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Development of new vaccines and drugs for TB: limitations and potential strategic errors

Ian M Orme

Department of Microbiology, Immunology & Pathology, Colorado State University, Colorado, CO 80523, USA

Abstract

The concomitant HIV and TB epidemics pose an enormous threat to humanity. After invading the host *Mycobacterium tuberculosis* initially behaves as an intracellular pathogen, which elicits the emergence of acquired specific resistance in the form of a T-helper-1 T-cell response, and involves the secretion of a myriad of cytokines and chemokines to drive protective immunity and granuloma formation. However, after that, a second phase of the disease process involves survival of bacilli in an extracellular state that is still poorly understood. This article briefly reviews the various strategies currently being used to improve both vaccination and drug therapy of TB, and attempts to make the argument that current viewpoints that dominate [both the field and the current literature] may be seriously flawed. This includes both the choice of new vaccine and drug candidates, and also the ways these are being tested in animal models, which in the opinion of the author run the risk of driving the field backwards rather than forward.

Keywords

BCG vaccine; cellular immunity; drug discovery; tuberculosis; vaccines

TB

TB is caused by the facultative bacterial pathogen *Mycobacterium tuberculosis* (it is usually referred to as an ‘intracellular pathogen’ but this is not fully accurate, as will be discussed below). Given the current pandemic and global burden [1] one can regard *M. tuberculosis* as a highly successful organism. In 2008, there were an estimated 8.9–9.9 million cases of TB, between 10 and 13 million prevalent cases of TB, somewhere in the region of 1.5 million deaths from TB among HIV-negative people plus an additional half million or more deaths among HIV-positive individuals. Most of the estimated number of cases (in 2008) occurred in Asia (55%) and Africa (30%). India and China alone account for an estimated 35% of TB cases worldwide (~2 million and 1.5 million, respectively) followed by South Africa, Nigeria, and Indonesia, all with approximately 0.4–0.5 million cases each. Not only is this putting an enormous strain on healthcare resources, but many cases are not being treated

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Tel.: +1 970 491 5777, Fax: +1 970 491 5125, ian.orme@colostate.edu.

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Adapted from [69].

Adapted from [47,50,53].

under the current Directly Observed Therapy programs (where a health care worker directly ensures the patient is compliant), and the great majority of patients with multidrug-resistant (MDR) TB are not being correctly diagnosed and treated [1].

There are thought to be approximately 0.5 million new cases of MDR TB per year [2–4]. There appear to be multiple potential mechanisms whereby such strains arise, but inadequate chemotherapy is often cited as a primary reason. At present (mainly because most basic science laboratories are loath to study them in virulence assays), it is unknown if MDR strains are any different to drug sensitive strains in terms of virulence, pathogenicity, subversion of immunity and so forth; indeed, one idea has been that as a result of becoming drug resistant the bacillus has ‘lost fitness’ [5,6]. This possibility was one of many serious gaps in our knowledge recently highlighted by an expert panel at the NIH [7], an article that sadly has had little impact.

Data to date seems to indicate that MDR strains do not appear to cause infection or disease more readily than drug sensitive strains, but HIV-positive individuals infected with MDR strains have a high level of mortality [8]; moreover, because HIV infection may cause malabsorption of (TB) drugs, this can actually lead to acquired drug resistance. As often as not, the number of drugs needed to be given daily to such coinfecting patients can be ten or more. TB has now become the leading cause of death in HIV-positive patients and in fact may accelerate the progression of HIV disease [9–11]. If the infection is MDR this can require up to 24 months of continuous drug therapy to be successful, compared with 6–9 months for drug sensitive TB. As a result, the cost of drugs can be up to 300-fold higher [12–14]. A further complication is that many countries have a limited ability to diagnose MDR TB [15]. Not surprisingly, the TB rate is falling in countries able to spend more money on health issues (e.g., Eastern Europe), or in higher income countries with less immigration and lower HIV rates. However, while control programs have significantly reduced mortality, there is no evidence as yet this has significantly impacted transmission rates or the overall incidence [16].

Of even greater concern, isolates are being found that are ‘extensively’ drug resistant (XDR), where the isolate is further resistant to fluoroquinolones and at least one of the injectable second-line drugs. The numbers are still low, but such strains have been found on all major continents. In a rather spectacular example, an outbreak of XDR in a Church of Scotland funded hospital in Tugela Ferry in the KwaZulu province in South Africa was rapidly fatal in over 50 patients [17], an event causing widespread concern [18]. The worldwide incidence of XDR is not known as yet, but a figure of approximately 40,000 cases has been proposed [19].

The bottom line is that we urgently need new vaccines to combat this growing menace. However, as I will expand upon below, the decision to focus on developing recombinant forms of BCG may not be the way to go, and the available data on the lead candidate is far from compelling. Similarly, we need to optimize the drugs we have, at least in the context of MDR TB, but even now the newest of the drug regimens recently recommended (by the TB Alliance, Berlin, Germany 2010) for treating MDR TB have only been tested in mice (which do not generate necrosis, a central site of bacterial persistence) and only against a modestly virulent completely drug-susceptible laboratory strain.

The host response to TB

Acquired specific resistance to TB is mediated by the orchestrated expansion of cell-mediated immunity, which seeks to control and contain the infection [20]. The observation that mice lacking T cells were highly susceptible to infection provided the first important clue, along with the observation that containment required the movement of monocytes

needed to initiate the granuloma out of the bloodstream [21]. Subsequent studies in gene-disrupted mice allowed the clear distinction between control (influx of cells mediating protection) versus containment (florid influx of macrophages to wall off the infection) [22].

In fact, in far earlier studies, it was shown that spleen cells from *M. tuberculosis*-infected mice transferred immunity, an ability that was lost if the cells were first depleted of T cells using a lytic antibody [23]. Soon after, these cells could be further enriched for CD4 and CD8 T cells, resulting in transfer of immunity not only by CD4 T cells as was predicted, but (surprisingly at the time) also by CD8 T cells [24].

