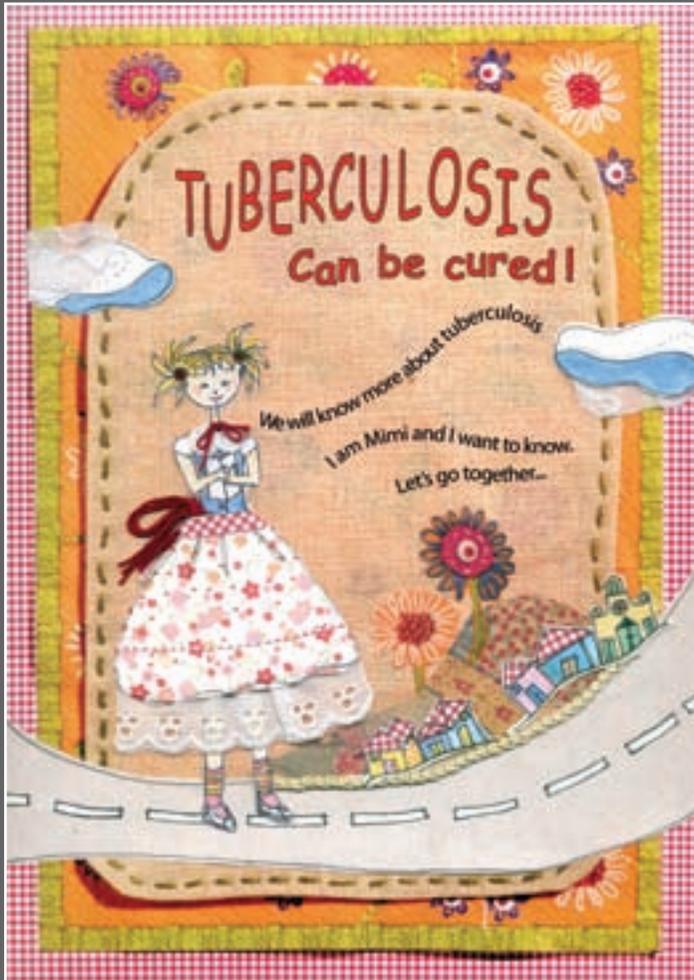


Although TB can be cured, and the cost of the whole medical treatment is cheap, a high percentage of TB patients, in many places of the world, still do not receive suitable treatment



**Tuberculosis can be cured! – Lili Sarmiento**  
Poster

'I send my voice into your mouth  
You return the compliment...'

***The Ventriloquists (Poem) – Harold Pinter***

**Lidia Sarmiento Garcia San Miguel**  
La Habana, Cuba

Lidia Sarmiento Garcia San Miguel studied and graduated in Architecture from CUJAE (La Habana, Cuba) in 1979. She did her Doctorate Course in restoration and monument conservation at the Catedra Gaudi in Barcelona. Her Master Course in restoration and museology was from CENCREM (La Habana, Cuba). She lead the restoration project of historical areas in Old Havana from 1979 to 1995. She serves as Adviser of the Department of Patrimony of the State Secretariat, Ceara State, Brazil. She also works as a researcher in Patrimonial and Museologic Education. She did an educational brochure on TB, 'Tuberculosis se cura, si', for the TB Vaccines/TB Art 07 at Varadero, Cuba in 2007.

# CHAPTER 19

## Therapeutic Vaccines

Pere-Joan Cardona

### Introduction

The term ‘therapeutic vaccine’ always seems to be controversial, as we understand a vaccine as a prophylactic product that must avoid an infection process. Historically, this is not the case, as one of the first vaccines was actually therapeutic. This was the vaccine against rabies designed by Louis Pasteur to stop the progression of this virus to its target at the central nervous system, and thus to avoid the development of that disease (1). Nowadays, this term is even more popular, because it represents one of the strategies to stop another lethal disease—AIDS. Effectively, the design of a therapeutic vaccine is an open field to stop the progression to disease in people already infected with HIV (2). Unfortunately, the link between this concept and TB appears to be less clear but in fact it is a question of trying to produce the same beneficial effects, as in the case of the referred diseases, that is, to avoid the development of a disease in those people already infected by MTB. In particular, the objective of these vaccines is to avoid the development of TB in those persons that already have a latent LTBI.

### The Diagnosis of LTBI

Generally speaking, individuals with LTBI are those who develop a certain size of local induration 72 hours after the intradermal inoculation of a

pre-established quantity of an extract of MTB (known as TST), in the context of a lack of symptomatology and a lack of any infiltration in the chest X-ray (3). Usually, the positivity is indicated by an area of induration ranging in size from 5 to 15 mm depending on different clinical aspects including BCG vaccination, risk of non-TB mycobacterial infections or concomitant toxic habits or immunodepression contexts, and even geographical aspects related to the annual risk of infection. All these variables have been recently framed in a web-based algorithm to aid the interpretation of TST results (4). The term 'tuberculin' was first adopted by a German doctor, Pohl Pincus (5), to refer to the MTB extract in the context of a therapy against infectious diseases based on the inoculation of the extract of each infectious agent. But it was Robert Koch who first demonstrated that inoculation with MTB extract (which he initially called 'lymph' for political reasons) could be useful for the diagnosis of MTB infection (5). Nowadays, the most commonly used tuberculin is the variant known as PPD, and in particular the batch RT-23 of this product generated in the 1950s by the Statens Serum Institute. The quantity of PPD RT-23 inoculated for the TST is quantified in International Units (IU). Each IU has 0.02 µg of protein content (6), and the IU inoculation in each TST can also vary, usually from 2 to 10.

## The Gold Standard Treatment of LTBI

The origin of this treatment can be found at the very beginning of the introduction of TB chemotherapy to try to find a solution to a terrible dilemma: what to do with breastfed infants whose mothers were diagnosed as suffering from a highly infective form of TB, that is, with positive acid fast bacilli stain in the sputum. These infants had a high probability of being infected and, instead of waiting for the TB to develop, the physicians concerned intuitively decided to start a prophylactic treatment with isoniazid (INH) to avoid it, and they succeeded (7). The length of the treatment was not well defined until cost-benefit studies were undertaken by GW Comstock, which finally demonstrated that the best choice was a nine-month duration period, obtaining a rate of 90 per cent effectiveness (8). Unfortunately, this treatment represents a 'logistical' problem in terms of compliance, as subjects must be strongly motivated to complete the full treatment, mostly because they have no symptomatology at all, and because the administration of INH generates a remarkable hepatic toxicity risk. In fact, the WHO recommends a six-month period of administration, considering that subjects have better prospects if this period is completed, even when its effectiveness falls to 60 per cent (8, 9).

