

New Insights on the Nature of Latent Tuberculosis Infection and its Treatment

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Abstract: Nowadays, there is no conclusive theory explaining the latent tuberculosis infection (LTBI). LTBI is reviewed herein as a standard progression of *M. tuberculosis* in the context of the usual microaerobiosis present in the host's tissues and displaying their main virulent factors: slow metabolism; cell wall thickness and ability to induce intragranulomatous necrosis. Therefore, latent bacilli (LB) would be generated by the irruption of specific immunity forcing bacilli to remain in a stationary phase (SP) inside the necrotic tissue. This tissue is crucial because it maintains a stable LB population and prolongs the production of foamy macrophages which facilitate the LB escape to the alveolar spaces. In the alveolar spaces, LB will regrow and, once freed in this privileged space, they will induce new granulomas –less developed because they are better controlled by immunity. This explains the ability of LB to face the chance to be drained as a consequence of the constant cellular turnover, and to survive for a long time in the lung. This activity also supports the hypothesis that generation of active TB highly depends on the probability of the LB regrowth in a favorable zone (i.e., in the pulmonary apex). This “dynamic” hypothesis faces a more classic one (or “static”) essentially based on the presence of a “resuscitation” factor that would reactivate “dormant” bacilli in old lesions in the apex. Current possibilities for LTBI treatment are reviewed according to this “dynamic hypothesis”, from the standard chemotherapy to the introduction of therapeutic vaccines and anti-inflammatory treatments.

Keywords: *Mycobacterium tuberculosis*, mice, latent tuberculosis, active tuberculosis, immunotherapy, DNA vaccines, RUTI, stationary phase.

1. INTRODUCTION: WHAT IS LATENT TUBERCULOSIS INFECTION (LTBI)?

LTBI occurs when *Mycobacterium tuberculosis* is able to grow within an alveolar macrophage, thus establishing an infection. Empirically, this is demonstrated through the tuberculin skin test [i.e., the intradermal inoculation of PPD (protein purified derivative from a *M. tuberculosis* culture)] and the induction of delayed-type hypersensitivity [1]; or by detecting the production of interferon-gamma (IFN- γ) in the supernatant (with an ELISA technique) or the presence of IFN- γ cell producers with an ELISPOT method in peripheral blood mononuclear cells stimulated with specific peptides [2].

The following step is to get a thorax X-ray image of the patient in order to detect a lesion. When no lesions are found, the patient is classified as having a LTBI. When the lesion seems to be new or active, then the patient is considered to have an active tuberculosis (TB) (i.e., TB disease) [1]. This is very crucial, as in the case of LTBI it means that the host is able to control bacillary concentration; this is not the case in TB. In the first case the patient must be treated with only one chemotherapeutic agent. In patients with active TB, the bacillary concentration is high enough to generate spontaneous mutations that may induce drug resistances. In this case, the patients must be treated with at least three drugs in order to avoid it [3].

In both cases, the treatment schedule requires a drug administration for nine and six months to control LTBI or TB, respectively [3,4].

2. THE ORIGIN OF LTBI: THE LATENT BACILLI (LB)

The interest in LTBI and its treatment is based on the fact that in a 10% of the cases, a bacillary regrowth occurs that causes a TB [1].

Classical observations had demonstrated the presence of bacilli in old TB lesions after a long period of *in vitro* culturing [5], and the resistance of *M. tuberculosis* in old cultures that were able to regrow *in vitro* after years of being in stress conditions [6]. These observations had led to the origin of the mechanism of “resuscitation”, which was recently supported by the finding of “resuscitation” proteins that may induce regrowth of latent bacilli (LB) that have been in stationary phase (SP) conditions for a long time, during which its viability in culture cannot be detected by other methods [7]. This is a very interesting process, although it is barely able to explain how this LB from old TB lesions can recover their activity to generate an active TB, taking into account that the resuscitation factor is only made by active growing bacilli [7].

In fact, for a long time it has been postulated that LB should be the one able to resist an anaerobic environment [8]. This might be the case of the huge lesions present in active TB, or even of those fibrosed in the way to being calcified, but to date this environment has never been found in any granulomatous lesion holding *M. tuberculosis* bacilli. At the most, a microaerobic environment has been demonstrated [9] that nevertheless is the usually one within normal tissue

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cells [10]. Besides, an exponential bacillary growth has been detected in experiments conducted on knock-out mice that lack the expression of clue cytokines like IFN- γ , TNF, or CD4 T cells and develop the largest and most necrotic lesions (and hence it is expected to have a lower oxygen pressure), therefore supporting the hypothesis that not only LB but also growing bacilli must resist such an environment [11,12].

Essentially, most of what we know about the origin of LB comes from the experimental models of TB infection. Classical studies demonstrated in a murine model that bacilli from infected lungs in a chronic phase were more resistant to heat stress than those isolated from an acute phase. As bacilli from a SP culture were also more resistant to this stress than those obtained from exponentially growing cultures (Fig. 1A), the author concluded that resistance to stress by the bacilli from the chronic phase was due to their slower metabolism as a consequence of being in SP. This theory has been recently confirmed by other authors [14]. The comparison between the concentration of viable bacilli and chromosomal DNA, a parameter that reflects both viable and dead bacilli, found a slight difference in the evolution and the amount of both parameters (Fig. 1B). Another scenario would be a high bacilli growth controlled by a high mortality induced by immune responses. In this case, taking into account that DNA can persist in the tissues for a long time, higher levels of DNA would be found. This datum reflects that bacilli in the chronic infection are mainly in SP as predicted by Wallace [13].

Therefore, it can be concluded that the bacillary population able to survive the host's specific immunity and persists

in the chronic infection are LB, and that these are mainly in SP. However, does it explain how a process like LTBI is able to persist for years?

2.1. On the Cycle of Life of *M. tuberculosis*

If we consider the growth dynamics of *M. tuberculosis* in the infection site as a culture in a media with limited nutrients, we will see the classical life cycle of bacteria as pictured in Fig. 2A [15]. *M. tuberculosis* bacilli, like any other bacteria, are constantly immersed in the cycle of *lag*, *log*, *stationary* and *death* phases. 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 SP. If the environment does not change, bacillary population decays and starts to die.

Interestingly, this cycle must start even before infection takes place in the tissue because once the bacilli leave the pulmonary cavity of a TB patient, stressful conditions surround the bacilli in the trachea and, to some extent, in the external milieu causing at least a decrease in temperature and humidity, the presence of oxygen radicals, ozone, or UV rays. Then, a small percentage of bacilli will reach the alveolar spaces after returning to the right temperature and humidity values that will allow them to face the alveolar macrophages. At this point, these bacilli must be quick enough to start the metabolic activity in order to avoid the phagosome-lysosome fusion, and hence the stressful environment that would be fatal for them [16]. This assumption is based on the evidence that phagosome-lysosome fusion is only avoided by *M. tuberculosis* with an active metabolism. Dead bacilli