Over the next decade cell transfer approaches were superseded by the development of gene disrupted mice, as well as the discovery of multiple additional cell subsets involved in host immunity. This has led to the realization that there are several other cell types that can at least contribute to immunity to TB, of varying levels of importance. For example, natural killer populations respond (probably very early on) and are a potent source of IFN- γ , however, if depleted, they are not critical [25]. Cells expressing the $\gamma\delta$ TCR also respond; they are sources of IFN- γ and especially IL-17 [26], and in their absence the integrity of the granuloma is altered and becomes more pyogenic [27]. Over the past few years, in addition, we have realized that two more CD4 T-cell subsets are not only involved, but may be centrally important. These are the Th17 cells, and Foxp3⁺ regulatory CD4 cells; both seem to play very important roles in controlling the overall inflammatory response (not always to the long term benefit of the host) [20,28,29]. Such incremental increases in our knowledge regarding these new subsets emphasizes the point that we still do not know with certainty the relative importance of each of (this growing list) of T-cell subsets.

CD4 cells are the master regulators of the host response to TB, emerging as effector cells, but also giving rise to effector memory and (probably to a much lesser degree) central memory T cells after the initial infection has been contained. By contrast, what do CD8 cells actually do, and how important are they? Reviews have a tendency to state that their role is unknown, but this is not correct. I was able to show by the classical T-cell transfer technique that CD8 T cells could transfer immunity in the mouse model (the reader should remember that at that time CD8 T cells were considered ‘suppressor T cells’) [24]. Several laboratories then found these cells to be a potent source of IFN- γ suggesting this was their primary function. These cells can also be cytolytic (at least *in vitro*, which is the only way to measure this) and opinion is divided as to whether this happens *in vivo*. I favor the concept that if CD8 cells were cytolytic, they would significantly contribute to lung damage. However, a recent study reaches the conclusion that cytotoxic T cells actually outnumber IFN- γ secreting CD8 in this model [30].

Studies then showed that mice deficient in the β 2-microglobulin molecule, and therefore incapable of any form of Class-I response, were highly susceptible to high dose intravenous infection with *M. tuberculosis* [31]. This was not an immediate event, with increases in the bacterial load in the spleen and liver only becoming apparent after 8 weeks, and was far less obvious in the lungs, presumably because CD4 mediated immunity had had plenty of time to be expressed. On the other hand, can the CD8 subset mediate protection by itself? My earlier studies showed that CD8 cells, that had transferred by themselves, had some limited degree of protective activity [24], and more recently it was shown that CD8 T cells induced by a DNA vaccine was sufficient to protect mice at 4 weeks after challenge, although this protection was already disappearing after another 4 weeks [32].

If one accepts the hypothesis that the role of CD8 T cells in the host response is that of an IFN- γ -secreting protective Class-I MHC-restricted T cell, then the next question is ‘when is this critical?’ The answer to that question was provided by studies in mice lacking CD8 cells

(or at least, lacking the CD8 molecule). After low dose aerosol infection no initial differences in host resistance could be seen in CD8-knock-out mice, indicating that the CD4 response is sufficient [33]. However, as the infection moved into the chronic phase of the disease, these mice started to exhibit a loss of control of the lung bacterial load, with necrosis and neutrophil influx starting to occur in this organ. This suggests that the key role of CD8 T cells is not in initial protection (although they obviously contribute) but in maintaining the integrity of the later-stage granuloma. How this works has not been further investigated, although a clue was suggested by the observation that an interesting facet of the mouse granuloma is the curious spatial distribution of CD8 T cells, which accumulate preferentially on the outer rim of these structures [34]. This does not appear to be just simply due to slower influx kinetics, and suggests instead that these cells stop at this point owing to chemokine signals (or lack of them).

Animal models of TB

There are several well-established animal models of TB (reviewed in [20,35–37]). Even today there is still an element in the field that promotes ‘our model is better than your model’ but as I have pointed out before [35,38], each model can provide useful information provided that it is recognized. To do this, however, one must weigh the deficiencies of each model as well as its relevancy.

There are three primary factors driving such models: cost, reagents and access to level-III biosafety facilities. As a result the mouse model has provided the great bulk of information, given its low cost coupled with a huge arsenal of immunological reagents. Nor is this model static, with new strains becoming available that model susceptibility to disease [39,40]. Further information has been gained from the rat model [41,42] and the rabbit [43,44], which represent the two extremes of resistance and susceptibility. At the top of the size scale is the cow, and this model has also provided useful information [45,46].

Particularly in the context of testing vaccines, the guinea pig and nonhuman primate (NHP) models are of importance. Guinea pigs have a long historical record (they were used by Koch) and they develop pathology similar to that seen in infected humans [47–50]. For many years information coming from this model was based almost solely on bacteriology, but this has changed in the last decade due to the development of real-time-PCR assays (almost single-handedly) by McMurray [51], the first description of flow cytometry for this species by Ordway [52], and the first comprehensive descriptions of the pathological process [53,54], including descriptions of elements of this process never previously appreciated [47]. In addition to its classical use for vaccine testing [55] it has recently been used to test drug regimens [56], which required solving some daunting animal husbandry problems.

The NHP models are the logical end point for vaccine testing, and this model has undergone considerable refinement over the past decade [57–60]. In addition, NHP infected by bronchoscope have been shown to develop lesions/symptoms that are potentially consistent with a state of latency (a state lacking in small animal models of the disease) [59]; although one should note that a more recent study, in which the infection was given by a more realistic aerosol exposure method failed to reproduce these findings, showing instead that the successful establishment of infection was dose-dependent, with all animals subsequently developing active disease [61].

In fact, many species can be infected with mycobacteria, and may not always require biosafety conditions needed for *M. tuberculosis*. These include goldfish, frogs and zebrafish. The zebrafish model is of course very cheap, and it may prove very useful in genetic screens [62] but it cannot be infected with *M. tuberculosis*, instead the cold-adapted saprophytic

organism *Mycobacterium marinum* is used. This animal also lacks lungs, but I will leave it to the reader to decide if this deficiency has importance.

Pathogenesis of the disease process

Reviews often describe the mouse granuloma as ‘disorganized’ and the guinea pig granuloma as ‘organized’; in fact the reverse is true. In the mouse, these structures are not necrotic, and fill with well-organized aggregates of lymphocytes (B cells as well as T cells) and macrophages [63]. Most of the T cells are CD4, whereas CD8 cells also aggregate, but usually on the perimeter of the granuloma [34]. In guinea pigs CD4 and CD8 seem to mix randomly, and are pushed to the periphery by the developing central necrosis [54]. As this continues to develop the actual numbers of T cells declines, as shown by flow cytometric analysis [52].

