Protection against tuberculosis: How close are we to a perfect vaccine?

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Tuberculosis (TB) is one of the most challenging global health problems. BCG, the only vaccine in use against TB, has not performed satisfactorily and most efforts to develop a new TB vaccine have met with little success. In this review, without revisiting the stories of failed TB vaccines, we focus on what has prevented the development of a perfect TB vaccine and issues that need immediate attention in order to succeed.

Since the time people have realized tuberculosis is preventable and since they have learned how to avoid infection, mortality rates caused by tuberculosis have declined in industrialized countries and signs are starting to appear that it can be eliminated. This is the right time to combat tuberculosis.

Robert Koch, the discoverer of the tubercle bacillus had made these optimistic observations more than a century ago. A vaccine against tuberculosis (TB) was developed about 80 years ago. Various drugs for control of the disease were developed around four decades ago and the combination chemotherapy has been in place for about two decades. Yet, today we do not seem to be any closer to eliminating this disease than we were a century ago. A perfect vaccine against TB, which would be most effective in the control of this disease, has eluded us all the time. TB claims approximately 2 million lives every year and one-third of the world's population is latently infected with *M. tuberculosis*. Every year, eight million individuals develop active disease[1]. The advent of HIV epidemic and the multi-drug-resistant strains of *Mycobacterium tuberculosis* has greatly accentuated the problem. TB has been mostly associated with developing countries. But with globalization and facilities for air-travel in an interconnected world, transmission of the disease from one part of the globe to another has become rapid.

Effective drugs are available against TB, but long period of intake (4–6 months) is required to ensure their maximum efficacy. This long-drawn schedule often results in poor compliance, which in turn can lead to the development of drug-resistant forms of the pathogen. WHO had initiated the DOTS programme[2], which involves getting healthcare workers in TB hot spots to prescribe TB drugs and supervise patients in order to ensure completion of the course. However, in 1990, the aim was to get 22 worst-affected countries detect 70% of TB cases and cure 85% of them by the year 2000. Only two countries, Peru and Vietnam have successfully met the target[2]. Besides, outbreaks of drug-resistant (and often multi-drug-resistant) TB have underscored the limitations of currently available treatments and control programmes, and have led to the general view that the real control of TB on a worldwide basis is unlikely until an effective vaccine becomes available. In this article, we are attempting not to compile the results obtained with various candidate vaccines in the past (almost none of which has shown an improvement over BCG), but to focus on the problems that need attention in order to develop a more effective vaccine against TB.

What to expect from a perfect TB vaccine?

Complete eradication of TB requires a vaccine that is cheap, effective and can be employed for mass immunization. BCG (live, attenuated *M. bovis* BCG), which represents the only vaccine currently available against TB is not satisfactory. It is the most widely administered of all vaccines in the WHO Expanded Programme for immunization[3], but has been estimated to prevent only 5% of all potentially vaccine-preventable deaths due to TB[4]. It has been shown to be protective against disseminated and meningeal TB in young children[5]. However, its efficacy in preventing adult pulmonary TB, that is responsible for the major burden of morbidity and mortality from this disease, varies dramatically as revealed by carefully conducted studies throughout the world – from 77% in the UK to 0% in Chinglepet, India[6]. The factors underlying this discordance continue to be debated. Due to the variable efficacy and interference with skin test screening, thereby thwarting discrimination between BCG vaccination and an infection with *M. tuberculosis*, BCG vaccination is not recommended for general use in many countries. Moreover, in developing countries, the vaccine(s) needs to be effective in an endemic population,

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which includes uninfected individuals, infected individuals, HIV-infected individuals and people with active disease. Besides, majority of people in such countries are exposed to a variety of pathogenic and non-pathogenic mycobacteria in addition to *M. tuberculosis*, *M. leprae* and *M. bovis*. Thus, we urgently need a vaccine that protects consistently against adult TB in all populations.

A perfect TB vaccine should desirably have the following characteristics.

- Should be safe.
- Should be inexpensive.
- Should prevent reinfection as well as reactivation TB.
- Should be long-lasting and be able to generate adequate memory responses.
- Should be stable and have a long shelf-life.
- Should be easy to transport and administer.
- Should be protective, preferably as a single dose.
- Should not interfere with other vaccines.
- Should work effectively in all populations.
- Should work effectively in all age-groups.
- Should be effective in HIV-positive population.

Given the difficulties faced by various investigators in the development of a TB vaccine during the last one and a half decades and the complexity of the problem, it will be highly optimistic to expect that a single vaccine will fulfil all these criteria. Besides, in view of the complicated scenario of TB, there has been a great deal of thinking whether more than one type of vaccine may be needed; for example, one vaccine may be best suited for use in newborns and infants who have never been exposed to TB, another one for adults who have been exposed, yet another designed to treat those already infected and perhaps one specifically designed for use in HIV infected persons. However, considering the current precarious scenario, a few candidate vaccines fulfilling some of the above criteria will also go a long way in reducing morbidity and mortality associated with TB.

Difficulties in the development of a vaccine against TB

While the objective of developing a perfect vaccine against TB is unambiguously clear, the path to realize this objective has been fraught with difficulties and uncertainities. In spite of rapid progress in the last 15 years in our understanding of the biology of mycobacteria, if we still have not succeeded in the development of a vaccine against TB, it certainly has not been due to lack of efforts. However, there are questions which are still seeking answers. We still do not understand the nature of protective immunity, which is a pre-requisite for the development of a vaccine. Besides, even to test the new candidate vaccines we do not, as of today, have surrogate markers. There are no clear leads to set parameters that define protective antigens despite the wealth of information from genomics and proteomics. The role of various animal models in the evaluation of a candidate TB vaccine is again a question, as protection in any existing animal model does not guarantee protection in humans. Besides, the issue of host immune response is still open and finally after 80 years of its use, we still do not clearly understand why BCG fails in some populations.

What can we do?

