Rational design of vaccines against tuberculosis directed by basic immunology

Stephen T. Reece, Stefan H.E. Kaufmann*

Max Planck Institute for Infection Biology, Charitéplatz 1, D-10117 Berlin, Germany

Abstract

Tuberculosis represents a serious problem for public health worldwide, and effective vaccines are urgently required. This represents a significant challenge as the causative bacterial agent, *Mycobacterium tuberculosis*, has developed strategies to persist in infected hosts despite the presence of potent T-cell-mediated immune responses. New advances in basic immunology are giving us improved understanding of what constitutes a protective immune response and ways this response is manipulated by the bacillus. Such insights should inform us how to design more effective vaccination strategies against intracellular pathogens.

Keywords: Tuberculosis; Vaccine; BCG; Immunity

Introduction

Tuberculosis represents a serious problem for public health worldwide. It has been estimated that one-third of the world’s population is infected with the causative bacterial agent *Mycobacterium tuberculosis* (Mtb), and roughly 10% of these individuals will develop active tuberculosis within their lifetime. Of these individuals, approximately 2 million per year do not survive (Kaufmann and McMichael, 2005). While antimicrobial drugs are available, treatment is both costly and lasts a minimum of 6 months, while the increasing number of cases of multidrug-resistant tuberculosis (MDR-TB) and extensively resistant tuberculosis (XDR-TB) either increases the cost of treatment by a factor of 100 or renders it ineffective (Glynn et al., 2002). Unsurprisingly, such regimens are difficult to implement in developing countries where effective public health infrastructure is often lacking, and as a result the major burden of tuberculosis is borne by the developing world, where the number of AIDS and tuberculosis co-infections continues to rise (Kaufmann and McMichael, 2005). Effective implementation of vaccination programmes could help reduce this global health burden. Bacillus Calmette-Guérin (BCG) is widely administered worldwide as a vaccine against tuberculosis; however, its efficacy remains somewhat controversial. This is largely due to the fact that while BCG effectively protects infants from tuberculosis, immunity declines with age and fails to protect adults against pulmonary tuberculosis, the primary source of dissemination (Fine, 1989; Rodrigues et al., 1993; Colditz et al., 1994). Our current control measures for tuberculosis rely largely on discoveries of the past 125 years. We use acid-fast staining of sputum samples for diagnosis of tuberculosis and skin testing with tuberculin (PPD, purified protein derivative of tuberculin) developed by the discoverer of the aetiology of tuberculosis Robert Koch (1843–1910) in 1882 and 1890, respectively. BCG was developed by Albert Calmette.
(1863–1933) and Camille Guérin (1872–1961) between 1906 and 1919 and first administered to a toddler in 1921. Streptomycin was the first drug developed against tuberculosis, discovered in 1945 by Selman Waksman (1888–1973), and development of additional drugs was mostly carried out before the 1970s (Kaufmann and Parida, 2007). These measures are not sufficiently controlling the disease and our complacency has meant a lack of development of alternative approaches in the past few decades. For these reasons, a more effective vaccine against tuberculosis would be highly desirable.

We live in an age where new discoveries in biology promise to deliver huge improvements in global health. One branch of biology developing exponentially is basic immunology and it should be feasible that improved understanding of immune mechanisms directs us to design more effective vaccines. Interestingly, especially for infections caused by intracellular bacteria, this has so far not been the case. A reason for this is perhaps the understandable lack of a constructive dialogue between microbiologists and basic immunologists as knowledge and methodology in both disciplines have expanded rapidly recently, and are often mutually incomprehensible to these specialists. By concentrating efforts on basic immunology, molecular biology and microbiology, work in our laboratory attempts to establish such a dialogue so that we might rationally design vaccines against tuberculosis. Though challenging, this approach is beginning to bear fruit, with the construction of a new tuberculosis vaccine candidate about to go into clinical trials, and with improved understanding of the mechanisms by which protective immunity against tuberculosis is generated. This review attempts to summarize the current progress in vaccination against tuberculosis and the role basic immunology could play in the made-to-measure vaccines of the future.

