime T cells. This strategy has been tested by Malowany et al who infected DC with recombinant adenovirus expressing the mycobacterial protein Antigen 85A (AdAg85A) [37]. Vaccination of mice with AdAg85A-infected DC induced a persistent CD4⁺ and CD8⁺ T cell response to Ag85A, which was clearly superior to the T cell response induced by DC pulsed either with Ag85A protein or with the synthetic peptides corresponding to the class I and class II MHC-restricted Ag85A epitopes. The Ag85A-specific T cells produced interferon- γ (IFN γ) and had cytotoxic activity in vivo. Interestingly, the T cell response following vaccination was dominated by CD8⁺ T cells. This type of vaccination induced protection that was comparable to BCG and clearly better than DC pulsed with Ag85A peptides or protein. In a similar study, DC that were retrovirally-transduced with Ag85A were used to vaccinate mice [38]. Vaccination of mice with transduced DC induced Ag85A-specific CD4⁺ and CD8⁺ T cells. The frequency of antigen-specific T cells was not determined so the relative efficiency of retrovirally-transduced DC can not be compared to the use of adenovirus-infected DC. Of note, protection induced by Ag85A transduced DC under these conditions was modest, with ~2-fold reduction in the pulmonary CFU 4 weeks after IV infection with H37Rv. The approach of immunizing with DC infected with replicative-defective recombinant viruses appears to be a promising way to elicit antigen-specific CD4⁺ and CD8⁺ T cells.

While the use of DC infected with recombinant viruses is efficient at activating CD4⁺ and CD8⁺ T cells, these studies have not addressed whether the CD8⁺ T cells generated by these methods are protective. Adenovirus-infected DC could be used to answer this question, since antigen-specific CD8⁺ T cells are efficiently stimulated. Another way to address the relative importance of CD4⁺ and CD8⁺ T cells following DC vaccination is to use synthetic peptides corresponding to well-characterized epitopes from Ag85A recognized by CD4⁺ or CD8⁺ T cells. McShane et al used Ag85A peptide-pulsed DC to vaccinate mice and found that DC pulsed with synthetic peptides recognized by both CD4⁺ and CD8⁺ T cells induce protective immunity against *M. tuberculosis* comparable to BCG [20]. In contrast, DC pulsed separately with either the class I or class II MHC-restricted peptide could not induce protection. Apparently, both peptides need to be presented by the same DC to induce optimum immunity since mixing DC that had been separately pulsed with each peptide did not protect mice from challenge with *M. tuberculosis*. Only DC pulsed with both peptides simultaneously were capable of inducing protection. Under these conditions, the frequency

of Ag85A-specific CD8⁺ T cells was higher in mice vaccinated with DC pulsed with both peptides compared to mice vaccinated with a mixture of DC pulsed with each peptide separately. This may reflect a dependence of the CD8⁺ T cell response on CD4⁺ T helper cells and provides evidence for the critical role played by conventional class I MHC-restricted CD8⁺ T cells.

Lastly, it is important to remember that tuberculosis is a disease that in large part is a consequence of immunopathology. Enhancement of the immune response always has the potential to worsen the outcome of infection. Intranasal vaccination of mice with DC pulsed with Ag85A protein led to priming of antigen-specific CD4⁺ T cells but was not associated with a reduction in CFU upon *M. tuberculosis* challenge [39]. However, mice that received Ag85A/DC, had a dramatic increase in the recruitment of CD4⁺ and CD8⁺ T cell recruitment into the lung and greater tissue pathology compared to control mice that were vaccinated with ovalbumin pulsed DC. The increase in tissue pathology without any evidence of improved bacterial control, suggests that this strategy led to an over-exuberant immune response that may have been detrimental for the host. Thus, an ideal vaccine would reduce the bacterial burden and diminish the tissue inflammation.

Intracellular bacteria

Bacteria, especially those that are intracellular pathogens, elicit potent CD8⁺ T cell responses and can be used in the development of vaccines that generate both CD4⁺ and CD8⁺ T cell responses. As detailed above, both *M. tuberculosis* and *M. bovis* BCG elicit CD4⁺ and CD8⁺ T cell responses [40]. In fact, given the extensive clinical experience with BCG, it is being used as a antigen-delivery system for the development of other vaccines. For example, HIV and hepatitis C proteins are being expressed in BCG and other mycobacterial strains [41]. Vaccination of experimental animals with these recombinant BCG strains elicits antigen-specific CD8⁺ T cell responses [42].

