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Ten Questions to Challenge the Natural History of Tuberculosis

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1. Is Mtb a naked emperor?

Making a parallelism with the short tale of Hans Christian Andersen "The Emperor's New Clothes", this first question wants to address a primordial question in the Mtb infection: how Mtb looks at the very initial moment when is about to be phagocytosed by the alveolar macrophage (AM).

The origin of infective Mtb is in general infected aerosols from a patient with active TB. More frequently those that carry such a high concentration that the bacilli can be observed directly in the sputum using the acid fast stain. Recently it has been discovered that a vast proportion of them are in a stationary phase, or latent phase according to their transcriptomic signature and the ability to accumulate lipid bodies [Garton 2008]. This accumulation can we a strategic activity for the bacilli in order to resume as soon as possible their growth when noticing that is embedded in a proper milieu. As one of the characteristics of Mtb is to build a thick cell wall [Torrelles 2010] the lipid accumulation appears to be a paramount activity.

Overall, what we can deduce is that stressed Mtb are the responsible of starting the infection. This speculation is supported by the fact that stressed bacteria have in general more capacity to resist further stress [Wallace 1961], and before infecting the AM, the bacilli must suffer at least the physical agents from the external milieu (i.e. the UV light action or desiccation). What is probably less taken into account is that immediately after "laying" in the alveolar surface, these bacilli are embedded in the pulmonary surfactant, which is plenty of hydrolases. Interestingly enough recently it has been discovered that surfactant reduce the cell envelop from up to the 80% [Arcos 2011] thus reducing very much one of the natural defensive mechanisms of the bacilli: its cell wall. In a way we can answer to the question affirmatively. Mtb is not presented as that pathogen with a huge indestructible armour, which together with the stressed status appears to be an irreducible enemy. On the contrary, this new input shows that AM face this pathogen as the children of the tale: naked and probably quite fragile. This process has quite annoying consequences for the bacilli, as the envelope changes correlate with a decrease in AM phagocytosis, early bacterial intracellular growth, and induction of proinflammatory responses with release of TNF-a from AMs, as well as an enhancement of phagosome–lysosome fusion.

2. Do the bacilli reside in the cytosol of the AM?

Classically, intracellular Mtb growth has been related to its growth inside the phagosome [Armstrong 1971], and this was the base for understanding the immune response based in the stimulation of CD4, and even for explaining the capacity of induce a chronic infection: as CD8 cells were not enough stimulated [vanPinxteren 2000]. ESX-1 complex became a crucial as a virulence factor, able to avoid the phagosome maduration [Xu 2007] as it was before the ATP-ase pump control to avoid the acidification of the phagosome [Sturgill 1994], or the production of ammonia by Mtb [Gordon 1980]. Then the concept of autophagy came to be essential for avoiding Mtb destruction [Deretic 2009]. Finally, it appears that Mtb is also able to disrupt the phagosome and reside into the cytosol [van der Wel 2009], in a way that has recently interpreted by Ian Orme as a natural way for Mtb tending to necrotize the macrophage to become extracellular and at the end growth extracellularly in the liquefacted tissue, which is the final target of the bacilli [Ian Orme, personal communication]. In this regard, it could be interpreted the pass to the cytosol as the beginning of the end of AM: i.e. to become necrotic.

3. Polymorphonuclear cells? If you explain me what they do, I will put them in my system!

Well, this was the answer when I asked to a systems' modelist why they didn't consider the presence of polymorphonuclear cells (PMN) when building a model to reproduce virtually the induction of the Mtb granuloma. *Why? If you explain me what they do, I will put them in my system!*. This concept comes to everybody naturally: how a cell that lives for 6 hours in the tissue can control Mtb which doubles every 24 hours? First impression is that if they play a role, they would kill Mtb immediately. Then there is the issue that Mtb is mostly intracellular thus the opportunity to be seen by PMNs is really reduced compared with all those pathogens that effectively growth in the extracellular milieu. But this is not accurate, taking into account that Mtb is able to destroy the AM becoming extracellular thus leaving a window. But for a long time it has prevailed the concept that before the onset of adaptive immunity, when there are a lot of PMNs in the granuloma, the bacilli apparently grow without resistance, in a exponential way. So far this is not accurate as recently a substantial bacillary destruction has been demonstrated in this period [Gill 2009]. But what is the role of PMNs? This bactericidal effect can be induced by Natural Killer cells, for instance. It can be said that as in any other process where a destruction of the tissue takes place, PMNs appears, so that their presence is incidental… but of course they play a role. In fact, this has been recently thought as anti-inflammatory [Zhang 2009], although bactericidal effect was effectively demonstrated when apoptotic [Tan 2006, Persson 2008]. This apparent contradictory data can be explained by the recent demonstration that immature granulocytes play a regulatory effect, and this precisely appears when there is a damage in the tissue [Gabrilovich 2009]. Likewise, PMN necrosis may also occur in the extracellular matrix, thereby curtailing bacterial dissemination [Brinkmann 2007] and contributing to the formation of a granulomatous structure that can support sudden cellular entrance [Lenzi 2006].PMNs can also carry bacilli to the lymph nodes through the lymphatic capillars thus favoring the adaptive immune response [Abadie 2005]. On the other hand, little information is on the role of microabscessification inside of the granulomas, which can be also an antiphagocytosis strategy or just increasing the local inflammatory response, thus favoring

granulomatous formation. Be what it could be, a new actor appears linking the presence of PMNs to the adaptive response. It has been described the induction in Mtb infection of Th17 cells, which also promotes the attraction of the PMNs to the granulomas [Bettelli 2007].