I propose here a different model in which the two elements, the cellular response and the lesion necrosis, develop separately but then converge. As recently reviewed in [47], there is probably a lot going on early in infection that we still do not fully understand, and this occurs long before the T-cell response arises. In fact, it is now well understood [64,65] that bacilli must be carried to the draining lymph nodes (presumably by dendritic macrophages, of which there are plenty in the lungs) to sensitize T cells, and this will obviously take a finite amount of time to happen.

What is becoming increasingly apparent is that there is an early response involving granulocytes, which probably arrive very early in response to the local inflammation. Whether these can actually kill the bacilli is unclear (perhaps sometimes, as an early study by Pedrosa *et al.* on this issue showed [66]), but they can certainly release oxygen radicals, and as they die probably release nuclear material (neutrophil extracellular traps) that could serve as a scaffold within which extracellular bacteria can survive and even potentially build small clusters or bio-films [Basaraba R, Unpublished Data]. However, a major consequence of the radical production, is damage to the local vasculature, which begins to bleed and break down [47,50]. This, I would propose, is a key event in defining the structure of the lesion; T cells and macrophages are now arriving but their influx can only happen on the periphery of the damaged vasculature, hence the developing ‘circular nature’ of the granuloma. In other words, I believe that these two sets of events are developing separately; despite its continuing popularity [67–69], I see no evidence that the developing necrosis is driven by an exuberant T-cell cytokine/chemokine response. In fact, how could it?

These events initiate a vicious cycle of increasing inflammation and necrosis, with a second, probably much larger wave of neutrophil influx after the T-cell response nose-dives, an event that occurs after 30–40 days in the guinea pig model, as now clearly shown by flow cytometry [52]. What happens to the T cells? Are they short-lived owing to a lack of antigen, the remaining bacteria now being trapped in the caseum? By this point there is a developing periphery of fibrosis, a thin, disorganized area in which lymphocytes can still be seen in lower numbers, a thin acellular eosinophilic area, and then a central area of necrosis with growing dystrophic calcification [47], see Figure 1. In addition, draining lymphatic vessels also become filled (and presumably blocked) by granulomatous inflammation [70].

Both the acellular region and the central necrosis contain significant numbers of bacilli, both single and in small clusters [71]. Differential staining reveals that at least three populations may be present, as described in [72]. In our previous studies using chemotherapy, we thought these remaining bacilli had to be dead, given our colony-forming unit counts, but we are now faced with the troubling possibility that at least some of these are alive but ‘unculturable’ (Figure 1). This critical point is discussed further below.

The prevailing view of the pathogenesis of TB, leading to granuloma formation and necrosis, has changed little over the past few decades, and was described recently in May 2010 in a special section in the journal *Science* [69]. In this model, inflammation drives cellular recruitment and granuloma formation, with extensive neovascularization of this structure. Lymphocytes are kept at the periphery, with the center of the lesion gradually developing necrosis. In humans (and in guinea pigs infected with virulent clinical strains) such lesions can cavitate, releasing bacilli into the airways.

I believe that several elements of this model are incorrect. First, lesion necrosis, often quaintly referred to as due to ‘excessive delayed type hypersensitivity’ (despite the absence of T cells), is regarded as an end point event. Instead, as noted above one can easily argue it is a very early event, visible as early as day 10 [54], and develops independently of the generation of acquired immunity. Second, granulomas do not become neovascularized, quite the reverse, and it is the collapse of the local vasculature, driven by early oxygen radical production, that sets up conditions in which bacilli can persist, T cells (and drugs for that matter) fail to penetrate, and the local oxygen tension drops dramatically [73]. Far from being a ‘balanced state’ as the aforementioned review argues, there is a progressive degeneration of the lesion, to which both the bacillus and the host tries to adapt. In the case of the bacillus, survival in the caseum is critical to subsequent transmission (a bacterium in a phagosome in a macrophage in an early granuloma is not going to infect anybody else) and it adapts accordingly [74]. At the host level, T-cell immunity drops off alarmingly (either owing to lack of antigen or downregulation) and so the host reverts to dystrophic calcification to try to seal off the lesion. A comparison of these two models is described in Box 1.

Box 1

Pathogenesis of TB: two viewpoints

Popular model†

- Resident macrophages ingest inhaled bacterium
- Infected cells invade subtending epithelium
- Monocytes arrive from blood
- Site becomes extensively neovascularized
- Macrophages differentiate into epithelioid cells, multinucleate giant cells and so on
- Fibrous cuff begins to form around structure
- Lymphocytes restricted to outer areas
- Granulomas persist in a balanced state. Some suffer loss of vascularization, show increased necrosis and accumulation of a central caseum
- Cavity collapses into the lung, bacteria are released

Alternative model‡

- Macrophages ingest bacteria, spread and adhere to the alveolar epithelium. Bacilli replicate, break through to underlying interstitial space using ESX-1 secretion system
- One or more bacteria picked up by dendritic cells, taken to lymphatics and lymph nodes (causing rapid disease in the latter)

- Early lesions contain neutrophils, macrophages, a few lymphocytes. Fibrin exudation starts
- Neutrophils and macrophages release reactive oxygen radicals; these have little effect on the bacteria, but initiate irreversible damage of the local vasculature. Small microfoci of necrosis appear
- Cell death occurs. Dead neutrophils release neutrophil extracellular trap material (potential attachment scaffold for bacilli). Macrophages ingest dead ones, forming multinucleate giant cells. Granulomatous inflammation now extends into lymphatic vessels. Collagen deposition begins on periphery
- Necrosis coalesces to form central sphere. Disorganized (disrupted?) groups of CD4, CD8, and B cells flux around this central structure
- Lesions steadily progress, coalesce in some cases. Dystrophic calcification begins. A second wave of purulent neutrophil influx occurs. Secondary (non-necrotic) lesions in the lungs become apparent
- Substantial numbers of bacilli survive in acellular rim and central caseum (even after chemotherapy). Some are in small clusters (biofilming?)
- Some lesions erode into adjacent airways or blood vessels, allowing dissemination and escape

Current drug pipeline

While epidemiology models indicate that a highly effective vaccine would boost the prevention of TB (something we are nowhere near achieving) early diagnosis and effective drug therapy are the top priorities for disease control [75]. However, the drugs we regularly use have been in use for four decades, and the recent emergence of MDR and XDR forms of the disease pose an extremely dangerous threat. Given the ease of air travel, this is a global problem, not a developing country problem.