To improve the ability of BCG vaccination to prevent tuberculosis, recombinant BCG is being developed that over-expresses immunodominant antigens that have the potential to elicit both CD4⁺ and CD8⁺ T cell responses. Thus, BCG over-expressing Ag85B has been shown to be superior to conventional BCG at protecting guinea pigs from virulent tuberculosis [43,44]. In addition to over-expressing selected mycobacterial antigens, BCG vaccine strains have been engineered to co-express certain host genes, primarily cytokines such as GM-CSF, which may provide an adjuvant or immunostimulatory effect [45]. One of the principal advantages of vaccines based on mycobacteria is that they are more antigenically “diverse” compared to subunit vaccines. From an immunological perspective, a *M. tuberculosis* based vaccine should have a greater chance of eliciting a protective immune response in a genetically diverse population than a subunit vaccine, which may have only a limited number of epitopes, presented only by a restricted number of HLA alleles. One hypothesis to explain why BCG vaccination fails to prevent pulmonary tuberculosis is that its loss of certain genes during attenuation included key targets of protective immunity. Consequently, recombinant BCG strains have been developed that overexpress the immunodominant antigen ESAT-6, which is expressed by *M. tuberculosis* but not by BCG [46]. Pym et al have shown that ESAT6-expressing BCG is superior to BCG alone at inducing protection [46]. BCG has been engineered in other ways to improve its ability to elicit protective immunity. For example, Stefan Kaufmann’s group has created a recombinant BCG strain that should be better able to prime CD8⁺ T cells. Listeriolysin is a protein normally expressed by *Listeria monocytogenes*, which functions to help the bacteria translocate from the phagosome into the cytosol. Grode et al engineered urease C⁻ BCG to express listeriolysin. Deleting urease C from BCG prevents alkalization of the phagosome, leading to a more optimum pH for listeriolysin activity [47]. Vaccination with this engineered recombinant BCG strain led to improved protection against virulent *M.*

tuberculosis as measured by lung CFU and survival. Macrophages infected with the listeriolysin-expressing BCG also underwent more apoptosis compared to macrophages infected with conventional BCG, which could also lead to greater cross-priming of CD8⁺ T cells [48,49]. These findings highlight the possibility that pro-apoptotic vaccines may generate a better CD8⁺ T cell response. It should be noted, however, that it has not yet been addressed whether the increased protection afforded by the engineered BCG is due to an increased CD8⁺ T cell response, it's proposed mechanism of action [50,51].

The desire to have a vaccine strain that closely resembles the pathogen *M. tuberculosis* has led to the development of attenuated vaccine strains of *M. tuberculosis* that have well-defined mutations. Laboratory selected mutants are generally the consequence of single gene defects that are amenable to precise characterization. A important class of these mutants are auxotrophs of *M. tuberculosis*. Auxotrophic strains are unable to grow under conventional conditions unless media is supplemented with certain nutrients. Thus, a leucine auxotroph requires growth media that is supplemented with leucine. Auxotrophs of *M. tuberculosis* and other well-defined mutants such as the *phoP* deletion, potentially have even a better safety profile than BCG, which may be advantageous for use in HIV-infected individuals; yet, except for the defined defects, they express a full complement of antigens comparable to pathogenic *M. tuberculosis* [52–55]. Nevertheless, safety concerns over using attenuated *M. tuberculosis* in people, particularly in those that may be immunocompromised because of HIV infection, has led investigators to develop strains with a least two independent mutations. One potential problem with attenuated *M. tuberculosis* is their susceptibility to growth restriction by the innate immune mechanisms may lead to an impaired induction of adaptive immunity. Although attenuated *M. tuberculosis* strains exist that are capable of inducing protection, the relationship between bacterial persistence, induction of memory immunity, and protection, have not been clearly delineated in these examples [52,53,56]. Furthermore, pre-existing immunity to environmental mycobacteria may limit the effectiveness of attenuated *M. tuberculosis* strains.

Other non-pathogenic mycobacterial strains have also been considered as vaccines such as the nontuberculous bacteria *M. vaccae* and *M. smegmatis* [41,57]. All of these various strains have the potential to elicit both CD4⁺ and CD8⁺ T cell responses. However, whether bacterial strains that are unable to persist long-term are capable of eliciting long term protective immunity remains to be determined [58]. If this is true, the less virulent, and hence safer, vaccine strains, may not elicit truly long-lived immunity. Other bacteria, including *Listeria monocytogenes* and *Salmonella* species, have also been developed as delivery systems that elicit CD8⁺ T cell responses. Although these bacteria elicit potent CD8⁺ T cell responses, they are also human pathogens and thus they require engineering so they are safe for human administration.