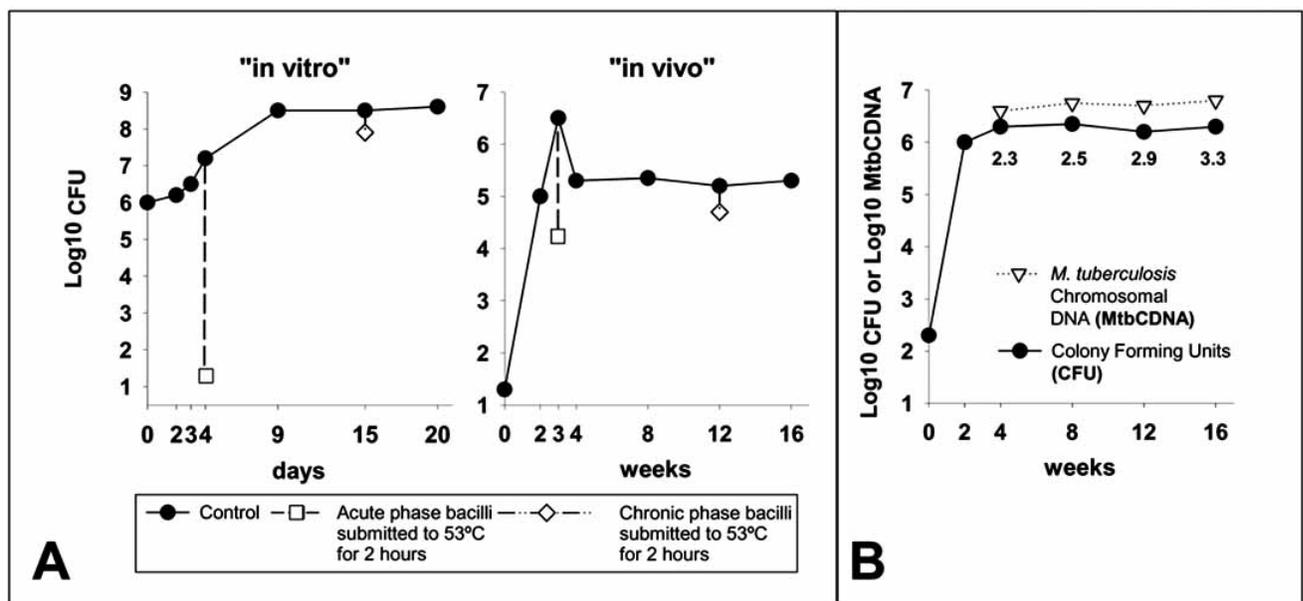


Fig. (1). Chronic phase bacilli are more resistant to stress than acute phase bacilli. (A) Bacilli actively growing *in vitro* (left) are more sensitive to heat stress (incubation at 53°C for two hours) than bacilli from SP. The greater resistance to heat stress shown by chronic phase bacilli *in vivo* (right) is then a consequence of their decreased metabolism. This fact is confirmed by comparing between viable bacilli [i.e., colony forming units (CFUs)] and *M. tuberculosis* chromosomal DNA (as a count of viable and dead bacilli) (B) [14]. This reveals a minimal accumulation of DNA during chronic phase infection in lungs obtained from an *in vivo* murine experimental model of tuberculosis, thus supporting the concept that the rate of bacterial cell division is very low during the chronic infection. Numbers below the curves indicate MtbCDNA/CFU ratios at the corresponding time points. (from [13,14]).

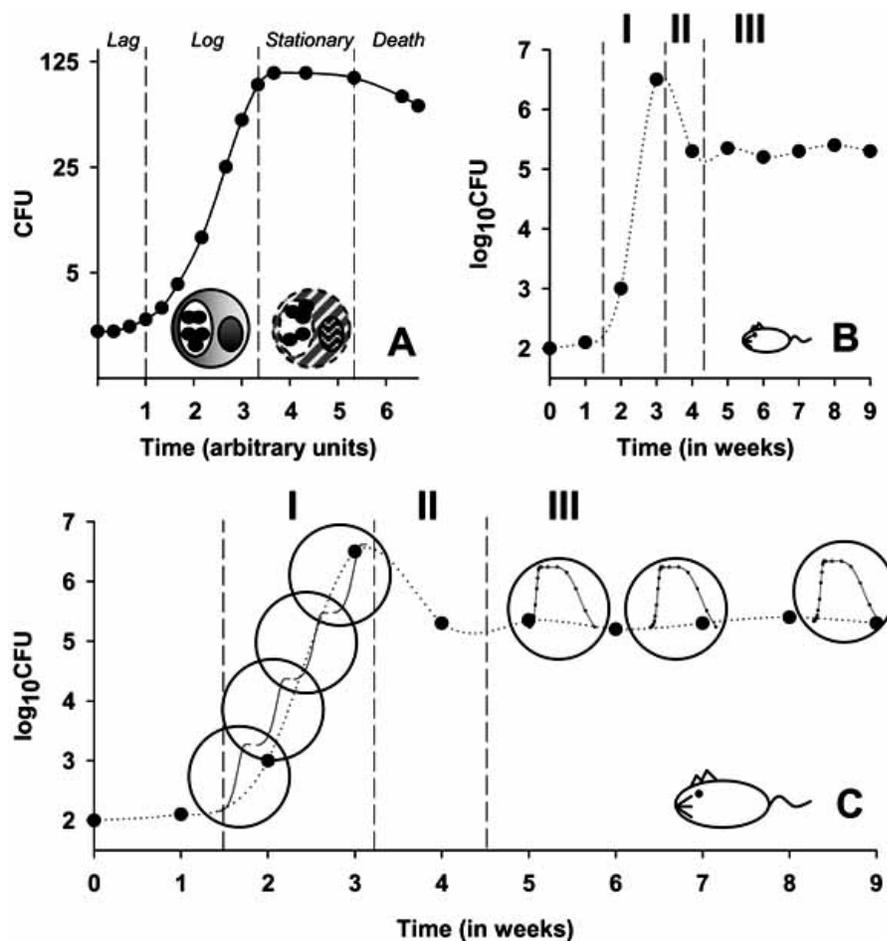


Fig. (2). Hypothesis on the evolution of the bacillary growth of *M. tuberculosis* in a human host. Picture A shows a classical *in vitro* bacterial life cycle in a culture with limited nutrients. In the *log* and *stationary* phases, a macrophage with growing bacilli and a necrosed macrophage are represented, respectively. Picture B represents the usual kinetics of the bacillary concentration in the experimental murine model. An exponential growth can be distinguished (I); a bacillary control induced by the specific immunity that induces the destruction of 90% of the bacillary bulk (II); and the chronic phase, in which bacillary concentration remains stable (III). Picture C shows a magnification of picture B to show the theoretical dynamics of the growing bacilli taking into account the life cycle of the bacteria represented inside the circles.

cannot stop such fusion [17]. Although it could be argued that SP bacilli could also do it, there are a lot of reasons to think it unlikely. It has been demonstrated that a powerful activity is required to produce large amounts of urease to avoid the pH decrease [18]; or to produce enough lipoarabinomanan [19] and ESAT-6/CFP-10 complex [20] to impair the phagosome-lysosome union. As the ESAT-6/CFP-10 production is closely related to growing bacilli [21] and active metabolism is closely linked to the *log* phase [15] it can be assumed that the bacillus will survive if it begins, or is going to begin the *log* phase before the phagosome-lysosome fusion.