## The Risk of TB Development

It is traditionally considered that once LTBI is established, there is a lifetime risk of developing TB, on the known principle of ‘once infected always infected’ (10). This lifetime risk is around 10 per cent and it decreases with time, being more frequent in the first four years after infection, which represents roughly 50 per cent of cases (11). This known fact also adds more uncertainties for the compliance of the treatment. The only way to ensure that somebody has been recently infected is to have a ‘TST conversion’. This means that technically speaking, to receive the most benefit from INH treatment, so that the subject will be highly motivated to follow it, a previous negative TST should be known in any case. This is not a typical scenario. This means that INH treatment is usually discouraged by medical staff, unless dealing with a clear high-risk case.

On the other hand, the concept ‘once infected always infected’ encouraged the concept of ‘protection’ against reinfection. Nowadays, molecular biology techniques have demonstrated that this is not the case, because it has been demonstrated that reinfection is possible (12). But still, this ‘protection’ against reinfection has also discouraged INH treatment in those places with a high infection risk, as INH treatment has been linked to the elimination of bacillary counts and thus to a reduction of immune memory against reinfection (13).

## What Causes Latency? The Non-Replicating Bacilli

### *The Silent Bacilli in Old Lesions*

The extraordinary capacity of MTB to remain viable but ‘silent’ in tissues was demonstrated in different studies from the very beginning of the twentieth century. Well-known studies demonstrated the recovery of bacilli from old calcified lesions, although in a very low proportion, thus revealing a crucial capacity to survive for a long time in tissue in a non-replicating state, keeping the ability to reactivate their growth when cultured in proper culture conditions (14). This property was linked to the adult TB form, called postprimary TB, which tended to develop in the upper lobes because the high oxygen pressure found there benefited the growth of the bacilli (15). This form was linked to the reactivation of old lesions, because at that time 100 per cent of the subjects were infected at the age of 20 years old and there was the concept that the reinfection in adults was not possible (15). Thus, the only explanation for this phenomenon was that the adult form of TB was always a consequence of the reactivation of an old lesion.

### ***Short-Course Chemotherapy and Low-Growth Bacilli***

The introduction of chemotherapy and the empirical test of different drugs and drug combinations led to the concept of bacteriological relapse after treatment not explained by the induction of resistance to drugs through spontaneous mutations. With the discovery of rifampicins, and thanks to the design of a series of 'in vitro' assays based on changes of temperature and of cultivation periods, a hypothesis was postulated for the presence in the tissues of a bacillary population with a low growth speed that was refractory to the drugs' bactericidal mechanisms (16, 17). The presence of this population led to the design of the 'short course chemotherapy' (which is still the gold standard today) in which there was a 'continuation phase' therapy (for four months) exclusively designed to kill this population. The rationale for this therapy was that some of this 'low growth speed bacilli' could start 'growing spurts' and then be susceptible to the action of rifampicin which would then be able to exert its bactericidal activity. This result was difficult to achieve through use of INH, as the latter was only active against 'high growth speed bacilli' (18, 19).

### ***Anaerobiosis and Non-Replicating Bacilli***

The observation of large TB lesions, highly fibrosed and even calcified, led to the concept that the remaining 'silent' bacilli adapted their metabolism to an anaerobic environment, which was considered the only stressful factor in the necrotic tissue (20). This led to the exploration of this hypothesis and the origin of a large series of 'in vitro' experiments to demonstrate that MTB could actually adapt their metabolism to these conditions. And this was indeed demonstrated. Through a system in which there was a progressive lack of oxygen, MTB was able to remain in anaerobiosis through a metabolic adaptation based mainly on the glyoxylate shunt, induced mainly by the overexpression of isocitrate lyase (21). In these conditions, the exposition of the bacilli to anaerobical drugs, like metronidazole, led to their destruction (22). These observations led to more experiments trying to demonstrate the utility of anaerobical drugs in the treatment of this infection in different experimental models, which had controversial outcomes. In particular, the experiments on mice did not really demonstrate the utility of this strategy, and this was linked to the lack of proper hypoxic lesions (23, 24). This in turn led to the use of experimental models 'closer' to humans, such as rabbits or monkeys, which produced results that better resembled human TB lesions. In these models, the presence of hypoxia has been recently demonstrated, thanks to the use of pimidazole (25). In fact, the accuracy of this kind of technique has been widely questioned in human tissues because there is no correlation between the direct measure of oxygen pressure and these markers (26–28). Inasmuch, these techniques have led to controversial outcomes in the murine model (29, 30). Be that as it may, there

are still currently ongoing clinical trials to attempt to ascertain the usefulness of this strategy in human TB.

### **Why Non-Replicating Bacilli Reactivate?**

LTBI has been classically explained (15) as a consequence of the presence of dormant bacilli in an old lesion generated at the beginning of the infection, when the bacilli disseminate throughout the body. Once the specific immune response is developed, there is a control of this dissemination and also of the bacillary growth. The lesions then become well structured, and the surviving bacilli are 'trapped' inside, becoming dormant. Several genes have now been identified (known as DosR genes), which are expressed after submitting the bacilli to stressful conditions (31) and which are linked to the development of dormancy. This status can be maintained for a long time, until a change in the immunological status allows the destruction of the granuloma structure and the liberation of the bacilli (32); the liberated bacilli in turn detect this more favourable situation and reactivate their growth through the expression of the resuscitation factors (31).

As described above, the most 'genuine' form of reactivation can be found in the adult form, known as postprimary TB. The most characteristic feature of this clinical manifestation is that it usually takes place in the upper lobes because at this location high oxygen pressure favours the growth of the bacilli (10). Thus, as observed in other infectious processes, there is a clear regional tropism in the pathogenesis of TB. Interestingly, in the study of TB, this concept has usually been neglected, giving greater relevance to the concept of immunodepression or even immunological dysregulation. This is probably a consequence of the fact that apparently nothing can be done to avoid the colonization of the upper lobes, and something can be done to modify the immunological response.

It is for this reason that there is a great deal of information available trying to compare the immunological scenarios in LTBI and TB patients, based on the premise that the former are able to control the bacilli, and that the latter are unable to do so. As TB patients seem to have a higher Th2 response, this has led to the theory that the induction of this response led to the beginning of TB (33). In this regard, the failure of BCG vaccination in the countries with a higher incidence of TB has been linked to the concomitant influence of non-TB mycobacterial infection and helminthiosis (34). On the other hand, so far no prospective study has been published monitoring LTBI subjects who finally develop TB, probably because such a study would require significant financial resources. In the same way, little consideration has been given to the actual circumstances of cavitory lesions, characterized by a high parenchyma destruction caused by a strong inflammatory response. This leads to a massive

necrosis which finally liquefies, triggering a strong bacillary extracellular growth, as there is not enough time to structure the fibrosis (35). In this context, the induction of a Th2 response, characterized by an increased fibrotic reaction and a higher humoral response (36), may be really necessary in order to stop the progression of the lesion. Incidentally, the cavity formation allows an 'outdoor' contact and the dissemination of bacilli, while for the host it allows drainage of the bacilli out of the body (39). In fact, Lurie described liquefaction and cavitation as '... nature's more rapid but more hazardous manner of eradicating the disease' (40).