Viral Vectors

Viruses are intracellular pathogens that assemble new daughter virions in the cytoplasm of infected cells. Viral proteins enter the class I MHC pathway leading to the presentation of viral antigens to CD8⁺ T cells, which are an important component of immunity to most viruses. Certain attenuated viruses can be engineered to express mycobacterial proteins and vaccination with these recombinant viruses stimulates CD8⁺ T cells. Two viruses with these properties that are being developed as vaccines are adenovirus and vaccinia.

Recombinant Adenovirus

Many microbial pathogens invade the host at mucosal sites and mucosal vaccination may be more effective at defending against these infectious diseases. Although it is debatable whether *M. tuberculosis* is a true mucosal pathogen, much of the respiratory system,

particularly the upper airway, is a mucosal site. This has led to interest in whether mucosal based vaccines would provide superior protection against tuberculosis. One encouraging approach has been the use of recombinant adenovirus. Adenovirus is a human pathogen that causes mild upper respiratory infections and infects respiratory epithelial cells. Replication defective adenovirus has been engineered as a vehicle for gene transfer and has also been investigated as a vector for vaccination against a variety of infectious diseases including AIDS and malaria. Zhou Xing has tested the feasibility of developing an adenovirus-based vaccine for tuberculosis. A secreted form of Antigen 85A was cloned into a replication-deficient serotype 5 human adenovirus (AdAg85A) [59]. Intranasal vaccination of mice with AdAg85A activated both Ag85A-specific CD4⁺ and CD8⁺ T cells, which accumulated in the lung, and protected mice in a *M. tuberculosis* respiratory challenge model. Treatment of vaccinated mice with anti-CD4 or anti-CD8 impaired protection only slightly, whereas treatment with both antibodies significantly impaired vaccine-induced protection. The performance of AdAg85 is extremely encouraging, both alone and following priming by a DNA vaccine. Follow-up studies have investigated the mechanism of protection in more detail. Santosuosso et al showed that compared to IM administration, AdAg85A specifically induced Ag85A-specific CD4⁺ and CD8⁺ T cells in the airway, and these T cells were cytolytic and produced IFN γ upon activation [60]. Furthermore, these airway lumen derived T cells were capable of protecting naïve recipient mice against respiratory tuberculosis. Based on the potent antigen-specific CD8⁺ T cell response elicited by this vaccination strategy, this approach may provide further insight into the relative contribution of CD4⁺ vs. CD8⁺ T cells in vaccine-induced protection against tuberculosis.

Pre-existing immunity to all vaccine vectors is a real consideration that may lead to the blunting of effective vaccine-induced protection via premature clearance of the vaccine or a skewed memory response towards vector antigens only. Indeed pre-existing immunity to environmental mycobacteria is a prevailing hypothesis to explain the failure of BCG in certain geographical regions [61]. Since adenovirus serotype 5 is a common viral infection among people, recombinant adenovirus serotype 5-based vaccines are particularly vulnerable to failure because of pre-existing immunity. The development of rAd systems that use relatively rare adenovirus serotypes [62], or replace rAd5 coat proteins with those from rare Ad serotypes [63] are strategies currently being pursued to circumvent anti-vector immunity. Additionally, phase II trials using rAd5-based HIV vaccines will provide additional information about the extent to which pre-existing immunity negatively affects the efficacy of these vaccines.

Recombinant Vaccinia Virus

Vaccinia virus is an enveloped virus of the poxvirus family. It has a linear double-stranded DNA and is unique in its cytoplasmic site of replication. Through its successful use in the worldwide eradication of smallpox, vaccinia virus has proven itself as an important and effective vaccine. Although protective immunity to vaccinia virus is incompletely understood, infection with vaccinia induces virus-specific CD4⁺ and CD8⁺ T cells [64,65], which have been shown to mediate protective effects against lethal infection, in the absence of protective antibodies [66,67]. Recombinant vaccinia virus (rVV) has been appreciated as a highly valuable expression and cloning vector for mammalian cells for nearly twenty-five years. Almost immediately following the advent of rVV, development of rVV-based vaccines began. The ability of rVV to generate effective heterologous antigen-specific CD8⁺ T cell responses following immunization focused much of the early work on the development of anti-viral vaccines for influenza, hepatitis B, and herpes simplex 1 and 2 viruses [68–70]. Indeed, in several cases the protective effect of rVV has been specifically associated with pathogen-specific CD4⁺ or CD8⁺ T cells [71–73].

