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RUTI: A new chance to shorten the treatment of latent tuberculosis infection

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Summary Treatment of latent tuberculosis infection (LTBI) requires a long period of chemotherapy (9 months), which makes treatment-compliance extremely difficult. Current knowledge of latent bacilli and of the lesions with which they are associated suggests that these bacilli survive in granulomas with a central necrotic core and an outermost layer of foamy macrophages (FM) that represent an important immunosuppressive barrier. The presence of FM, which is especially strong in mice, explains not only the kinetics of the drainage of dead bacilli, debris and surfactant, but also how latent bacilli can escape from the granuloma and re-grow in the periphery, particularly in the alveolar spaces where they can disseminate easily.

RUTI, a therapeutic vaccine made of detoxified, fragmented *Mycobacterium tuberculosis* cells, delivered in liposomes, was used to assess its effectiveness in a short period of chemotherapy (1 month). The rationale of this therapy was first to take advantage of the bactericidal properties of chemotherapy to kill active growing bacilli, eliminate the outermost layer of FM and reduce local inflammatory responses so as to avoid the predictable Koch phenomenon caused by *M. tuberculosis* antigens when given therapeutically. After chemotherapy, RUTI can be inoculated to reduce the probability of regrowth of the remaining latent bacilli.

RUTI has already demonstrated its efficacy in controlling LTBI in experimental models of mice and guinea-pigs after a short period of chemotherapy; these experiments in animals showed the induction of a mixed Th1/Th2/Th3, polyantigenic response with no local or systemic toxicity. Local accumulation of specific CD8 T cells and a strong humoral response are characteristic features of RUTI that explain its protective properties; these are particular improvements when compared with BCG, although the regulatory response to RUTI may also be an important advantage.

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Further experiments using bigger animals (goats and mini-pigs) will provide more data on the efficacy of RUTI before starting phase I clinical trials.

Introduction: what is a latent bacillus?

Despite this being a simple question, finding an appropriate answer is crucial to demonstrate the validity of any new treatment against latent *Mycobacterium tuberculosis* bacilli; latent bacilli are responsible for the long period of treatment currently required for sterilizing lesions infected with *M. tuberculosis*.

The nature of latent bacilli and their ability to survive "in vitro" in low pO_2 conditions, and even in anaerobiosis,[1] has been widely studied. To survive in low pO_2, bacilli appear to acquire a state of nonreplicant persistence (NRP).[2] Huge lesions with intragranulomatous necrosis (IN) that are induced in tuberculosis (TB) may develop such an extreme anaerobic environment,[2] supporting the idea that latency induced by low pO_2 occurs in lesions. However, direct measurements have never demonstrated the presence of anaerobiosis in TB lesions and factual support for the hypothesis is lacking. Furthermore, IN is a "living" tissue, full of collagen fibers, that also needs a physiological environment for survival. Anaerobiosis, therefore, appears unlikely in such a setting. Even if such atmosphere with low pO_2 be induced in calcified lesions, experimental models[3] demonstrate that the period in which the bacilli may survive in this environment is definitely finite. Moreover, microaerobiosis is usual in the host tissues[4] and, therefore, not only latent bacilli but also actively growing cells may adapt to this environment. Furthermore, experiments in knock out (KO) mice lacking functional genes essential for triggering a competent immune response demonstrated that active growing bacilli can be found inside massive lesions with abundant IN, expected to have a low pO_2.[5,6]

Some authors defined latent bacilli as those present in the tissues of mice treated with chemotherapy for a long time, whose presence was not detected in cultures until a few months after the end of the treatment; this process was favored by the administration of cortisone. This experimental model, characterized a long time ago, is known as the Cornell model.[7] In the Cornell model bacilli do not have to resist an extremely low pO_2. However, patients with latent tuberculosis infection (LTBI) face circumstances different from those in the Cornell model.[8] Usually, people with LTBI display a cell-mediated and antibody-mediated immune response that controls progression of the infection in the initial focus of infection and in the local draining lymph nodes (the "Ghon complex") by inducing a strong granulomatous infiltration,[9] a process which does not happen in the Cornell model. Besides, It is currently accepted that the population obtained with the Cornell model is special and is not considered to be a good model of latent bacilli, but rather of "persistent" bacilli.[10,11] Persistence implies that a special population of bacilli subsists the period of chemotherapy, probably through acquiring tolerance to antibiotics,[11,12] although its mechanism remains poorly understood.

Other authors focused on the hypothesis that latent bacilli have to adapt to a lack of nutrients, thus resembling the bacterial population found in the steady state of conventional liquid cultures.[13] Thus the term "dormant" was established to define bacilli that were "in a state of low metabolic activity and unable to divide or to form a colony without a preceding resuscitation in liquid medium, which may either occur spontaneously or require the provision of compounds (growth factors) present in the supernatant of growing cells".[13,14] These authors considered the in vitro "dormant" form as the equivalent to the one obtained in vivo with the Cornell model, representing an extreme form of viability, close to the death of the cell. However, the exquisitely fastidious requirements needed for regrowth of these "dormant" bacilli are difficult to reconcile with the conditions faced by latent bacilli in vivo, bacilli which are, however, able to reactivate after a long period of time to induce TB disease.

Finally, there is the observation made a long time ago that the bacillary population obtained from the lungs of chronically infected mice has a greater resistance to heat stress (53 °C) than the bacillary population in the acute phase.[15] The same author submitted "in vitro" cultures, young (exponential phase) and old (steady-state phase) to the same conditions and obtained a higher resistance to heat stress in the latter. This observation led to the conclusion that a slower growth rate made bacilli from the chronic phase more resistant to stress. Consistent with this hypothesis, Muñoz-Elias et al.[16]

recently reviewed this work and demonstrated that the chronic phase of infection in murine TB was accompanied by a reduction in the rate of bacterial cell-division.

The current consensus is that the chronic phase in the experimental murine model is a consequence of the specific immunity elicited against growing *M. tuberculosis*.[17] This immunity triggers a number of mechanisms that induces the death of most bacterial cells (i.e. low pH, reactive oxygen intermediates, reactive nitrogen intermediates, etc.). As a consequence of this strong immune response, the population in the chronic phase comes from bacilli able to adapt to stress and thus, a high proportion of these bacilli are the same as so-called "latent" bacilli. Therefore, in our opinion latent bacilli are those that can resist the immunological response triggered by the host. However, it is noteworthy that not all bacilli from the chronic phase are in a latent state, consistent with the observations in patients with LTBI.