Furthermore, there is a point when macrophages cannot support a certain bacillary concentration and die. One of the characteristics of *M. tuberculosis* infection is the capacity to induce necrosis in the centre of the granuloma. The origin of this reaction is linked to some components of its cell wall, like the cord-factor [22] and to the induction of a local Schwartzman reaction-like [11]. In the necrotic milieu bacilli are once again submitted to stress, by being immersed into a bizarre mixture of toxic radicals, catalytic enzymes and a

low pH that probably will kill a little proportion of them [23]. This will force bacilli into a new SP. This phase does not last long, as both the infected macrophages and necrosis cause a strong inflammatory response favoring the accumulation of new macrophages that will phagocyte a proportion of these extracellular bacilli. Then, after a short *lag* phase, a *log* phase will start again. It must be highlighted that some of the SP bacilli will remain trapped in the necrotic tissue for years. As shown in Fig. 2C, this cycle will be repeated uninterruptedly, causing an extensive necrosis that would finally result in the host's death within a short period of time [11,12]. However, specific immunity may activate infected macrophages, quickly forcing the union between phagosomes and lysosomes, killing bacilli [16-18] and stopping progression.

2.2. The Role of the Local Scenario During the Chronic Phase: The "Bacillary Escape"

The study of histopathology in the experimental murine model of TB has demonstrated how interesting the local scenario is during the chronic phase of the infection, when the bacillary concentration is controlled by the immune re-

sponse. Macrophages and neutrophils accumulate at the beginning of the infection. Lymphocytes then start appearing until the central area with infected macrophages is surrounded by an external ring. This is when the immune response reaches its highest expression, approximately four weeks after a low-dose aerosol infection [24]. At this point, another kind of cells, the foamy macrophages (FM), begins to accumulate at the external side of the lymphocytic ring and occupy the alveolar spaces. This is a common reaction based on the activity of those macrophages that go into the lesion in order to “clean” it, and once they have metabolized enough cells, bacilli and surfactant and become plenty of lipidic vacuoles (which are not able to metabolize), they

leave the lesion through the alveolar space in order to go to the main bronchi and trachea, where they will be removed by swallowing to the stomach or just expelled to the exterior [25] (Fig. 3). In *M. tuberculosis* infection, FM start appearing mainly once the specific immunity is triggered [25]. This may be because at the beginning of the infection there are hardly no necrotic macrophages, and because the new incoming macrophages immediately phagocyte the bacilli and are subsequently destroyed. In other words, there is little chance to generate FM. At this point, immunity can activate the infected macrophages and destroy the bacilli and then, both previously infected and new macrophages can become FM without being killed by the bacilli.

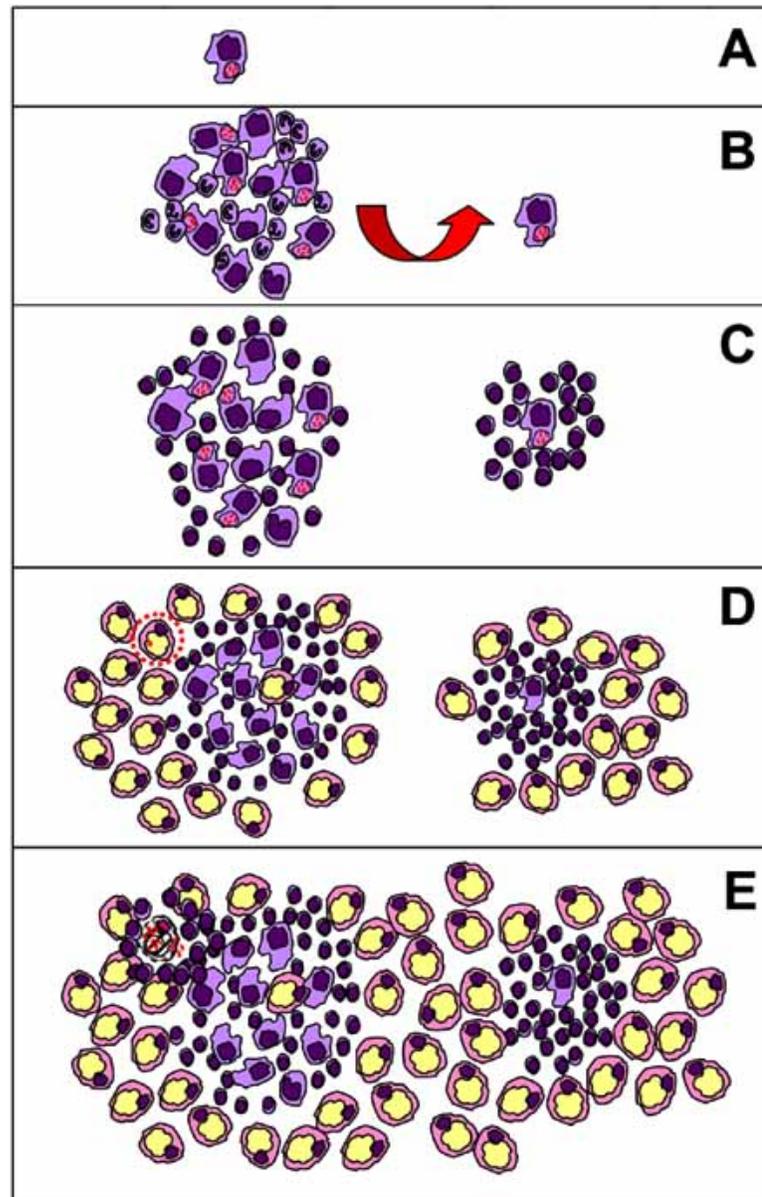


Fig. (3). Evolution of lung granulomas in the murine experimental model of tuberculosis, from the initial infection of alveolar macrophages (A); formation of pre-granulomas and lung dissemination (B); acquisition of specific immunity revealed by the formation of the outer lymphocytic ring (C); and appearance 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. (from [31]).

At this stage, acid-fast bacilli are difficult to find in the center of the granuloma, where they can be easily detected during the acute phase of the infection (Fig. 3D,E). During the chronic phase, acid-fast bacilli can only be seen in those FM that leave the granuloma towards the alveolar spaces, where they can be seen growing afterwards, destroying the FM and thus becoming extracellular again [24,25]. This is a crucial phenomenon, because then reinfection starts anew (Figs. 2C,3E) and explains the low but continuous production of IFN- γ during the chronic phase of the infection. Since the immune response against *M. tuberculosis* is mainly focused on growing bacilli [26], this scenario could not be seen with a bacillary population based only by SP bacilli.

2.3. “Dynamic” Versus “Static” Hypothesis in the Origin of LTBI

This scenario supports a “dynamic hypothesis” of LTBI, which suggests that LB is a consequence of their usual “cycle of life”. *M. tuberculosis* would simply remain in a SP for a long time (years), as demonstrated in other bacterial species [27], being retained in the necrotic tissue. Thanks to the usual kinetics of FM cells, these bacilli will be phagocytosed with the necrotic tissue and drained to the alveolar spaces, where they can reactivate again. Then, it is feasible to consider that the length of this phenomenon will be correlated with the degree of the initial necrosis extension accumulated in the pre-immune stage, and also by the ability to chronically generate new necrotic lesions afterwards.

This hypothesis is also supported by the latest diagnostic methods used to detect LTBI. The overnight incubation of whole blood with ESAT-6 mainly detects effector T cells able to recognize this antigen, which is linked to the active growth of *M. tuberculosis* [2], but it does not detect memory T cells, which would require a 14-day incubation [28]. This point counterbalances the skepticism generated by the fact that no LB have been ever detected in LTBI, and the diagnosis would only show the presence of memory T cells [29].

Taking into account this hypothesis, the chances of reactivation would obviously decrease with time, the same as the necrotic tissue (the main source of LB). The constant reactivation of bacilli would probably not be frequent enough to counterbalance the lost of the initial necrotic tissue, since the presence of the specific immunity does not allow for an excessive growth [30]. This idea agrees with the extensive epidemiological data available, demonstrating that the risk of reactivation and TB induction has a negative correlation with the period of time after the initial infection, and supports the theory suggesting that LTBI is limited to a certain period of time, instead to the “whole life” as classically postulated [31,32].