Vaccinia virus has many advantageous characteristics as a vaccine. It is highly immunogenic with a broad host cell tropism. The vaccinia genome does not integrate itself into the host genome. Recombinant technologies allow precise insertion, via homologous recombination, into its large (~190 kbp) viral genome large that can accommodate large inserts. In contrast to other large genome viruses, vaccinia does not appear to disrupt class I or class II MHC processing and presentation.

Although, vaccinia virus has a strong efficacious history, safety concerns of this live vaccine have lead to the development of attenuated vaccinia strains. Modified vaccinia virus Ankara (MVA) was developed by greater than 570 passages in chicken embryo fibroblasts as a safer smallpox vaccine. MVA has lost the promiscuous tropism of wild-type vaccinia virus, and is unable to grow in human cells, as well as most other mammalian cells, in culture (Reviewed in [74,75]). A safer vaccinia virus vector is of special importance considering the high incidence of *M. tuberculosis* in HIV-infected individuals, and an early study of MVA safety in HIV-patients is encouraging [76].

Although much of the initial vaccine development using rVV was focused on viral pathogens, rVV expressing *M. tuberculosis* antigens have been studied since 1990 [77]. Administration of rVV expressing secreted Mtb 19kD or 38kD glycoproteins protected mice against *M. tuberculosis* challenge although the immunological basis for protection was not elucidated [78]. In 2000, Malin et al characterized the *M. tuberculosis*-specific cellular immune response following vaccination with rVV expressing an *M. tuberculosis* antigen [79]. Splenocytes isolated from mice immunized with rVV expressing Ag85A or Ag85B secreted IL-2 and IFN γ in recall responses to the Ag85 complex, suggesting the stimulation of *M. tuberculosis*-specific T cells. The first evidence that *M. tuberculosis*-specific CD8⁺ T cells could be elicited by vaccinia immunization was shown with rVV expressing the *M. tuberculosis* early-secreted antigen MPT64 (rVV-MPT64) [80]. Feng et al reported the generation of CTL specific for a MPT64-derived peptide vaccination of mice with rVV-MPT64 [80]. Interestingly, the CTL generation was similar between rVV-MPT64 (the Western Reserve vaccinia strain) and attenuated MVA-MPT64 (MVA expressing MPT64). Furthermore, a prime-boost strategy in which mice were first immunized with a DNA vaccine encoding MPT64 and then boosted with rVV-MPT64 or MVA-MPT64 further increased the frequency of MPT64-specific CD8⁺ T cells. Although it was never determined whether vaccination with rVV-MPT64 or MVA-MPT64 could provide protection in an *M. tuberculosis* challenge model, a similar DNA-rVV prime-boost strategy enhanced the protective efficacy of MVA expressing ESAT-6 [71]. However, in this study, *M. tuberculosis*-specific CD8⁺ T cells could not be detected and the vaccine-induced protection was presumably mediated by the measurable *M. tuberculosis*-specific CD4⁺ T cell response.

The use of prime-boost strategies to enhance the efficacy of rVV may be of potential value in improving *M. tuberculosis* vaccine design. Indeed, priming animals with BCG and then boosting with MVA expressing Ag85A (MVA-Ag85A) enhances survival and decreases bacterial burden after aerosol *M. tuberculosis* challenge compared to BCG alone [81,82]. MVA-Ag85A boosting of mice previously vaccinated with high doses of BCG enhanced CD4⁺ and CD8⁺ T cells responses to Ag85A peptides. However, when a sub-optimal BCG dose was used, MVA-Ag85A administration led to a boost in *M. tuberculosis*-specific CD4⁺ T cells but not of the CD8⁺ T cells. Because the boost did lead to an increase in protection, this data argues that Ag85A-specific CD4⁺ T cells were responsible for protective effect. Regardless of the cellular basis for the protection mediated by MVA expressing *M. tuberculosis* antigens, its success, particularly in cooperation with BCG, has propelled MVA into clinical trials. Phase I trials show a good safety profile. People vaccinated with MVA-Ag85A generated IFN γ -secreting CD4⁺ T cells specific for Ag85; however, no Ag85A-

specific CD8⁺ T cells responses were detected. Ag85-specific T cells were increased in subjects pre-exposed to BCG or environmental mycobacterium [83,84].

