Lessons from the murine experimental model of tuberculosis: the need to reconsider some topics

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ABSTRACT

*Mycobacterium tuberculosis* is probably one of the microorganisms best adapted to evade the human immune system. Several key mechanisms allow it to remain inside the tissue cells of the host for a long time. Among them, its low growth rate, and the ability to face the stress conditions triggered against it by adapting its metabolism and stopping its growth. The murine experimental model of tuberculosis has been deeply studied, and it is the basis for progressing on the knowledge of its pathology, diagnosis, prophylaxis and treatment. But far from the classical infallibility of the murine resistance against *M. tuberculosis*, nowadays its response is being reconsidered. Susceptibility is induced by several factors, among them, the «escape» of latent bacilli from the granuloma vehicled by foamy macrophages, which has been poorly studied. Such mechanism could be a good explanation for the origin of tuberculosis latency.

KEY WORDS: Tuberculosis / Murine experimental model / Macrophages / *Mycobacterium tuberculosis*.

INTRODUCTION

Tuberculosis is caused by the infection with the *Mycobacterium tuberculosis*. It is estimated that 30 million people will die from tuberculosis during the first decade of the XXI century, and that 80 million of new cases will be generated. It is worth mentioning that nowadays a third of the total population (2000 million people) is already infected, as they have latent tuberculosis. The 90% of these people will never develop tuberculosis. However, 5% of infected people will suffer from it during the first 5 years after the infection (85% in the first year). These are usually immunodepressed people or children, who endure in some cases the most dangerous forms of the disease, such as disseminated (milliary) disease or meningitis. The other 5%
can acquire it along their life, because *M. tuberculosis* can reactivate and cause a post primary disease, which typically affects adult immunocompetent people who usually develop a big lesion in the pulmonary apex with a huge necrotic centre that liquefies and becomes a cavity\(^{(1)}\).

### IMMUNOPATHOLOGICAL SPECTRUM OF TUBERCULOSIS

Human tuberculosis has a wide histopathological spectrum. First, the well known Ghon complex, which is a primary granuloma in the pulmonary parenchyma, predominantly in the middle and lower lung fields in a subpleural location, also affecting the draining lymphatics. From there, the bacilli are able to disseminate locally and systemically through all the organs, generating new granulomas. The typical granuloma in human tuberculosis is made by a necrotic nuclei surrounded by macrophages and epithelioid cells (activated macrophages) and a thick lymphocyte based mantle. The presence of multinucleated macrophages, the Langhans cells, is also characteristic. In the majority of the cases, granulomas become well circumscribed by a layer of collagen and suffer fibrosis and scarification, or calcification depending on the size. Usually, there are no clinical symptoms at this point. The only way to identify infected patients is through the tuberculin test. This is based on the stimulation of a delayed hypersensitivity reaction after the intradermal inoculation of PPD (Protein Purified Derivative), a protein extract from *M. tuberculosis* cultures. Unfortunately, in some cases (approximately 10-15%) such lesions are not well controlled and the necrotic tissue enlarges, generating a progressive primary complex, or becoming a tuberculoma. When this process is generated in the pulmonary apex, the necrotic tissue (caseum in the classical nomenclature) may liquefy. This very common process is called pulmonary tuberculosis of the adult, and seems to be related to the high local oxygen pressure, and to the proteases secreted both by the bacilli and the macrophages. Be whatever could be, liquefaction of the necrosis is a convenient culture media for the bacilli. They start to grow, increasing the inflammation and the size of the lesion until the process erodes bronchi. At this point, with the introduction of oxygen, bacilli grow even more, and the liquefied material drains into the bronchial tree and the patient becomes infective. This lesion is called «cavity».

There is also another kind of evolution: the miliary tuberculosis. In this case there is a massive dissemination of small necrotising granulomas and it is related to immunodepression.

Looking at different experimental animal models of tuberculosis (Table I) the one that best resembles the pathogenesis of the lesions in human is the rabbit, which is able to generate a real cavity with liquefaction. Guinea pigs are the more susceptible hosts. They also generate a strong tuberculin reaction that best resembles the human one. Mice are the most used for economical reasons and because they are the host in which the immunological responses have been best studied. As it can be seen in Table I, mice develop a soft tuberculin response, neither develop necrosis nor Langhans cells, and are classically considered the suffers of a low mortality, at least when experiments are followed during a short period of time. Figure 1 represents the differences between human and mice granulomas.