In fact, the usual slow metabolism of *M. tuberculosis* (even during the *log* phase) favors such a situation, especially when compared with other pathogens like *Escherichia coli* (100 times quicker) [33] that do not favor the “escape” out of the granuloma. In this case, reactivation would have more chances to take place inside the granuloma, instead of the alveolar space.

There are also other “slow” pathogenic mycobacteria, like *Mycobacterium avium*, but much less efficient if compared with *M. tuberculosis*. This is because they cannot in-

duce necrosis [34] and thus they cannot accumulate SP bacilli for such a long period of time and have enough chances to reactivate in a privileged zone (Table 1).

Table 1. Virulence Factors of *M. tuberculosis*

- | | |
|----|--|
| 1. | Slow metabolism, x100 times slower than <i>E. coli</i> |
| 2. | Thick hydrophobic cell wall |
| 3. | Ability to induce intragranulomatous necrosis |

The “dynamic hypothesis” would be in contrast with the classical view (which could be referred to as the “static hypothesis”) which suggests the presence of “dormant” bacilli trapped in the granulomas that generate a special metabolism to counterbalance an anaerobic atmosphere. In this case, a “resuscitation” factor is required to reactivate LB growth. This hypothesis has many weak points: mainly that the resuscitation factors are made from active growing bacilli, and that the resuscitation of “dormant” bacilli requires a very rich media [7] that is not present in old lesions that have become fibrosed or calcified over the years. Also, *in vitro* experiments supporting the theory that anaerobiosis is the clue to induce LB show that survival of bacilli subjected to anaerobiosis is very limited in time, even when a metabolic change has been induced by slowly decreasing oxygen pressure [35]. Furthermore, *M. tuberculosis* must also face the fast cell turnover in infected tissues [36,37] making implausible the idea of a static bacilli in a “dormant” state that suddenly reactivates.

Interestingly, it has been described that LB would just remain “dormant” outside the granuloma structure, inside the epithelial cells and in fibroblasts. This idea is based in a retrospective study in which DNA from *M. tuberculosis* was searched for in paraffined samples from old autopsies of non TB patients who lived in a country with a high TB endemia [38]. To date, no one has been able to explain the mechanisms of such persistence.

However, the “dynamic hypothesis” also has some weak points, because it is based on the experimental murine model of TB. Little is known about lesions in humans with LTBI. Humans are known to induce a strong fibrotic ring around the granulomas, which must control the massive presence of FM in mice. However, the exact timing of these lesions remains unknown. Even though considering the possibility that the hematological spreading would be the mechanism, it is known that whereas this is constant in mice, it takes place mainly during the beginning of the infection in humans [39] (Table 2).

Finally, it must be taken into account that the immune status of the host plays a crucial role in the maintenance of LTBI regarding both “dynamic” and “static” hypothesis, as it curtails the reactivation probability of the bacilli. This factor will be discussed in posterior chapters when reviewing the induction of TB. In addition, the “tolerance” of the host to the presence of bacilli must be taken into account. The presence of bacilli is higher in small hosts as the induction of a strong inflammatory response would compromise their life, when compared with bigger hosts who can better tolerate it (reviewed in [32]).

Table 2. Main Points in Dynamic and Static Hypothesis in LTBI and TB

<p>A. In the Induction of LTBI</p> <p>A.1. Static View</p> <ul style="list-style-type: none"> • “Dormant” bacilli trapped in a necrotic tissue for years (up to all life) • Trapped in an anaerobic environment • Requires a “resuscitation” factor <p>A.2. Dynamic View</p> <ul style="list-style-type: none"> • Stationary-phase bacilli able to survive for years (up to 10) in necrotic tissues. • Bacilli must face the constant turnover of the pulmonary cells • “Drainage” by FM of SP state phase bacilli out of granulomas • Inability of FM to be activated, thus leading to bacillary re-growth and the presence of extracellular bacilli in the alveolar spaces • Constant induction of new granulomas • Diagnosis of LTBI is based on the demonstration of specific T effector reacting against ESAT-6 and CFP-10, antigens related to the active growth of <i>M. tuberculosis</i>. • Gold standard therapy requires a long lasting period (9 months of isoniazid) and it is only active against growing bacilli. <p>B. In the Induction of TB</p> <p>B.1. Static View</p> <ul style="list-style-type: none"> • Sudden resuscitation in an old granuloma remaining in the apex years after (even all life) the initial dissemination from the primary lesion. • Resuscitation is helped by a Th2 polarization of the immune response <p>B.2. Dynamic View</p> <ul style="list-style-type: none"> • Constant reactivation from granulomas of different generations, led to the occupation of the apex. • The development of the cavity is a consequence of local conditions. Th2 polarization plays a restricted role.

2.4. The Nature of LB

SP cells are slow-growing cells with an infinite interdivision time, smaller, and with less DNA and RNA and fewer nucleoids per cell than fast-growing cells [40]. The lower growing rate is a defense by itself, as it hides potential weaknesses of the metabolical machinery, as can be seen in the usually lower activity of antibiotics against these cells, compared with *log* phase cells [41]. Furthermore, SP cells also show certain phenotypic changes, in the cell wall composition for instance. It has been proven that *Escherichia coli* cells suffer changes in the peptidoglycan composition [42] and penicillin-binding proteins [43]. *Nocardia asteroides* SP cells also develops a significant change in the mycolic acid composition [44] and a decrease in the cord factor concentration [45]. This is interesting because this bacteria is filogenetically very close to mycobacteria, and because mycolic acids represents the 50% of the *M. tuberculosis* dry weight [44].

M. tuberculosis SP bacilli increase the cell wall thickness when stressed at a low oxygen tension, which is related to the increase of the cell wall localization of the 16 kDa alpha crystalline protein [46], as well as its expression, as previously described by other authors [47]. Recently, it has been isolated the electrodense molecule responsible for the in-

crease of the cell wall thickness: the anaerobically produced pigment 1 (APP1), an ethanol-soluble low weight aliphatic compound [48].

The development of intracellular lipophilic inclusions (ILIs), which are mainly made of triacylglycerols, has been also associated with SP cultures [49]. Interestingly, the application of a combined acid-fast (auramine-nile red) stain to sputum samples from patients with TB indicated that the ILI component appears in *in vivo M. tuberculosis*, thus suggesting a virulence factor since these lipids could provide the initial energy source before initiating the active growth inside the alveolar macrophages. This could also explain how LB can survive for a long period of time in the necrotic tissue in SP.

Many means are nowadays being invested to demonstrate a specific genomic expression profile in *M. tuberculosis* SP cells and thus discover a particular antigenic profile [50,51], but so far no definite profile has been found.

3. END POINT: THE INDUCTION OF TB

Cavitary TB in the pulmonary apex is the most frequent form of TB in adults. Classically, the natural history of cavitary TB has been explained as the enlargement of a previous granuloma, the liquefaction of the necrotic tissue and the erosion of the bronchial tree. This permits the drainage of the bacillary population and the creation of the cavity. This is also an excellent environment for the bacilli, as the cavity is in contact with the inspired air, thereby increasing the oxygen pressure [52].