Experience with BCG provides proof of the principle that vaccination can provide protection against TB. However, the credibility of any potential vaccine candidate lies in the demonstration of its ability to prevent caseating disease and induce a cellular response in the lungs, similar or better than that induced by BCG[7]. Around the world, as many as 60 million people suffer from TB. This high figure may apparently lead to the false conclusion that protective immunity is totally insufficient for the control of this disease unless we consider the fact that more than 1.7 billion individuals[8] are currently infected with tubercle bacilli, globally. Protective immunity is extraordinarily efficacious in preventing the disease, but it is highly inefficient in complete clearance of the bacilli from the system, thereby resulting in latency and thus possible reactivation at a later stage. If we accept the notion that active TB develops due to weakening of the immune mechanisms designed to keep the pathogen in check, it also provides evidence that *M. tuberculosis* induces some immunity that provides protection. This notion is further supported by a significant increase of TB incidence in AIDS patients. However, that the protection induced by *M. tuberculosis* is insufficient, is evident from the fact that the bacilli are not completely cleared and that some infected individuals do develop active TB. A perfect vaccine indeed would transform the TB control programmes worldwide. Yet, such a vaccine does not appear to be in sight within the next couple of years. Thus, under the circumstances, we can only do the following:

- We can use new tools that technology has provided us to generate potential vaccine candidates and screen them in a rather empirical way in existing animal models.
- Focus our efforts on several aspects such as understanding the nature of protective immunity, development of surrogate markers, identification of new antigens, improvement of existing animal models, development of new animal models, strengthening of the infrastructure, preparation for vaccine trials, etc. for long-term success in the development of effective vaccine(s) against TB.

Specifically, we need to focus our attention on the following.

Understanding the basic immunology of TB

One of the major areas that requires extensive research is mycobacterial immunology. There is still no clear understanding about the nature of protective immunity, which is an essential requirement for any rational approach for the development of a vaccine. The immunological control of *M. tuberculosis* infection is complex and multi-faceted (Figure 1). However, given the intracellular nature of the pathogen, the role of cellular immunity in the control of *M. tuberculosis* infection has been established beyond doubt. T-lymphocytes play a central role in the protection against TB; thus it is imperative that future vaccine designs focus on T-lymphocyte populations. Most of the present-day vaccines against infectious diseases, such as MMR and DPT, protect against the disease and not infection[9]. Similarly, no vaccine to date has protected animals from infection with the tubercle bacillus – rather they protect against development of clinical disease. It is important for us to answer whether sterilizing the system is possible by immunization against TB or should we focus our attention on the development of vaccines that are capable of resolving clinical disease and preventing reactivation. In the light of the above observations, development of a vaccine against TB demands reconsiderations that are related to the following aspects:

- Since one-third of the world's population is already infected with *M. tuberculosis*, vaccine development would have to take into consideration the presence of post-infection targets. In this case also, the phenomenon of reactivation threatens to add ambiguity to the results of post-infection vaccine testing.
- The development of therapeutic vaccination demands special attention in the light of MDR–TB and HIV–TB co-infection scenario.

Since the bacilli reside within the macrophages, an obvious outcome of the infection is the MHC class-II

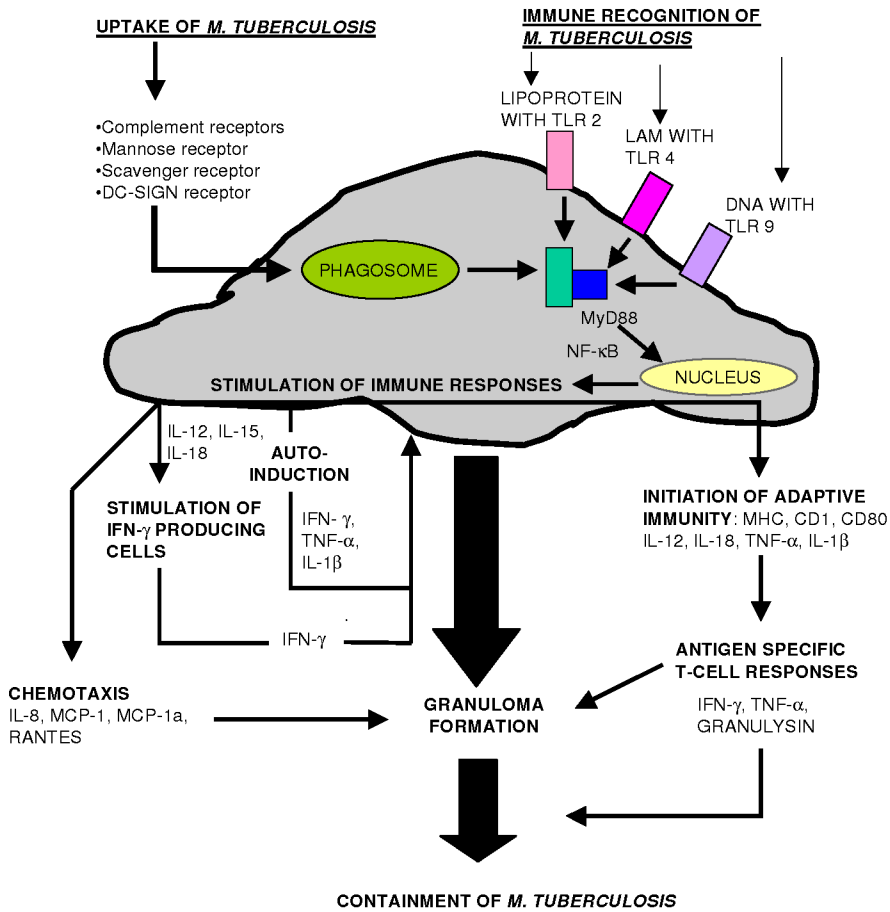

Figure 1. Immune mechanisms in response to mycobacterial infection. Intricate immune mechanisms that are involved in the containment of an infection with *M. tuberculosis* are illustrated. Internalization and immune recognition of *M. tuberculosis* by macrophages and dendritic cells result in the induction of cellular responses. Cytokines and cell-surface markers are the key players in this response. An intricate balance between the pro-inflammatory and anti-inflammatory (not shown) responses decides the outcome of the infection.

presentation of the mycobacterial antigens to CD4+ T-cells. Hence, these cells have been rightly implicated to be the most important ones in protective immune response against *M. tuberculosis*. The use of techniques such as antibody depletion of T-cells[10], adoptive transfer[11,12] and the use of gene-disrupted mice[13,14] confirms the role of CD4+ T-cells in the murine model. In humans, the best experiment is conducted by nature itself, i.e. natural infection with HIV (which is known to target the CD4+ T-cells and makes these patients more susceptible to both acute and reactivation TB), thus again confirming the involvement of CD4+ T-cells in protection against TB.