**Vaccinologists – what has basic immunology done for you lately?**

It is often suggested that vaccination is the crowning achievement of immunology. This actually stretches the truth slightly. The pioneers of vaccination, such as Edward Jenner (1749–1823) and Louis Pasteur (1822–1895), would be better described as infection biologists. In both these cases, their methods preceded a precise knowledge of immunology and were based largely on imaginative insight and the fact that killing the infectious agent before administration gives protection without disease. It is important to mention that all successful vaccines developed thus far against acute diseases like smallpox, anthrax and rabies, function largely through production of specific antibodies. Design of vaccines against intracellular bacterial pathogens like Mtb remains more challenging since the pathogen hides from immune attack by antibodies within cells, and protection requires mobilization of the cellular arm of the immune response mediated by T cells, which is notoriously difficult to accomplish. Preceding a knowledge of T-cell immunity, BCG was generated in the early 20th century by continual passage of virulent *M. bovis* (the causative agent of TB in cattle) both using primitive but ingenious in vitro culture methods and in experimental animals, to generate an attenuated strain still capable of inducing protective immunity. The resultant strain, BCG, proved safe and efficacious in both animals and humans, and, with 4 billion doses given, it has become one of the world’s most highly administered human vaccines. Surprisingly, for a human vaccine used for almost a century, the mechanisms by which BCG generates protective immunity remain largely undefined and the vaccine is believed to be only partially effective. However, it remains a good place to start unravelling the mechanisms of protective immunity against tuberculosis. Moreover, every new vaccine starts with animal models and BCG is the golden standard by which all new vaccines are measured for protection in the mouse, guinea pig, rabbit or non-human primate models of experimental infection. The last 20 years have seen the development of a number of novel vaccines against tuberculosis, and the current crop of new vaccines entering clinical trials (Table 1) can be broadly divided into subunit vaccines, on the one hand, and live attenuated vaccines, on the other. Subunit vaccines constitute one or two antigens, delivered with powerful adjuvants or by novel systems such as modified vaccinia virus Ankara (MVA), so the immune response during infection is directed against these antigens. Such antigens are selected on the basis of criteria such as strong recognition by the immune system during tuberculosis, high abundance or active secretion by Mtb during infection. This supposes that a protective immune response can be constituted by potent reactivity against a limited number of antigens. The Mtb72F and Ag85B-Esat-6 fusion proteins represent subunit vaccines in the protein+adjuvant format. Another subunit approach utilizes delivery of a DNA transcript enabling expression of Ag85A via a recombinant MVA construct (McShane et al., 2001). These approaches have met with some success with all three subunit vaccines showing efficacy comparable to BCG in animal models and largely aim at improving protection by being administered after BCG vaccination, effectively boosting BCG-primed immunity (Weinrich Olsen et al., 2001; Skeiky et al., 2004; Williams et al., 2005; Dietrich et al., 2007).

Secondly, attempts have been made to genetically modify BCG to improve immunogenicity and thus vaccine efficacy. Yet, these vaccines are aimed at replacing BCG and must therefore be either safer or more effective than BCG. One such approach attempts to combine the live and subunit approaches by
constructing BCG expressing high levels of Ag85B, thus leading to improved protective capacity (Horwitz et al., 2000). The BCG-based approach taken by our laboratory differs in this respect. We have attempted to utilize increasing knowledge of how protective immunity against tuberculosis is generated during natural infection or BCG vaccination and harness this in the design of new vaccine strategies.