Immunopathology of the scenario of local infection

At the beginning of *M. tuberculosis* infection, the development of IN suggests the occurrence of the Koch phenomenon, observed in most mammals.[18] The Koch phenomenon probably arises from a local Schwartzman reaction.[19,20] The following sequence of events are postulated: infected macrophages in the granuloma produce high concentrations of TNF; the presence of this cytokine makes the site responsive to endotoxin-like molecules (like trehalose dimycolate[21,22]), which exist in the cell wall of *M. tuberculosis* when the lack of specific immunity allows an exponential growth of bacilli; the corresponding reaction triggers a "first wave" of latent bacilli (i.e., bacilli surviving the initial inflammatory response), which are then trapped in the collagen fibers that "take the place" of the destroyed infected macrophages.

A "second wave" of latent bacilli may be triggered by the induction of specific immunity, which activates the infected macrophages to destroy most of the initial bacillary bulk. This response to specific immunity is observed in the experimental murine model of aerosol-induced TB, where IN is not usually induced and where antibody-mediated immunity is not triggered during this initial phase of infection. In this model, a 90% reduction of the bacillary concentration is caused by the activation of infected macrophages by T cells that produce IFN-γ and by the cytolysis induced by specific CD8 cells.[23–25]. However, during the chronic phase, new cells appear at the outermost layer of the granulomas, occupying the alveolar spaces: these cells are the foamy macrophages (FM).[26,27] FM are usually seen in all inflammatory responses in the lung, and not only in infectious inflammatory responses.[28] The function of FM is to phagocyte the necrotic material produced after an interstitial inflammatory response (i.e. dead host cells, the surfactant secreted locally and the dead *M. tuberculosis* cells remaining), to rebuild the original parenchyma.[29] These newly accumulated macrophages can leave the lesion through the alveolar spaces to reach the upper bronchi, where they are swallowed and enter the stomach. In the context of *M. tuberculosis* infection, the FM may be also originated from the initial infected macrophage population, following the killing of most of bacilli.[29] The aerosol murine model presents an interesting scenario because large quantities of bacilli are observed in granulomas that are initially ill-defined, during the acute phase of infection, whereas no acid fast bacilli are seen in their center during the chronic phase (at 6 weeks post infection); instead, single bacilli are present in the periphery, inside FM located in the alveolar spaces. Further characterization of these structures demonstrated a progressive increase of inducible nitric oxide (NO) synthase (iNOs) during the chronic phase of infection, which is the main enzyme responsible for NO production and which is localized almost exclusively in FM.[27] Hence, since FM produce NO and may suppress the activating specific T cells, both Th1 and Th2,[30] they constitute an immunosuppressive outermost ring around the lesion.[31–34]

In the acute phase of *M. tuberculosis* infection, production of RNI is linked to a Th1 activation, and is paramount for the control of the infection[34] including the control of chronic infection in the murine model of TB.[35] However, at the same time, NO production, which is stimulated not only by IFN-γ but also TNF[36–38] and IL-4 in human monocytes[39] and pulmonary epithelial cells,[40] also generates a negative feed back in the production of these cytokines thus generating a local immunosuppression both for Th1 and Th2 responses.[41,42] The anti-inflammatory role of NO has been suggested by others,[43] taking into account that RNI are also extremely toxic to the host cells[44] and not all of them are able to kill *M. tuberculosis*, (for example, peroxynitrite anion,[45] which, at the same time exerts a negative feedback against NO production).[46]

FM have been extensively studied in the induction of atherosclerosis. In this case, their origin is

linked to an inflammatory process. In particular, it has been demonstrated that TNF is able to upregulate the lectin-like oxidized LDL receptor (LOX-1)[47] which increases the uptake of Oxidized LDL (OxLDL)[48] thus contributing to lipidosis and FM formation. In this case, the increase of LOX-1 was linked to a NO deficiency. Thus, the presence of NO does not favor the formation of FM. On the other hand, it has been recently demonstrated that oxidation of LDL is not a limiting factor for induction of FM as they can be also formed with native LDL[49] through a mechanism of macropinocytosis. Additional evidence in support of the view that inflammatory response is paramount in the induction of FM comes from the studies with IL-10 KO mice, which exhibited a threefold increase in cholesterol accumulation in macrophages, whereas over-expression of IL-10 in either C57BL/6 or *Ldlr*$-/-$ mice resulted in a significant decrease.[50] Furthermore, treatment of *Ldlr*$-/-$ mice with the nonsteroidal anti-inflammatory drug indomethacin or a COX-2-selective inhibitor, rofexocib, resulted in a significant reduction in lesion size.[51]

In the context of the lung infection it must be taken into account that the presence of large amounts of a lipoprotein surfactant complex may also play an important role. The name ''surfactant'' is derived from its well-defined ability to lower surface tension at the interface of alveolar gas and the liquid hypophase,[52] and also participate in the control of parenchyma destruction.[53] In this regard, when alveolar macrophages are chronically exposed to high quantities of surfactant they become FM.[54] The presence of killed *M. tuberculosis* bacilli or cell wall fragments from them also induces the formation of FM.[29] Furthermore, some components of *M. tuberculosis* are also known to induce iNOS production.[55] This may explain why apparently incoherent circumstances may coexist in the chronic phase of *M. tuberculosis* infection in mice, i.e. the presence of FM in the context of NO expression and the continued local immunosuppression over a long period. Besides, as NO production in the FM may occur in the context of insufficient macrophage activation, as a consequence of the suppression of Th1 response, this could explain why the bacilli that shelter in them can survive. It is known that RNI bactericidal effect is concentration dependent.[56] Thus, paradoxically, RNI are known to be able to even induce mycobacterial growth, and have been implied in the hormesis phenomenon, i.e. stimulatory effects caused by low levels of a potentially toxic agent.[57]

Recently, several reports have shown that low or nontoxic concentrations of NO donors (DETA-NO) modulate the expression of a 48-gene regulon, which is expressed by *M. tuberculosis* both in vivo and in vitro and prepares bacilli for survival during long periods of in vivo dormancy. In conjunction with low concentrations of oxygen in tissues, NO can play a role in the initiation and maintenance of the latent state of *M. tuberculosis* within granulomas, which could be a mechanism for the tubercle bacillus to persist in FM of the host.[58–61] In addition, Darwin et al. have shown that mutation of the proteasome of *M. tuberculosis* sensitized bacilli to NO, suggesting that mycobacterial proteasome serves as a defense against nitrosative stress.[62]

FM also produce large quantities of transforming growth factor-beta (TGF-β) and thus may induce apoptosis of effector T cells.[63] They are known also to display high levels of DEC-205, (i.e., a marker characteristic of dendritic cells), but down regulate MHC class II markers, thus rendering them inadequate for antigen presentation (although no data on MHC Class I markers are available). Besides, FM appear to resist apoptosis.[64] Thus, FM appear to be a kind of sanctuary for those bacilli that survive the immunological response, in addition to also being a source of local immunosuppression. Furthermore, we must also consider the hypothesis that FM cannot produce mature endosomes and induce stress conditions due to having too much material to process. This in turn, may encourage bacillary growth at the periphery of the granuloma.