The main role that the CD4+ T-cell population plays in the combat against the pathogen is the secretion of the cytokine IFN-γ. Other cytokines which activate macrophages, play the role of secondary effector molecules. Besides, the production of IFN-γ and other cytokines, CD4+ T-cells have been implicated in other important roles in controlling *M. tuberculosis* infection. This assumption has been corroborated by the finding that gradual depletion of CD4+ T-cells by the use of anti CD4 antibodies, in a murine model of chronic persistent TB, resulted in a rapid reactivation of infection, even though IFN-γ levels remained similar in CD4+-depleted and control mice. Also, there was no apparent change in macrophage NOS 2 production or activity in the CD4+ T-cell-depleted mice[15]. Hence, this is one of the most important areas that requires further investigation.

Although the role of CD8+ T-cells in controlling disease has been well established, it was not without its share of incongruity. When the existence of cytolytic cells in mycobacterial infections was first described, the idea that these cells would have a protective function was not acceptable to many because it was felt that it was more likely that such activity would directly disseminate the disease[16]. However, investigations in this area have revealed that such cells peak at about a month into the experimental infection, making sure that cell lysis and bacterial release will occur after an intact, extensive granuloma has formed at the site of infection, thus making it unlikely that the bacteria can escape[17]. Also, one of the reasons why CD8+ cells did not receive much attention as potential players in protection against *M. tuberculosis* infection was the belief that *M. tuberculosis* resides within vacuoles in the macrophage and MHC class-I presentation is efficient with cytoplasmic antigens[18]. However, recent studies have provided evidence for a mycobacterium-induced pore or break in the vesicular membrane surrounding the bacilli, thereby permitting the entry of mycobacterial antigens into the cytoplasm. It has been suggested that mycobacteria utilize this pore to obtain nutrients or introduce toxic molecules into the cytoplasm[19].

The role of CD8+ T-cells in controlling TB was confirmed by data generated from experiments employing adoptive transfer[11,12] or antibody depletion[10] in mice. A major hurdle in the acceptance of the role of CD8+ T-cells, however, was the difficulty in isolation of *M. tuberculosis*-specific CD8+ CTLs from infected mice or humans[20]. This has been overcome in the recent years. A recent study by Turner *et al.*[21] reveals that the development of the necrotic core of the granuloma is an early event and almost certainly precedes the emergence of the acquired immune response. This in turn suggests that innate mechanisms are the basis of the early lesions and that subsequent acquired responses are unable to moderate them. This hypothesis differs from the current dogma that excessive activity of T-cell mediates delayed-type hypersensitivity and that cellular cytolysis is the root cause of necrosis[21].

The identification and validation of surrogate markers of protective immunity are a prerequisite for initiation of human vaccine trials. This is the most crucial aspect of mycobacterial immunology that requires extensive research.

A common notion related to mycobacterial infections is that the generation of a Th-1 type of an immune response is involved in protective immunity[22,23]. Since, the Th-1 and Th-2 responses down-regulate each other, an enhancement in a type-2 immunity would be expected to be detrimental for host immunity. These observations have, however, faced protest from the findings of experiments in which *IL-10* gene-deleted mice incapable of generating a Th-2 response were no more capable than wild-type mice at dealing with *M. tuberculosis* infection[24].

Production of IFN-γ by *M. tuberculosis* has long been proposed as a marker for protective immunity. Symptomatic pulmonary TB patients, however, produce high levels of IFN-γ, suggesting that IFN-γ alone may not correlate well with protection. Recent studies by Cappelli *et al.*[25] have revealed that when human macrophages were infected with *M. tuberculosis* $H_{37}Rv$, high IFN-γ transcription resulted in a decrease in the number of *M. tuberculosis* genes expressed without affecting the bacterial growth. The ambiguity about the importance of the role of IFN-γ results from various observations, such as the production of IFN-γ by healthy PPD+ subjects as well as by those with active TB. One study with the 27-kDa antigen revealed that although the vaccination regimen was able to induce a typical Th-1 response with significant levels of IFN-γ, no significant protection was observed[26] against *M. tuberculosis* challenge.

One aspect of the immune response frequently measured while evaluating an anti mycobacterial vaccine is IL-10. IL-10 is known to possess macrophage-deactivating properties, which include the down-regulation of IL-12 that in turn decreases IFN-γ production[20]. IL-10 is also known to be capable of suppressing the transcriptional activation of genes necessary for the activated state of macrophages[27]. There have been demonstrations of IL-10 knockout mice generating an increased ability over wild-type mice to control the growth of virulent *M. tuberculosis* at an early stage and this was related to an increased ability of spleenocytes to synthesize IFN-γ[28].

However, as is the norm for any mycobacterial immunity-related issue, controversies exist in this case also. North *et al.*[29] have reported that IL-10 knockout mice are no more capable than wild-type mice at controlling *M. tuberculosis* infection. Thus the role of IL-10 in down-regulating the Th-1 mediated immunity to *M. tuberculosis* remains controversial.

In the light of the existing controversies, the role of the immunosuppressive action of IL-10 to explain progressive TB in humans reflects the exigency of a clear understanding of the role of IL-10. This role has been assigned to IL-10 on the basis of observations that T-cells capable of producing IL-10, as well as IFN-γ, are more numerous in the lungs of humans with active rather than inactive tuberculosis[30]. Also, neutralization of IL-10 with anti-IL-10 antibodies enables human peripheral blood mononuclear cells from persons with active disease to make more IFN-γ and IL-12 in response to *M. tuberculosis* antigens[31].

The gravity of the situation is clearly reflected from the example of tuberculin sensitivity, which, long thought to be a measure of protective immunity, is now being shown to be associated with high risk of disease and vice versa. Correlates such as lymphocyte proliferation and cytokine production in response to antigenic stimulation have never been validated in humans. Hence, due to the complexity of the immune response to TB, distinct correlates of protection have not been determined.

An important aspect of mycobacterial immunity that needs extensive investigation is related to the role of memory T-cells. Immunological memory is a cellular recall mechanism for the rapid and efficient mobilization of the immune system against previously encountered organisms. It has recently been shown that the development of memory is crucially dependent upon the type of response, cytokines produced and the differentiation state of activated cells[32]. Using a combination of cytokine-capture techniques and *in vivo* adoptive transfers, it has been demonstrated that the persisting memory T-cell population is derived from activated cells that are not producing IFN-γ[33]. These findings have profound implications on vaccine design, where it might be useful to establish conditions that do not promote full differentiation of Th1 effector cells, in order to promote a long-lived memory response[32]. It would be wise to include such experiments in future testing of potential vaccine candidates because till now it was thought that the induction of IFN-γ would be useful in providing anti-mycobacterial immunity, but what role this plays in the induction of memory T-cells has never been studied.