Surprisingly, little focus has been given to malnutrition factors. Historical data from populations which suffered specific periods of dietary restrictions (e.g. during the First and Second World Wars) showed an increase in the incidence of TB (37). Furthermore, there is at least one cross-sectional survey in a representative sample of the US population from 1971 to 1975 that clearly demonstrates that people with a body mass index (BMI), an average skin-fold thickness, or an upper arm muscle area in the lowest decile had a sixfold to tenfold increase in the adjusted risk of developing TB (38).

## The Dynamic Hypothesis of LTBI

### ***Life Cycle in Bacteria: The Adaptation of 'In Vitro' and 'In Vivo' Scenarios***

MTB is a non-sporulating bacterium and thus behaves accordingly. Its cycle in the classical 'in vitro' culture with limited nutrients includes *lag*, *log*, *stationary*, and *death* phases (41). In the *lag* phase, the bacilli introduced in a new environment rearrange their metabolic machinery to start growing actively. This growth becomes exponential (*log* phase) until stopped by a lack of essential nutrients (starvation) or a stress factor, and then the bacilli enter the *stationary* phase. If the environment does not change, the bacillary population decays and starts to die.

How to adapt this cycle to an in vivo scenario? The most important point is that MTB grows mainly intracellularly. All its growing machinery seems to be adapted to be phagocytosed by macrophages in order to start its growth (42). Evidence of this adaptation is that it is able to avoid phagosome-lysosome fusion, at which point the *log* phase starts. Meanwhile, MTB even tries to avoid macrophage apoptosis, which would prevent bacterial growth (43). But usually, the macrophage becomes necrotic once the bacillary concentration is high enough to induce it, probably because of the presence of toxic molecules. The fact that MTB reaches high levels of density provides a major distinction when comparing with bacteria with a similar phenotype, for instance with *M. avium* (44). Once in the extracellular milieu, the growth stops. We can consider then

that the stationary phase starts. We can also consider this point as the beginning of the non-replicating status (or dormancy) since at this point the extracellular milieu is abundant in reactive oxygen and nitrogen intermediates (ROI and RNI), bactericidal enzymes, and has a low pH, as a consequence of the high destruction of neutrophils and macrophages that liberate all their intracellular content (45). This non-replicating status must end once the extracellular bacilli are phagocytosed by new incoming macrophages, at which point the bacilli probably go through a *lag* phase to start the *log* stage once again.

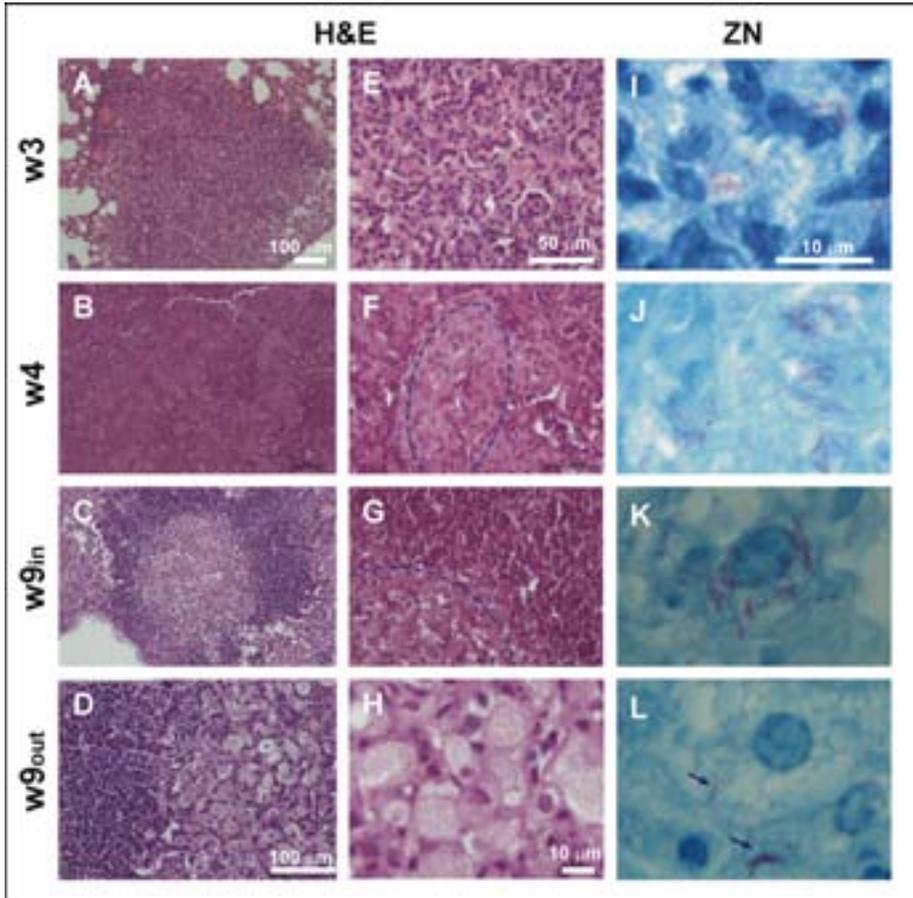
### ***The Immune Response is Mainly Generated against Growing Bacilli***

It is well known that immune response against MTB bacilli is mainly directed against growing bacilli (46). In particular, against antigens secreted to the external milieu, and thus those obtained after filtration of 'in vitro' cultures in the *log* phase, known as 'culture filtrate proteins'. Among them, the ESAT-6 and Ag85 complexes are the ones against major specific lymphocytes are generated. The initiation of the immune response to MTB depends on transport of live bacteria from the lungs to the mediastinal lymph node; MTB may delay this process to expand the bacterial population in the lungs and to evade immune effector mechanisms, establishing chronic infection (47).