Finally, it has been reported that treatment with isoniazid (INH) and rifampicin results in the elimination of the FM outermost layer,[65] but it is still not clear if this phenomenon is linked to the inhibition of the regrowth of the bacilli and thus the reduction of the inflammatory response, or by the direct reduction of the inflammatory response caused by the antibiotic, as demonstrated for moxifloxacyn.[66,67]

Figure 1 represents a time-related description of the murine model of TB with regards to growing/dead bacilli, synthesis of surfactant, IFN-γ and TNF, and FM. All these data are based on published work.[16,26] However, the kinetics of surfactant has been interpreted based on its function, as a component of the innate immunity and considering its role against tissue destruction. The peak of surfactant occurs before the peak of IFN-γ and TNF and its production is sustained as a consequence of cumulative infiltration of the lung parenchyma,[27,28,68] causing constant injury to this tissue. In this model, the presence of FM is a consequence of the accumulation of parenchymal debris, surfactant and dead *M. tuberculosis* cells.

Note that if latent bacilli remain in the macrophage initially infected, which has been activated

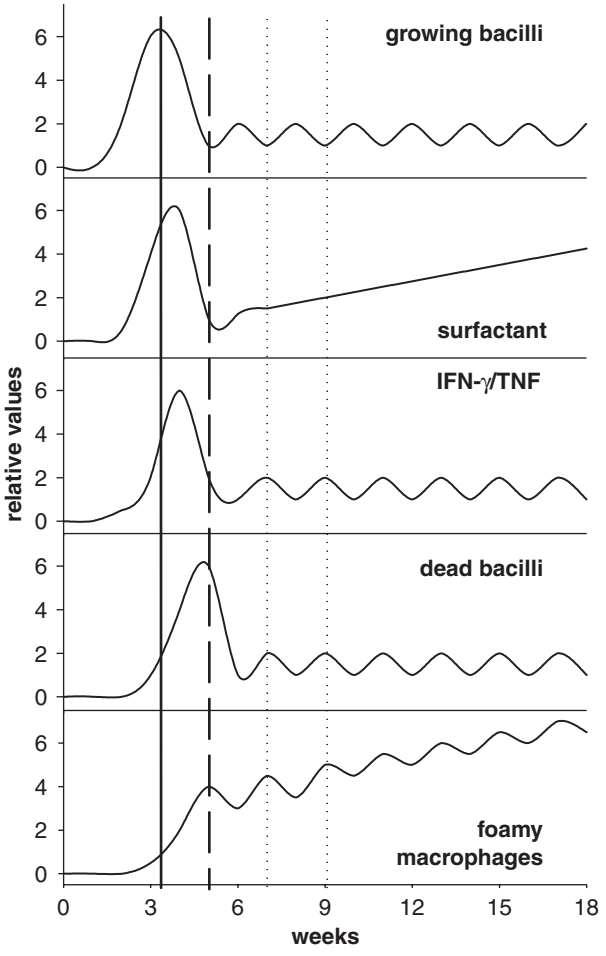

Figure 1 Evolution model in experimental murine TB, focusing on the local presence of growing/dead bacilli; surfactant, IFN-γ and TNF production; and presence of FM. Continuous and dashed lines mark the peak and the base of growing bacilli. Dotted lines represent a cycle in the evolution in the chronic phase.

and has destroyed most bacilli, and it becomes a FM, it is reasonable to assume that, with time, low pH will be neutralized and the bacilli will re-grow when FM are at the periphery of the granuloma. However, if these infected macrophages become apoptotic or necrotized in the center of the granuloma, what happens when the latent bacilli is phagocyted by new macrophages? These bacilli are metabolically inactive and they cannot avoid the phagosome-lysosome fusion, as it requires a strong metabolic effort.[69] These latent bacilli are then considered "dead bacilli" and must face, once again, a low pH that will further prolong their latent state until stressful conditions disappear. This hypothesis may also explain why very few bacilli remain in FM and why so few are drained to the periphery where they finally re-grow. Interestingly, in both situations the antigens of the latent bacilli can be easily processed due their "dead like" status,[70,71] and if they are presented inside apoptotic vesicles, they would enhance antigen presentation through MHC I and CD1 molecules.[72] Paradoxically, latent bacilli might trigger a specific immunity. Why does this not happen? The answer may be "due to local immunosuppression induced by FM" or "because the macrophages harboring them do not reach the lymph nodes to present the antigens, as this is not their role".

Finally, we must also consider the hypothesis that FM cannot produce mature endosomes and induce stress to bacilli, due to having too much material to process, thus encouraging bacillary regrowth at the periphery.

In fact, the presence of these FM is crucial in the murine model, as they allow continuous infiltration of the pulmonary parenchyma during the chronic phase of the infection.[26,27] However, this phenomenon seems to be limited in bigger hosts, such as guinea pigs[73] or humans,[74] where the host triggers a strong inflammatory response at the beginning of the infection, inducing IN and quickly surrounding the granuloma with a fibrotic mantle. Nevertheless, these foamy cells have also been observed inside the granuloma harboring single bacilli.

All these data support the hypothesis that latent infection in humans is sustained by a static population in the necrotic tissues and by a constant reactivation of latent bacilli phagocyted by macrophages trying to reabsorb the necrotic tissues. *M. tuberculosis* recognizes the absence of stressful conditions by modulating their gene-expression depending on external conditions (i.e. *icl* production is triggered with acid pH),[75] and must also detect when the adverse conditions disappear (i.e. when phagocyted by FM) and thus when they can start growing.

The very low metabolic activity of *M. tuberculosis*[76] also favors their survival. These bacilli may reactivate far from the initial infection focus, away from the presence of effector T cells, and inside the alveolar space where, after growing and destroying the infected macrophages, they can easily disseminate to be phagocyted by other naïve alveolar macrophages and reinitiate infection. The focus of the immunological response is on growing bacilli, which pool is constantly regenerated; in contrast, a response against latent bacilli, if it is ever triggered, remains relatively weak.