Rapid progress in our understanding of immunology associated with mycobacterial infections has led to the emergence of several new areas of investigations; for example, the discovery of the receptor referred to as DC-SIGN, which is identified to be employed by HIV to infect the dendritic cells. It is hypothesized that the HIV uses this receptor to 'hitch a lift' to the target cells[34]. DC-SIGN is now known to be a universal pathogen receptor that also recognizes Ebola, cytomegalo virus and mycobacteria. It has also been demonstrated that *M. tuberculosis* also targets DC-SIGN to escape immune surveillance[35] (Figure 2). What is particularly enthralling about the discovery of this receptor is the optimism that it raises about the prevention of infection by both *M. tuberculosis* and HIV by blocking this receptor.

Absence of correlation between production of IFN-γ and protection has now been explained by the observation that *M. tuberculosis* is capable of preventing macrophages from responding to IFN-γ and hence limiting the activation of macrophages by it. Studies have also shown that mycobacterial components as well as live mycobacteria inhibit IFN-γ signalling in human macrophages by disrupting the association of the transcription activator STAT-1 with CREB binding protein and p300 (ref. 36). This results in a situation where the levels of this crucial cytokine fail to predict the outcome of the response.

The basic research in immunology is needed to answer the following:

- A better understanding of innate immunity.
- A better understanding of T-cell memory induction *in vivo*.
- Improved understanding of human protective immune responses to *M. tuberculosis* infection, including the potential role(s) of various T-cell populations and the molecular signals that activate the protective immune response.
- Identification and validation of surrogate markers of protective immunity in animals and humans.
- Screening of population for genetic mutations associated with disease resistance/susceptibility and subsequent immune response.
- Elucidation of mechanisms employed by *M. tuberculosis* and the host response against this to cause tissue damage.
- The role of antigens other than proteins (such as lipid and carbohydrate antigens) in protection against TB.

Understanding the latent form of TB

Latency and bacterial persistence continue to be the mysterious focus of infection with *M. tuberculosis* and require extensive study. *M. tuberculosis* is an enormously successful pathogen that has evolved numerous mechanisms for evading elimination by the host immune response. We are still in the process of learning which immune responses are important in controlling *M. tuberculosis* during the latent and acute phases of infection. Experiments reveal that *M. tuberculosis*-infected macrophages appear to be diminished in their ability to present antigens to CD4+ T-cells[18], which may be achieved by

down-regulation of MHC class-II molecules on the surface of the infected macrophages. The mechanisms for directing the organism to enter into latent phase or for subsequent reactivation are not clearly understood. Possibly, low oxygen tension in the granuloma may force the bacteria to use anaerobic metabolic pathways for production of energy. Activation of glyoxylic acid pathway in the pathogens on exposure to a hypoxic environment *in vitro* supports this hypothesis. Some genes have been identified as latent phase genes (*hsp X*), which seem to provide a helping hand to the bacteria to survive in the latent phase, but what conditions trigger the bacteria to enter latency are not well defined.

It has been well established that only 10% of the infected individuals develop active disease and the remaining 90% are able to overpower the pathogen. A better understanding of the immunological correlates of protection requires a comparative analysis of the immune mechanisms in systems that are capable of inducing a host protective immune response and those that are not. Comparative analysis of the various effector molecules such as IFN-γ, IL-10, IL-12, IL-4 and TNF-α, which have been assigned essential roles in the outcome of the immunological battle between the host and the pathogen, in both the healthy human subjects and patients, will go a long way in improving our under-