DNA vaccination

Since DNA vaccination can effectively induce cellular immunity, especially CD8⁺ CTL and Th1 response, genetic immunization is a promising approach for developing vaccines against intracellular pathogens including *M. tuberculosis*. In the past few years, several plasmid DNAs encoding an immunodominant antigens from *M. tuberculosis* have been reported to induce protective immunity in animal models. Huygen et al showed that DNA vaccines based on Ag85A reduced bacterial burdens in organs after aerosol or IV *M. tuberculosis* challenge by establishing a cellular immune response characterized by antigen-specific IFN γ production and CD8⁺ CTL activity [85]. Vaccination with plasmid DNA encoding the 38 kDa protein of *M. tuberculosis* also elicited antigen-specific CD8⁺ T cell responses [86]. Similarly, immunization of mice with DNA encoding Mtb72 fusion protein elicits a strong CD4⁺ and CD8⁺ T cell response and this vaccine prolongs the survival of guinea pigs following aerosol challenge with *M. tuberculosis* [87]. Additionally, DNA vaccines have a therapeutic effect in mice infected with *M. tuberculosis*. When *M. tuberculosis* infected BALB/c mice were given four doses of plasmid DNA encoding HSP65 of *M. leprae*, the number of viable bacteria in the spleen and lungs was dramatically reduced up to 5 months later [88]. The therapeutic effect was associated with CD8⁺ lung cell activation and restored production of IFN γ induced by HSP65 DNA [89]. However, it has been noted that HSP60/lep DNA vaccines induce severe pulmonary necrosis in an immunotherapeutic model [90,91]. These findings raise a safety issue with regard to DNA vaccines. Furthermore, in contrast to the encouraging results using mice, the clinical experience with DNA vaccines has been disappointing [92,93] and new methods are needed to enhance the immunogenicity of DNA vaccines.

DNA vaccines have a number of potential advantages compared to more conventional vaccines such as peptide or attenuated virus. DNA vaccines are easily prepared and stable. Plasmid DNA does not induce vector immunity and therefore can be repeatedly administered without side effects. Furthermore, DNA is able to be maintained in cells for long-term expression of the encoded antigen. Thus, maintenance of immunologic memory is possible. However, the level of protection induced by DNA vaccines has varied for different antigens and generally is less than that induced by BCG immunization. Therefore, strategies to increasing the effectiveness of DNA vaccines are needed. Prime-boost immunization consisting of plasmid DNA and BCG have been developed in order to increase the immune responses, particularly the CD8⁺ T-cell responses. Priming with Ag85B-expressing DNA and boosting with BCG was more effective than BCG immunization in protecting B6 mice against aerosol *M. tuberculosis* challenge [94]. The enhanced protection observed following prime-boost was diminished after CD8⁺ T cell depletion, suggesting that the augmentation of host resistance was partially mediated by CD8⁺ T cells [94]. Several factors may contribute to the improved protection. First, priming with DNA vaccines can focus the immune response toward the dominant mycobacterial antigen, and away from potentially harmful or non-protective antigens. Second, DNA vaccine priming is capable of increasing antigen-specific CD4⁺ and CD8⁺ T cell responses and may be more potent than mycobacteria at priming naïve CD8⁺ T cells. Third, BCG immunization may effectively amplify mycobacterium-specific Th1 and CD8⁺ T responses primed by DNA immunization. Prime-boost strategies can increase protection against *M. tuberculosis* either by priming with DNA or by boosting with DNA vaccines. This may have practical implications since Derrick et al reported that waning BCG-induced anti-tuberculosis protective immunity could be boosted in aging mice by vaccinating with a DNA vaccine expressing an ESAT6-Antigen 85B fusion protein [95]. Interestingly, the number of CD8⁺ cells secreting IFN γ post

challenge with *M. tuberculosis*, was substantially elevated indicating that BCG vaccination induced memory CD8⁺ T cells that could be expanded by the DNA boost and ultimately activated by the infection [96].