As represented in Fig. 2A, the suggested scenario of LTBI includes a static population of latent bacilli that periodically re-grows at different sites of the parenchyma. For a bacillus in this context, reaching a convenient site for growth is crucial (e.g., the apical zones of the lung with high oxygen pressure to induce a large granuloma and a cavern).[77]

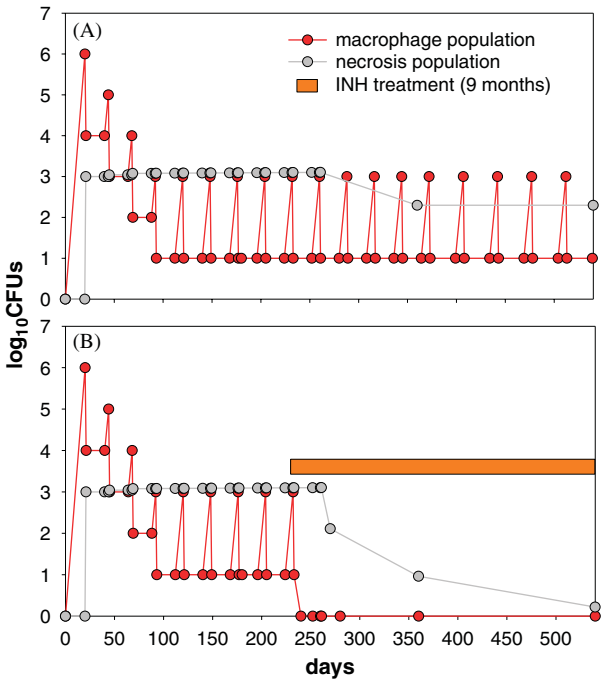

Figure 2 Mathematical approach to latency in TB based on the constant reactivation of latent bacilli. There is an acute phase where bacilli reach maximum concentration after 20 days of constant growth that lasts until the immune response is triggered and 99% of the bacillary bulk is destroyed (A). This reaction originates from 0.1% of latent bacilli in necrotic tissue. The remaining 1% of bacilli in macrophages re-grow after a resting period of 20 days, 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 in humans of 2 $\log_{10}$. The number of bacilli in necrotic tissue decreases while the resting period between reactivations increases proportionally to historical data[81] of untreated tuberculosis-infected household contacts. This data suggests that the disease occurs at a rate of 0.74% per year in years 1 and 2; 0.31% per year in years 3–5; and 0.16% per year in years 6 and 7. (B) shows the end of constant re-growing with chemotherapy and the elimination of latent bacilli from necrotic tissue calculated in relation to the protection obtained with several periods of treatment with INH.[129] The number of bacilli inside macrophages is indicated in red, whereas the bacillary number in necrotic tissues is in gray. The time of chemotherapy is presented in orange.

As a consequence of all the above, the strategy of inducing a strong Th1 response is not relevant for designing an immunotherapeutic strategy against LTBI, especially if we take into account that humans, who are the hosts that manage to control *M. tuberculosis* infection most effectively, trigger a Th1–Th2 response. Th1 response kills growing bacilli whereas Th2 "walls off" latent bacilli, the latter being responsible for granuloma fibrosis,[78,79] together with the presence of TGF-β,[80] a Th3 response. However, it has been suggested that TGF-β is the only factor required for fibrosing the granulomas,[80] i.e. to ensure that latent bacilli do not escape. In this scenario also, a "pure" Th1 response, such as occurs in mice, does not "close" the granuloma sufficiently and therefore allows constant dissemination of the infection.[26,27] Even more, as NO local production is paramount in chronic phase granulomas and inhibits both Th1 and Th2 responses[30] it is nonsense to increase any of them as it will be useless.

Interestingly, histopathology studies on human TB lesions have demonstrated that the presence of IL-4 may not be an indicator of poor prognosis in such patients but rather may be an integral feature of tuberculous granuloma formation with a role in controlling tissue damage.[81] In the same study, those patients exhibiting the highest percentage of TNF-positive granulomas also have IL-4-positive granulomas and a lower percentage of granulomas with caseous necrosis.

Why does conventional chemotherapy of LTBI need such a long period of treatment?

According to the field-work carried out by Comstock,[82] the gold-standard treatment for LTBI is the administration of INH for 9 months.[8] Interestingly, the study of early bactericidal activity (EBA) of INH in patients with pulmonary TB shows that the highest bactericidal activity is achieved within the first 2 days of treatment (with a 0.7 $\log_{10}$ reduction).[83] Essentially, the highest bactericidal effect of INH occurs within the first 15 days post administration. Therefore, why is such a long treatment period required? This is because INH, as most bactericidal drugs, is only effective against actively growing bacilli. In fact, in the case of cavitary TB, INH is effective because there is an important extracellular population with many actively growing bacilli.[84,85]

The long period of chemotherapy required in LTBI treatment may be explained by the ability of chemotherapy to stop constant regrowth of bacilli (Fig. 2B): the lack of growing bacilli reduces local inflammatory and immunological responses[65,86] allowing reabsorption of the necrotic debris, surfactant and dead bacilli, thus significantly reducing the presence of FM and latent bacilli. Considering that this decrease in local immune responses facilitates future re-growing of latent bacilli if these bacilli are not completely removed,

it is crucial to maintain long chemotherapy periods so as to allow total removal of latent bacilli from the pulmonary parenchyma.

What does RUTI do?

The design of RUTI

RUTI was designed to fill the immunological gap left by short-term therapy for *M. tuberculosis* infection. In fact, our group demonstrated in previous investigations and in different experimental models of TB in mice that *M. tuberculosis* grew again after short-term chemotherapy, although this was not followed by an immediate stimulation of immunity. Moreover, this delayed immune stimulation was only triggered when the bacterial load reached a value of $4 \log_{10}$ (after a period of 2 months),[65] which had clear implications for the "old issue" of "tolerance" of mice to larger bacillary exposure than humans, and the need for more bacilli to boost immunity.[20] The original idea on introducing RUTI, therefore, was to "boost" the immunological response against *M. tuberculosis* that already existed in the host.

RUTI was also designed to trigger a new immunological response against antigens of the latent bacilli, i.e. the so-called "structural" antigens[87] as well as those associated to stress responses. Protective immunity arises against antigens that are actively released by growing bacilli[88] and it is likely that the "focus" of the immunological response only on growing bacilli allows nonactive bacilli to remain "invisible" to the specific immunity of the host.