Mother nature knows – the argument for naturally acquired immunity acquired unnaturally

Ninety percent of individuals exposed to Mtb never develop active disease. This suggests that naturally acquired immunity against tuberculosis is highly efficient and, to some degree, BCG recapitulates this immunity without pathological effects. During natural infection of the lung, Mtb most likely enters alveolar macrophages and traffics to draining lymph nodes. Here macrophages and dendritic cells (DCs) present mycobacterial antigen to T cells which become activated and traffic back to the lung, where waves of infiltrating cells wall off infected macrophages present in structures called granulomas, resulting from the dynamic interaction between Mtb and the macrophages and T cells trying to kill them. Thus, priming of T cells and rapid migration into the infected lung are of critical importance in protection. BCG is almost exclusively administered in humans via the dermis for vaccination, and analogous events likely take place in lymph nodes draining the dermis after vaccination, resulting in the generation of memory T cells specific for mycobacterial antigens capable of mediating this process. The priming of specific T cells requires efficient presentation of mycobacterial antigens, and both Mtb and BCG are able to interfere with these processes to persist in the host. Firstly, on entering macrophages Mtb and BCG reside in vacuoles called phagosomes, which, under normal circumstances, undergo maturation, become acidified and fuse with the lysosomes, organelles containing aggressive antimicrobial enzymes. This results in a hostile environment in which the bacillus cannot survive. Mtb and BCG arrest acidification of the phagosome, enabling them to grow and persist within this vacuole (Russell, 2001). Despite this, the host responds by priming both CD8 and CD4 T cells via major histocompatibility complex (MHC)-I and MHC-II presentation pathways, respectively. Mtb is likely to induce apoptosis in infected macrophages via multiple pathways. One such mechanism revealed by our group involves the small molecule methylglyoxal (MG) (Rachman et al., 2006), which modifies proteins resulting in so-called advanced glycation end-products (AGEs). AGEs accumulate in cells due to aberrant catabolic and metabolic activity during diseases such as diabetes. MG induces both tumour necrosis factor α (TNF-α) and reactive oxygen intermediates, which in turn cause apoptosis and inhibit the growth of Mtb in macrophages in vitro. Identification of AGEs as well as upregulation of glyoxylase in Mtb, which functions to reduce AGE formation, residing in lesions of tuberculosis patients is consistent with a role of MG during infection. Thus, the degree of apoptosis in granulomatous lesions among other mechanisms involves MG as well as its detoxification. Others have shown that cell death due to mycobacterial infection occurs by different pathways depending on abundance of bacteria in infected macrophages. At low multiplicity of infection (MOI), macrophages die via TNF-α-mediated apoptosis, which progresses relatively slowly and likely reflects an innate mechanism of protection used by the host to prevent infection (Keane et al., 1997). Relative to BCG, Mtb appears able to inhibit this process to perpetuate its survival in host cells. Conversely, at higher MOI, virulent Mtb induces more potent TNF-α-independent cell death that progresses rapidly from apoptosis to necrosis and could facilitate dissemination of bacteria.

### Table 1. The most advanced vaccine candidates against TB

<table>
<thead>
<tr>
<th>Candidate</th>
<th>Type</th>
<th>Current status</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Ag85-Esat-6 fusion protein in IC31</td>
<td>Subunit (protein/adjuvant)</td>
<td>Clinical phase I trial started 2005</td>
<td>Weinrich Olsen et al. (2001)</td>
</tr>
<tr>
<td>r-MVA-Ag85</td>
<td>Subunit (recombinant-virus)</td>
<td>Clinical phase I trial in BCG-vaccinated and unvaccinated healthy uninfected volunteers completed</td>
<td>McShane et al. (2001)</td>
</tr>
<tr>
<td>r-BCG-Ag85</td>
<td>Viable</td>
<td>Clinical phase I trial in healthy uninfected volunteers completed</td>
<td>Horwitz et al. (2000)</td>
</tr>
<tr>
<td>r-BCGAure:Hly</td>
<td>Viable</td>
<td>GMP production initiated, clinical phase I trial planned for 2007</td>
<td>Grode et al. (2005)</td>
</tr>
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Lee et al., 2006). These processes result in the formation of apoptotic bodies containing both mycobacterial cell wall components capable of engaging Toll-like receptors (TLRs) and mycobacterial antigen. Uninfected DCs can engulf this material, become activated and prime potent T-cell responses via multiple pathways including MHC I and II pathways in a process termed cross priming (Schaible et al., 2003; Winau et al., 2004). There is evidence that this process has major importance in the generation of immunity as apoptotic bodies generated in vitro by BCG infection of macrophages have been demonstrated to induce a protective immune response equivalent to BCG vaccination (Winau et al., 2006). Thus, uptake of apoptotic bodies produced during infection differs remarkably from events occurring during physiological homeostasis. Apoptotic bodies generated in the course of homeostatic cell death are quiescent and do not stimulate DCs and macrophages which engulf them. In contrast, apoptotic bodies produced during infection sound an alarm via TLR signalling within the DCs and hence stimulate the antigen-presenting capacities of these cells.