Other approaches have been investigated to increase the effectiveness of DNA vaccination against tuberculosis including the codelivery of plasmid expressing cytokines, such IL-12 [97] or IL-2 [98], or coimmunization with DNA vectors encoding multiple antigens [99]. Alternatively, multiple CpG motifs can be added to the DNA backbone to increase its adjuvant effects. Furthermore, the development of new DNA vaccines that incorporate the Sindbis virus RNA replicase increase the immunogenicity of conventional DNA vaccines and allow lower doses of DNA to be administered [100]. For example, Derrick et al found that a Sindbis virus-based DNA vaccine encoding the *M. tuberculosis* Ag85B was able to provide protection comparable to that of BCG based both on a reduction in CFU and a prolongation in survival [29]. Based on data showing that apoptotic vesicles prime CD8⁺ T cells, there is interest in developing pro-apoptotic vaccines. DNA vaccines encoding fusion proteins in which the antigen is fused to a pro-apoptotic protein such as CD95 may increase their effectiveness [101]. Finally, coadministration of DNA and proteins or live vectors in prime-boost strategies have been used to augment the protective response to *M. tuberculosis* infection [102]. A combination of these approaches may be necessary to obtain clinically significant long-lived protective efficacy for DNA vaccines in humans.

HIV⁺ individuals co-infected with *M. tuberculosis* are very frequently associated with a drop in CD4⁺ T cell counts. The development of a new vaccine against *M. tuberculosis* for use in HIV⁺ persons has been considered unlikely because of the presumed essential roles that CD4⁺ T cells play in controlling *M. tuberculosis* infections. Derrick et al demonstrated that immunization with a DNA vaccine cocktail protected CD4^{-/-} mice against an aerosol infection with *M. tuberculosis* [103], suggesting that it may be possible to develop effective *M. tuberculosis* vaccines in HIV-infected populations. By contrast, DNA vaccines encoding mycobacterial antigens Ag85A, Ag85B and PstS3 from *M. tuberculosis* were ineffective in mice lacking CD4⁺ T cells [104]. Although the specific reasons for these disparate results are unclear, the use of different mouse strains, *M. tuberculosis* antigens, the mode and timing of infectious challenge may have contributed to the differing outcomes. In the latter study, reconstitution of CD4⁺ T cell compartment in CD4^{-/-} mice restored DNA vaccine-mediated anti-mycobacterial immune responses and protection [104]. CD4⁺ T cell counts are known to increase under effective and highly active anti-retroviral therapy in HIV-infected patients. Taken together, the findings provide evidence to support that DNA vaccination is a promising direction for the treatment of HIV and *M. tuberculosis* co-infected patients.

Finally, an interesting hybrid approach uses attenuated intracellular bacteria as a delivery system for DNA vaccines. Dietrich et al cloned Ag85A, Ag85B or MPB51 into a plasmid that was stably carried by the *L. monocytogenes* Δ2 mutant [105]. Following infection of APC, this mutant escapes from the phagosome into the cytosol, and in the cytosol it undergoes self-destruction but releases its plasmids. This approach to DNA vaccination appears to be able to induce protective immunity against *M. tuberculosis* [106]. Additional work including a detailed immunological analysis will be required to determine whether this ability to target plasmid delivery makes immunization more efficient.

Subunit vaccines

The most effective and practical way to elicit a CD8⁺ T cell response, whether experimentally or clinically, is to vaccinate with a viral vector or with plasmid DNA (for example see [107]). However, Skeiky et al have evaluated the vaccine 72F. The 72F vaccine consists of the Mtb39 protein inserted into the middle of the Mtb32 protein. Vaccination

with plasmid DNA encoding 72F elicits a robust CTL response against Mtb32_{93–102} and generates protective immunity in both mice and guinea pigs [87]. Interestingly, when the vaccine is formulated as a polyprotein vaccine, it elicits a strong CTL response against Mtb32_{93–102}, an epitope that elicits CD8⁺ T cells after DNA vaccination. A critical variable appears to be the adjuvant – and the adjuvant AS01B seems to be particularly effective at stimulating a CD8⁺ T cell response. Although it is not yet clear whether the elicited CD8⁺ T cells contribute to the protective effect induced by the DNA vaccine or the adjuvanted polyprotein, it raises the possibility that protein subunit vaccines coupled with appropriate adjuvants can stimulate CD8⁺ T cells in addition to CD4⁺ T cells.