### ***The Observation of Non-Replicating Bacilli***

The study of the histopathology of chronic infection in murine experimental models led to the discovery of the accumulation of foamy macrophages (FM) at the outermost ring of granulomas, inside alveolar spaces (Figures 19.1 and 19.2) (48, 49). This cellular response may be a common phenomenon, as it has been widely related to different inflammatory processes (50). The most important finding in these studies was that acid fast bacilli was difficult to be seen in the centre of the granuloma, in contrast with the acute phase of the infection, but easily to be found as single forms, precisely inside those FM. This observation was very interesting because FM are produced after alveolar macrophages start to phagocytose all the debris generated inside the inflammatory foci, at the immune phase, precisely when they could not be destroyed, and they could phagocytose and accumulate enormous amounts of cellular debris. This means that those single bacilli were drained out of the granuloma at the chronic phase, as though 'confused' among the debris. Interestingly, some of those single bacilli were able to reactivate inside the FM. This was a key process to explain the constant increase of pulmonary granulomatous infiltration in infected mice, at the same time that mice manage to control the bacillary count. This provided the first evidence of the presence of non-replicating bacilli and, intriguingly, these bacilli were drained, not remaining static and well-controlled inside a fibrotic granuloma.

**Figure 19.1** Evolution of Lung Granulomas in the Murine Experimental Model of TB, from the Initial Infection to the Chronic Phase (weeks 3 to 9)



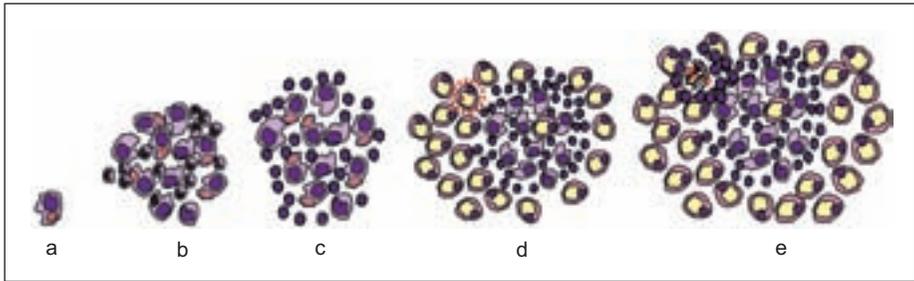
*Note:* Acute infection reveals the formation of a pregranuloma characterized by the mixture of different cell types with a non-clear spatial structure (pictures A, E, and I).

Acquisition of specific immunity is revealed by the formation of the outer lymphocytic ring which starts on w4 as thin lines that surround infected macrophages that become the nuclei of the granuloma (dotted blue line). Note that from this point, lymphocytes tend to avoid the contact of macrophages and accumulate at the periphery of the granuloma (picture F). This effect is even stronger at the chronic phase (picture C).

Foamy macrophages (FM) accumulate at the outermost ring (pictures D and H). Some of these carry single bacilli (picture L). Note that in the chronic phase (pictures D and E), the concentration of acid fast bacilli decrease in the nuclei of the granuloma.

### ***Chronic Phase Resembles Atherosclerosis***

Recently, it has been demonstrated that the origin of FM is inside the infected granuloma (51). From the very beginning of infection (one week post aerosol infection) formation of FM can be seen, in the form of macrophages accumulating

**Figure 19.2** Evolution of Lung Granulomas in the Murine Experimental Model of TB

*Note:* From the initial infection of alveolar macrophages (**a**); formation of pre-granulomas (**b**); acquisition of specific immunity revealed by the formation of the outer lymphocytic ring (**c**); and appearance of foamy macrophages (FM) at the outermost ring to remove all the debris generated with the infection. Some of these carry single bacilli (highlighted with a red circle) (**d**), which can reactivate and destroy the FM in the alveolar space and reach the alveolar space, although usually those cells are surrounded by lymphocytes, which apparently are not able to activate the FM (**e**). Note that in the chronic phase (pictures **d** and **e**), almost no acid fast bacilli can be seen in the centre of the granuloma.

lipid bodies (LB) in their interior. The accumulation of these LB increases with time, until some of the macrophages harbour high amounts of LB. The quality of LB also changes as FM start to produce cholesterol crystals, in a process that has a high resemblance with what happens in atherosclerosis (52). In fact, in atherosclerosis, phagocytosis of oxidized lipids is crucial to developing cholesterol crystals. This process resembles what happens in the chronic phase of the murine model of TB, considering that the source of LB must be mainly cellular debris immersed in a highly oxidized environment (46).

### ***FM: Key Cells for Maintaining Latency and Dissemination***

Careful observation with electronic microscopy demonstrates the presence of bacilli inside FM. These bacilli have a non-replicating status, as every one is present inside a single phagosome. Interestingly also, these non-replicating bacilli accumulate lipids, the so-called intracellular lipid inclusions (ILIs), which are mainly composed of triglycerides (51, 53, 54). This triglyceride accumulation by bacilli has been demonstrated in the sputum of a majority of patients with active TB (53, 54). This observation may reflect an interesting scenario, in which FM would be responsible for maintaining the non-replicating status of the bacilli, probably by inducing some kind of bacteriostatic stress, but giving, because of their high LB content, the chance to bacilli to accumulate ILIs, in a process also linked to the non-replicating state (54–56). Interestingly, accumulation of triglycerides has been linked to a high dissemination through the community, as it is strongly stimulated in highly virulent strains like Beijing (57). This view of bacilli with ILIs in the sputum is revolutionary because it contradicts the classical view that tended to consider this lipid reservoir as an energy source

maintaining the dormancy status of bacilli, which could then remain viable for years in host tissues (58). All this information led to the hypothesis that the induction of non-replicating bacilli is key for MTB dissemination, as it would help to face future stressful conditions like the outside environment, as has been well demonstrated (59). Thus, in this scenario, FM are responsible for the bacillary drainage towards the alveolar spaces, where bacilli form part of infective aerosols. In fact, the presence of FM harbouring non-replicating bacilli has also been demonstrated in humans (60), although FM accumulation outside granulomas is not as apparent in humans as in mice models because of the larger size of the alveolar spaces in humans, which do not allow the massive accumulation of FM as seen in mice.

### ***The Production of Aerosols by Inspired Air is Crucial for LTBI***

The production of aerosols is usually linked to the spread of the disease in the human population; what is often forgotten is that the production of aerosols serves the function of conditioning the inspired air (61). Effectively, aerosols are made mainly at the upper bronchial tree, from the alveolar fluid, to provide humidity and to warm the rapidly inspired air. This fact implies that there is the possibility of a constant endogenous reinfection.