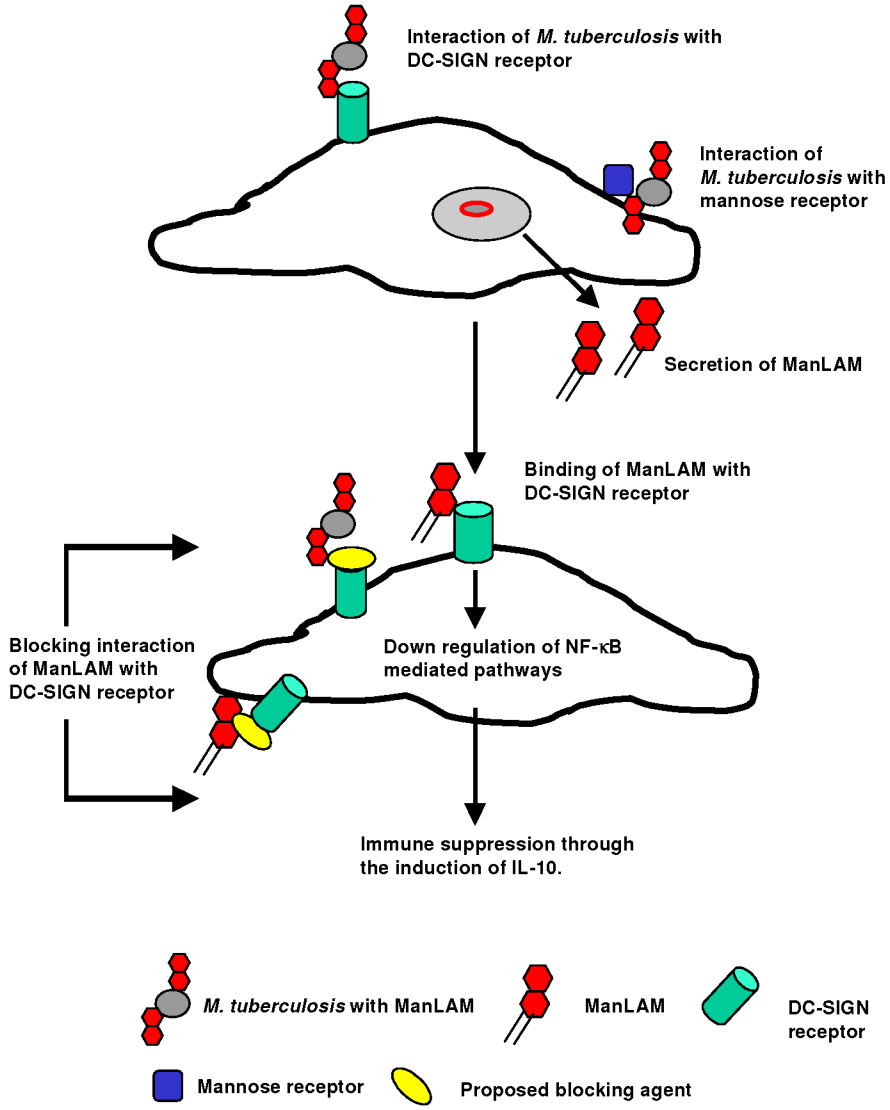

Figure 2. Role of DC-SIGN receptor in mycobacterial infection. *M. tuberculosis* also interacts with the DC-SIGN receptor on dendritic cells. Internalization is followed by a series of steps, including secretion of the virulence factor ManLAM. The latter interacts with the surrounding dendritic cells and down-regulates the NK-κB-mediated pathways, resulting in immuno-suppression. Blocking the interaction of *M. tuberculosis*/ManLAM with the DC-SIGN receptor presents an interesting strategy for preventing such an immuno-suppression.

standing of the strategies that are employed by the rivals to emerge triumphant.

Basic research is thus required in these areas for the following reasons:

- To understand the mechanism(s) employed by mycobacteria for their survival in the latent phase for a prolonged period of time.
- To understand the host immune responses during latent infection and reactivation.
- To understand the factors that govern reactivation of this latent infection.
- To develop diagnostic assays for latent infection.
- To develop diagnostic assays to distinguish between reactivation and exogenous reinfection.
- To develop better models of latent infection as close as possible to the *in vivo* situation with standardized protocol for establishing latency.
- To develop vaccines that can eliminate the reservoir pool of these latent mycobacteria from the people.

Identification of antigens as potential vaccine candidates

The success of the subunit vaccine approach has been shown to depend on both the choice of the delivery vehicle as well as the mycobacterial antigen that is delivered. Since the induction of a mycobacterium-specific immune response rests solely on the choice of the antigen included, the most careful consideration of a subunit vaccine is the selection of the antigen.

Several strategies have been followed for the selection of antigens for their inclusion as vaccine candidates. One of these is based on the inclusion of the 'immunodominant antigens' which are selected on the basis that they contain human T- and B-cell epitopes. Proteins present in the filtrates of *M. tuberculosis* cultures are also given preference for inclusion in candidate vaccines on the basis of assumption that the secreted proteins would be available for immune recognition at early stages of infection and the consequent early response would be associated with protection. Immunization with culture filtrate proteins as subunit vaccines was shown to confer protection comparable to that of BCG in the mouse model[37,38]. Similar results have also been observed in the guinea pig model[7,39]. Important culture-filtrate proteins include members of the antigen 85 complex[7,38,40,41]. Low molecular-weight proteins are also important targets for T-cells from infected individuals and from immunized animals[42,43], of which the 6-kDa protein has gained special attention[44,45]. Demonstration of an important role in virulence by proteins that are exported via specialized secretion systems in other bacterial pathogens, has led to the identification of two such proteins. These include ESAT-6 (ref. 45), and iron-containing superoxide dismutase[46–48].

The role of antigens other than proteins (lipids and carbohydrates) also needs a detailed investigation, because these are known to be potent inducers of cytokines and chemokines.

The sequencing of the whole genome of *M. tuberculosis* has led to the opening of the 'Pandora's box' with respect to the expanded pool of antigens. Some useful parameters to establish the antigen potentials include: abundance (relative amounts of different antigens and their protease sensitivity), location (access to antigen processing pathways), specificity (since the immune response is likely to get influenced by sharing of the immunological determinants) and natural immune response (recognition of antigens by T-cells from TB patients)[49]. 'Never underestimate your enemy'; this is particularly relevant when referring to an enemy as ominous as *M. tuberculosis*. Hence, these above stated criteria should not be an exclusive requirement because if there are 'protective antigens' which elicit a particularly effective immune response, mycobacteria can divert the attention of the immune response to some alternate antigens for their benefit[49].

Use of genomics and proteomics for identification of new candidates for vaccines

Availability of the genome sequences of two strains of *M. tuberculosis* and one strain of *M. leprae* promises to provide a solid foundation for comparative functional analysis and identification of the antigens that confer protection against TB. More than 100 vaccine candidates have emerged in the last decade alone. While the evaluation of the current crop of candidates should be carried out at a rapid pace, basic research in future should focus on the use of new technologies to identify new antigens that may serve as potential candidates for vaccine development. Library immunization facilitates the screening of the entire genome for protective antigens. Identification of gene products specifically expressed during different stages of infection is now possible through the use of techniques such as microarray. This may also help in the identification of antigens that are involved in dormancy and persistence.

While the identification of new antigens as prospective vaccine candidates is an essential first step, this will have to be followed by an understanding of their role in mycobacterial infection. In addition to comparative functional genomics, the microarray and proteomics technologies should be used for the following:

- Identification of new antigens.
- Identification of gene products specifically expressed *in vivo*.
- Identification of gene products expressed during different stages of the disease process.

- Development of simple diagnostic methods to distinguish between *M. tuberculosis* infection from vaccination and from environmental mycobacteria.

Lack of appropriate animal models

Koch recognized the spectrum of pathology of TB in different animal species. Examination of clinical specimens from infected humans, cattle, badgers and possums confirmed the extreme variation in the pattern of pathological reactions in different species[50]. Guinea pigs are innately susceptible, whereas humans, cattle, rabbits, mice and deer express varying levels of resistance depending upon their genotype. Although studies in laboratory animals such as mice, rabbits and guinea pigs have significantly enhanced our understanding of the etiology, virulence and pathogenesis of TB[51], they have limited use for the study of protective immune responses. While humans and ruminants are relatively resistant, fewer than five virulent organisms introduced by the aerosol route into guinea pigs can consistently produce lung lesions, bacteremia and fatal disease, thereby impeding the extrapolation of the results of such experiments to humans. Rabbits are relatively resistant to *M. tuberculosis* but like in the case of humans, produce pulmonary cavities as a consequence of the liquefaction of caseous foci and subsequent extracellular multiplication of tubercle bacilli. This model, however, is again limited by non-availability of the reagents.