Expert Commentary

An important issue in designing tuberculosis vaccines is to effectively incorporate advances in immunology. This is particularly relevant for vaccines designed to elicit CD8⁺ T cells because the target antigens are still being defined. Furthermore, the way CD8⁺ T cells are elicited may affect both their antigen specificity and function. During the past five years, the progress in defining mycobacterial antigens recognized by human and murine CD8⁺ T cells has led to the identification of several vaccine candidates that can now be tested for their ability to protect experimental animals against virulent *M. tuberculosis* (reviewed in [13]). Despite the availability of other animal models, the mouse is still the most facile model to determine induction of adaptive immunity and protection as measures of vaccine efficacy. Establishing whether a particular antigen elicits protective immunity is a task that is subject to various pitfalls. A critical issue is whether vaccination elicits an adaptive immune response. For antigens recognized by CD4⁺ T cells, this can be measured post-vaccination by stimulating the subject's leukocytes with the immunizing antigen and assaying the T cell recall response either by a cytokine assay (e.g., IFN γ production) or by measuring T cell proliferation. If pure recombinant protein is not available, crude mixtures of antigen such as *M. tuberculosis* sonicate or PPD are often sufficient, as long as the vaccinating antigen is represented in the crude antigen preparation. In contrast, this strategy will not work for CD8⁺ T cells as it is necessary to get the antigen into the cytosol of the APC. For investigators committed to the study of a single antigen, the cDNA encoding the antigen can be used to transfect tumor cell lines to create artificial APC expressing the antigen. However, this limits the choice of APC. A more versatile approach uses defined peptides. As more mycobacterial epitopes recognized by CD8⁺ T cells are defined, investigators will no longer be forced to empirically determine whether vaccine-induced CD8⁺ T cells are protective.

Although defining the precise epitopes recognized by CD8⁺ T cells is important, particularly during the early stages of vaccine development, it is important to recognize that most T cell epitopes specifically bind to a single MHC molecule. One of the strengths of the mouse model, the availability of genetically defined inbred strains, is also an important limitation for vaccine studies. Most tuberculosis vaccines are tested in one or two commonly used mouse strains. The majority of studies use C57BL/6 mice and this practice significantly reduces the potential immunogenetic diversity of the host. This is because C57BL/6 mice express a single class II MHC molecule (I-A^b) and two class I MHC molecules (K^b and D^b). Humans have three distinct class I MHC molecules (HLA-A, -B, -C) and three distinct class II MHC molecules (HLA-DP, -DQ, -DR), and since most people have two different alleles that encode these antigen-presenting molecules, each individual potentially expresses 12 distinct MHC proteins. Thus, the probability that a peptide derived from a mycobacterial protein will bind to a MHC molecule is greater for human APC than for murine APC. This effect is demonstrated by CD8⁺ T cell recognition of CFP10. Neither C57BL/6 nor BALB/c mice generate a CD8⁺ T cell response to CFP10; in contrast, nearly 30% of the CD8⁺ T cells in the lungs of infected C3H (H-2^k) mice are specific for CFP10 [18]. After infection with

M. tuberculosis, many people with diverse MHC haplotypes, mount a CD8⁺ T cell response to CFP10 [108,109]. Thus, the CD8⁺ T cell response of C57BL/6 mice is not predictive of the human CD8⁺ T cell response, probably because of its limited MHC diversity. In vivo models with greater MHC diversity may better predict true “antigenicity” albeit with the risk of significantly complicating the immunological analysis.

As more effort is applied to developing tuberculosis vaccines that elicit CD8⁺ T cells, new challenges are encountered. CD8⁺ T cells induced by vaccination may recognize different mycobacterial epitopes than CD8⁺ T cells elicited following natural infection. After administration of an Ag85A DNA vaccine, the array of epitopes recognized by both CD4⁺ and CD8⁺ T cells is broader compared to the number of epitopes recognized by T cells from *M. tuberculosis* infected mice [110]. In fact, CD8⁺ T cells from DNA vaccinated mice recognize three peptide epitopes from Ag85A that were not recognized by CD8⁺ T cells from *M. tuberculosis* infected mice. One explanation is that the frequency of Ag85A-specific CD8⁺ T cells significantly increases following vaccination, whereas it remains very low after infection. If this were true, these vaccine-induced CD8⁺ T cells may enhance protective immunity, but only if the relevant Ag85A peptide epitopes are processed and presented by *M. tuberculosis* infected cells.