RUTI is made with bacilli grown under the stressful conditions of starvation, low pO_2 and low pH, conditions achieved gradually by culturing on solid media.[89,90] Progress to stressful conditions (e.g., low pO_2) has been shown to "prepare" the bacilli for even more stressful conditions (e.g., strict anaerobiosis),[3] while progressive starvation is known to lead to stationary growth in old cultures in which slow metabolism makes bacilli more resistant to stress, as in the chronic phase of the murine infection.[15] In summary, bacilli used to make RUTI are subjected to conditions that are probably found in the granuloma of hosts with active immunity: a low pO_2 in fibrotic structure of the granuloma and the low pH and starving conditions inside the phagolysosome of the activated macrophage.

The choice of fragmentation of the bacilli to make RUTI was to allow optimal presentation of cell wall antigens, as was the choice of composition into liposomes.[91] The average diameter of these fragments, which is 0.1 μm, allows the antigens in the cell wall to be well presented and thus provide an easier recognition of latent bacilli. Moreover, it is well known that the cell wall of *M. tuberculosis* has adjuvant properties[92] that ensure the induction of an immunological response without further adjuvant measures. In addition, the induction of a polyantigenic response is postulated to help recognize latent bacilli.

Another relevant feature of RUTI is the "detoxification" of the cell fragments obtained. In previous experiments, our findings supported the hypothesis that the characteristic IN in granulomas of *M. tuberculosis* was induced nonspecifically by endotoxin-like molecules, essentially glycolipids located in the outermost layer of the cell wall[20] instead of being induced by a specific immunological response such as delayed type hypersensitivity (DTH).[93] Our hypothesis arose from previous work by the group of G.A.W. Rook, suggesting that IN induced by the "Koch phenomenon" resembles a local "Schwartzman reaction".[19] This necrosis is not usually seen in murine experimental models, maybe due to the relative "tolerance" of these small hosts to *M. tuberculosis* antigens. For the response to infection in mice to behave like other, larger, mammals that spontaneously develop this necrosis,[18] mice would require a higher amount of endotoxin-like molecules.[20] Therefore, to avoid the potential risk of developing a Koch phenomenon caused by a therapeutic vaccine against LTBI[94,95] we decided to remove endotoxin-like molecules from the surface of the fragments using Triton X-114,[96] a detergent that minimizes the denaturation of antigenic proteins. Finally, we also removed the lipidic phase to enhance the presentation of those "hidden" antigens by the outer coat and to increase the range of the immunological response.

The protection mechanisms of RUTI

By using a western blot technique, we observed that the inoculation of 3 doses of RUTI (of 185 μg each) 2 weeks apart, after chemotherapy, induced a strong polyantigenic response, triggering a strong Th1–Th2 immunity against at least 13 known *M. tuberculosis* antigens, as well as a Th3 response.[87] In addition, studies on the cellular immunity induced by 2 doses of RUTI (245 μg 3 weeks a part) and compared with the inoculation of BCG in mice previously treated with chemotherapy, revealed that both inoculation of BCG or RUTI induced a 10-fold increase in the CD4 IFN-γ+ population induced by PPD stimulation. Interestingly, RUTI also enhanced ten times the

CD8 IFN-γ+ population (Fig. 3), whereas this increase was not detected with BCG[97] which induced no protection, a difference already found by other authors.[98] This difference may explain the improved effectiveness of RUTI, since the CD8 IFN-γ population is crucial for the control of latent bacilli.[99] Additionally, antigenic differences between BCG and *M. tuberculosis* also help to explain differences in protection,[100] which was another important factor for the design of RUTI.

Although the exact mechanism by which RUTI increases the CD8 IFNγ+ population in the lungs remains to be elucidated, its delivery as liposomes appears crucial; alternatively, the induction of apoptosis in macrophages that phagocyte RUTI,[101] may also favor a CD8 response.[102]

We were surprised to find that polyclonal antibodies obtained after RUTI administration provided protection against *M. tuberculosis* reactivation after chemotherapy in SCID mice.[103] In these experiments, we allowed aerosol infection to progress for 3 weeks, at which point chemotherapy with INH and rifampicin was administered for 5 weeks. After chemotherapy, two experimental groups were defined by the inoculation of serum obtained from infected immunocompetent mice treated with chemotherapy and RUTI (serum-therapy group), or the inoculation of serum from noninfected untreated immunocompetent mice (control group). After 2 weeks, necropsies revealed a significant difference between both groups: the serum-therapy group reflected a weaker bacillary reactivation (Fig. 4), 100 times less than the control, and a decreased granulomatous infiltration in the lungs (3 times less than the control). In fact, mice from the serum-therapy group developed fewer abscesses. These data support the notion that IgG antibodies are also able to control dissemination of the infection in the pulmonary parenchyma, as was earlier suggested from experiments of infection of bacilli pre-coated with specific IgG,[104] or by inducing active IgA production.[105] Interestingly, it has been recently shown that inoculation of nonspecific IgG also increases the effectiveness of chemotherapy when administered therapeutically.[106] Furthermore, a strong response of specific antibodies against lipoarabinomannan[107] and Ag85[108] were also associated with better prognosis in patients with active TB, further supporting the current view that mere production of IFN-γ is insufficient for induction of protection against TB, as reflected in the paradoxical protection achieved by *Cynomolgus macacca* with BCG vaccination, which could not be linked to the production of IFN-γ.[109]

A combined cell/antibody-mediated immunity and combined Th1/Th2/Th3 immunity is thought to provide the protection elicited by RUTI. This type of response is coherent with the concept of global immunity against latent bacilli, with an aggressive Th1 cellular response probably mediated by CD8+ T cells; Th2 and Th3 responses that wall off the granuloma[79,80,110]; and an antibody Th1/Th2

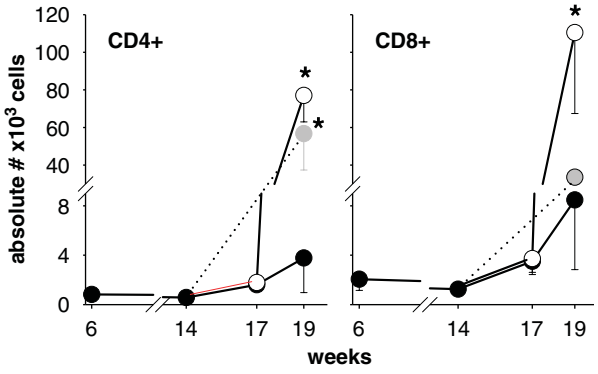

Figure 3 Evolution of TCD4+ and TCD8+ IFNγ+ cells from lungs in mice treated with chemotherapy from weeks 6 to 14, and treated with 2 subcutaneous (s.c.) inoculations of RUTI (245 μg) at weeks 14 and 17; 1 s.c. inoculation of BCG (10e6 CFUs) at week 14 or 1 s.c. of empty liposomes (control)—white, gray and black symbols, respectively. The results are given as mean values with standard deviations obtained from 4 mice for each time point. Differences with control were significant when marked with * for $p<0.05$.[97]