4. How the granuloma can ever be considered as a foe? The Citadel paradox

If a student interested in granulomatous processes had the opportunity to take a look at the city map of Barcelona around the second half of the 18th century he would appreciate a magnificent "granuloma-like" structure attached to the East wall of the city. This is the Citadel: a pentagonal wall fortified by extra triangular fortifications that result in a symmetric star-like structure (Figure 1). The first impression is to interpret this as a defensive structure, although if our student would like to extend his knowledge on it, he would realize that this is not the case. Indeed, at the beginning of the 18th century, Barcelona, the capital city of Catalonia, was fiercely besieged for a whole year. This siege resulted in such a large number of casualties among the attackers that, once they took the city, they initially decided to completely destroy it. Fortunately, an engineer proposed to build the Citadel instead in order to prevent the likely future riots of Barcelona's citizens against the new rules imposed by the victors, who had decided to abolish the Catalan State (Figure 2).

Fig. 1. **Map of Barcelona in 1719** showing a nice granuloma-like structure attached to the East wall. *Taken from Ròmul Brotons. "La ciutat captiva", Albertí Editor. Barcelona 2008.*

This historical perspective illustrates a common question about the role of the granulomas, which although built by the host to face the infection appears also to hide and to allow the persistence of the bacilli inside the body. Early data strongly support a defensive role in the case of TB, as after building the granuloma, there is enough chemokine production to attract specific lymphocytes, a fact that would not be possible in the case of isolate infected macrophages [Bru 2010]. On the other hand, the special structure of the lung parenchyma of bigger mammals requires the presence of intralobar septae to support the inflated structure

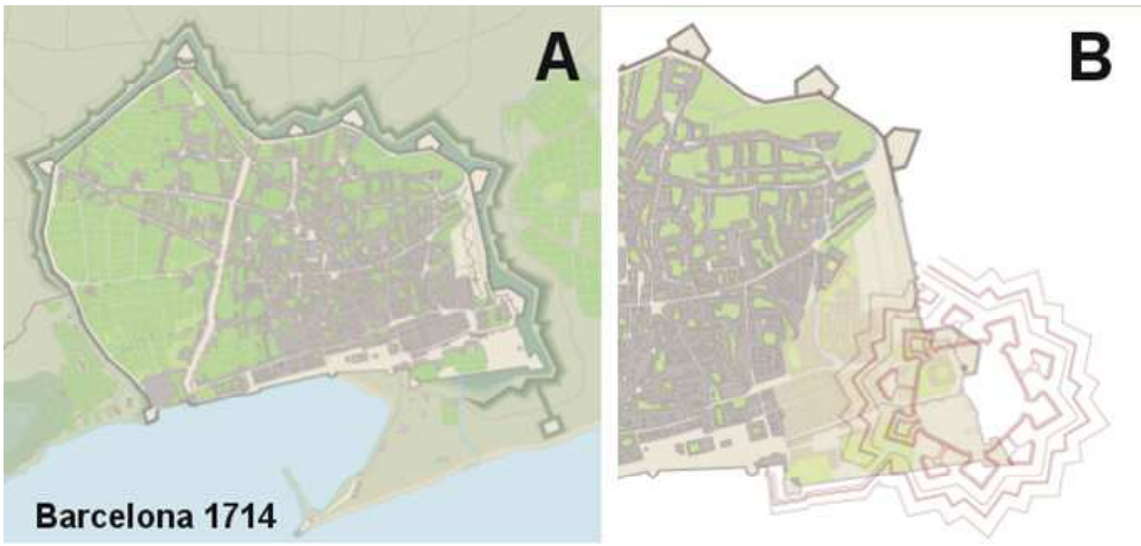

Fig. 2. **Map of the previous situation of Barcelona on 1714** before the siege settled by the Borbon Army (Picture A). Picture B shows the works of the neighbors of the East wall that were forced to fall down their houses in order to clean the space at the end of the Citadel to better bomb the city. *Taken from Ròmul Brotons. "La ciutat captiva", Albertí Editor. Barcelona 2008.*

of the lung. These septae, when teased by a disruption of the usual mechanical forces, i.e. because of the presence of a lesion, proliferate and tend to encapsulate it [Gil 2010]. We do believe this encapsulation is also responsible for avoiding the drainage of non-replicating bacilli towards the alveolar space, and thus the constant endogenous reinfection which allows the persistence of the bacilli through time [Cardona 2009; Cardona &Ivanyi 2011] (Figures 3 and 4).