In another dramatic example of how the way a vaccine is delivered can alter which epitopes elicit immune T cells, Peter Andersen developed a fusion protein consisting of ESAT6 covalently linked to Ag85B as a subunit vaccine [107]. This vaccine elicits a CD4⁺ T cell dominated response to ESAT6_{1–15} and Ag85B_{241–255}, and induces protective immunity in C57BL/6 mice. To potentially enhance vaccine efficacy, the gene construct encoding the ESAT6/Ag85B fusion protein was inserted into adenovirus. Interestingly, rAd_{ESAT6/Ag85B} elicits T cells that recognized a significantly different repertoire of epitopes, including a CD8⁺ T cell dominated response to ESAT6_{15–29}. In contrast, the frequency of CD4⁺ T cells recognizing ESAT6_{1–15} and Ag85B_{241–255} were markedly reduced. Interestingly, CD8⁺ T cells specific for ESAT6_{15–29} have never been detected following *M. tuberculosis* infection. Despite eliciting antigen-specific T cells capable of producing IFN γ , the rAd_{ESAT6/Ag85B} form of the vaccine failed to protect mice. Perhaps this is due to the failure of ESAT6_{15–29} presentation by *M. tuberculosis* infected cells, so that even though abundant, these CD8⁺ T cells are unable to recognize infected macrophages and therefore can not contribute to host immunity. The rules governing peptide selection for presentation by class I MHC have not been completely defined. Thus, even with the precise definition of epitopes recognized by CD8⁺ T cells, the task of developing a vaccine that elicits the desired CD8⁺ T cells is still formidable. With our increasing appreciation that CD8⁺ T cells serve to protect the host against tuberculosis, there is renewed interest in developing immunization strategies that can induce protective CD8⁺ T cells. Testing this hypothesis, i.e., that immunization strategies designed to elicit CD8⁺ T cells, such as rVV and listeriolysin-expressing BCG, actually induce protective CD8⁺ T cells, is required as these vaccines move forward in clinical development.

Five-year View

The appreciation that CD8⁺ T cells play an important non-redundant role in host immunity against *M. tuberculosis* infection has led to a greater interest in their stimulation by vaccination and their role in vaccine-induced immunity. During the past few years, several mycobacterial antigens that are recognized by human and murine CD8⁺ T cells have been defined, some of which are immunodominant antigens after experimental infection ([18,111] and reviewed in [13]), and large-scale antigen discovery efforts are underway, particularly to define the mycobacterial epitopes recognized by human CD8⁺ T cells. These efforts will

define the prospective vaccine candidates but also will provide reagents that can be used to monitor the immunological success of eliciting antigen-specific CD8⁺ T cells.

In parallel, because of the complexity of the immune response elicited by many vaccines, more in vivo models are beginning to be applied in a more sophisticated way in order to definitively demonstrate the protective potential of vaccine elicited CD8⁺ T cells. Because CD4^{-/-} mice have an unusual population of CD4⁻CD8⁻T cells that are class II MHC-restricted and can provide Th1-like function, their usefulness in assessing protection mediated solely by CD8⁺ T cells is more limited than previously thought. Instead, traditional adoptive transfer models using flow cytometry to sort pure populations are being introduced into the BL3 setting, and the function of CD8⁺ T cells can be better evaluated in class II MHC or RAG-1 knockout mice.

We have recently learned that the development of both CD8⁺ T cell memory and certain CD8⁺ T cell effector functions are dependent upon CD4⁺ T cells. We expect that during the next few years, a greater emphasis will be placed on understanding the synergy between CD4⁺ and CD8⁺ T cell responses and elucidating how to optimally stimulate these T cells simultaneously. This is an important goal since many of the vaccines that are in clinical development, including recombinant BCG, *M. tuberculosis* auxotrophs, DNA vaccines, and recombinant viruses, have the potential to elicit both antigen-specific CD4⁺ and CD8⁺ T cells.

Perhaps most important, as tuberculosis vaccines start clinical phase I/II trials, we will have a unique opportunity to learn about both the CD4⁺ and CD8⁺ T cell response, not in animal models, but in people. This data will provide invaluable insight about how host immunogenetics, antigen diversity, and specific vaccine strategies all interact to establish a protective T cell response.

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Key issues

- Antigen-specific CD8⁺ T cells are elicited as part of the adaptive immune response following infection with *Mycobacterium tuberculosis* and contribute to host defense.
- Optimum expression of CD8⁺ T cell effector functions require help or licensing that is dependent upon CD4⁺ T cells.
- CD8⁺ T cells are elicited by several of the vaccine strategies that are being considered for use in people at risk for tuberculosis. These include recombinant BCG and *M. tuberculosis* auxotrophs, viral vectors such as recombinant adenovirus and vaccinia, and DNA vaccines.
- Data exists to indicate that CD8⁺ T cells contribute to the protective efficacy of vaccines used against tuberculosis, and argues for the development of vaccines that will stimulate both *M. tuberculosis*-specific CD4⁺ and CD8⁺ T cells.
- Before the role of vaccine-induced CD8⁺ T cells in protection can be assessed, the mycobacterial antigens they recognize need to be defined so the immunological efficacy of vaccination can be determined.