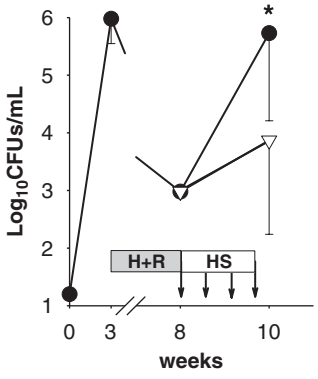

Figure 4 Control of CFUs after serum-therapy in the lung of SCID mice. After infection, mice were treated with INH+RIF from week 3–8 (in gray), and treated with 4 intraperitoneal (i.p.) inoculations of serum (hyperimmune serum, HS) from mice treated with a short period chemotherapy and RUTI (in white) or from normal noninfected mice (in black). The results are given as mean values with standard deviations obtained from 4 mice for each time point (apart from week 19, where 10 mice were treated with immune serum and 11 mice were given normal serum). Differences with control were significant when marked with * for $p<0.05$.[103]

production that neutralizes the extracellular bacilli that escape through the alveolar spaces.

These conclusions do not agree with the most common hypothesis suggesting that immunity against *M. tuberculosis* infection must be induced by a Th1 response, whereas Th2 responses are detrimental. This hypothesis is based on the Th1/Th2 response triggered by TB patients compared with the responses seen in patients with LTBI, who are thought to avoid reactivation of their latent infection.[111] In this regard, since Th2 response plays a relevant role in the immunopathology of TB lesions in controlling and reclosing a large lesion (i.e. a cavern) by building a strong fibrotic mantle around it, a complete absence of any Th2 response would be surprising. In any case, since most of these findings are incidental, we consider that the hypothesis suffers from a "chicken and egg" problem: are these patients suffering TB because of Th2 polarization, or do they develop a Th2/Th3 response to face the disease?

Another interesting point is the lack of efficacy of BCG vaccination in newborn babies in the Third World due to the lack of a strong Th1 response. This seems to be caused by the *in utero* sensitization to filariae, which results in a Th2 bias, leading to a Th1/Th2 response instead of a stronger Th1 response as observed against PPD-driven IFN-γ production.[112] On the other hand, the immunological environment at the fetomaternal interface is Th2 dominated in order to protect the fetoplacental unit, and at this age the induction of a Th2 immunological response is generalized.[113] Furthermore, experimental mice models do not support this hypothesis: no difference in either protection or in the progression of *M. tuberculosis* infection has been found after vaccination with different doses of BCG that induced either pure Th1 or mixed Th1/Th2 responses.[114] Even more so, no differences in the evolution of *M. tuberculosis* infection could be demonstrated in IL-4, IL-4 plus IL-13 KO,[115] and IL-10 KO mice compared with wild-type mice.[116] Finally, long-term chronic murine TB in the lungs was not influenced by the absence of B lymphocytes or IL-4.[117]

The role of the Th3 response triggered by RUTI must be clarified. It is possible that Th3 plays an important role beyond the final fibrosis of the granulomas through the action of TGF-β.[79,80,111] In fact, immunotherapy with DNA encoding for stress proteins like hsp 65 has proven effective against *M. tuberculosis* infection when combined with chemotherapy.[118,119] It is widely accepted that these proteins induce regulatory T cells that can suppress autoimmunity induced by Th1 or Th2 responses, thus counterbalancing the immune response.[120] In this regard, these proteins may help to "synchronize" the immune response (i.e. activating the infected machrophages or walling off the granulomas) rather than just inducing a strong Th1 response[121] or reducing the Th2 response, similar to the postulated mechanism of *Mycobacterium vaccae* immunotherapy.[122]

Why is previous short-term chemotherapy still necessary before LTBI therapeutic vaccination?

At this point, it is very important to study LTBI in human patients, because we currently ignore which bacillary population the lesions harbor. Are most of these bacilli actively growing or are they latent? And which is the relative proportion of these populations? The presence of heterogeneous populations in a lesion may explain why a short period of chemotherapy (e.g. 4 weeks), which could "harmonize" or "standardize" the bacillary population in all patients toward a latent population, is advantageous for therapy.

Furthermore, this short period of chemotherapy also allows a decrease in the immunological constant boost against growing bacilli and removes FM (Fig. 5) and thus the source of local immunosuppression. Moreover, chemotherapy in patients with LTBI has also been associated with the increase of Th1 specific effector cells in peripheral blood within the first 4 weeks of LTBI conventional chemotherapy (9 months of INH). This rise in Th1 effector cells arises from antigen presentation and the release of effector T cells from the infected sites.[123] These data also support the hypothesis that local changes in the granuloma favour recognition of latent bacilli. Chemotherapy also increases reabsorption of the lesion, therefore reducing the inflammatory response in the lungs,[65,86] reducing the chance that the therapeutic vaccine will induce a Koch phenomenon, because the infected site will no longer be a "prepared site".

In conclusion, the need for chemotherapy indicates that once latent bacilli are induced, vaccination is useless because these bacilli remain invisible to the immune system. Moreover, even if the host's immunity is already prepared to control infection, it cannot prevent the reactivation of these cells in a site which is privileged for the bacilli (such as the pulmonary apex), thus being unable to prevent the induction of pulmonary TB. Therefore, it is possible that chemotherapy allows the presentation of latent bacilli to the host immunity by "rediscovering" their presence.

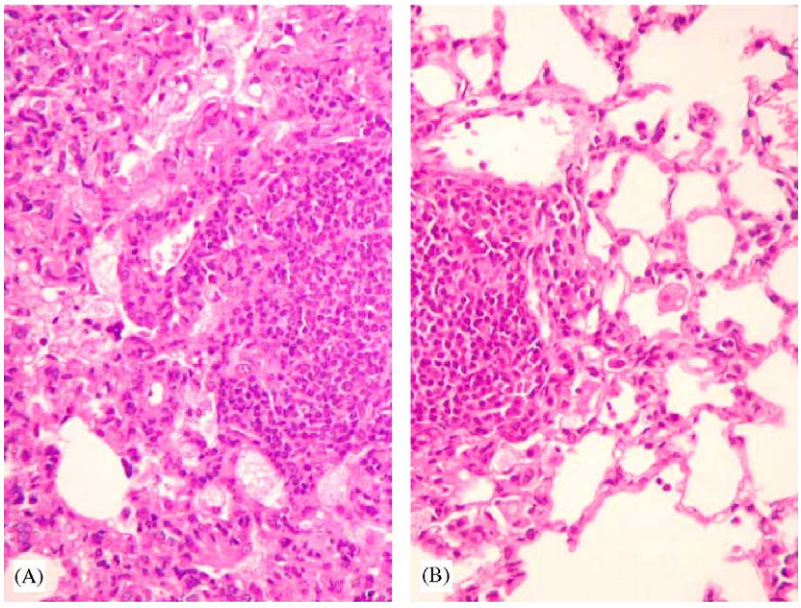

Figure 5 Effects of 8-week chemotherapy on the structure of granulomas. Note the lack of FM in the periphery of treated mice (B) compared with mice before starting chemotherapy (A), at week 8 post-aerosol infection. Slides were stained with hematoxylin–eosin. Original amplification is 400x (data not published).