### ***Chronic Infection Granulomas are Anergic***

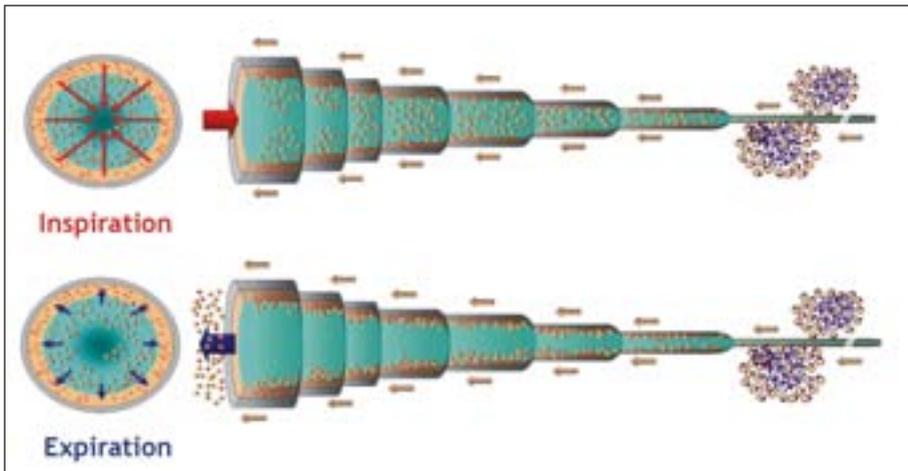
Once activated, alveolar macrophages cannot be reactivated again. This lack of reactivation is caused by the secretion of nitric oxide (NO), which suppresses the activity of effector T lymphocytes (62). Presumably thanks to this feedback, the potential for a local necrosis caused by an excess of activation is avoided (63). On the other hand, this inability of alveolar macrophages to be activated twice probably prevents the recognition of other antigens other than the ones related to the growing bacilli. The predominance of specific T lymphocytes able to recognize the growing bacilli, which activate the infected macrophages, may lead to the suppression of specific T lymphocytes able to recognize non-replicating bacilli. This suppression might be the reason why specific immunity is just selected against replicating bacteria and not against non-replicating bacteria (46). Further, it could also explain why the granuloma has such a structure in which the presence of lymphocytes at the centre of the granuloma is mainly avoided, and why there is in fact a local anergy (Figure 19.1) (64). Jointly, the structure of the granuloma and the phenomenon of anergy may be the keys to understanding the process of perpetuation, in which many non-replicating bacilli are drained towards the upper bronchial tree allowing cyclical reinfection through aerosols. It is noteworthy that this perpetuation cycle is usually broken with the bacilli being finally destroyed in the stomach or drained with the faeces.

### ***End Point: The Delicate Balance between the Bacilli and the Host Led to the Dynamic Hypothesis***

Together, the observations described above led to the enunciation of the ‘dynamic hypothesis’ of LTBI. The ‘dynamic hypothesis’ is based largely on the key finding that the host only triggers the immune response against growing bacilli, which are, in fact, the most dangerous, and which are easier to destroy than non-replicating bacilli. Non-replicating bacilli would be simply drained to be destroyed in the stomach. However, there is a host mechanism, that is, the production of aerosols by inspiration, which gives another chance to bacilli to come back to the alveolar spaces and reinitiate their growth (Figures 19.3 and 19.4).

The assumption of a constant endogenous reactivation makes the phenomenon of LTBI easier to explain. This explanation takes into account many observations, including that all lesions tend to be removed by curing, making a fibrotic net in order to rebuild the original parenchyma. The notion of static, dormant bacilli retained in old lesions, that can reactivate after an unknown environmental trigger, is hard to reconcile with the attempt of the body to drain all the bacilli and rebuild the original parenchyma. Further, the dynamic hypothesis would explain the effectiveness of the actual treatment of LTBI, based in the

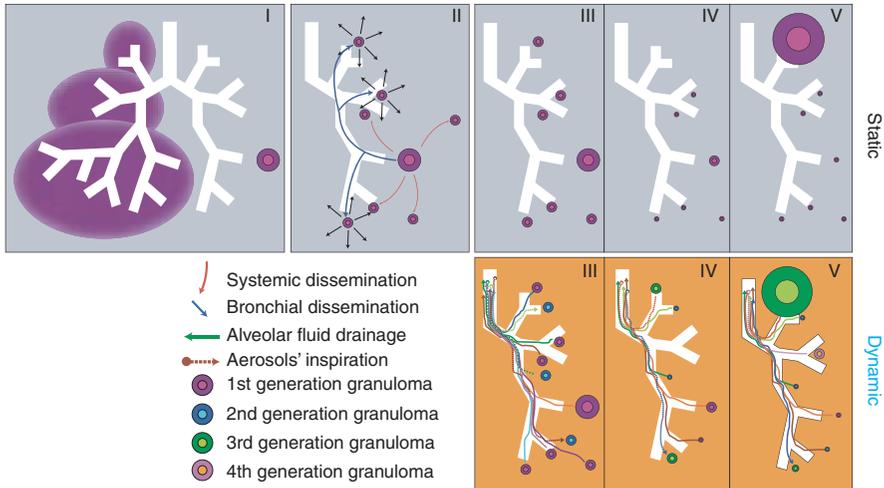
**Figure 19.3** Mechanism of Reinfection



*Note:* In this picture the bronchial tree is represented by tubes of different diameters. The smallest one represent the alveoli and the largest the pharyngeal cavity. In this scenario, foamy macrophages (FM) harbouring non-replicating bacilli reach the alveolar fluid, which is drained up to the bronchial tree (orange arrows). Inspired air (red arrow) generates aerosols to condition the air. As the aerosols are generated from the alveolar fluid, non-replicating bacilli are included in, and thus have the opportunity to come back and reinfect the parenchyma. Expiration is characterized by the return of the aerosol particles to the alveolar fluid, although some of them can be lost to the open air, depending on the maneuvers of the host (i.e. talk, sing, and sneeze).

administration of INH, a drug that is only active against growing bacilli. In the scenario of the dynamic hypothesis, the effectiveness of INH therapy can be explained by the natural drainage of non-replicating bacilli, as it avoids the chance of constant reactivation (Figure 19.5).

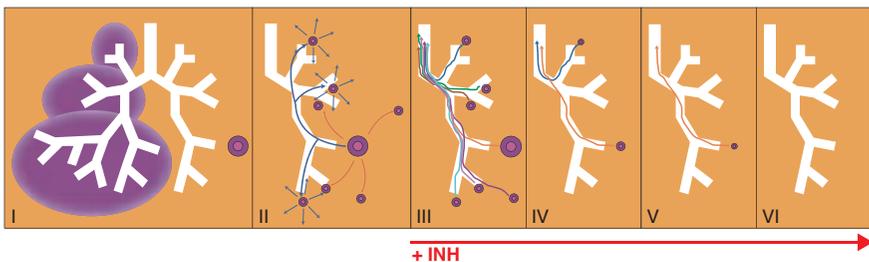
**Figure 19.4** Latent TB Infection (LTBI) and Generation of Active TB (TB)



*Note:* Comparison between the traditional 'static' theory and the dynamic hypothesis. Once the initial lesion is generated (I), there is a bronchial (blue arrows) and systemic (red arrows) dissemination that generates new secondary granulomas. This process is stopped once the specific immunity is established (III). Lesions remain from then (IV) keeping dormant bacilli that have the ability to reactivate its growth after a long time (V).