### Table I. Responses of several animal species to *M. tuberculosis*, based on Lefford\(^{(2)}\)

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<th>Mortality</th>
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Figure 1. Typical granuloma structure in humans (A) and mice (B). Note the necrotic tissue and the multinuclear Langhans cells (A), as well as the lack of necrosis and the presence of foamy macrophages outside the granuloma (B).
For a long time, mice have been a kind of «standard» on the resistance against *M. tuberculosis* infection. Effectively, mice seem to trigger a highly effective non-toxic response, by building non-necrotic granulomas based on Th1 lymphocytes\(^1\). By synthesising interferon-gamma (IFN-γ), such cells are able to activate infected macrophages, enabling them to destroy *M. tuberculosis* bacilli by generating radical nitrogen intermediates (RNIs). These mechanisms drive the infection towards a chronic, controllable episode\(^2\). Therefore, mice have been the standard host in which all new treatments or vaccines have been tested. The paradox of this model is that firstly, in our opinion, mice are not the so-called resistant host that best mimics the resistance triggered by humans, as all of them develop a progressive disease with a constant increase of the pulmonary infiltration, and finally die as a consequence. Secondly, because researchers have not studied the reason of this low resistance, which must be clearly related to the evident presence of foamy macrophages in their lesions.

**THE MURINE EXPERIMENTAL MODEL OF TUBERCULOSIS INDUCED BY AEROSOL**

*M. tuberculosis* infection usually begins with the entrance of the bacilli into the alveolar space through little droplets of water (1 µm on average) from the aerosols coming from the coughing, sneezing or talking of infected patients. Once in the alveolar space, bacilli are phagocytosed by alveolar macrophages. At this point, it is possible for macrophages to destroy them under unspecific activation conditions. As a primary pathogen, *M. tuberculosis* is usually able to overcome this attack. The paramount mechanism is its ability to suppress the link of ATPase pumps to the phagosome, avoiding not only acidification, a well recognised mechanism of bacillary destruction, but also the fusion between phagosomes and lysosomes\(^3\). Afterwards, *M. tuberculosis* will start to grow until it will destroy the infected macrophage, reaching the extracellular milieu. Since infected macrophages synthesise a bulk of chemokines and cytokines, new macrophages will be attracted that will phagocyte the extracellular bacilli\(^4\).

This situation induces an interstitial granulomatous lesion, which alters the parenchymal structure of the lung\(^5\). On the other hand it seems clear that some of the monocytes that migrate to the pulmonary parenchyma will become dendritic cells instead of macrophages. The basic difference between both cell types relies on their respective different kinetics and capability to destroy pathogens and to present antigens\(^6\). Macrophages usually stay in the parenchyma until they are destroyed or filled with necrotic material, becoming foamy macrophages. These are then drained to the alveolar spaces and carried up to the bronchi, trachea and throat by the ciliary epithelium and the peristaltic movements\(^7\). On the contrary, dendritic cells have a lower capacity to destroy pathogens. They phagocyte and drive them to the regional lymph nodes to trigger a specific immune response. That is the reason why little interstitial lesions are hardly seen in the parenchyma while mediastinic lymph nodes are plenty of infected dendritic cells with growing bacilli. From these foci, bacilli reach the blood stream and, potentially, all the organs of the host.

Once the specific immunity is induced by week 3 after the infection, the cell population of granulomas start to change. From a bizarre amalgam of neutrophils and monocytes, granulomas become infiltrated by specific lymphocytes, mostly CD4+ (some CD8+ and B cells). The lymphocytes activate infected macrophages to kill a high percentage of bacilli, and build a kind of peripheral crown. One of the particularities of the granuloma resides on the appearance of the primary granulomas. Looking at «resistant» mouse strains like C57BL/6 or BALB/c, such granulomas, which appear to be the original foci, are quite different compared to those that develop in «susceptible» strains like 129/sv or DBA/2. Effectively, the former have higher macrophage nuclei than the latter. Such difference could be related to a higher capability of resistant strains to attract monocytes to the infectious focus. Accordingly, a higher secretion of RANTES has been reported in C57BL/6\(^8\). Subsequently, monocyte differentiation to macrophage might limit the infectious foci by inducing a higher inflammatory reaction and destruction of the highly connected alveolar spaces that ceases the dissemination of the bacilli through the parenchyma. At the same time, it seems reasonable to think that a high proportion of them become dendritic cells, making more feasible the presentation of antigens in the regional lymph nodes and thus a quicker induction of immunity\(^9\).

Specific immunity against *M. tuberculosis* seems to be triggered against peptides released by growing bacilli. Effectively, by assaying the capability of inducing proliferation and IFN-γ production *ex vivo*, the response against antigens obtained from the filtration of young cultures of *M. tuberculosis* in exponential growth has been demonstrated\(^10\). This fact might explain the sudden decrease of IFN-γ secretion in the parenchyma, even though the bacillary concentration is still high. Effectively, after such activation, a 1 to 1.5 log decrease of the bacillary concentration occurs, making difficult to observe acid fast bacilli in the macrophage nucleus of the granulomas. Again, striking differences from this very
In the beginning of the infection between resistant and susceptible mice strains exist. IFN-γ secretion is higher, while lower concentration of bacilli is detected in C57BL/6 compared to DBA/2 mice.