In addition to the conventional MHC class II-restricted CD4 T cells and the MHC class I-restricted CD8 T cells, so-called unconventional T cells are stimulated during MtB infection and are glycolipid-specific and CD1-restricted (Ulrichs et al., 2003). This group includes T cells which, analogous to CD4 and CD8 T cells, use the conventional α/β chain combination T-cell receptor. The group also includes γ/δ T cells, which use an alternative T-cell receptor composed of a γ/δ chain combination and recognize non-proteinaceous phosphorylated ligands without need for a presentation molecule (Kaufmann, 1996). From the vaccination perspective, immunity generated by BCG vaccination comprises a wide spectrum of T-cell phenotypes stimulated by protein, lipid and carbohydrate antigen.

In contrast, the response generated by subunit vaccination comprises mainly CD4 and CD8 T cells responding to protein antigens (Fig. 1).

**Vaccinologists – what has the mouse done for you lately?**

All attempts to evaluate vaccines start with assessment in animal models, and the widest array of immunological tools to study protection against infection have been established for the mouse. As a result, attempts to establish new vaccine strategies tend to, at least initially, lean rather heavily on the mouse model of infection. Typically, mice are infected via aerosol with 20–100 organisms and the bacillus proliferates unfettered for the first 30 days of infection, after which a TB-specific T-cell immune response contributes to a standoff between bacillus and host, resulting in the continued presence of approximately 1 million bacilli in the lungs until susceptible mice eventually succumb at around 250 days post-infection. BCG vaccination protects adult mice with a 90% reduction in the number of MtB bacilli in lungs after infection. Infection of genetic knock-out (GKO) mice indicates that both CD4 and CD8 T cells are of major importance for protection (Flynn et al., 1992, 1993; Cooper et al., 1993). Classically, CD4 T-cell responses can be divided into Th1 and Th2 lineages, which inhibit the development of each other. Th1 cells secrete interferon (IFN)-γ, interleukin (IL)-12 and TNF-α cytokines, and Th2 secrete IL-4, IL-5 and IL-13. Studies in mice have demonstrated that a Th1 cytokine profile is critically required for control of infection, with Th2 cells having little or no protective capacity. Moreover, IFN-γ and TNF-α are key cytokines in that they activate macrophages to control bacterial proliferation by increasing fusion of the phagosome with the