Mice are generally the animal models of choice for studying the immunology of mycobacterial infections. It has come a long way in establishing the role of various immunological mechanisms, including those of Th-1, Th-2, and the role of CD4+ and CD8+ T-cells. However, the concept of a 'rational' animal model of TB is based upon the selection of a test system that mimics the important aspects of human disease. The resistance of mice to TB, however, represents one of the reasons for inadequacy of the mouse model. To add further ambiguity, different strains of mice exhibit variations in resistance. In this context, the similarity of the guinea pig model to the progression of clinical disease in humans offers several advantages[52]:

- There is reproducibility in the infection of guinea pigs by the pulmonary route and this can be attained with a small number of virulent human bacilli.
- The progression of the disease following pulmonary infection, including the stages of bacilemia and hematogenous reseeding of the lung exhibits extensive similarity to that in humans. The hypersensitivity responses of guinea pigs and humans to *M. tuberculosis* are also comparable.
- The similarities in the course of clinical infection between guinea pigs and humans allow us to model different forms of TB and evaluate the protective efficacy of the candidate vaccines in such systems.

The only limitation of this model, however, is the dearth of immunological reagents that are required for the qualitative and quantitative evaluation of the immune responses, with special reference to cytokines and cell phenotypes. Another limitation is the higher cost of guinea pigs compared to mice. However, its biological relevance more than justifies the efforts required to improvise the guinea pig model to ensure the following:

- It allows understanding of the role of environmental mycobacteria in skewing the efficacy of a vaccine.
- It provides understanding about the influence of earlier vaccination with BCG (any new vaccine will have to work with this common BCG background, at least in a large number of countries, if not all).
- It allows evaluation of protection due to reinfection as well as reactivation.
- It would enable us to carry out screening of new vaccine candidates in HIV-positive population.

Alternatively, if a candidate vaccine is found to be protective in humans, it can be tried out in various animal models. The animal model which mimics the results obtained in humans can then be considered as closest to humans.

Development of immunological reagents for guinea pigs

Until an alternate animal model is developed, efforts should be directed towards the enhancement of the guinea pig model. In this respect, the deficiency of immunological reagents to study the immune responses deserves attention. When challenge experiments are performed in guinea pigs, the evaluation of efficacy of a vaccine is based upon the ability of the vaccine to decrease the bacterial burden in the different organs. However, the immune response that is responsible for such a decrease cannot be evaluated. To study the underlying immune mechanisms of protection, the cytokines induced and the type of T-cells that are activated have to be identified. This requires various reagents and monoclonal antibodies. Although such reagents that aid the analysis of immune responses in mice are available, they need to be developed for the guinea pig model. This will go a long way in the identification of the immune correlates of protection. Development of reagents that allow the histopathological analysis of the fixed specimens of various organs will help in revealing the histological correlates of protection.

The current procedure for the evaluation of candidate vaccines is to evaluate the immune response in the murine model and generally choose those candidates that induce high IFN-γ levels and low IL-10 levels. Hence, the gene-

ral rule is to consider those candidates that are capable of inducing a Th-1 type of a response (which is evaluated on the basis of cytokine profile) and to proceed with those for challenge experiments in the guinea pig model[4]. However, in the present situation where distinct correlates of protection have not been validated, adoption of a reverse sequence for vaccine testing is being suggested[4]. According to this approach, the ability of the vaccine to protect against *M. tuberculosis* challenge is first evaluated and followed by the evaluation of the immune response induced. Besides, allowing for a way of detecting immune correlates of protection, it will also prevent the missing out of certain candidates that though are unable to generate the typical Th-1 responses, might be protective against mycobacterial challenge.

Development of an alternate animal model

Although monkeys are closely related to humans, due to high cost and handling difficulties, they have not been exploited for TB research to a large extent. Since all existing animal models fail to mimic the human disease perfectly, efforts should be focused on the development of monkey as an alternate animal model for TB. Since monkeys naturally develop TB, enhancement of the monkey model that perfectly mimics human TB will aid greatly in the screening of a large number of vaccine candidates for efficacy as well as toxicity studies.

Development of expression system/vaccine vehicle

Subunit vaccines can be directly used for immunization. However, in the case of several other forms of candidate vaccines such as recombinant BCG, auxotrophic vaccines, atypical mycobacterial vaccines and DNA vaccines, exposure of host immune system to the protective antigens is mediated via living or non-living carriers. Not withstanding the concerns over the use of live vaccines in potentially immuno-compromised individuals, live vaccines are still favoured as the experience with mycobacterial vaccines in animals indicates that viable organisms are generally more protective and better able to induce cell-mediated immune response. Thus, it is imperative that new expression systems be developed and the existing ones improved. Recently, *Salmonella typhimurium* has been used as a vaccine vehicle for development of a vaccine against TB. Several studies have used BCG as a vehicle of choice for the development of vaccines against TB and other diseases. BCG is one of the safest vaccine vehicles with a proven history of safety, as exemplified by its use for immunization of more than three billion individuals. Due to inefficiency of BCG to induce a significant CD8+ response, strains of BCG expressing listeriolysin have been developed and shown to result in a better MHC class I presentation of antigens. There have been questions regarding the safety of BCG vaccine in HIV-infected individuals, but there is not enough evidence for such complications of BCG in HIV sero-positive cases. Hence, this has to be verified with more studies. Expression systems are already available for expression of genes in BCG. Some of these vector systems make it feasible to regulate the level of expression based on the promoter chosen for transcription of genes. However, the plasmid vector has to be integrated into the mycobacterial chromosome for its continuous perpetuation without loss from the cell in the absence of an antibiotic. The presence of the plasmid in a single-copy format in this manner would lead to reduced expression of antigenic protein due to dosage effect. Thus, it would be preferable to have a high copy number plasmid vector in the extrachromosomal form, which would yield high levels of expression. For this, auxotrophic mutants of BCG should be developed and used to maintain the plasmid in the absence of an antibiotic. This can be achieved if the plasmid vector harbours a gene that complements the mutation. Besides, expression vectors should be developed which can express the genes for cytokines such as IL-2 and IL-12 to help induce the Th1 response in a preferential manner. To avoid prolonged expression of IL-2 and IL-12, which may create complications, it will be advisable to use non-replicating plasmids for such work. New expression systems should be developed to meet the special needs of the HIV positive population, if necessary.