5. Is the disturbance of a proper antibody response the main strategy of Mtb to survive? Why we can be constantly reinfected?

As posed in the previous question, attraction of specific lymphocytes appears to be paramount to stop the bacillary growth. Immune response against Mtb is mainly based on the induction of specific Th1 lymphocytes able to activate infected AM, but this leaves a huge window in which the bacilli can grow freely inside naïve AM before they are detected. This is clearly seen by looking at the low dose aerosol model in mice: no lesion can be seen until week 3 post infection, although meanwhile the bacillary load has increased 1000 times. The only way to avoid this phenomenon would be to induce the production of specific antibodies that would be able to directly destroy the bacilli; or at least to favor the immediate destruction of them once phagocytosed [Casadevall 2004]. But it is not the case ! Mtb infection is characterized by the lack of antibody formation [Davidow 2005]. That's why even when adaptive response is present, immune subjects can be constantly reinfected [Jung 2005] and that's why it is considered that in TB coexistence of lesions of different ages is possible [Cannetti 1955]. Interestingly, some authors have already demonstrated that production of those antibodies can exert a control on the bacillary concentration [Guirado 2006]. But apparently, this approach has not been enough fashionable, and still today the

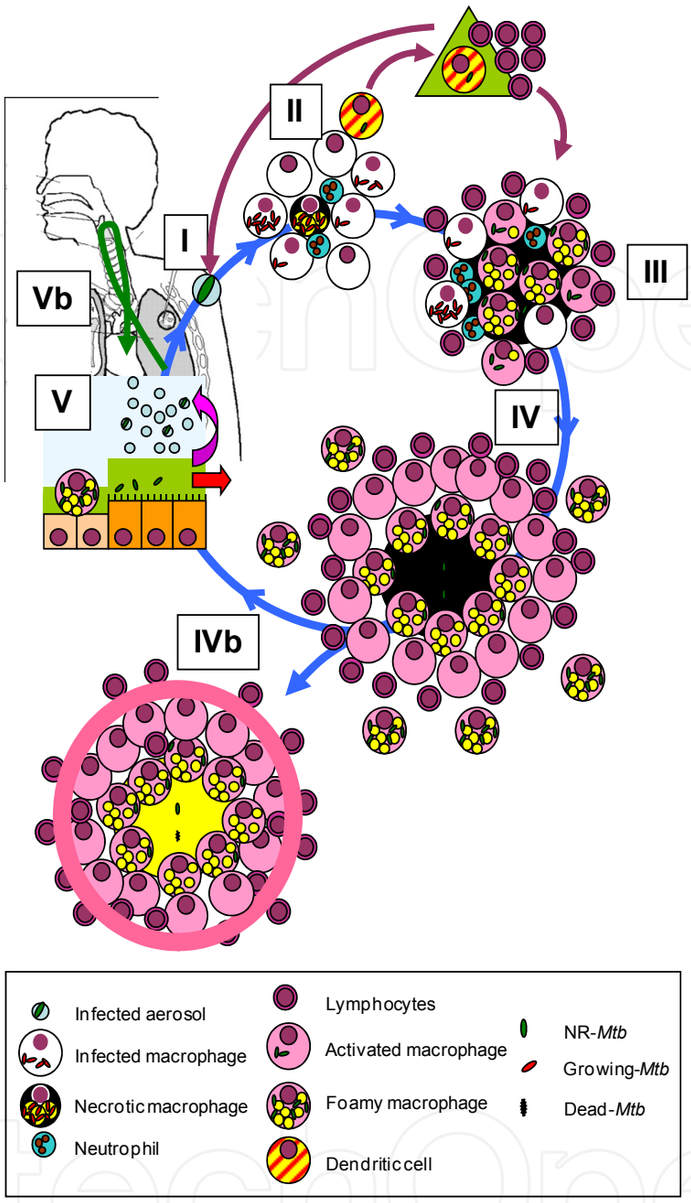

Fig. 3. **Lung pathology changing the life cycle of *Mtb* in the lungs. I.** *Mtb* transmitted by aerosol settles in the alveoli. **II.** *Mtb* growing inside macrophages, causing their necrosis. Infected monocytes become dendritic cells, that are drained to the lymph nodes (green triangle) for antigen presentation. **III**. Neutrophils, NK cells, lymphocytes and new macrophages are attracted to the granuloma**;** infected macrophages, bactericidal or bacteriostatic develop into FMs. *Mtb* changed to NR-*Mtb* in necrotic tissue are drained by FMs towards alveoli. **IVb**. Encapsulated necrotic granuloma, starting to mineralize; NR-*Mtb* cannot drain. **V**. NR-*Mtb*-infected alveolar fluid generates aerosols with the inhaled air or is swallowed and killed/drained in the gastrointestinal tract (**Vb**). Drainage of bacilli from infected lymph nodes through the thoracic ducts to the right atrium to be pumped back to the lung across the pulmonary artery also contributes to the reinfection process. Symbols: black = necrotic tissue; yellow = mineralized tissue. *Obtained from Cardona & Ivanyi 2011.*