If one is lucky enough to contract drug-susceptible TB, the treatment is well established and consists of rifampicin, isoniazid, pyrazinamide and ethambutol for 2 months, and rifampicin and isoniazid for the following four. While there are some (often dogmatic) statements about the basis of this treatment choice, the reality is that we still do not completely understand exactly how some of these drugs are actually working (see the literature on the molecular action of isoniazid as an example). This argument also extends to persisting bacilli that re-grow after chemotherapy has ended, resulting in disease relapse, and our animal models (of ‘sterilizing drugs’, ‘persistence’, and the like) may be deluding us (see below).

When such regimens fail there are second-line drugs to fall back on. Many of these are toxic, and expensive, and often require directly supervised therapy to ensure compliance [76]. There are various categories, including new generation rifamycins, injectable agents (such as kanamycin and amikacin), fluoroquinolones (gatifloxacin, moxifloxacin), oral bacteriostatics (cycloserine, ethionamide) and more modestly efficacious drugs (clofazimine, linezolid). In the context of MDR and XDR, regimens based on these drugs require long duration, have high toxicity, poor tolerance, high cost and poor outcomes [77,78].

The good news, such as it is, is the development of a drug pipeline by the Stop TB Partnership and allied entities. As a result, approximately a dozen compounds have reached

clinical evaluations, and a far larger group are at various stages of discovery, preclinical or clinical development (see [76,79] for a far more comprehensive description).

Recent reviews on this topic contain the usual mantra. We need simple, optimized regimens that are effective, potent, that will reduce the duration of therapy and minimize relapse of the disease (in other words, sterilize persisting bacteria). Such regimens will be badly needed for MDR, and now XDR TB. There is reason for optimism here, given the above pipeline, but where optimism diminishes is when we consider that when such compounds get to the animal testing stage the number of laboratories capable of *in vivo* testing is sadly minimal (and decreasing), not to mention the considerable inter-laboratory differences in the ways such tests are done [80]. Even laboratories capable of large scale *in vivo* testing balk at the idea of including MDR strains in such testing, relying instead on laboratory strains only. With increasing identification of active compounds, and fewer resources to test them properly *in vivo* against relevant (high virulence) clinical strains; well, the reader can do the math.

It is also completely baffling to some of us just how some decisions to take experimental regimens to clinical trials are actually made. Two examples come to mind. The first consists of the observation in mice that as the bacterial load in the lungs gets to relatively low levels, a regimen consisting of rifampicin/pyrazinamide plus moxifloxacin was superior to the standard RHZ regimen [81]. Our own laboratory could not reproduce these findings, and neither could a (presumably expensive) clinical trial [82] (one should note however, that replacing isoniazid with moxifloxacin would presumably be efficacious against isoniazid-resistant infections). The second example is more disturbing. Four studies had provided solid evidence from three separate laboratories clearly showing that the drug metronidazole had no activity against *M. tuberculosis* [83–86]. In our hands, in a guinea pig study, not only was this drug inactive, but it was toxic and seemed to be increasing the severity of lung necrosis [85]. However, in another study, in which rabbits were infected with a virulent strain of bovine TB, this drug was found to be ‘highly effective’ [73]. As a result, metronidazole was added to a treatment regimen for MDR patients in South Korea. As we understand matters, this trial has been abandoned.

One would think that a sensible plan to establish and conduct clinical trials would arise only after a consensus that animal studies clearly warranted this; that is, more than one laboratory, and preferably more than one animal species, illustrated efficacy. Not only is this not happening, but one can argue that basic scientific information from *in vivo* observations is taking a back seat to what funding organizations appear to want to hear; moreover, this is destructive because it just fuels the argument made by those who think animal models are of no value.

Enigma of the persistent ‘latent’ bacillus

The fact that certain individuals harbor bacteria in some state of persistence or latency is incontrovertible. In classical studies this was usually observed in elderly people, who presumably were exposed many decades earlier before they reactivated [87]. As the HIV epidemic arose, many exposed (much younger) people suffered from reactivation disease. This reflects the fact that people who are exposed to TB but are not actively infected are at risk of harboring latent disease [88,89]. Such people have an approximately 10% chance of developing active disease in their lifetimes, but this risk goes up alarmingly if they then contract HIV. A further, now very large, reservoir of people harboring ‘persistent’ bacilli, are those that have completed a drug treatment regimen, but then develop secondary TB. This often represents relapse of disease, but in the current era it can also be as a result of

being exposed to a new isolate of the bacterium, an event that is becoming more and more prevalent [90].

What are these 'persistent' bacilli, and where are they exactly? Such questions were initially framed in the context that such bacteria were somehow 'asleep', a concept I have previously challenged [91]. In fact, there are two opposing viewpoints regarding the ability of bacilli to persist despite chemotherapy; the 'drug tolerance' theory, and the 'location' theory. The latter idea, the reader might note, gets little attention despite that (in my view at least) it provides a much simpler explanation.

In the prevailing theory [88,92,93] bacteria have to undergo some sort of adaptation allowing them to survive drug treatment, and multiple mechanisms have been suggested as to how they do this. By contrast, however, over the past few years the pathology of the disease process has been studied in the relevant guinea pig model, including in the context of drug treatment, and these observations instead seem to be supporting the 'location' theory. A careful examination of the granulomatous process in this species shows the development of a 'rim' of acellular debris adjacent to viable (and presumably normoxic) tissues, a structure that starts to become evident after approximately 20–30 days of the disease process, inside of which is an area of central necrosis in which dystrophic calcification is progressing [47,50]. There is evidence that that this necrosis is initially triggered by oxidative damage to the vasculature, further promoted by neutrophil influx. After 30–40 days or so, a central region of necrosis has visibly developed, and the associated inflammation now exacerbates this by attracting in a further, much larger wave of neutrophils, as flow cytometry clearly demonstrates [52]. These cells are not especially noticeable under the microscope and thus we think these cells are dying after crossing the viable tissue region into the more hypoxic rim (they would not be able to get any further). There appear to be a large number of bacteria present, both in this rim region and in the central caseum itself.