Problems related to testing of vaccine efficacy in animal models

Experience with BCG indicates that factors other than the inherent potency of the vaccine play a decisive role in the outcome of vaccine trials. The same holds true for efficacy evaluation in animal models. The role of such factors was highlighted by an international collaborative study carried out by seven laboratories, each of which used its local animal model and test systems to rank seven vaccine candidates against TB. The results showed that no two laboratories ranked the vaccines in the same order and not a single vaccine was ranked top by more than three laboratories[53]. The factors responsible for such variable results include animal species (with the three common choices being mouse, guinea pig and primates), vaccination route, vaccine dose, vaccination-challenge interval, virulence of challenge strain, challenge route, challenge dose, challenge-necropsy interval and the determinants of host response[54] (Figure 3).

This anomaly can be resolved by arriving at a logical consensus regarding the choice of these factors. For example, only the aerosol route of challenge should be used which mimics the natural route of infection most closely. Extensive studies to establish the kinetics of the

immune response should form the logical basis for determining the vaccination-challenge and the challenge-necropsy interval. If the animal is challenged too soon after the vaccination, it is possible that the animal is still bathing in the cytokine milieu that is generated by the vaccination and this would be contrary to the situation in natural infection. The virulence of the challenge strain and the dose of challenge should definitely remain constant. Regarding vaccination route and dose, it can be varied and should be varied in order to arrive at the best possible option that is capable of providing protection. Restriction to any one choice is not a requirement for these variables since we can use the best-identified route and dose for any further trials. Unlike the route of infection that occurs naturally by the aerosol route and hence binds the researchers to use only this route for any extrapolation of the animal studies to humans, vaccination is in our hands and we can try out all possible choices to determine which one works the best. Regarding the determinants of the host response, potential endpoints of vaccine-induced protection should include the following:

- Prolonged survival.
- Reduction in clinically apparent disease (e.g. weight loss, fever and respiratory disease).
- Decreased bacterial burden in target organs (lymph nodes, lung, spleen and liver).
- Anatomical restriction of bacilli to the point of entry (i.e. prevention of extra pulmonary dissemination).
- Decreased histopathological damage in the target organs.

Human trials

Before a new vaccine can be recommended, it must be evaluated in human trials. But with the present status of TB cases, we cannot afford a large-scale, long-term trial. Previous trials were conducted on large populations and followed up over a period of ten to twenty years[55]. With rising mortality associated with this disease, we need to design trials that would answer our queries within a short period of time and which would also be statistically significant to show the vaccine efficacy as well as side

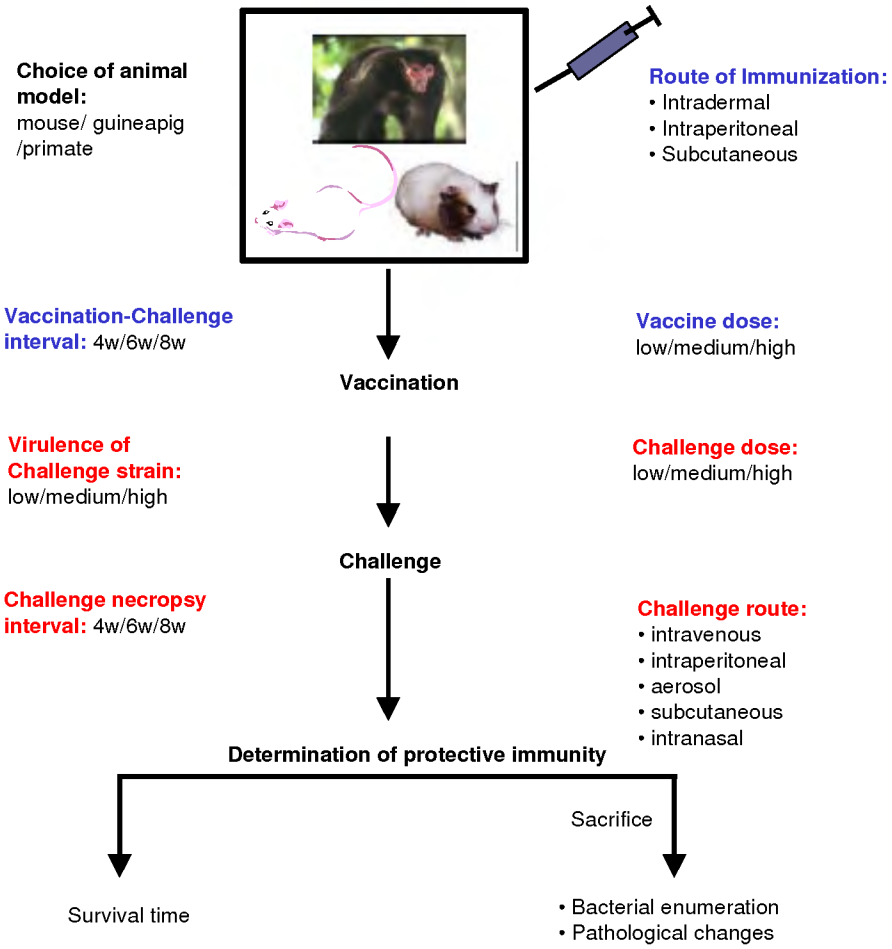

Figure 3. Variations in the screening of vaccine candidates. Reasons that account for the observed variations in the efficacy of a vaccine candidate are depicted.

effects. Several types of study designs can be used to evaluate a new vaccine in a human population. However, many issues have to be sorted out for the effective completion of trials[9]. First of all, the efficient translation of the basic research findings into the clinical arena would require a focused, coordinated effort involving multiple types of expertise. The needed participants include mycobacteriologists, animal model developers and vaccine testers, epidemiologists, clinical trial specialists and vaccine biologists, on-site physicians, nurses and support staff[9]. The establishment of multidisciplinary teams or networks will definitely go a long way in expediting the goal of progression from the laboratory bench to clinical trial. Complete evaluation of a vaccine would require three different phases of trial. In the first phase, the vaccine would be evaluated for the maximal dose that can be tolerated along with the evaluation of safety issues and adverse effects. The second phase would focus on further evaluation of safety issues in detail to point out any adverse effects that might occur at lower frequency, in addition to evaluation of immunological responses against different doses and regimens of the vaccine. The third phase is usually related to the evaluation of protective efficacy of the vaccine. However, TB being a complex disease, it is recommended that this phase of vaccine trail be carried out in three different formats[9].