How has the effectiveness of RUTI been assessed?

The research of new drugs and vaccines against TB is especially difficult because no direct correlation exists between the results obtained in commonly used experimental models and what really happens in humans. For instance, testing new prophylactic vaccines requires an efficacy at least similar to that obtained with BCG vaccination, which reduces bacterial concentration in mouse lungs by 1 $\log_{10}$ at 3 weeks after infection compared to nonvaccinated animals and allows infected guinea pigs to survive for up to 30 weeks instead of the 14 weeks usually seen in nonvaccinated animals.[124] Overall, prophylactic vaccines do not provide any prophylaxis but improve control of the infection, although they do not prevent death of infected animals as a result of TB development. The lack of clear correlation between effectiveness of vaccines in animal models and in humans means that the validation of new therapies in experimental models must be interpreted with caution. For example, in LTBI therapy, the gold standard treatment ensures a 90% efficacy against LTBI reactivation in humans[82] but at most it only achieves a 10% efficacy in mice.[125]

Another striking and relevant question already discussed above relates to the type of experimental model that needs to be used for testing the efficacy of a new therapeutic vaccine, with the aim of mimicking what happens in humans. The Cornell

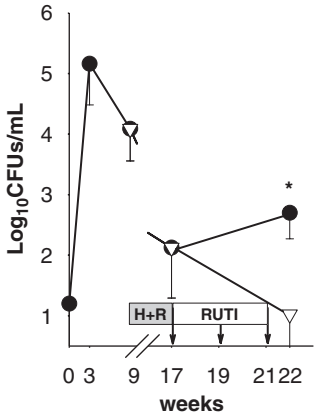

Figure 6 Bactericidal activity of RUTI in the lung of C57BL/6 mice. After infection, mice were treated with INH+RIF from week 9–17 (in gray), and with 3 subcutaneous (s.c.) inoculations of RUTI (185 μg per inoculation) at weeks 17, 19 and 21 (in white) or empty liposomes (in black). The results are given as mean values with standard deviations obtained from 4 to 6 mice for each time point. Differences with control were significant when marked with * for $p<0.05$ (modified from[87]).

model does not appear very appropriate because it is not clear if persistent or latent bacilli are present, and no "complete" granulomas and growing bacilli are detected at the onset of treatment. Thus it does not reflect the conditions of human LTBI. Instead, we chose chronic infection in mice as a starting point. Moreover, the "tolerance" shown by mice to *M. tuberculosis* infection

may underestimate new treatments, as the host does not display a strong inflammatory response against the bacilli, suggesting that evaluating the reduction of bacillary bulk and its control after chemotherapy should be the final target instead of sterilization. It is relevant that initial experiments showed that RUTI significantly reduced the bacillary bulk (Fig. 6)[87] and maintained this control for a long time, at least for up to 11 weeks after the last RUTI inoculation.[126]

Concerning the "tolerance" phenomenon discussed above, we decided to run new experiments with guinea pigs instead of mice. The rationale for this decision was based on the hypothesis that the bigger the host, the stronger the inflammatory response against *M. tuberculosis*, because more parenchyma can be destroyed in larger animals to stop bacillary growth (assuming that the size of immune cells is similar in all mammals). Fig. 7 exemplifies this idea by comparing the volume of a small human TB cavern with the total volumes of different mammals, and shows that a mouse will never develop such a lesion, as it represents its total volume. We thus started working with the guinea pig using a more "humanized", short chemotherapy regimen (4 weeks with INH and rifampicin), as well as a more virulent *M. tuberculosis* strain (H37Rv Pasteur). Histopathological analysis 4 weeks after infection showed that guinea pigs developed a structured and defined granuloma, with strong outermost fibrosis and IN; few acid fast-bacilli were detected, localized only in the periphery of the granuloma and inside FM (Fig. 8). In contrast, at this same time point, mice only

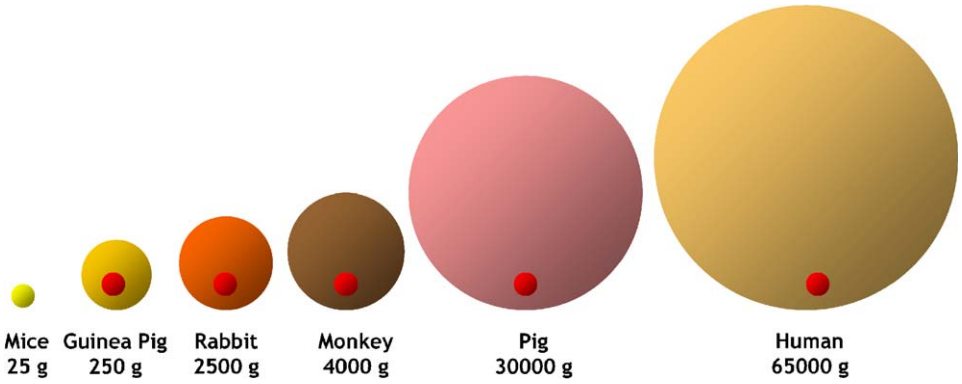

Figure 7 Comparison of different hosts according to their weight. The volume of a TB cavern weighing 25 g is shown in red.