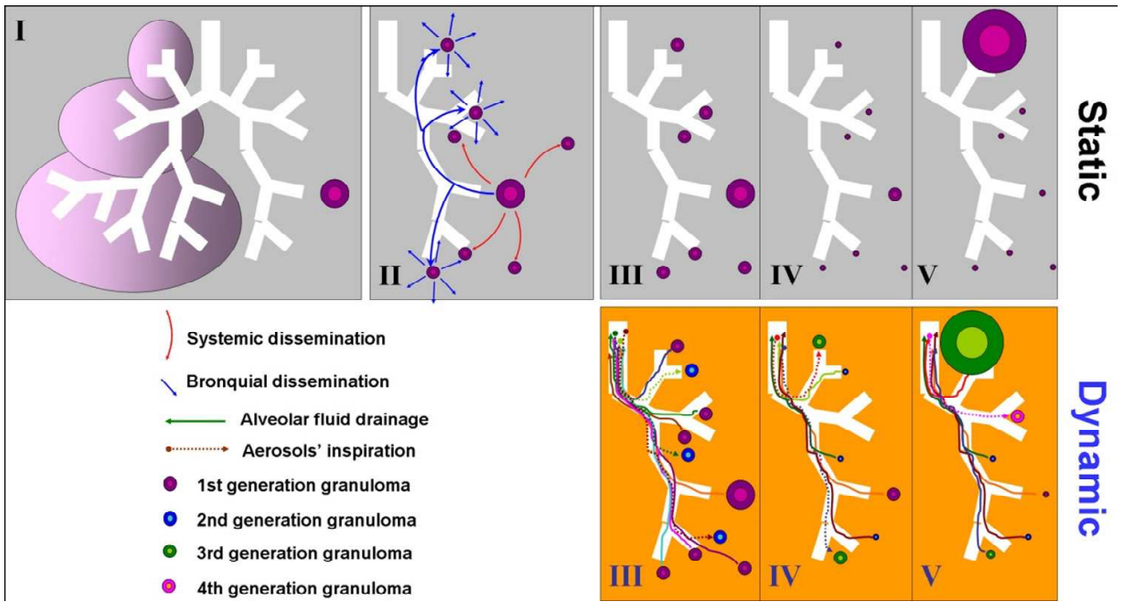

Fig. 4. **Latent TB infection (LTBI) and generation of active TB (TB).** Comparison between the traditional 'static' theory and the dynamic hypothesis. Once the initial lesion is generated (I), there is a bronchial (blue arrows) and systemic (red arrows) dissemination that generates new secondary granulomas. This process is stopped once the specific immunity is established (III). Lesions remain from then (IV) keeping dormant bacilli that have the ability to reactivate its growth after a long time (V).
In the dynamic hypothesis, there is a constant drainage of non-replicating bacilli towards the bronchial tree (solid arrows) but also the inspired aerosols (dotted arrows) can return the bacilli to generate new granulomas (III-IV). This process implies the induction of different generation of granulomas. In this process, if one of these reinfections takes place in the upper lobes, it has the opportunity to induce a cavitary lesion. *Obtained from Cardona 2009.*

majority of vaccine approaches are designed to build a strong cellular immune response giving no role to the antibody production. And what is the outcome: none of them avoid the infection by Mtb, at the most they can induce some reduction of the bacillary load [Kaufmann 2011]. That's all ! Should be resign to the fact that we will be never avoid Mtb infection?

6. What kills Mtb?

There was a time when taking into account the information coming from the experimental murine model reactive nitrogen intermediates (RNIs) appeared to be the clue to explain why after the activation of AM with interferon-gamma (IFN-γ) there was a control on the bacillary growth [Chan 1995]. This was also recreated in vitro. But the problem came when it was realized that in human AM production the role of RNIs might not be that important [Tufariello 2003]. The other mechanism could be the induction of apoptosis triggered by IFN-γ. In this case, once in an apoptotic vesicle Mtb can be effectively destroyed by any other AM regardless they activated status [Lee 2006]. This factor is also supported by the fact that Mtb tries to avoid AM apotosis [Lee 2009]. On the other hand, there is at least

another mechanism less studied but much more apparent: induction of granulomatous calcification. This is probably the oldest described bactericidal mechanism against Mtb, and very well described in human lesions [Feldman 1938]. This is a complex mechanism that our group has recently reproduced in the minipig model. Encapsulation of the lesions and turn to a fibrotic response promotes the accumulation of apoptotic vesicles in the necrotic center of the granulomas. This fact promotes the accumulation of calcium and thus the local induction of a polystress effect, based in a increased pH, hypoxia, starvation and osmotic stress [Gil 2010]. In this regard, the growing issue on the protective effect of vitamin D should be also related to this mechanism, not only devoted to the ability of trigger immunological mechanisms [Liu 2007]. Again, the obsession constantly seen by the majority of the authors to induce Th1 responses is not correct at all. This can be useful at the beginning of the infection to induce the apoptosis… but at the end, a fibrotic response is also need to induced calcification; and also to avoid the drainage of "latent" bacilli.