In the dynamic hypothesis, there is a constant drainage of non-replicating bacilli towards the bronchial tree (solid arrows) but also the inspired aerosols (dotted arrows) can return the bacilli to generate new granulomas (III-IV). In this process, if one of these reinfections takes place in the upper lobes, it has the opportunity to induce a cavitory lesion.

**Figure 19.5** Mechanism of Long-Term Isoniazid (INH) Treatment of the Latent TB Infection (LTBI) According to the Dynamic Hypothesis



*Note:* This treatment allows the drainage of the non-replicating bacilli, but once the inspired aerosols reach the parenchyma, the bacilli have no chance to reactivate. In this case the lesions disappear with time and the opportunity to reach the upper lobes and generates the cavitory lesion is avoided.

### ***The Dynamic Hypothesis, New Therapeutic Scenarios, and Difficulties***

The dynamic hypothesis gives a new comprehensive scenario for the LTBI that makes feasible the design of novel therapeutic approaches (65). Until now, the classical notion based on the presence of dormant bacilli trapped in an impermeable structure, which is hypoxic-anaerobic and practically non-vascularized, gave practically no chance to any therapeutic approach, except the design of a drug able to diffuse into this structure and able to recognize the hidden enzymatic ways that allows the bacilli to resist there for years. Inasmuch, the acceptance of this classical notion makes the effectiveness of INH administration, the present gold standard treatment (9), totally incomprehensible.

The dynamic hypothesis gives at least a rationale for the effectiveness of the INH treatment. This hypothesis is based on solid, well-proven observations in experimental models and even in human tissues. Of course, these observations need to be extended in order to fully support (or disprove) the hypothesis. Unfortunately, this hypothesis draws a really complex reality that renders difficult the design of therapies to trigger an effective immunity that is able to stop progression of infection. Future therapy designs should avoid three major difficulties:

- 1 The local immunosuppression generated by the natural immune response triggered against LTBI.

On one hand, this immune response ensures the control of reactivation in 90 per cent of the cases (12), which can be considered a real success. So, eradication of this energy can be a danger in itself. On the other hand, this immune response prevents the recognition of non-replicating bacilli, as it appears to suppress the less abundant specific T lymphocytes that are able to do so.

- 2 The intrinsic resistance of non-replicating bacilli.

These bacillary forms are really more resistant to stress than growing ones (60). In the scenario drawn, non-replicating bacilli would be mostly phagocytosed by already activated macrophages. It seems clear that many of these bacilli can resist the stress generated by these macrophages. An explanation for this phenomenon may also reside in the progressive decrease of bactericidal activity due to the simultaneous phagocytosis of cellular debris and the accumulation of LB. This phenomenon leads to the induction of FM, which can only reinforce the non-replicating status of the bacilli.

- 3 The slowness of the cellular immune response, which enables constant endogenous reinfection of the host.

Effectively, once those non-replicating bacilli come back to the alveolar spaces through inspiration of infected aerosols, they can start to grow because the attraction of specific T lymphocytes able to activate the newly infected macrophages takes some days, even when the host already has this immunity (66). This delay in immune response makes possible the generation of new infective foci and allows the bacilli to remain.

## Therapeutic Vaccines

### The Old Therapeutic Vaccines: The Tuberculins

#### ***The Historical Context: The Rivalry between Pathologists and Bacteriologists***

As explained previously, when Robert Koch was forced to describe his remedy against TB, after his timid reference at the 10th International Medicine Congress in Berlin in 1890, he talked about a 'lymph' instead of talking openly about 'tuberculin'. Although this gesture has usually been interpreted as a way to somehow hide the nature of his therapy in order to gain extra time to better define it and to protect it with a patent (67), this also reflected how weak the young new science of Microbiology was compared to the traditional discipline that had the monopoly of the interpretation of human diseases (Pathology). Effectively, the name 'tuberculin' was first used in 1884 by Pohl Pincus who read a paper at the Berlin Medical Society in which he proposed a method of immunization and treatment by means of bacterial extracts, and the use of various terms (tuberculin, variolin, scarlatinin) to describe these extracts. The paper was so much ahead of its time that the *Deutsch Med Wochenschr*, when reporting it, added a special disclaimer explaining that the views expressed were not accepted by other pathologists (6). The appearance of Microbiology, and the irruption of a new science able to exactly relate a disease with a microorganism, led to the end of the 'monopoly' of the pathologist and gave an admittedly cool welcome to the concept of demonstrating the bacteriological origin of different diseases (68). In fact, the father of Pathology, Rudolf Virchow, regarded the tubercle as a tumour, and found no difficulty in accounting for its genesis, evolution, and structure in much the same terms that were used for the description of cancer (69). Another example of such competition was also seen in Spain, where Microbiology remained under the control of pathologists even when it was already a well-established subject. In one case, the eminent pathologist, Santiago Ramon y Cajal prevented the creation of a Chair of Bacteriology at his university, so that the subject could be kept under his own control. This fact is explained because the new academic position should have

been given to the best known of Spanish bacteriologists, Jaume Ferran, the creator of the first cholera vaccine (70).

### ***The ‘Fiasco’ of the TB Therapy Announced by Koch***

When, finally, Robert Koch recognized that the ‘lymph’ was a tuberculin, another political conflict occurred. A kind of ‘triumph crisis’. At that point, Koch wanted to devote his energies to the development of his new therapy, a task that would bring him greater financial advantages. But, at that point, the Prussian government was more interested in establishing a prestigious National Infectious Disease Institute, of which Koch would become the director, and at the same time maintaining state control of the new therapy. There was a major confrontation between both sides, very well described by Burke (71). In the middle of the negotiations, the Prussian government set up a statistical survey to determine the actual benefits of the tuberculin therapy. This led to a hastily prepared clinical survey that demonstrated that the benefits of the tuberculin therapy were not conclusively proved (Table 19.1). This clinical survey was focused only on tuberculin-treated patients suffering from the disease with different grades of severity, despite the fact that even Koch himself had previously highlighted that the treatment should be given at the beginning of the disease (72). The irresponsibility of Koch was to give expectations about the treatment of a disease that was the leading cause of mortality at that time. The blow to tuberculin would not have been so serious had the remedy not first been heralded all over the world. Everybody had heard of tuberculin; consequently, everybody knew that it had failed to be the magic cure for TB that had long been looked for and was hoped for when it was announced. Probably one of the severest blows to tuberculin was the pathological findings, which were made by Virchow. He found that miliary tubercles were present in the lungs of patients who died of TB after treatment with tuberculin; therefore, the idea

**Table 19.1** Effectiveness of the Tuberculin Treatment from the Guttstadt’s Report on February 1891

	Tuberculosis Form	
	Pulmonary	External <sup>a</sup>
Total	1061	708
Cured	13	15
Substantially improved	171	148
Improved	194	237
Unimproved	586	298
Died	46	9

Source: Burke 1993 (71).