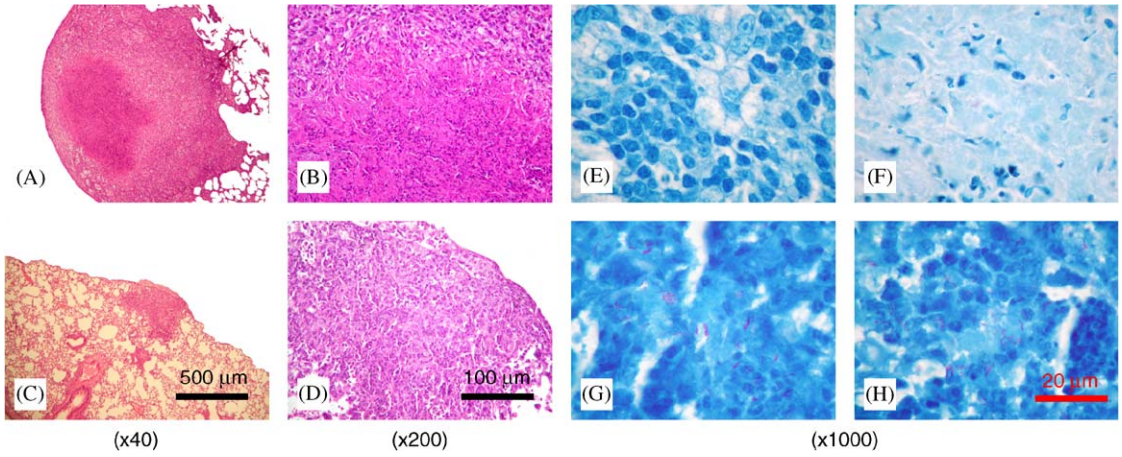

Figure 8 Comparison of lesions in guinea pigs (A–D) and mice (E–H) at week 4 post-aerosol infection. Pictures A, B, E and F show the structure of the granuloma stained with haematoxylin–eosin. Note the highly organized guinea pig granuloma, the presence of a central necrotic tissue surrounded by a mantle of fibrotic tissue and an outermost layer dominated by lymphocytes. In contrast, the granuloma of mice is just a mixture of PMNs, macrophages and some lymphocytes with no apparent organization. Pictures C, D, G and H show the result of Ziehl Neelsen staining in these granulomas. Acid-fast bacilli are scarce inside FM included in the fibrotic mantle (C), and inside the necrotic center (D); on the contrary, acid fast bacilli can be easily seen in mice granuloma (G and H).

developed a tiny pre-granuloma full of acid-fast bacilli, with no outer fibrotic content, thus leaving more time for further pulmonary dissemination. In fact, bacillary counts were higher in mice than in guinea pigs (1 $\log_{10}$ more),[127] which agree with previous reports.[128] In this context, administration of 3 doses of RUTI (180 μg) at weeks 8, 10 and 14 after shorter chemotherapy than in mice experiments (between weeks 4 and 8) significantly decreased the bacillary load at week 16 (at the time of necropsy) even when bacillary bulk in control guinea pigs did not increase (Fig. 9). In contrast, bacillary counts in control mice increased by 2 log 10 when the same *M. tuberculosis* strain was used.[97]

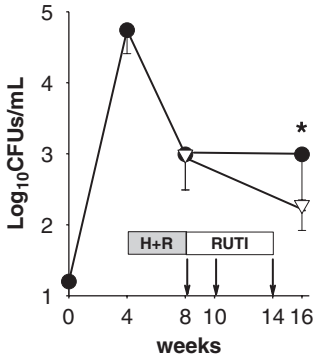

Figure 9 Bactericidal activity of RUTI in the lung of guinea pigs. After infection, animals were treated with INH+RIF from week 4–8 (in gray), and with 3 subcutaneous (s.c.) inoculations of RUTI (185 μg per inoculation) at weeks 8, 10 and 14 (in white) or empty liposomes (in black). The results are given as mean values with standard deviations obtained from 8 animals for each time point. Differences with control were significant when marked with * for $p<0.05$.[127]

Note that control of the bacillary count in guinea pigs requires a tremendous inflammatory effort that finally kills the host faster than it kills mice. Therefore, we cannot assume that mice are more resistant to *M. tuberculosis* infection than guinea pigs, but instead, we assume that mice are more "tolerant" to these bacilli, allowing an abundance of bacilli in their lungs by triggering a weaker inflammatory response. This tolerance allows mice to survive longer to *M. tuberculosis* infection, but in the end the bacilli also kill the host due to constant dissemination throughout their lungs.[26,27,68] However, it is obvious that the evolution of infection is not equivalent in guinea pigs and in human hosts because the latter control bacillary progression better by walling them off in the granulomas. The formation of granulomas in humans will also allow administration of weaker chemotherapy (e.g. INH alone) rather than the combination of INH and rifampicin required for controlling infection in guinea pigs.

Table 1 Properties of the LTBI.

A. The nature of latent bacilli
   1. *Mycobacterium tuberculosis* has a innate slow growth-rate.
   2. Once submitted to stress, bacilli slow metabolism to near-zero activity, in which situation the bacilli can resist stressful environments.
   3. A relevant capacity to survive in necrotic tissue.
   4. Bacilli can grow at the periphery of the granulomas once the stress has resumed.

B. The host immunological response
   1. The host develop active immunity largely against growing bacilli.
   2. CD8 T cells may play a relevant role in the control of latent bacilli.

C. The role of foamy macrophages
   1. Are a consequence of the accumulation of necrotic debris, surfactant and dead *M.tuberculosis cells*.
   2. Have a decreased ability for presenting antigens.
   3. Suppress effector T cells that try to activate them.
   4. Harbour latent bacilli.
   5. Represent the outermost layer of the granuloma.
   6. Render the induction of a specific immunological response against latent bacilli useless.

Key aspects for the destruction of *M. tuberculosis* latent bacilli
   1. Removal of the outermost layer of foamy macrophages that exert local immunosuppression.
   2. Induction of a polyantigenic immunological response not only against antigens of actively growing bacilli, but also against structural antigens.

Regarding the possible toxicity of RUTI, in both experiments with mice and guinea pigs, only a local transient inflammatory response at the site of inoculation was found. No systemic toxicity (weight loss or increased granulomatous response) were detected.

The future

Based on current data, new experiments are being carried out to gain further understanding of all the mechanisms involved in the protective benefits of RUTI. These experiments include more ''humanized'' models with larger mammalian hosts. In this regard, we have started some field experiments with naturally infected goats and experimental models such as mini-pigs. Our aim is to use hosts with a volume more comparable to humans than models used hitherto, to determine the protective effects of RUTI and also to confirm lack of toxicity, because larger hosts are more likely to develop greater inflammatory responses and the Koch phenomenon.

In conclusion, we expect to achieve a better chemotherapeutic treatment of LTBI patients, by administering INH for a short period of time, i.e. 4 weeks, with only 2 doses of RUTI; this improved regimen is also expected to ameliorate treatment compliance. (Table 1)

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