In fact, a newly developed staining protocol [72] reveals very large numbers; many of these are single cells, but there are also clumps of up to 20 bacilli evident. This study combined auramine-rhodamine staining with immunofluorescence targeting bacterial proteins using an anti-whole cell lysate polyclonal antibody. Evidence was obtained in lung sections from at least three populations, which were either exclusively acid-fast positive, exclusively immunofluorescent positive or acid-fast and immunofluorescent positive.

This eosinophilic rim of residual necrosis is still evident even after chemotherapy [71], and continues to contain acid-fast bacteria. In fact, it was observed some time ago that chemotherapy readily clears the infection in secondary lesions, but not so in primary lesions where this residual necrosis remains [94]. Are these remaining bacilli the elusive persisting/latent population? They are by now extracellular, swimming in a carbon-rich soup left behind by the dead neutrophils, and while almost certainly in a hypoxic environment are not that far away from normoxic tissues. Another, recently identified, aspect of these necrotic regions is the apparent accumulation of ferric iron in these same areas, potentially directly by siderophore or ferritin release by these bacteria [95], presumably to support bacterial enzyme systems. As for the clusters or clumps of bacteria that can still be seen in this rim, could these be some sort of primitive biofilm? New evidence suggests this is a real possibility [96]. Therefore, one can easily imagine that the bacteria in these areas of residual necrosis continue to survive because the vasculature has been destroyed and drugs cannot penetrate in sufficient concentration into this necrosis, coupled with the possibility that even if they do, they cannot penetrate the putative biofilm, especially if this releases a shell of mycolic acid as the *in vitro* data shows [96]. Hence this 'drugs don't get to the bugs' hypothesis is not only very simple but it also does not require the bacillus to undergo any

sort of drug tolerance adaptation; moreover, it also easily explains the outcome of the classical Cornell mouse model. In addition, one must face the disturbing fact that if drugs cannot penetrate, vaccine-induced T cells certainly could not either.

In the Cornell model mice were treated with drugs until no bacteria could be cultured from the organs of these animals. Then, after being immunosuppressed, many reactivated. If some sort of clustering or biofilming does indeed explain why these bacteria survived, it also raises a serious worry about the way we test drugs in animals. In our guinea pig model, as an example, we can record colony-forming unit levels at or just above zero after 'successful' chemotherapy, but staining of primary lesions at this time still reveals an apparently far higher number of bacilli in these sites. Admittedly, many are probably dead, but we make this assumption because we cannot culture these on agar. If they are truly bio-filming, and hence will not grow planktonically, then this means we are seriously undercounting the real number of bacteria that are still surviving in these lesions in the standard assays the field currently uses.

These alternative ideas also have relevance in drug discovery aimed at these persisting bacteria. There has been a considerable emphasis in the recent literature promoting the idea that persisting bacteria are under anoxic (rather than hypoxic) conditions in which drugs like metronidazole should work well (as it does in the *in vitro* Wayne model). The fact that the recent trial did not work not only tells us that the Wayne model does not reflect reality *in vivo*, but also seriously questions the high-through-put drug screening systems being used that also make this assumption. There is no doubt that a new drug that efficiently destroyed 'persisting' (hypoxia surviving) bacteria that could be added to conventional regimens would be of great value, but it seems that the high-through-put assay systems are making a series of assumptions that may not actually reflect reality. This is illustrated in Box 2.

Box 2

High-throughput screening systems for 'anti-latency' drugs: a critique of current strategies

Favored systems

- Intracellular screens (in macrophages or cell lines)
- Carbon starved
- Anoxic environment
- Assumes dormancy

Alternative systems

- Persisting bacilli are almost certainly all extracellular
- Dead neutrophils release cell membranes, cholesterol, and so on, providing a carbon-rich medium in which extracellular bacilli can survive, and even potentially form biofilm
- Bacilli are spread out across a hypoxic gradient, the rim of which is close to viable tissues under conditions that must be approaching normoxia
- Persisting bacilli are probably trying to avoid using as much energy as possible by keeping replication to a minimum, accumulating iron. Their ATP synthase is still operational, explaining the rapid action of the drug TMC207

I believe these approaches have the potential to move us away from the central goals of the current research field, and may be directing future research investment unwisely. In my experience, even the best and most potent drug regimens do not fully sterilize all treated animals, and some of these then undergo reactivation disease. Whatever the viewpoint of the reader (whether you believe it to be drug-tolerance or biofilm-like microclusters) unless we face the issue of these ‘persisters’ all our endeavors may be futile.

Current vaccine pipeline

As with drug discovery, there is a considerable vaccine pipeline, and in fact this has steadily developed over the past two decades. Despite this, the pyramid of ideas and candidates has become very narrow at the apex of this pyramid, and it seems to this observer that not only will many of the very innovative ideas proposed over this time never reach fruition, but the current dreadful funding environment will kill any new innovative ideas dead in their tracks (astonishingly, a very recent article on this topic [97] described the field as ‘re-invigorated’). At the Global Forum on TB Vaccines conference held in September 2010 a questionnaire was circulated as a prelude to publishing a ‘Blueprint for TB Vaccines’ document, and it will be illuminating to see if other workers in the field share my viewpoint.

As this field grew, it became apparent that there were multiple types of vaccines that had some sort of protective response, and which could potentially replace BCG. This was necessary, most agreed at the time, because BCG simply did not work. There were some positive effects in children, but this was completely lost as the vaccinated individual approached adulthood. Now, a decade or so further on we appear to be back at square one, with an improved recombinant (r)BCG as the prime candidate.

The spectrum of candidates is in itself very impressive, and was initially driven by the ‘must replace BCG’ idea (an idea that might soon reappear, see below). Ideas, and subsequent candidates, ranged from using key secreted proteins, which soon transformed into the manufacture of fusion polypeptides, to DNA-based vaccines (an idea that died owing to the concern that these would not work in larger animals, including humans), to the use of viruses to deliver key antigens, all the way to taking the *M. tuberculosis* organism itself and removing genes to make it less virulent or auxotrophic [38,98–102].