The cavity allows an “outdoor” contact and the dissemination of bacilli, while for the host it allows drainage of the bacilli out of the body [53]. In fact, Lurie described liquefaction and cavitation as “...nature’s more rapid but more hazardous manner of eradicating the disease” [54].

The positive correlation between high oxygen pressure and development of TB is well known, as it favors the growth of *M. tuberculosis* in *in vitro* [55], *ex vivo* [56] and *in vivo* experimental models [57]. Recent studies indicate a strong inverse correlation between above sea level altitude and the incidence of pulmonary TB [58]. Actually, the location of TB lesions in the apex is not a coincidence, because this area has limited vascular irrigation and the highest oxygen pressure [59]. However, the chances of being infected are scarce due to its lower volume. That’s why it is widely accepted that the infection of the pulmonary apex is usually a consequence of the dissemination from an initial infectious foci located at the base of the lung, where volume is larger (Fig. 4). However, once again this phenomenon may be explained by at least two different hypotheses.

On the one hand, the classical hypothesis, which is related to the “static” concept of LTBI, suggests that an initial systemic dissemination occurs before triggering a specific immunity. Then, a granuloma enters the apex but its development is stopped due to immunity. Bacilli become “dormant” and, at some point, reactivation takes place. This re-growth is possibly related to some “resuscitation” factor [60,61] and, of course, it can be linked to a decrease in the host’s immune response. Therefore, since resuscitation seems to be related to a sudden capacity to liquefy the ne-

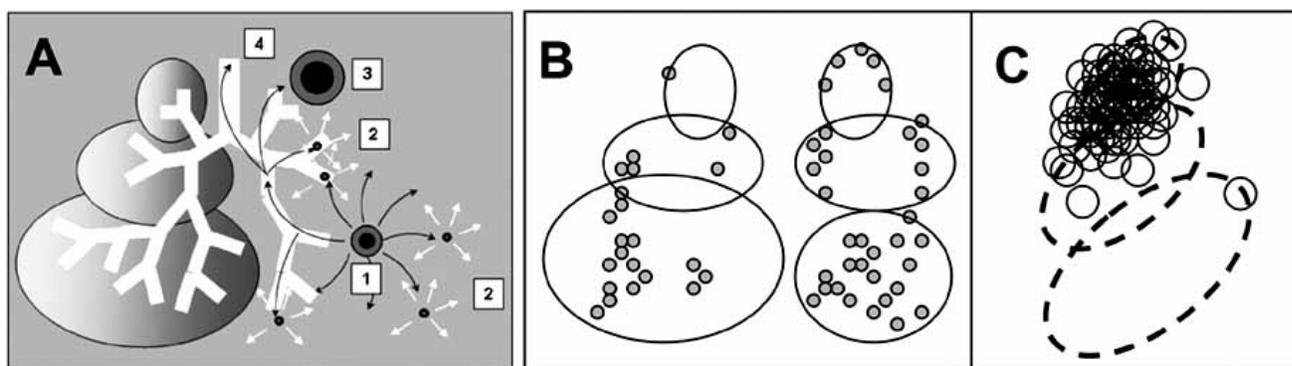


Fig. (4). Bacillary dissemination of LTBI in the lung and induction of TB. The right lung shows the differences on the volume of the basal lobes, compared with the apical lobes. This explains why the primary lesion usually takes place in the basal lobes (1). Constant dissemination would constantly result in secondary granulomas (2), which would be smaller because they develop in specific immunity until they appear in the pulmonary apex, where they generate a cavity (3); however, they could also be removed from the lungs (4). Pictures **B** and **C** reflect the location of calcified lesions corresponding to primary lesions and cavitory lesions, respectively, obtained from a large clinical study [39]. Frontal views are represented in pictures **A** and **B**, whereas a lateral view is shown in picture **C**.

cretic tissue, this raises the question on who is responsible for this resuscitation: the bacilli or the macrophage [62].

On the other hand, the “dynamic hypothesis” related to a dynamic concept of LTBI, is based on the constant dissemination of bacilli and the production of secondary granulomas, both before and after the acquisition of specific immunity. The demonstration of the bacilli escaping through the alveolar spaces carried by FM permits the constant generation of new granulomas in the lung, although they would be better controlled (smaller and with a lower bacillary concentration) than before due to immunity. In fact, the chance of its induction will decrease with time, as SP bacilli will decrease with the constant drainage of necrotic tissue.

Thus, according to this hypothesis, TB induction would be a matter of probability, i.e., the chances of the bacilli to reach and reactivate in the appropriate place, like the pulmonary apex, a zone with high oxygen pressure that allows a quick bacillary growth and creates a local immunosuppressive area [56,59,63] (Fig. 4, Table 2). Once the lesion has been generated, a late but strong local immune response would induce a bigger lesion that would not be structured on time and will liquefy. This would allow the growth of extracellular bacilli and will increase significantly the volume of the lesion until eroding the bronchial tree. At this point a cavity is created, and the introduction of inspired air will promote even more the extracellular bacilli growth and thus the expansion of the lesion [64].

The probabilistic mechanism of the origin of TB also explains why severe immunodepressed hosts (e.g., patients with AIDS) have more chances for developing an active TB. A 10% every year is widely accepted [65]. This is explained by their restrained ability to control the usual constant reactivation of bacilli, thus generating lesions not only in the apex, but also in other lung areas. It is also widely accepted that patients with AIDS have less probability of harboring bacilli in their sputum and generating cavitory lesions as the inflammatory response is not strong enough and liquefaction does not develop [53].

Regarding on the induction of extrapulmonary TB, which represents a third of the TB cases, it is clear that a systemic

dissemination from the pulmonary foci is required, thus being less probable and usually linked to immunodepression states, being localized into a well irrigated extrapulmonary tissues [52].

3.1. The Th1/Th2 Paradigm

For a long time it has been suggested that polarization to a Th2 response provokes the generation of TB. This idea initially came from the concept of “Listeria-like” and “Koch-like” responses against intracellular pathogens. In the first case, non-necrotic granulomas are generated, as in *Listeria monocytogenes* infection. However, the intragranulomatous necrosis generated by *M. tuberculosis* infection was considered to be “toxic” and thus to be avoided to better control infection [66]. This concept was the origin of the treatment with killed *Mycobacterium vaccae* (KMV), as its inoculation could induce “Listeria-like” responses, even in the presence of *M. tuberculosis*, and should improve the treatment of TB.

Years later, the demonstration of the polarization of T CD4 cells in Th1 and Th2, defined schematically by the ability of these cells to produce interferon-gamma (IFN- γ) or IL-4, led to adapt the theory to make it corresponding to “Listeria-like” and “Koch-like” responses, respectively [67]. In fact, some studies demonstrated that a Th2-polarized environment was responsible for inducing intragranulomatous necrosis, whereas Th1 tended to avoid it [68]. Again, the inoculation of KMV showed a marked ability to produce IFN- γ [69]. As a consequence, extensive clinical trials were launched, although KMV inoculation showed little or no effectiveness in the treatment of TB [70].

Moreover, this theory has led to conduct many studies comparing the polarization of Th2 between LTBI and TB patients and considering that the former are presumably “resistant” to the acquisition of TB. Usually, these studies have been conducted studying the IFN- γ /IL-4 ratio in peripheral blood using different techniques, and controversial data have been obtained [71]. To date, the recent finding suggesting that δ -IL-4 is a cytokine that may counterbalance IL-4 has added a new factor that would explain this controversy: i.e., the slight difference, if any, in the production of IFN- γ between LTBI and TB patients [72]. Recently, it has been

demonstrated that intestinal helminth co-infection has a negative impact on both anti-*M. tuberculosis* immunity and clinical response to TB therapy [73] not only because of a Th2 polarization, as usually stated [71], but by causing a decrease on absolute frequencies of CD3+, CD4+, CD8+, natural killer and CD4CD25+ T cells, when compared to either TB patients or healthy controls.