![Fig. 1. BCG vaccination aims at recapitulating naturally acquired immunity against TB. BCG and subunit vaccination stimulates both CD4 and CD8 T cells via protein antigens, but only BCG stimulates so-called unconventional T cells via glycolipid and non-proteinaceous phosphorylated ligands.](image-url)
lysosome (Schaible et al., 1998; MacMicking et al., 2003). It has therefore often been argued that a potent Th1 T-cell response against a limited number of antigens could be enough to mediate protection, and, as a result, vaccines are often evaluated on their ability to elicit high levels of these cytokines. Recent data suggest that extra layers of complexity contribute to CD4 T-cell-mediated responses that may be missed by studying infection of GKO mice. Tumour growth factor (TGF)-β has recently been demonstrated to play a key role in development of CD4 T-cell lineages distinct from the Th1/Th2 axis. TGF-β alone favours the development of suppressive T cells termed T regulatory (Treg) cells, which produce IL-10 and TGF-β, and function by dampening pro-inflammatory responses to reduce host tissue damage (O’Garra et al., 2004). Conversely, in the presence of IL-6, TGF-β mediates generation of Th17, a recently described T-cell lineage producing IL-17 and IL-6. Th17 cells are strongly pro-inflammatory and have been implicated in autoimmunity (Harrington et al., 2005; Park et al., 2005). These two T-cell lineages appear to have opposing functions and could play key roles in the regulation of protective immunity in tuberculosis. A recent study by our group demonstrated that a mixed population of T cells from naive mice were highly effective in providing protection to RAG KO mice against tuberculosis via adoptive transfer when depleted of Treg cells (Kursar et al., 2007), suggesting that presence of Treg cells could dampen an otherwise effective T-cell response allowing Mtb survival. IL-23 is required for full development of Th17 cells and has been shown to be dispensable for protection from primary tuberculosis in the mouse (Khader et al., 2005). However, it has recently been shown that Th17 cells are recruited to the lung after vaccination and rapidly respond to tuberculosis infection, increasing recruitment of Th1 cells into the lung (Khader et al., 2007). IL-17 is primarily secreted by γ/δ T cells in the lungs of mice in the early stages of tuberculosis infection (Lockhart et al., 2006), which might also facilitate Th1 cell influx into the lung in the absence of Th17 memory, although this still remains to be fully established. Therefore, both limiting the function of Treg cells and/or maximizing IL-17 production via vaccination strategies may help optimize Th1 responses to control Mtb in the lung (Fig. 2). However, care must be taken to avoid risks of damage to the host via uncontrolled inflammatory processes. Optimization of these processes for protection against tuberculosis represents a fresh challenge for the vaccinologist.

Making BCG better

Clearly, the key in optimizing vaccination strategies against tuberculosis lies in improving access of antigen to presentation pathways and thus enabling high-quality T-cell priming to mycobacterial antigens, whether they be protein, lipid or carbohydrate. In light of new tools for the genetic manipulation of mycobacteria (Glickman and Jacobs, 2001), it is feasible to genetically modify BCG to dictate how it interacts with macrophages to optimize antigen presentation by infected cells. Clues on how this might be accomplished have come from insights into how non-mycobacterial intracellular pathogens modulate host cell processes. Listeria monocytogenes is an intracellular bacterial pathogen that avoids lysosomal killing in infected macrophages by escaping from the host cell phagosome into the cytosol. This phenotype is mediated by a single secreted protein listeriolysin (Hly) (Glomski et al., 2002). Although this phenotype primarily facilitates cell-to-cell spread of...
bacteria, the host responds by loading antigen present in the cytosol onto MHC-I to prime a potent CD8 T-cell response, eventually eradicating the pathogen by direct killing of infected cells. A novel BCG construct expressing Hly (r-BCG Hly) was generated in our laboratory with the original rationale that it would result in increased presence of BCG antigens in the cytosol of the host cell causing enhanced CD8 T-cell priming. Compared with wild-type BCG, r-BCG Hly was unable to survive for equivalent duration, and gold-conjugated antibody labeling studies indicated the presence of Hly in vacuoles either in the presence or in the absence of BCG. However, r-BCG Hly was not identified in the cytoplasmic compartment (Hess et al., 1998). As described previously, BCG is able to block acidification of the phagosome to prevent fusion with lysosomes, and this is achieved by secretion of ureases which function by increasing levels of ammonia within the vacuole allowing the maintenance of a neutral pH. However, an acidic pH (5.5) is required for full functional activation of Hly in the phagosome. To improve this, urease C was deleted from the r-BCG Hly, with the resulting strain termed r-BCG ΔureC:Hly (Grode et al., 2005). This strain was unable to prevent acidification of the phagosome and it indeed appears to mature and fuse with lysosomes. Moreover, electron and confocal microscopy revealed an increase in the cytosolic presence of mycobacterial antigens in macrophages harbouring r-BCG ΔureC:Hly compared with parental BCG. A second striking observation was that macrophages infected with r-BCG ΔureC:Hly showed an increased tendency to undergo apoptosis in comparison to parental BCG or r-BCG:Hly. Further elucidation of this and other mechanisms of apoptotic cell death mediated by mycobacteria and their relative contribution to the generation of immunity during tuberculosis remains an exciting prospect, since levels of apoptosis might be adjusted to enhance the outcome of vaccination with recombinant BCG.