To give a few examples, HyVac-4 is a fusion of Ag85B and TB10.4 and gives excellent results in mice using IC31 adjuvant [103]. M72 (or Mtb72F) consists of a 72-kDa polypeptide genetically linked in tandem in the linear order Mtb32(C)–Mtb39–Mtb32(N) [104]. It was first identified in a murine screen conducted by the author under the NIH TB Vaccine Contract program at Colorado State University, CO, USA. Further studies in guinea pigs illustrated its potency further [104], and it was shown to significantly prolong the survival of guinea pigs in a BCG prime boost model compared with BCG alone [105]. In the latter case, in some animals the lung pathology even suggested disease reversal. More recently, it was tested in an AS02A adjuvant formulation in the cynomolgus monkey model [106], where it was immunogenic and caused no adverse reactions. In a BCG prime boost protocol protection superior to that afforded by using BCG alone was achieved, as measured by clinical parameters, pathology and survival, and as in the previous guinea pig study evidence of disease reversal was observed. The immunological pro-file in these animals indicated IL-2, IFN- γ and TNF- α secretion by T cells, suggesting central memory generation.

A new generation vaccine, ID93, was recently described [107]. It is a fusion protein using antigen targets implicated by human T-cell line recognition analysis [108,109], in this case Rv1813, Rv3620 and Rv2608. When administered with the adjuvant GLA-SE, a stable oil-in-water nano-emulsion, this vaccine candidate was shown to be immunogenic in mice,

guinea pigs and cyno-molgus monkeys. In mice, the ID93/GLA-SE combination induced polyfunctional CD4 Th1-cell responses and protected these animals from challenge with virulent strains including an MDR isolate [110]. Similar results were observed in guinea pigs. What is particularly important is that this candidate was identified by screening cloned human T-cell lines and contains none of the 'immunodominant' antigens, yet it works. Avoiding these may be critical, as explained below.

A potential drawback to the efficacy of any nonliving vaccine is the need for adjuvant delivery vehicles; these can be complex to formulate (liposomes, for example) and also very expensive to make. Progress is now being made on less expensive materials including synthetic derivatives of monophosphoryl lipid A; this material is a good Th1 adjuvant because it signals through TLR-4, and can be further formulated with other materials, including TLR9 agonists to drive a strong Th1-type immune response.

Using nonreplicating viruses to deliver antigens, either as prime or boost, continues to be very promising. The author has previously expressed his admiration [38,111] for the team leading the development of the modified vaccinia Ankara (MVA)85 vaccine candidate, and their careful, sequential testing of this virus in animal models and then humans. However, as this line of research progresses, additional data regarding the immune response to this candidate is growing, not all of it positive. Recently, it was observed that following vaccination with MVA85A, antigen-specific IL-17A-producing T cells were induced in the peripheral blood of healthy volunteers [112]. These T cells appeared later than IFN- γ ⁺ cells. In individuals with pre-existing immune responses however, there were higher numbers of cells with a regulatory T-cell phenotype, and a concomitantly lower number of Th17 CD4 cells [112]. A new strategy to possibly improve this further (in mice at least) used MVA expressing both the cytokine IL-15 (to boost memory T cells) plus an ESAT/Ag85 fusion in a prime boost study. This improved the number of 'polyfunctional' T cells, but otherwise was not any more protective than the BCG control [113].

One would think that solid, reproducible animal efficacy data would be absolutely essential before any vaccine candidate would advance to clinical trials, let alone climb to the very top of the pipeline (of course it depends on who controls that pipeline). Certain candidates, notably MVA85, rBCG30, HyVac-4, and Δ ureC hly⁺ rBCG, have undergone very considerable animal efficacy testing for all to see. While I am certain the lead Aeras candidates have also undergone appropriate testing, it is actually hard to find much data in the literature.

The lead rBCG (Aeras-403) consists of BCG expressing the (pH-independent) perfringolysin molecule, coupled with overexpression of the molecules Ag85A and Ag85B, and TB10.4. In a mouse study it was shown that control mice infected with the W-Beijing strain HN878 lived nearly 300 days [114], this was improved if they were first given BCG, and this survival was improved further if they were given Aeras-403 instead.

If the astute reader finds this result strange, then he/she is not alone. When HN878 was first tested (by Kaplan) it was described as 'hypervirulent' [115]. This description is apt; it is highly virulent both in mice and guinea pigs. In our hands, HN878 kills mice (infected by low dose aerosol) in approximately 75–90 days [29]. This isolate is a very potent inducer of regulatory T cells [29], and this completely interferes with BCG vaccine-induced immunity. While mice are protected at day 30, by day 60 this protection is completely lost [116; ORWAY DJ AND ORME IM, UNPUBLISHED DATA]. Thus, to claim that control mice live 300 days is baffling.

The insertion of a membrane perforation molecule in Aeras-403 is based on an earlier elegant study by Grode *et al.* [117]. They inserted the gene for listeriolysin into BCG; the

idea here was that this lysin is known to drive the pathogenesis of listeriosis by perforating membranes, and thus could promote escape of antigens into the cytoplasm and thus into the Class-I pathway.

To their credit, this group compared the protective effect of the Δ ureC hly⁺ rBCG against both the laboratory strain H37Rv and a W-Beijing strain. The authors stated that in the H37Rv challenge model the rBCG showed greater protection, but the results they present only show a minor difference between the rBCG and the control BCG at a very late time point; other than this there was no difference at all. In mice challenged with the W-Beijing strain the BCG control was initially protected but this was soon lost, whereas protection by the rBCG was extremely rapid and sustained. This is an important observation, with the caveat that the growth kinetics of the two challenge infections were identical (i.e., the clinical strain was not of any higher virulence than the laboratory strain, growing only to ~5.2-log despite deposition of >200 bacilli in the lungs).

Whereas Aeras has clearly made the decision that CD8 induction is a critical component of a new rBCG vaccine, and have thus engineered Aeras-403 to include an endosomal membrane disruption molecule, one can easily argue that any hard evidence that a CD8 response to a vaccine is essential is actually hard to find. In fact, is endosomal disruption actually needed? It seems that *M. tuberculosis* at least can promote a perfectly good CD8 response without this, as I pointed a few years ago [38], cross-talk between Class-II endosomal presentation and Class-I is actually a well-known phenomenon. One such mechanism involves Sec61, a protein translocation channel complex that is present on the phagosomal membrane where it can transport proteins from the phagosomal compartments across to proteosomes where peptides ready for Class-I presentation can be processed. Moreover, a recent study revisited the idea that virulent strains of *M. tuberculosis* can actually progressively translocate from phagolysosomes into the cytosol in nonapoptotic cells [118]. This event was dependent upon secretion of the mycobacterial gene products CFP-10 and ESAT-6. This resulted in significant cell death within a week. This is consistent with an earlier study showing that infection of macrophages with virulent strains causes necrosis rather than apoptosis [119].