7. How an aircraft carrier be hidden? Does really latency exists in Mtb infection?

Ian Orme challenged years ago the TB community with a paper entitled "Latent bacilli? (I'll let you know if I ever meet one)" [Orme 2001]. The concept latency comes very much from the latent viral pathogens. Those viruses that have the ability to effectively hide and become silent and apparently non-noticed by the host, using strategies like to become part of the host's DNA [Knipe 2008]. But this is a virus… it is not the case for a bacilli, a sort of "aircraft carrier" compared with a virus, that on the contrary could be considered as a children's toy boat. It is true that Mtb has a stress response that induces a defensive metabolism including a growth disturbance, that has been called "latency"… but there is nothing special in this, as it is an universal behavior [Buchanan 1918]. In fact considering the Mtb infection as a constant reinfection process, it is clear that the bacilli are constantly noticed by the host, and that in every case it triggers very specific and efficacious responses. Looking at Figure 3 once the bacillary growth stops with the immune response, the stressed bacilli, retained mainly in the necrotic tissue, is constantly drained towards the gastrointestinal tract in a organic way that considers the degradation of AM towards foamy macrophages (FM) and thus allowing the effective drainage [Cardona 2009]. It is true that a tiny window is left by allowing the reinfection process with the production of aerosols from the alveolar fluid, but this process is only important at the very begging of the infection [Gil 2010], becoming less and less frequent with time, lowering the chance to induce active TB. All this process means that contrary to what is generally accepted, the bacilli could never become "invisible" to the host as herpes virus can do… becoming the paradigm of latency.

8. So, how active TB is induced?

The most frequent manifestation of active TB is the induction of cavitation in the lung. This happens because a liquefaction process is induced locally, favors the extracellular growth of the bacilli and makes possible the induction of a big lesion [Grosset 2003]. One of the main factors is the tropism. Again, as in other pathogens, Mtb has a special site that favors their growth. This is the upper lobe.

Cavity formation has traditionally been considered to occur from solid caseum, and a lot of controversies were raised to understand who is the responsible of inducing liquefaction: the reactivation of the bacilli trapped in the caseum of old lesions? the macrophage through the extracellular release of hydrolytic enzymes?

We understand liquefaction as one of the three possible outcomes (the other two being control and dissemination) of the constant endogenous reinfection process which would maintain LTBI [Cardona 2011]. The induction of a higher number of new lesions would increase the probability of one of them occurring in the appropriate location to induce liquefaction as upper lobes (Figure 5). These lobes favor higher bacillary load before the