Finally, data obtained from murine experimental models of TB showed no evidence about a negative influence of Th2 cells on the Th1 protective response neither in IL-4/IL-13 KO [75] nor comparing the vaccination with different doses of BCG able to generate a "pure" Th1 response or a Th1/Th2 one [76].

Recently, new cells have appeared in the scenario that may affect this immunological balance: the T regulatory cells. To date, it has been hypothesized that these cells would counterbalance the Th2 response, and have tried to introduce this concept in the mechanism of KMV based therapy [74]. So far, it has been demonstrated that regulatory T cells are expanded in blood and disease sites in TB patients, and that suppress the Th1 immune response [77].

3.2. The Role of the Antibody-Immune Response

Cell-mediated immunity (CMI) by Th1-specific cells clearly plays a central role in the control of *M. tuberculosis* infection, despite the finding of B cells in *M. tuberculosis* granuloma (especially during the chronic phase of murine experimental TB infection [78,79]), and the characterization of specific patterns of antibody production in ILTB and TB [80]. For a long time it has been a solid statement regarding the presence of antibody-mediated immunity (AMI) due to the parallelism with human Hansen disease. In this case there is the dichotomy CMI vs AMI linked to the forms of the disease, i.e., better or worse controlled according to the presence of CMI (in Tuberculoid Lepra) or AMI (in Lepromatous Lepra) [81]. However, a recent review of classical studies has supported a protective role for AMI against *M. tuberculosis* [82].

A protective role of IgA monoclonal antibodies (mAbs) against the α -crystalline antigen of *M. tuberculosis* in early lung infection [83] has been reported in a murine model of TB, which also synergizes with IFN- γ in their bactericidal activity [84]. Moreover, a protective role of IgG mAbs in experimental models of murine TB has also been suggested in the literature in experiments using *M. tuberculosis* or BCG cells pre-coated with mAbs before inoculation in mice. Specifically, *M. tuberculosis* cells coated with IgG3 against arabinomannan (AM) enhanced granulomatous formation and survival of intranasal inoculated mice [85]; *M. bovis* BCG cells coated with IgG2a and IgG3 anti heparin-binding hemagglutinin adhesin (HBHA) mAbs before intranasal inoculation limited extrapulmonary dissemination and reduced the number of bacilli in the spleen [86], whereas *M. bovis* cells coated with IgG2b anti-MPB83 mAbs before intravenous inoculation increased long-term survival but did not reduce the bacterial load [87]. Finally, passive immunization using antibodies generated against fragmented *M. tuberculosis* cells (the RUTI vaccine) protects against reactivation of tuberculosis in SCID mice treated for a short period of time with chemotherapy, thus supporting the hypothesis that AMI plays a role in controlling *M. tuberculosis* infection [88].

In human tuberculosis, the presence of specific antibodies (Abs) against LAM or against antigen-85 complex correlated with the absence of disseminated tuberculosis in a pediatric population [89] and with a positive outcome in tuberculous Mexican Indians [90].

3.3. Malnutrition

The study of the mechanisms involved in the development of TB tends to avoid the mechanism that is probably the most important: malnutrition. The building of an effective immune system obviously requires huge protein supplies. From the political and social point of view, TB has always been related to poverty [91]. Just by looking at the estimated world incidence provided by the WHO [92], one can easily understand why the incidence of TB is especially high in countries where there is a high risk of malnutrition.

Nevertheless, despite using these premises to apply for project funding, most of the studies conducted by the scientific community do not take malnutrition into account. This may be due to the lack of specific data defining what is malnutrition from an immunity point of view, and of commonly agreed specific tools to measure it [93].

Historic data from people who suffered specific periods of diet restrictions (e.g., during World War I or II) showed an increase in the incidence of TB [94]. 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 six-fold to ten-fold increase in the adjusted hazard of TB [95]. Taking into account these data, it is natural to consider the measurement of malnutrition in future studies, including, for example, interviews and nutritional examination such as the Standard Global Assessment [96], which includes tests like subcutaneous tissue, muscle wasting, edema assessment or BMI in its comparisons [97].

4. IS THE MODEL OF CHRONIC INFECTION A GOOD MODEL OF LTBI?

This question is crucial, as the bacillary concentration in this model is much higher than the one expected in LTBI in humans (4-5 logs vs 2 logs). This can be explained by a more "tolerant" reaction of mice against *M. tuberculosis* infection, which allows the presence of a higher bacillary concentration, as pointed above. Besides, mice usually do not generate a necrotic reaction inside the granulomas, as is the case in humans or larger mammals. This can be a survival maneuver. In the case of large mammals, tissue destruction induced by the inflammatory response focused on the building of a strong fibrotic granuloma is not a problem. This is very important i.e. in mice, as their tissue volume is much lower, and thus they try to trigger a less toxic response, a Th1 one [11, 32]. In summary, this strategy keeps mice alive but harboring a high bacillary concentration and a progressive dissemination of the lesions in the lung [24, 25]. This dissemination is what finally kills all the infected mice. In contrast, large mammals such as humans trigger a strong innate response which, combined with a Th2 immunity and the presence of IL-4 and the so-called Th3 response (characterized by the presence of transforming growth factor-beta), controls the dissemination of infection by isolating the lesions with a thick fibrotic mantle [64,71], and also by a better control of

the bacillary concentration. In this case, a strong toxic reaction is generated, but the control of the infection is more effective. That's why 90% of LTBI patients will never suffer a TB [1].

The low bacillary concentration expected in LTBI in humans was the origin of the Cornell Model [98], which has been used for a long time, and still remains so. This model is based on a strong chemotherapy administered shortly after the beginning of infection. Then a sterile status is achieved in the tissues, as no viable bacilli are detected after *in vitro* culturing. This status is modified a long time afterwards, either spontaneously or after treatment with immunosuppressive drugs. In this model, the LTBI candidate must demonstrate its capacity to avoid reactivation; this often requires a very high number of animals to obtain a significant result, a point that usually has not been well addressed [99]. In this case, there are two critical assumptions: that in LTBI no viable bacilli and no granulomatous lesions are present in the host. The first assumption may be assumed, but not so the second one, as the control of *M. tuberculosis* bacilli is known to be based on the granulomatous response induced by the specific immune response [30]. In this regard, it has been suggested that the Cornell model might be a good model for studying the hypothetical tolerance to chemotherapy found in some *M. tuberculosis* bacilli [29].

5. TREATMENTS AGAINST LTBI

5.1. Chemotherapy

The origin of the LTBI chemotherapy with isoniazid is at the beginning of chemotherapy of active TB itself, trying to respond to an immediate problem: what to do with household dose contacts of open cases of pulmonary TB, which had a high infective capacity [100]. Nowadays, the 9-month period has been established as the best cost-effective schedule [4]. This is an empirical treatment supported by epidemiological data that raises interesting questions about its mode of action. The bactericidal activity of isoniazid is known to be optimal at the beginning of the treatment, i.e., during the first two weeks, when the higher concentrations of actively growing bacilli are present in the lesions [101]. Then, why is a 9-month period required?