At this time, Aeras has pushed ahead with NHP and clinical evaluations of their vaccine. A recent study compared BCG Danish with the Aeras-403 (rBCG/AFRO-1) candidate, with boosting provided by two subsequent immunizations with Aeras-402 (a nonreplicating adeno-virus-35 expressing the same antigens), in rhesus macaques [120]. The primary conclusions of this study were that Aeras-402 induced qualitatively and quantitatively different cellular immune responses as compared with BCG in the vaccinated monkeys. While this was so, the actual variation between monkeys was considerable; some responded and some did not in virtually every assay performed. However, two animals did show evidence for multifunctional T-cell generation, which was promising. However, since there was no data supporting a sustained memory CD4 T-cell response, this study instead emphasized responsiveness by CD8 $\alpha\alpha$ T cells, which the study regarded as encouraging. The reality though is that this is an unusual and poorly understood subset of CD8 T cells. There is some suggestion it is a memory T-cell precursor (in a study of acute viral infection in mice, and in a tumor study in humans) and one study showed these to be CCR7^{lo}, again consistent with an effector memory T-cell subset [121]. More analytical studies have shown it recognizes nonclassical MHC class I molecules (TL) and that while CD8 $\alpha\alpha$ cells can bind presenting molecules at equal frequencies to CD8 $\alpha\beta$, the β chain is essential to correct engagement of the MHC/peptide complex and to correct signaling [122]. Further studies suggest that CD8 $\alpha\alpha$ represents a differentiation stage that leads to negative regulation of T-cell activation [123]. If this is so, one could speculate that if these macaques in the above study were challenged, they would have no resistance at all.

In a more recent report, in humans, Aeras-402 was given alone to healthy volunteers. Here, the vaccine induced good CD4 and CD8 responses and evidence for multifunctional memory cells. The vaccine was safe and immunogenic, but even a single injection of this adenovirus induced seropositivity in 22% of the individuals [124].

In newly ongoing trials, infants will receive BCG and then MVA85 4 months later. There are plans to compare this with Aeras-402 used as a booster vaccine, providing a head-to-head comparison of the two virus-based boosting approaches. Both boosts improve the numbers of 'polyfunctional' T cells, indicating that they generate some degree of memory immunity (hopefully, central memory). But why the timing of these studies? It is generally agreed that BCG protects children but the overall durability and protective longevity of the vaccine is highly variable. Therefore, is it likely that giving the boosting vaccine so soon after the BCG prime in these (barely immuno-competent) infants will have a desired or even noticeable effect? I have hypothesized that BCG causes a gradual expansion of effector memory T cells in children but this is gradually lost through attrition [125]. If the viral vaccines expand central memory that should be a beneficial event, but what is the half-life of these cells? Would it not make more sense to wait maybe 6–8 years before boosting, when the memory response might be more amenable to effector memory to central memory transition, and the boosting effect potentially more likely to significantly extend protection?

Finally, a tremendous drawback in all these studies is the lack of an adequate biomarker of vaccine efficacy. Production of the cytokine IFN- γ was an initial approach, but this was soon questioned [126,127]. More recently, it was shown that the frequency and cytokine pro-file of antigen-specific T cells, including CD4 IFN- γ responses, appeared to be poor correlates of protection [128]. These ideas have now been replaced by the idea of poly-/multi-functional T cells, consistent with the knowledge that central memory T cells can often make IL-2 and TNF- α , as well as IFN- γ [129]. Further bad news was obtained in a NHP animal model; this used a new aerosol model to look for immunological and clinical readouts that could be used in vaccine evaluation studies. However, neither animal survival, nor were the IFN- γ profiles induced following vaccination found to correlate with protection. Interestingly, the only data that did correlate was MRI of lungs combined with stereology [61]. It should be noted, in this regard, that MRI seems a useful imaging technique both for small and large animal models [56,61,130] because it can detect dead tissue containing necrosis, whereas the much more heavily promoted PET approach [69,88] cannot.

Vaccination with BCG: are we doomed to failure?

As we have recently discussed in [125], while BCG has certain attractions – it is safe, immunogenic, cheap to make and so on – it may also have some serious problems that are only just now starting to be recognized. The fact that BCG just simply does not work in adults has been well known for some time, but what is harder to explain is the apparent waning of vaccine efficacy in teenagers/young adults. We can now think of at least three reasons why a rBCG vaccine may be a disastrous choice.

- In our recent studies we demonstrated that mice vaccinated subcutaneously with BCG establish antigen-specific (tetramer-positive) T-cell populations in the lungs (prior to any challenge) [131]. These are detectable for hundreds of days and have characteristics of memory T cells. The great majority of them are CD62L^{lo}CCR7^{lo}, indicating they are effector memory T cells, whereas very few are CD62L^{hi}CCR7^{hi} central memory T cells. This has led us to propose the hypothesis that BCG is adept at generating effector memory but not central memory [125,131]. This would be completely sufficient to protect vaccinated neonates and children from TB, but this population could potentially be lost over a decade or so due to attrition (exposure to

M. tuberculosis, or environmental mycobacteria (especially in India). With an inadequate central memory response to provide a backup at this point the individual would lose any resistance to TB infection (as is observed).