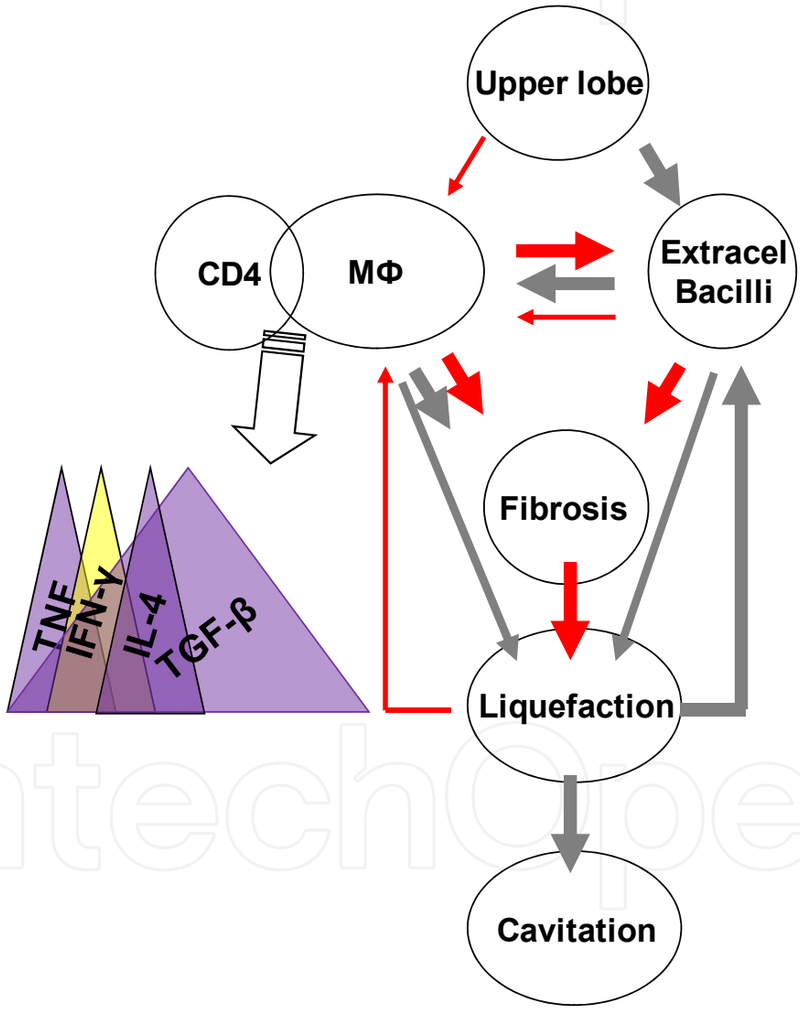

Fig. 5. **Interactions between the factors involved in the liquefaction process**. The colour of the arrows shows the ability to induce a process (in gray) or inhibit it (in red), and the thickness of the arrow is proportional to the intensity of this induction.
The upper lobe appears to be the *sine qua non* condition for the process to take place. Macrophage (MΦ) activation and the presence of CD4 is linked to the appearance of different cytokines with time: TNF initially, followed by IFN-γ and IL-4, and TGF-β from the

onset and peaking at the chronic phase. All those cytokines are profibrotic (in violet) except for IFN-γ (in yellow). This site mainly undergoes a profibrotic process, although there is also a nonspecific anti-fibrotic effect arising from the macrophages and their enzymatic activity. Extracellular bacilli also have antifibrotic activity and promote macrophage activation, although they are also thought to inhibit such activation to some extent. Fibrosis prevents liquefaction, whereas liquefaction is promoted by macrophages; the immune response, by promoting the apoptosis of infected macrophages; and extracellular bacilli. Liquefaction induces cavitation, inhibits macrophage activation (indeed, it appears to destroy them) and promotes extracellular bacillary growth.
Overall, liquefaction comes first, and then the extracellular multiplication of bacilli occurs. Fibrosis, and thus resume of the liquefaction would occur only after the number of extracellular bacilli is reduced sufficiently to allow attempts at healing to take place. Finally, a large number of extracellular bacilli results in tissue destruction, cavity formation and the death of the macrophages that attempt to inhibit such bacillary growth.
Obtained from Cardona 2011.

immune response appears by directly promoting bacillary growth and delaying the local onset of the immune response. Once this response appears, however, the synchronized induction of apoptosis/necrosis of infected macrophages together with a high IFN-γ concentration and the release of metalloproteinases by new incoming macrophages would be critical factors to promote the inhibition of localized fibrosis of the lesion and thus liquefaction. A high ability to generate a nonspecific inflammatory response, which is structurally present in males (i.e. high levels of ferritin), lower ability to produce collagen with age, or lack of proper healing of the lesions, as seen in diabetes mellitus were there is combination of local inflammation together with excessive production of metalloproteinases, could hypothetically promote this liquefaction.

Although this process can be redirected with time, with fibrosis finally taking place, another factor, the extracellular bacillary growth, even if slow, should be taken into account. Such growth might be essential to allow the irreversibility of the liquefaction process already triggered due to the so-called bacillus factor, i.e. fibrinolitic properties of proteins from the bacillary cell wall, or by infecting the macrophages surrounding the liquefaction. This would maintain the Th1 response favoring liquefaction to persist, whereby the presence of a large volume of liquefaction product leads to the destruction of new incoming macrophages (due to the high concentration of free fatty acids) and fibroblasts, thereby preventing the structuration of the site.

It could be said that liquefaction appears to be a stochastic effect due to disturbance in the organization of the intragranulomatous necrosis. The immune response and its magnitude, the bacillary load, the speed of the bacillary growth and the amount of extracellular bacilli, as well as mechanic and chemical factors (due to the distribution of the blood flow) are involved in it. Animal models have provided evidences to infer some of these factors, but more efforts on developing new models should be done in order to better mimic the human disease. Interestingly, this scenario supports the "damage framework" [Casadevall 2003] of infectious diseases that in the case of TB supports the fact that liquefaction and cavity formation is the cause of an excessive immune response against the bacilli [Cardona 2010] (Figure 6).