The only explanation is that the presence of constant levels of isoniazid avoids the possibility of reactivation of LB in the dynamic scenario marked by the constant reabsorption of the necrotic debris from the granuloma (Fig. 5), and thus the lesions disappear as well as the LB, which are removed from the pulmonary parenchyma until none of them are present and thus reactivation is not possible [32] (Fig. 6B).

Besides, chemotherapy also induces local immunosuppression [102]; this is why there is a chance of LB reactivation when chemotherapy is not prolonged enough.

To date, shorter schedules for LTBI treatment have been tested [1], including the shorter 6-month course of isoniazide, which is likely to be inferior to the 9-month course [4], and a 4-month course of daily rifampicin, which remains largely untested [1]. Unfortunately, the most interesting one, based on a 2-month treatment with rifampicin and pyrazinamide, which has a similar efficacy compared to treatment

with INH for nine months [103], induces strong hepatic toxicity which may cause the death of the patient [104].

5.2. Therapeutic Vaccines

5.2.1. Historical Approach

The use of therapeutic vaccines against infectious diseases is an old issue. Historically, its origin is closely linked to the idea that microorganisms are the etiology of infectious diseases. Louis Pasteur, the first and more relevant advocate of vaccines, was the first to prove their efficacy in the treatment of rabies, with attenuated virus [105]. Unfortunately, the prestige of vaccines suffered a sudden breakdown a few years later precisely in the treatment of TB. The use of tuberculin (based on killed *M. tuberculosis* extracts) was immediately banned and rejected as a consequence of a passionate fight between Robert Koch (its promoter) and the Government of Prussia. Governors wanted to subdue the interests and claims of Koch on the development of tuberculin by promoting a quick clinical assay that did not take into account the indications and conditions in which the efficacy of tuberculin had been proven (i.e., in localized and in initial pulmonary TB). By including advanced cases of TB, highlighting the mortality registered in the treated cases, and, of course, without considering any case control or statistical methodology (not available at that time), the Prussian Government discouraged the development of this therapy and subdued Koch's wills to the Government's wishes (i.e., the construction of a Pasteur's Institute-like project with Robert Koch as a director) (extensively reviewed in [106]).

Fortunately, therapeutic vaccination had another chance with Sir Almrod Wright, who not only continued the treatment of TB with therapeutic vaccines based on killed *M. tuberculosis*, but also used this strategy with other microorganisms, like *Staphylococcus aureus* or *Haemophilus influenzae*. In fact, this therapy represented a therapeutic revolution and these vaccines were distributed by the Parke and Davis Company. The benefits obtained with this collaboration allowed Wright to fund the Inoculation Department in the Saint Mary's Hospital in London, and extended the practice of this therapy during the first half of the 20th Century, until the availability of antibiotics reduced its use and finally disappeared [107,108].

5.2.2. Therapeutic Vaccines Against LB

Several antigens are known to trigger protective immunity against *M. tuberculosis* infection that is mainly aimed at avoiding its growth, such as the ESAT-6 group or the Antigen 85 complex [26], but little is known about the particular antigenic composition of LB. Even though, it is not clear whether the reduced metabolic activity implies a change in the antigenic composition or, on the contrary, it is precisely the decreased metabolism what makes them invisible to the immune response.

This explains why the approach to obtain an immunotherapy has been mainly empirical, while potent international consortiums try to better understand the exact metabolism of LB in order to design a rational vaccine against it using the most advanced and powerful tools in genomics and proteomics.

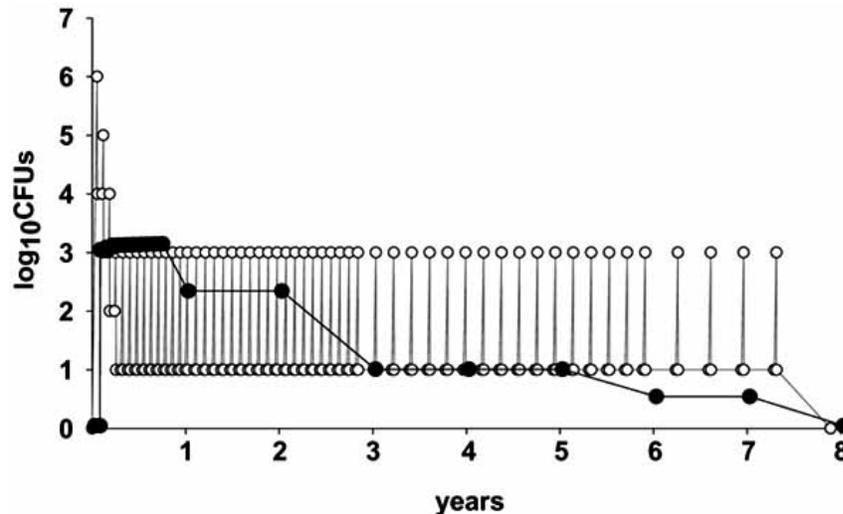


Fig. (5). Mathematical approach to latency in TB based in the constant reactivation of latent bacilli. There is an acute phase in which bacilli reach the maximum concentration after 20 days of constant growth lasting until the immune response is triggered and 99% of the bacillary bulk is destroyed (A). This reaction originates from 0.1% of latent bacilli in necrotic tissue. The remaining 1% of bacilli in macrophages regrow after 20 days at rest, when once again 99% are destroyed, originating another 0.1% of latent bacilli in a necrotic tissue. This dynamic continues until equilibrium is reached at the tolerance threshold of $2 \log_{10}$ in humans. The number of bacilli in necrotic tissue decreases whereas the period at rest between reactivations increases proportionally to historical data of untreated tuberculosis-infected household contacts [124] (from [32]).

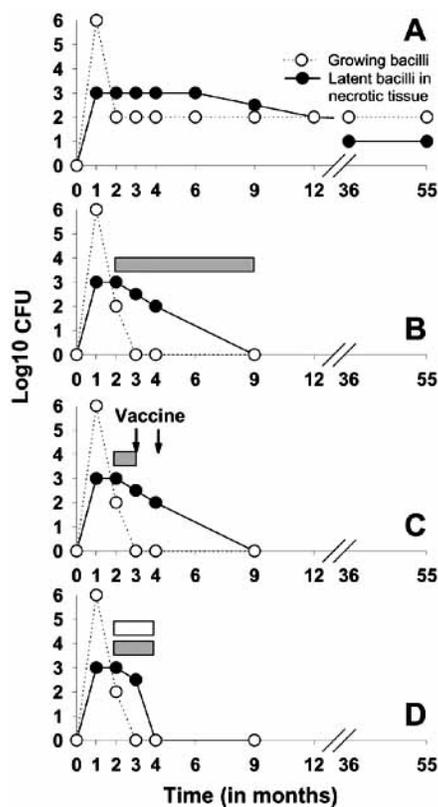


Fig. (6). Strategies for treating LTBI. Picture A summarizes the mathematical model shown in Fig. 5. The following pictures show the standard 9-month chemotherapy (gray box) (B); the combination of a short-period chemotherapy and a vaccine therapy (C); and the combination of a short-period chemotherapy and an anti-inflammatory therapy (white box) (D).