<sup>a</sup> Most part comprised TB of the skin (lupus), bones, and lymph glands

was suggested that tuberculin mobilized bacilli which had previously been enclosed in the TB foci and in that way caused dissemination and a miliary condition. Virchow did not say that the same thing can be found when patients are not treated with tuberculin. This form of TB had not been studied before and so this fact was not known. This was, however, soon demonstrated by Petruschky. While this 'mobilization of bacilli' was only a myth, yet it has been almost impossible to eradicate it from the minds of medical men (73). After the fiasco, Koch pleaded with the Prussian government to make him director of the National Infectious Disease Institute, a request that was granted. What happened about the tuberculin from this point onwards? Did it then vanish and completely disappear as a therapy? Not at all!

### ***The Revival of the Tuberculin Therapy***

First of all, Koch spent some years in making new formulations, which the Prussian government finally decided to license to Hoescht (67). After the fiasco, there was a period in which only a minority of physicians still used tuberculin. Until 1901, tuberculin therapy was purely academic in nature and restricted to large TB hospitals; later, with the appearance of new manufactured forms of tuberculin (there were more than 20 of them), there was renewed interest in Germany after it was observed that 'phthisis', as TB was often called, could be safely treated with large regular doses of tuberculin, if, instead of starting with a large dose, small ones were used to lead up to it (6). In England, the revival was mainly headed by Sir Almrod Wright, who developed a successful treatment of surgical forms of TB by means of small infrequent doses of the new tuberculins, a thousand or more times smaller than those employed 30 years later (74). In summary, two major different therapeutical strategies were established. One was to be used for adult TB forms, and mixed the concepts of immunity and tolerance induction, using frequent large doses of tuberculin; and Wright's method, focused on young children, based on inoculations with infrequent small doses of initial forms of the disease, looking to reinforce the immune response against MTB.

Although there are not many statistics available on the subject, in Germany the use of tuberculin in public institutions rose from 29 per cent (in 1905) to 70 per cent (in 1910) (6). It was widely in use from 1905 to 1935 at the St Mary's Hospital in London, in the context of a global development of vaccine therapy against different infectious diseases (75, 76). Two surveys carried out in the mid-1920s among 1,261 physicians in New York and 257 TB specialists in the United States revealed that 67 per cent and 30 per cent, respectively, currently used or had actively used tuberculin therapy. The majority of these physicians believed that they had obtained good results (76). At that time, a

real case control statistical record was established (Table 19.2) demonstrating the utility of the therapy (73). In fact, its use was finally standardized in terms of equivalence of the different tuberculin preparations (up to 15), dosification, and number of doses (6, 73, 77–79).

**Table 19.2** Effectiveness of the Tuberculin Treatment in Different TB Stages

Disease Stage <sup>a</sup>	Tuberculin Treatment	No. of Patients	Cured	Cured (per cent)
I	No	32	8	28.1
I	Yes	42	25	59.5
II	No	34	0	0
II	Yes	38	11	28.9
III	No	34	0	0
III	Yes	20	0	0

Source: Pottenger 1913 (73).

<sup>a</sup>Disease Stages: (I) Early TB (i.e. weak pulmonary, primary and local TB); (II) Pulmonary TB, clear symptomatology and Chest X-ray lesions; (III) Late TB, long lasting TB.

## The RUTI Vaccine

### *Production of RUTI*

RUTI is manufactured from bacilli grown under increasingly stressful starvation conditions with low pO<sub>2</sub> and low pH on solid media. Progressive starvation is known to lead to stationary growth in old cultures in which slow metabolism makes the bacilli more resistant to stress (59), as in the chronic phase of murine infection (80). The bacilli used to manufacture RUTI are therefore subjected to conditions that are probably found in the granulomas of hosts with active immunity: a low pO<sub>2</sub> and a low pH, and starvation conditions in the extracellular milieu.

Studies on the induction of a ‘human-like’ experimental model in mice to reproduce intragranulomatous necrosis in the animals led to the theory that this resulted from the triggering of a local Schwartzmann reaction by the endotoxin-like molecules present in the bacillary cell wall (81, 82), and not from adaptive immunity, as described by Dannenberg (83). During the production process, the cells are therefore treated with Triton X-114 to remove such endotoxin-like molecules. This is a very important point, as the induction of this toxic reaction (known as the Koch reaction) was one of the factors that led to unsuccessful research efforts in the field of anti-LTBI vaccines for a long time. The cells are also fragmented, giving the FCMTB (Fragmented Cells of MTB) and liposomed to favour better antigenic presentation (84).

### ***The Efficacy of RUTI in Preclinical Assays***

Inoculation with RUTI in infected mice treated with short-period chemotherapy induces a strong polyantigenic immunity that boost the specific Th1 immune response against secreted antigens (mostly ESAT-6 and Ag85 complex) generated in LTBI that is lowered after the short-period chemotherapy, but also against structural antigens. This effect avoids the reactivation of non-replicating bacilli seen after the short-period chemotherapy, and also induces bactericidal activity (85–87). The difference between the therapeutic administration of BCG and RUTI at a systemic level resides mainly in the stronger activation of IFN- $\gamma$ +CD4+ and CD8+ cells against PPD, ESAT-6, and Ag85B, although both vaccines also triggered a specific immune response against the MTB structural antigens 16 kDa, and 38 kDa (87). In the lungs, both vaccines induce a marked mRNA expression of IFN- $\gamma$ , TNF, IL-12, iNOS, and RANTES, but only RUTI is also able to induce marked accumulation (tenfold) of PPD-specific IFN- $\gamma$ -producing CD4+ and CD8+ T cells at this level, when BCG increases only the recruitment of CD4+ T cells (88).