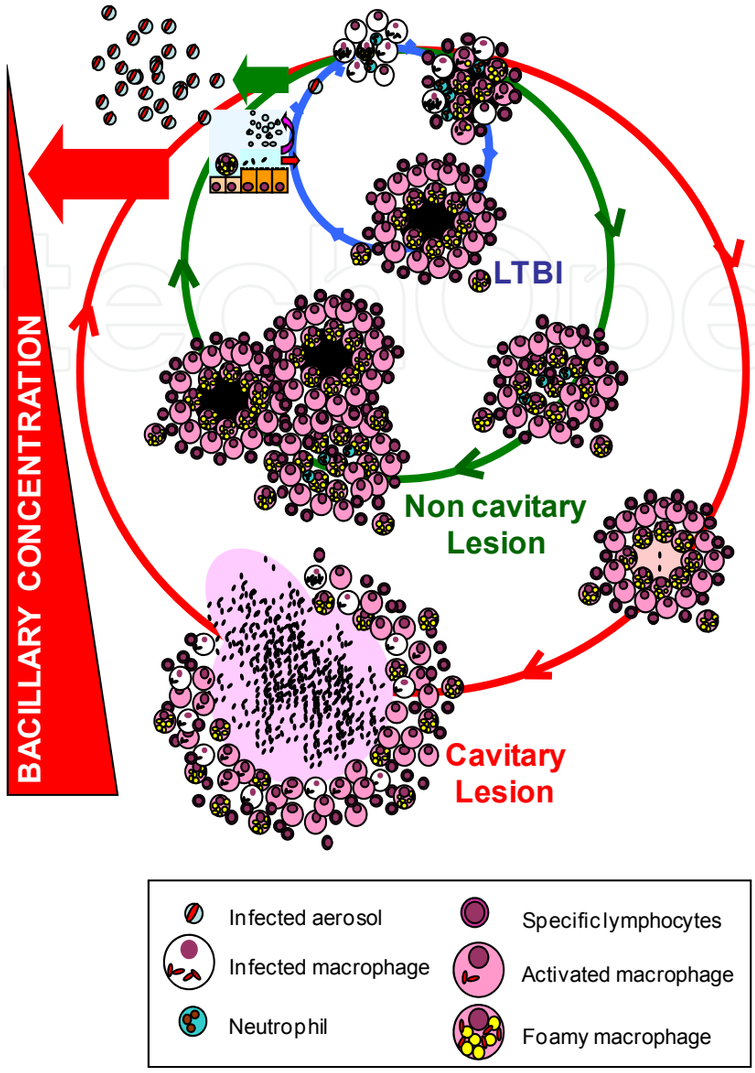

Fig. 6. **Transmission of *Mtb* infection.** LTBI (green circles) results from protracted endogenous re-infection of macrophages from drained NR-*Mtb*. Aerosol spread of drained NR-*Mtb* to susceptible hosts occurs from cavitary (i.e. in those patients that overreact against the presence of the bacilli) (red circles), and less frequently from non-cavitary (e.g. immunocompromised patients) (green circles) granuloma lesions
Symbols: black = necrotic tissue; pink = liquefacted tissue.
Obtained from Cardona & Ivanyi 2011.

9. Is Mtb fitness that important?

Considering that induction of active TB needs to be generated in that specific setting, and that tropism is that important, it appears that the most important fact comes from the chance of one person to have this site infected (Figure 6). Of course the best way is to be constantly reinfected, so the higher the prevalence of infection in the geographic region were the host resides, the higher the chance to infect the upper lobes and thus to generated active TB. In a way, also this depends on the index case. If the source of aerosols has a very intensive social

live, it has more chance to infect more people [Caminero 2001]; even more, if he or she is a good aerosol maker, the capacity to infect other people is even higher [Fennelly 2004]. So, the epidemiological factor is the most important. The host factor is also important in a way that the higher the reactivity the higher the chance to liquefact the tissue. In this regard, host polymorphism was soon detected as being a paramount factor in TB susceptibility [Dubos 1952], a fact that is nowadays clearly consolidated [Moller 2010]. Furthermore, far from the tropism issue, if the host has a depth immunosuppression and has a poor immunological capacity; or even a diminished capacity to heal lesions (i.e. to induce a correct fibrotic response) like in diabetes mellitus, the chance to develop active TB is huge.

At the end we have the third factor: the bacilli. In this case it appears that probably is the less important once taking into account the previous factors, as demonstrated by some authors [North 1999]. In this regard, the capacity to generate liquefaction by itself appears to be limited: it needs the special site and the inflammatory response generated by the host…, so it is not risky to predict that the variability of the bacilli is not really important to keep the life cycle of Mtb. That's probably why there are not really big differences among clinical strains, and that the bacilli has a very low mutation ration. It has no need so far… Figure 7.

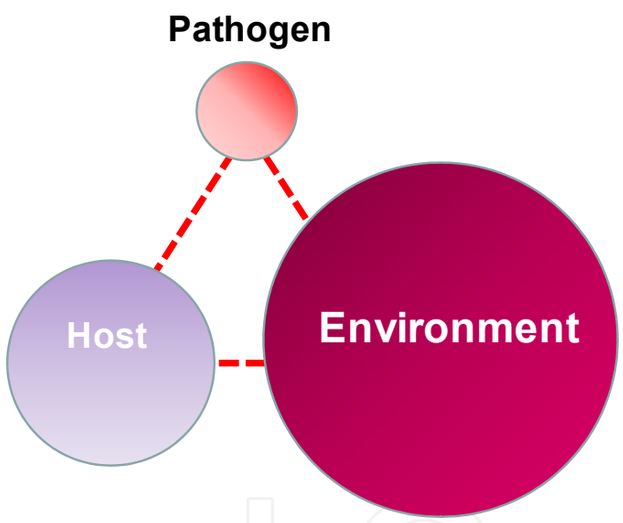

Fig. 7. **Relative importance of the different factors implied in the development of active TB.**

10. Towards new therapeutic approaches: Does host response ruin chemotherapy?

Mtb is a really slow pathogen. If *E. coli* divides every 20 minutes, Mtb needs about 24 hours, so it is 72 times slower. In this regard, if a standard antibiotic treatment of an *E. coli* infection requires 1 week, Mtb should require 72 ! Fortunately is not the case, the actual treatment needs "only" 24 weeks. This means that the actual drug combination is targeting very much very initial metabolic pathways, compared with the treatment of other bacteria. Of course the discover of a drug able to reduce even more this administration time would be desirable, but taking into account the global experience in quicker germens, it appears that we are reaching a kind of "glass roof" in this respect.