Based on current knowledge, we suggest that two mutually non-exclusive mechanisms are likely to occur. Firstly, higher quantities of mycobacterial antigen in the cytosol leading to more efficient loading of MHC I molecules with antigenic peptides for CD8 T-cell stimulation, and secondly, enhanced apoptosis of infected macrophages, formation of apoptotic blebs and subsequent cross priming by uninfected DCs. Perhaps more importantly, we have observed enhanced protection against tuberculosis in the mouse model with both the laboratory strain H37Rv and a virulent human isolate, Beijing W (Grode et al., 2005). With both strains, enhanced protection over wild-type BCG is most apparent at later time points post-infection (days 90–200), a good indication that protective immunity is sustained over a protracted period. In addition, SCID mice vaccinated with r-BCG ΔureC:Hly survived for longer time periods than those vaccinated with parental BCG, suggesting an improved safety profile of this vaccine. Further elucidation of the mechanisms by which protective immunity is generated in this context could contribute greatly to how T-cell-mediated vaccines against intracellular pathogens are constructed.

Concluding remarks

So the question remains, how do we effectively control tuberculosis by vaccination? An expanded population of T cells specific for one or two antigens primed by subunit vaccination or broader T-cell immunity further optimized by genetic modification of BCG? Clearly there are good arguments for both approaches. That the vast majority of Mtb-exposed individuals do not develop tuberculosis suggests that an improvement of the immunogenicity of BCG could reap huge dividends in terms of protection. Furthermore, a live recombinant vaccine also affords the opportunity to construct strains expressing additional Mtb antigens that were deleted during attenuation of BCG or even heterologous antigens. On the other hand, one of the most obvious advantages of subunit vaccines over recombinant live vaccines is the question of safety. One pertinent question would be whether BCG should continue to be administered to HIV-positive newborns and toddlers due to risk of disseminated BCG disease (Hesseling et al., 2006, 2007). Recently, the Global Advisory Committee on Vaccine Safety established by the World Health Organization recommended that BCG should not be administered to children known to be HIV infected (WHO, 2007). This could present a major obstacle to novel vaccine approaches as subunit vaccines rely on a BCG prime, because alone they are unlikely to be superior to BCG. The ideal scenario would be a combination of both live and subunit approaches to maximize the potential of both and aim at improving the safety of BCG. The new attenuated vaccine strains aimed at substituting BCG, therefore, need to demonstrate robust safety profiles. Although the r-BCG ΔureC:Hly developed by us shows remarkable safety in immunocompromised mice (Grode et al., 2005), we are in the process of creating an even safer vaccine candidate which survives in the host for a limited time period only. It is likely that an improved BCG vaccine can contain Mtb for long periods of time and this may be sufficient for disease prevention. However, with the advent of HIV, reactivation of persistent Mtb becomes possible even in vaccinated individuals. Once HIV has attacked CD4 T cells responsible for sustaining protective immunity, there is a risk that Mtb will reactivate. Hence, in HIV-prevalent regions such as Africa, a more potent vaccination regime is required. Further, increase in protective efficacy may be achieved by combining two
vaccination approaches—namely, substitution of BCG with a better recombinant BCG construct and boosting with an efficacious subunit vaccine. It is hoped that this strategy will result in sterile eradication of the pathogen, necessary in HIV-prevalent regions where tuberculosis rampages most in the world.

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