In guinea pigs, a bactericidal effect was also seen after treating them with INH+Rifampicin during four weeks, compared with the ones only treated with chemotherapy. Long progression experiments (i.e. 24 weeks evolution) showed no difference in controls of bacillary count, but survival and the extent of pathological pulmonary changes were better in RUTI-treated animals (87). This was also the case in *M. caprae*-infected goats in a field study. Short-period chemotherapy markedly reduced both pulmonary and extrapulmonary affectation, but only treatment with chemotherapy plus RUTI decreased the extrapulmonary dissemination and the pathology scores for the hilar lymph nodes, a key indicator for efficacy in humans with LTBI. The RUTI-treated animals in this study also showed a marked increase in the production of IFN- $\gamma$  in the peripheral blood induced by *ex vivo* stimulation with ESAT-6, PPD, or RUTI (89).

The effectiveness of RUTI was also demonstrated in the mini-pig experimental model. After intrapulmonary infection of 18 *spf* mini-pigs with a low-dose MTB, the animals (six each) were assigned to three treatment groups: (i) non-treated group; (ii) isoniazid-treated; and (iii) treated with isoniazid plus RUTI inoculations. The animals were closely monitored for immunological effects for 21 weeks. Histological and bacteriological investigations were also performed after killing at the end of the study. The results showed that RUTI triggered a specific immune response, increasing the effector T-cells secreting IFN- $\gamma$  against specific MTB antigens, also controlling intrapulmonary dissemination, decreasing the CFUs and the number of tubercular lesions and the extent of affectation (90).

RUTI has also demonstrated a prophylactic effect in two studies in mice and one in guinea pigs, both compared with the gold standard for prophylaxis, the

administration of BCG. In all the three experiments, after the administration of two inoculations three weeks apart, the animals were infected four weeks after the last inoculation by low-dose aerosol. The mice were killed and the bacillary counts in the lungs were found to be significantly lower than in the controls. There was no significant difference with the BCG group. Survival was assessed in the guinea pig experiment. Survival after RUTI was greater than in the controls, but again was lower than in the BCG group (data unpublished). This fact gives a new value to the RUTI therapy strategy, as one of the major concerns of a nine-month INH treatment is the risk of losing immune memory, and of being reinfected. In this case, this is avoided by the inoculation of RUTI.

Based on non-clinical results, a double-blind, randomized, placebo-controlled Phase I clinical trial has already completed, with a good safety and immunogenic profile. The objectives of that study were to evaluate the tolerability and the immunogenicity of four increasing doses of FCMTB in the subcutaneous RUTI formulation (91).

In summary, the strategy of RUTI therapy combines a short-term chemotherapy (one month) with INH plus the administration of therapeutic vaccine (RUTI). The rationale for this combination is based on the dynamic hypothesis: the clearance of growing bacilli, and thus inflammatory responses and presence of active macrophages and FM in the infective foci, is achieved by chemotherapy; in addition, immediate induction of a polyantigenic immune response able to recognize non-replicating bacilli present in old foci and newly induced ones is achieved by RUTI.

## **BCG Revaccination and the Heterologous Boost Strategy to Prevent Disease**

It is accepted that the protective effect of BCG wanes after an average of 15 years post inoculation. Attempts to boost this immunity through BCG revaccination to protect against TB incidence have failed and this revaccination is not currently recommended by WHO (92). It is well known that the protective effect of BCG only avoids the development of fatal TB forms in children, but not the induction of LTBI. So, in fact this strategy implies the use of BCG as a therapeutic vaccine because in 15 years' time the majority of these children will already be infected. Nowadays, this concept has been revisited in the form of the 'heterologous boost strategy', to differentiate it from BCG revaccination (the homologous version), as the new prophylactic vaccines that are currently in clinical development will be used as a booster for BCG in 15-year-old children (93).

It seems that this strategy is being adopted to avoid the development of TB in those countries where the gold standard treatment (i.e. six–nine-month INH treatment) is not feasible, but sooner or later its effectiveness will need to be compared with the latter's.

## ***Mycobacterium Vaccae***

Some years ago, the Stanford group developed another vaccine with heat-killed *M. vaccae*. Initially, its therapeutic effect was attributed to the capacity of the antigens present in the *M. vaccae* cell wall to develop a Th1 immune response and to avoid a Th2 response. This idea came from the concept of 'Listeria-like' and 'Koch-like' responses (94). The former was related to a typical Th1 mechanism and induced non-necrotic granulomas, while the latter was related to a Th2 mechanism, and thus responsible for the intragranulomatous necrosis generated by MTB infection, considered to be negative. Th2 response should be counterbalanced in order to control the infection better and to avoid the development of TB (81, 95). The comparison of IFN- $\gamma$ /IL-4 ratios measured by different techniques, following the stimulation of T cells, has generated controversial results (96); moreover, many theories have been postulated to explain the observed polarization of T cell responses. Recent findings suggest that IL-4 $\delta$ 2 may counterbalance IL-4 effects (97) or that T regulatory cells could counterbalance Th2 responses. Several clinical trials have been run with *M. vaccae*, demonstrating some benefits when given to TB patients receiving DOTS, like faster bacteriological conversion, increase in weight, and radiological resolution (98).

## **DNA Vaccines**

The therapeutic effect of DNA vaccines is associated with a switch from a predominantly Th2 to predominantly Th1 response. DNA vaccines enhance IFN- $\gamma$  production and CD8+ CTL, which can lyse macrophages infected with MTB, down-regulate IL-4 and eliminate persisting organisms (99). DNA vaccines encoding a mycobacterial (*M. leprae*) stress protein (hsp65) (99) or hsp70 fused to CD80 (100) are therapeutic when administered in murine experimental models of TB. A DNA vaccine expressing ESAT-6 in a flu vector is effective as adjunctive therapy when administered with chemotherapy (100). Immunotherapy with hsp65 as adjunct to chemotherapy is associated with a more rapid and efficient response to treatment of MDR-TB in mice (101). There is synergy between chemotherapy (moxifloxacin) and DNA vaccine in BCG-immunized, TB-challenged mice. DNA vaccination at the end of chemotherapy has a sterilizing effect in mice (102).

## **Conclusion**

Once MTB infects a person, it can persist for a long time in a process called latent tuberculosis infection (LTBI). LTBI has traditionally been considered to involve the bacilli remaining in a non-replicating state (dormant) in old lesions

but still retaining their ability to induce reactivation and cause active TB once a disruption of the immune response takes place. The present review aims to challenge these concepts by including recent experimental data supporting LTBI as a constant endogenous reinfection process. It also revisits the concept of therapeutic vaccination against LTBI, by reviewing the initial 'fiasco' of tuberculin therapy by Robert Koch, the flourish period of this therapy, and the recent proposals.

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