- Vaccines entering the current NIH/Aeras pipeline are almost invariably tested against laboratory strains of *M. tuberculosis* (H37Rv, Erdman). We recently observed that such strains absolutely pale in comparison with the newly emerging clinical strains in terms of virulence, lung pathology and inflammation [132,133]. Importantly, in the mouse model the H37Rv strain induces strong protective immunity, and while this is seen with the clinical strains, in the latter case this immunity decays. The reason for this, as we demonstrated [29] and discussed above, is because the clinical strains but not the laboratory strains potently induce the emergence of a population of CD4⁺Foxp3⁺ regulatory T cells capable of secreting the immunosuppressive cytokine IL-10. In the guinea pig model, in which the presence of Foxp3⁺ cells can be inferred by real-time PCR, all of the virulent clinical strains we have tested so far have this property [Ordway DJ and Orme IM, Submitted Manuscript]. This has also been observed in larger animals [134]. Although my laboratory has not had the resources to test a significant number of such strains, it appears that the W-Beijing strains seem to be particularly good at this, and it is very disturbing to think that widespread use of the BCG vaccine may have actually selected for this *M. tuberculosis* family in certain regions of the world [135]. Indeed, we have to face the un-nerving possibility that most or all of our vaccine candidates are doomed to failure because they will not be able to overcome the regulatory T-cell responses generated by highly virulent strains our previous vaccination policies have unwittingly selected for. Even highly active new attenuated vaccines (such as S02 as a pertinent example [136]) may not overcome this.
- A stunning paper recently demonstrated that disparate strains of *M. tuberculosis* from geographical diverse regions of the world have something in common: they all hyperconserve certain immunodominant epitopes, even beyond the conservation of essential genes [137]. This is very troubling, because a sinister interpretation of this phenomenon is that it favors the survival of the organism. In other words, the bacillus has gone to considerable effort to conserve such epitopes (in Ag85 and others) because these generate potent immune responses. This in turn ensures the continued expansion of the inflammatory process, an event needed by the bacillus if it is to escape to the next individual. Thus, by over-expressing immunodominant antigens (as Aeras is doing with their lead candidate) we may be doing exactly what the bacterium wants us to do. In fact, the recent NHP MVA85 boosting study may have unwittingly revealed this [61]. Having said that, as discussed above, much of the NHP data recently appearing are curious to say the least, and thus it may be that it is this animal model that is wanting, not the MVA85 vaccine, a candidate that seems to work well both in smaller animals and in initial clinical studies. This of course further illustrates the fact that not everyone is fully persuaded that animal models are a valid means of predicting vaccine efficacy in humans. This is a cross animal modelists such as myself have to bear (and acknowledge) and this issue will never be resolved until a novel and highly effective new TB vaccine has a dramatic effect on global disease rates. Only then could we look back and really see if the animal models predicted this. But, until then, this is the best we have.

Future perspective

A total of 50 years ago we started to see the emergence of TB as a paradigm for cell-mediated immunity, involving both acquired specific and nonspecific resistance beautifully described by the studies of Mackaness and others [138,139] at the Trudeau Institute, NY, USA. In the 1980s we saw the first definition of the role of T cells and macrophages [21], the first direct demonstration that CD4 and CD8 T-cell subsets could transfer immunity [24,140], and the beginnings of the realization that proteins produced by live bacilli as they replicated were key antigens [141,142]. Over the past two decades, as funding for TB research improved, we have seen a vast expansion of knowledge regarding the key roles of cytokines in what we now refer to as the 'Th1 response' [20], as well as a vast amount of information pertaining to the bacillus itself at the physiological, biochemical and genomic levels.

If the last few decades were the Golden Age for basic research into TB, what does the future hold? In terms of drug discovery, many activists point out quite correctly that getting new drugs into people is a glacially slow process, and while the current Global Alliance pipeline is expanding, progress is undoubtedly too slow. Moreover, how many new drugs do we need? In fact, those on the front lines have pointed out that while new drugs could be good, more effort should be placed on optimizing the drug regimens we already have. In addition, while there was a feeling at one point that regimens put together to treat MDR cases was haphazard at best, this seems to be changing and effective regimens are now being widely used. Having said that, drug resistance is clearly increasing, so we must continue drug discovery efforts. Even as we do so however, laboratories capable of screening have to show some backbone and test these drug combinations against high-virulence, newly emerging clinical isolate *in vivo*, including MDR strains.

In the vaccine field, it is very encouraging to see candidates finally reaching Phase III trials, but I will repeat my concern above that the available (minimal) data for the lead candidate seems rather questionable, in comparison to very solid data published by several other laboratories that seem to have sunk to lower priority in the current pipeline. Above all, the emphasis on rBCG vaccines may be a mistake. If they work (and it will take a while to truly determine this) then fine. But what if they don't? In the current funding climate I suspect very few laboratories will be left standing to try to come up with alternatives.

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Executive summary

TB

- TB is a disease of antiquity, which infects the lungs after inhalation, but can then cause disease in multiple organs.
- There is a current epidemic of TB, which is further exacerbated by the emergence of multidrug-resistant strains.
- The epidemic is driven by the concomitant HIV epidemic, which greatly increases the risk of active TB, as well as driving the reactivation of latent TB.

Host response to infection

- The host response to TB is cell-mediated, with CD4 T cells driving the process through the activation of infected macrophages.
- In addition, there is evidence that multiple other cell types can also respond to mycobacterial antigens, contributing both to protection and to the disease process.
- The cellular response is mediated by the production of large numbers of cytokines and chemokines, leading to the granulomatous inflammatory response.
- There are several useful animal models of TB that can be used to understand the disease process, and in which to test vaccines and drugs.

Drugs

- Treatment of TB is a lengthy process involving a minimum of four drugs; more so in the case of drug-resistant infections.
- There is a pipeline of new drugs moving through the various stages needed to reach clinical trials.
- Bacteria that survive initial chemotherapy and thus persist in a more latent form pose a difficult challenge in terms of effective treatment.

Vaccines

- Vaccines are traditionally tested in mouse and guinea pig models.
- There is a growing pipeline of potential new candidates but only a few are way down that path.
- The current emphasis on improving BCG can be questioned.

Figure 1. Primary granulomas in the infected guinea pig lung

(A) Typical primary lung lesion appearance at day 60 in the guinea pig. There is a large region of central necrosis that is undergoing dystrophic calcification, surrounded by a ribbon or rim of eosinophilic acellular debris. (B) A lung lesion visualized by combination staining with auramine O and rhodamine B to detect acid fast bacteria, hematoxylin QS (tissue stain), and 4'6-diamidino-2-phenylindole dihydrochloride (nuclear stain). There are multiple single bacterial cells and small clusters of bacteria scattered throughout the central necrosis and acellular rim.

(A) Colorado State University, CO, USA. (B) Courtesy of Anne Lenaerts and Gavin Ryan, Colorado State University, CO, USA.