5.2.2.1. The DNA Vaccine Approach

The first successful approach was obtained in 1999 [109] when Lowrie *et al.* achieved control of bacillary reactivation after immunotherapy with a DNA vaccine encoding for the heat shock protein 65 (hsp65). These results were recently confirmed by Silva *et al.* [110] using the intratracheal model of TB, which induces a massive pneumonia [111], and also by Nuermberger *et al.* [112] who obtained slightly better bactericidal results when combining moxifloxacin with this vaccine in the treatment of LTBI, although it did not prevent the regrowth after discontinuing moxifloxacin treatment. Besides, the authors highlighted the possibility that the efficacy obtained could be just a consequence of boosting the previous BCG vaccination induced to obtain the experimental model of LTBI [112]. The protection mechanism of this approach seemed to be related to the boosting of the immune response, favoring the Th1 response and decreasing the Th2 response. Bactericidal activity was also obtained when chemotherapy was combined with inoculation of a DNA vaccine encoding for the Ag85 A peptide [113] (Fig. 6C).

Unfortunately, DNA vaccines are not free of problems, as to date no direct relationship has been demonstrated between protective outcomes in mice and those in larger animals. The fear of autoimmunity or problems related to the integration in the host genome, as well as the high doses required (milligrams) economically complicates their development and are relevant issues that must be solved and will require a special regulatory approach [114]. Additionally, recent evidence suggests that immunotherapy with DNA vaccines may induce toxicity in the murine models of TB, at a histopathological level [115].

5.2.2.2. RUTI

The origin of the RUTI vaccine comes from the idea that LB are bacilli that are able to resist stressful conditions (like

a low pH) developed in the necrotic tissue, combined with the low oxygen pressure commonly found in infected tissues. The easiest approach was to culture the bacilli in a solid media where progressive decreases in oxygen pressure and pH were achieved [116,117]. Studies on the induction of a “human-like” experimental model in mice, which intended to reproduce intragranulomatous necrosis in those animals, led to the theory that this was a consequence of the endotoxin-like molecules present in the bacillary cell wall, that triggered a local Schwartzmann reaction [118], and not as a result of the adaptative immunity, as described by Dannenberg [119]. That’s why in the process of obtention, the cells are treated with Triton X-114 (i.e. to remove such endotoxin-like molecules). This was a very important point, as the induction of this toxic reaction (known as the Koch reaction) was one of the factors that misaimed the research on therapeutic vaccines against LTBI for a long time. Additionally, those cells were fragmented and liposomed to favor a better antigenic presentation [120].

The mechanism of action of RUTI is extensively reviewed elsewhere [32]. Briefly, RUTI induces of a strong polyantigenic response of a mixture nature (Th1/Th2/Th3) that should promote a better recognition of LB, not only through the induction of a Th1 response to a wide range of antigens (not published) but also through the production of specific antibodies, which have been shown to play a crucial role in controlling the reactivation of *M. tuberculosis* infection in SCID mice [88].

As described above, there is increasing evidence about the protective role of AMI in the control of infections caused by intracellular pathogens [121]. Its mechanism of action includes toxin and virus neutralization, antibody-dependent cellular cytotoxicity, and other activities that require other immune systems components, such as opsonization and

complement activation. Furthermore, other mechanisms have been related to AMI, like the capacity for amplifying or suppressing the inflammatory response, or to have direct antimicrobial activities [121].

The strategy to use RUTI requires the brief administration of chemotherapy (i.e., for one month) to “homogenize” the LTBI patients and essentially to kill any growing bacilli; to reduce the local inflammatory response and thus the potential Koch reaction; and to remove FM and with them the local immunosuppression and the “escape vehicle” of LB. In this regard, RUTI also works as a immune boost to compensate for the transient immunosuppression induced by the chemotherapy itself [32] (Fig. 6).

5.2.3. The Addition of Anti-Inflammatory Therapy

With the introduction of anti-TNF antibodies for the treatment of autoimmune diseases, a high increase in the reactivation of TB cases have been detected in LTBI patients. This phenomenon was expected, as the role of TNF in the control of *M. tuberculosis* infection is well known [122], and confirms the role of this cytokine in the maintenance of the balance between the host and the bacilli in order to avoid *M. tuberculosis* reactivation. Interestingly, a recent review of medical literature identifying new adjuvants for the treatment of pulmonary TB to shorten treatment regimens, demonstrated that, contrary to what could be expected, the addition of IFN- γ did not effectively increase the mycobactericidal capacity of lung macrophages. However, the addition of anti-TNF therapies and prednisolone accelerated the response to treatment, thus supporting the hypothesis that the granulomatous response triggered by the host may protect its bacillary population against the anti-TB therapy [123].

Although no experience is related to the treatment of LTBI, this could be achieved in the future. As indicated in

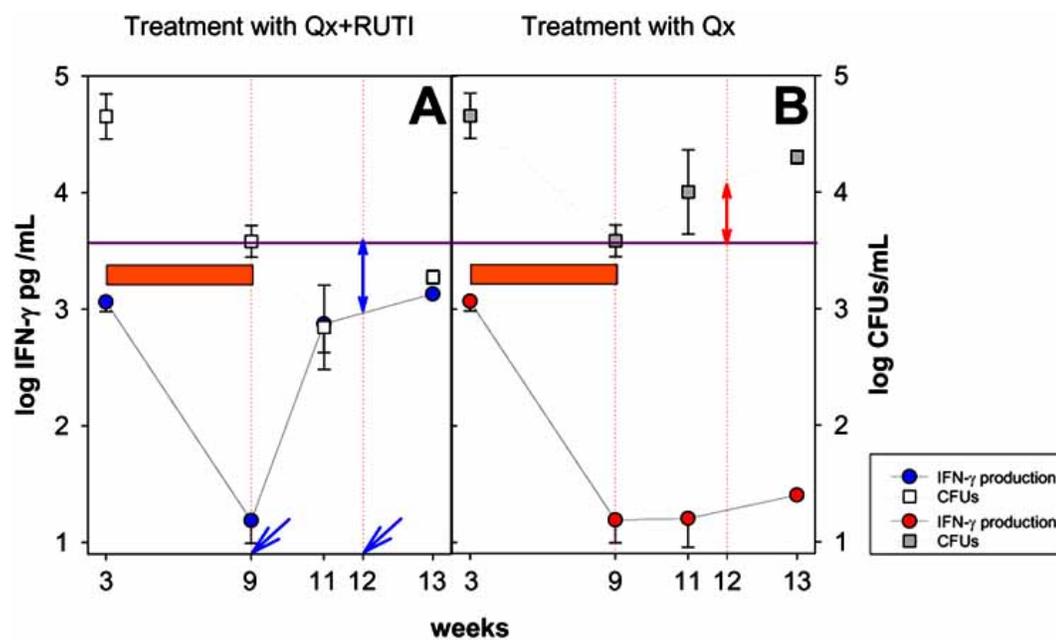


Fig. (7). The inoculation of RUTI (blue arrows) allows a short-period therapy (i.e., 6 weeks) by enhancing the immune response against *M. tuberculosis* decreased with isoniazid therapy (A). However, treatment with short-term isoniazid alone allows the reactivation of the remaining bacilli (B). (unpublished experimental model of murine tuberculosis induced by i.p. inoculation. Follow-up of bacillary concentration and IFN- γ production “ex vivo” after splenocyte-stimulation with PPD.

Fig. 6D, such a treatment should be carefully designed to guarantee complete sterilization, as the inclusion of anti-inflammatory treatment would enhance the same effect induced by chemotherapy, and thus it should remove all bacilli from the necrotic tissue. Otherwise, the reactivation of any remaining LB would curtail the success of this approach. In addition, it is interesting to note the relevance of such a design because it could accelerate the removal of necrotic tissue, although the treatment period required should be carefully quantified.

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