One growing issue is trying to lie Mtb by favoring artificially their growth stopping for instance the inflammatory response [Wallis 2005]. If the problem is that the stressful conditions change the bacilli metabolism in a way that make it less accessible to the drug targets, the solution should be the administration of anti-inflammatory drugs and even depress the immune response to lie the bug and "tell it" that it can finally grow !

My perception of the problem is that even in these circumstances, the reduction of the drug administration will be really neglectible. Why? Because the problem resides in that a majority of those non-replicating bacilli resides in the necrotic tissue, and to drain all of the bacilli require the elimination of all this material, and this takes time. In fact, in the case of LTBI, where the lesions are tiny, this requires up to 9 months… (Figure 8). In the case of active TB where the necrotic tissue is massive and this process would take years… So, again, the only hopeness to reduce the treatment would come from that ideal drug able to "make a hole" in the cell wall as soap, without needing any enzyme to disrupt… something very "physical" of course without hampering the much weaker host cell membranes…

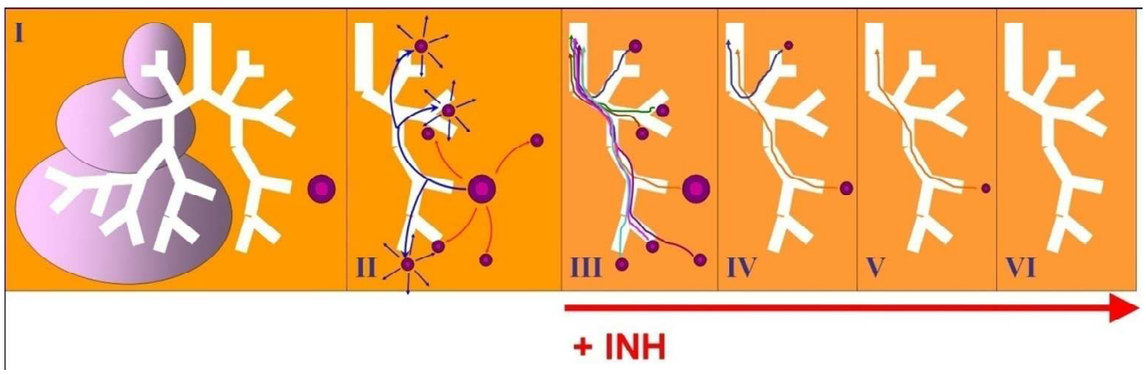

Fig. 8. **Mechanism of long-term isoniazid (INH) treatment of the latent TB infection (LTBI) according to the dynamic hypothesis.** This treatment allows the drainage of the nonreplicating bacilli, and in the case of endogenous reinfection through inspired aerosols reach the parenchyma, the bacilli have no chance to reactivate. In this case the lesions disappear with time and the opportunity to reach the upper lobes and generates the cavitary lesion is avoided. *Obtained from Cardona 2009.*

In this regard, our group promoted years ago the combination of short term chemotherapy together with a therapeutic polyantigenic vaccine (RUTI) [Cardona 2005], an approach that has already successfully finished a Phase II clinical trial [Vilaplana 2010, Archivel 2011]. The rational was to avoid precisely the sudden immunosuppression induced after chemotherapy, which is deleterious because the short time of antibiotic administration is not been able to cover all the bacilli drainage period. This attempt maybe does not induce a miraculous sterilization of the tissues but at least combines the destruction of growing bacilli, and avoids the sudden promotion of reactivation after finishing the chemotherapy. It also promotes a wider immune response, able also to help the detection of non-growing bacilli [Guirado 2008].

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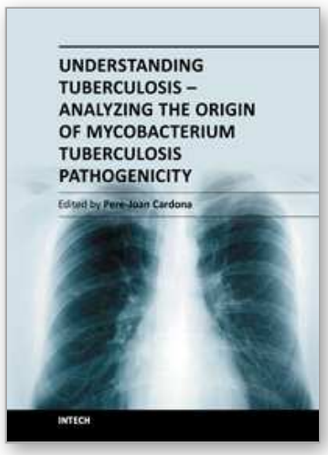

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Mycobacterium tuberculosis in an attempt to understand the extent to which the bacilli has adapted itself to the host and to its final target. On the other hand, there is a section in which other specialists discuss how to manipulate this immune response to obtain innovative prophylactic and therapeutic approaches to truncate the intimal co-evolution between Mycobacterium tuberculosis and the Homo sapiens.

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