Pre-infection vaccine trial

The objective of the pre-infection vaccine trail would be to evaluate if the vaccine can prevent primary infection or progression of a primary infection to disease, in addition to preventing a latent infection. This would be carried out by immunizing children or adults who are skin test-negative, with no prior infection with tubercle bacilli.

Post-infection clinical trial

A large number of healthy individuals are already infected with tubercle bacilli and can develop active TB at any point of time. The post-infection clinical trial would be designed to cater to this population. However, with slow progression of latent infection to the active disease, this format of trial would require a long wait of several years before its completion.

Total population vaccine trial

This format of trial would include non-infected as well as infected individuals (thus combining the features of the above two formats) and would be carried out in the populations that are subjected to a high risk of infection.

These populations should be screened epidemiologically as well as for known genetic markers, which would also be indicative of resistance/susceptibility to the disease[9]. To carry out effective trials, we require the following:

- Detailed epidemiological knowledge of TB infection in sites selected for the trial.
- Laboratory facilities for diagnosis.
- Laboratory facilities for immunological studies.
- Laboratory facilities for evaluating surrogate end-points.
- Trained personnel.
- Standardized protocols.
- Good coordination between participating centres.
- Quality control of reagents, methods and data.
- Supply of reagents.
- Extensive data interpretation and compiling facilities.

Such large-scale trials will also generate significant amount of data, which will help in determining the immunological correlates of protection and will also generate information on the rate of progression of the disease.

Continuous evaluation of upcoming candidate vaccines in existing animal models

In spite of all the existing limitations in the development of a perfect vaccine against TB, investigators all over the globe are continuously evaluating the already identified antigens. These efforts have also led to the availability of new technologies such as recombinant BCG approach and DNA vaccine approach. The developments of these candidate vaccines as well as the procedures to evaluate their efficacy have been empirical to a certain extent. It will be important nevertheless that these efforts are continued with a great vigour, as currently these represent our best chance to succeed. Simultaneously, efforts should be continued to generate knowledge that would be helpful towards a rational approach for the developments of TB vaccines (Figure 4).

Several approaches are being followed for the development of new TB vaccines, including strategies such as protein subunit approach, DNA vaccine approach, recombinant BCG approach, and atypical mycobacterial vaccines. New candidate vaccines developed should be evaluated in the existing animal models and optimization of promising candidate vaccines and delivery parameters should be carried out through expanded animal-model testing. The promising vaccine candidates then should be rapidly moved into clinical trials.

Concluding remarks

The task ahead is not simple, but we have the advantage of techniques allowing rapid increase in understanding of cellular immunity, knowledge of entire genomes of several mycobacterial species, including *M. tuberculosis* that provides access to the entire antigenic repertoire of the

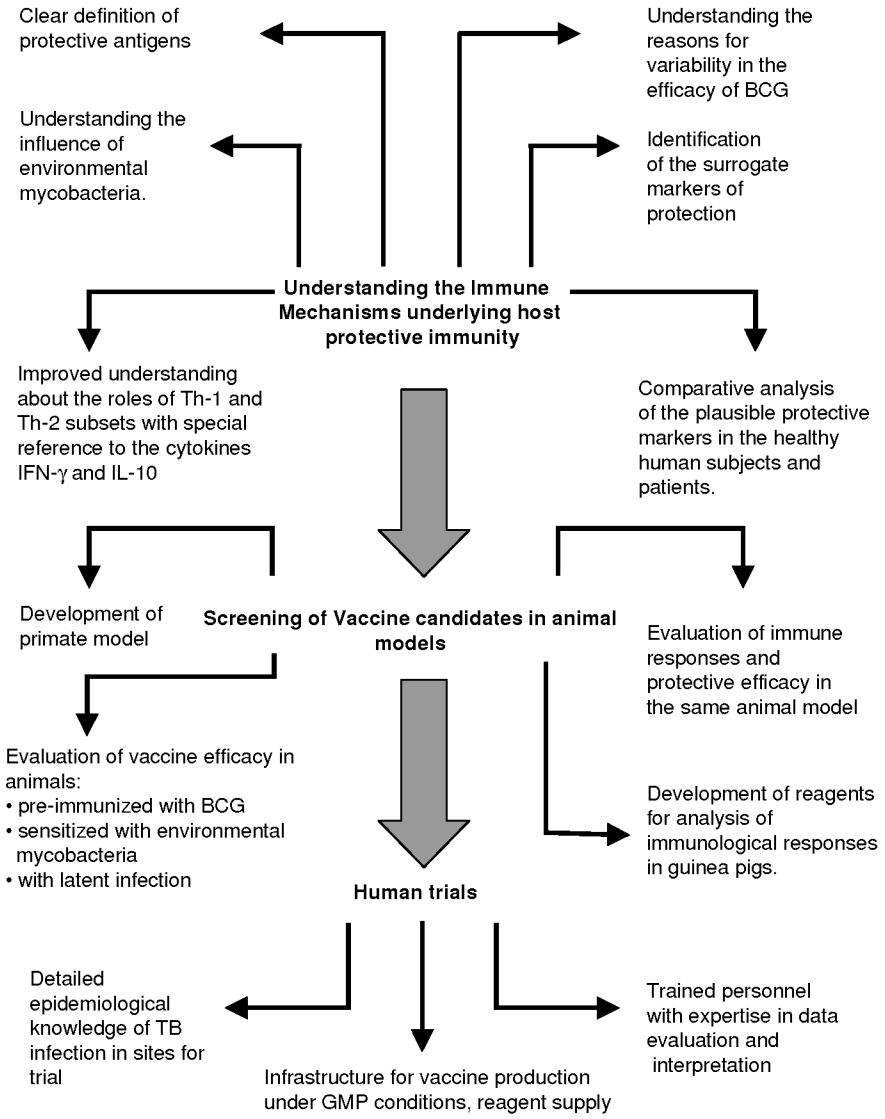

Figure 4. Inputs required towards a rational approach for the development of TB vaccines. Various aspects that require consideration, investigation and preparation towards a more informed approach for the development of TB vaccines are depicted.

tubercle bacillus and new approaches for studying global gene regulation patterns. Besides, we have new technologies for the development of vaccines and we have in the form of BCG, a proof that some vaccines do provide protection against TB in some populations. It is now essential that all the necessary resources are committed to develop an effective TB vaccine. Otherwise we will have to put ourselves up against more serious challenges by this dreaded disease.

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