Interestingly, by week 6 post-infection, foamy macrophages start building an outset crown, surrounding the well-defined crown of lymphocytes. This peripheral structure has a definitive difference: it is an alveolar lesion, there is no disruption at all of the pulmonary parenchyma. Another remarkable point is that it is constantly growing to the point that it is able to connect isolated granulomas. Finally, acid fast bacilli are hardly seen in the nucleus of the granulomas, usually alone and inside foamy macrophages.

These observations support the notion that *M. tuberculosis* is able to «escape» from the granuloma. Considering that foamy macrophages will be transported to the upper respiratory tract to be either expelled or swallowed to the stomach, it is reasonable to hypothesise that during this process, some bacilli might be able to reactivate, to grow and to start another lesion. This is, in fact, what happens in this experimental model, since the percentage of parenchymal infiltration grows arithmetically along time. The discovery of the intensity of this phenomenon is paramount. Effectively, it is accepted that there is a great systemic dissemination of *M. tuberculosis* at the beginning of the infection, without an immune response. With the acknowledgement of foamy macrophages as vehicles for bronchial dissemination when specific immunity is already triggered, evolution of infection should be revisited.

**THE INDUCTION OF LATENT BACILLI AND «LATENCY»**

It has been demonstrated that growing bacilli are highly susceptible to the stress generated by activated macrophages. After killing more than 90% of the bacillary concentration, stressful conditions trigger metabolic changes including reduction of metabolism, expression of factors like SigF or changes in the antigenic composition of the cell wall, such as accumulation of the 16 kDa α-cristallin protein. Under such conditions is not odd to suppose that bacilli become undetectable by the specific immunity stimulated against growing *M. tuberculosis*.

Special attention must be paid to the production of NO by infected macrophages. In the murine model, production of toxic nitrogen radicals (RNIs) by infected macrophages is a paramount mechanism of bacilli destruction. IFN-γ is the cytokine that triggers the production of these intermediates through the stimulation of the inducible NO synthase (iNOS). Interestingly, foamy macrophages produce the majority of iNOS (at late stages, from week 9 after infection). Comparison of the course of infection between «resistant» (C57BL/6) and «susceptible» (DBA/2) mice, has demonstrated that the production of iNOS is significantly higher in the latter, as it is the accumulation of foamy macrophages and the size of the granulomas. This observation is very surprising, as iNOS production has been considered the paradigm of macrophage activation in tuberculosis. On the other hand, production of iNOS might only have a mycobacteriostatic effect, depending both on the acidification of the phagosome, and on the quantity of RNI generated.

DBA/2 mice trigger a weak Th1 response, with significant lower RANTES and IFN-γ production, when compared with C57BL/6 mice. Therefore, it is logical to suppose that DBA/2 mice are not able to destroy as many bacilli as the «resistant» host. The superior presence of foamy macrophages in DBA/2 mice is also logical, because there is a more important presence of bacilli and thus, a prolonged inflammatory response, with a higher introduction of macrophages inside the infectious foci.

It must be taken into account that production of NO by macrophages has an immunosuppressive action on effector lymphocytes. Infected macrophages can only be activated once, and if this activation is not strong enough, a «second chance» to destroy the infective agent does not exist. These observations lead to the following hypothesis on what occurs in the granuloma. At the beginning of the infection, *M. tuberculosis* freely grows inside the macrophages until the immune response is triggered. Then, presentation of antigens promotes activation of macrophages, inducing the production of RNIs and oxygen toxic radicals or lowering the pH. However, a considerable percentage of the bacilli (almost 10%) are able to resist this reaction. The most probable scenario is that infected macrophages become foamy macrophages, and being full enough with phagocytosed necrotic material, they leave through alveolar spaces. If they carry any «resistant» bacilli inside there is a chance for reactivation, and probably at this point they will not be able to destroy them, as they cannot be activated again. Once these bacilli grow, they destroy the foamy macrophage and remain free at the extracellular milieu of the alveolar spaces, with a great chance of dissemination. When incoming macrophages reach this space and phagocyte them, they will have serious difficulties to be activated because of the presence of foamy cells inducing local immunosupression directed to the incoming lymphocytes which would try to activate them. This might be one of the reasons why finally, parenchyma of mice is all infiltrated, a fact that may be the cause of their death.
ANOTHER THEORY TO EXPLAIN LATENCY IN TUBERCULOSIS

Until now, the notion that *M. tuberculosis* is able to regrow from old lesions inducing a disease called "post primary", in immunocompetent people or people with a slight reduction in general immunity, such as people aged >65 years, has been fully accepted(19). So far, nobody has been able to explain how *M. tuberculosis* can escape from a fibrotic old lesion, or how it can suddenly grow inside and «break» it, or even how these bacilli are able to resist during all these years to finally grow again. Some authors have demonstrated that *M. tuberculosis* is able to resist anaerobic or microaerobic conditions and «resuscitate» once these conditions have finished(20). However, these were just «in vitro» assays, and resuscitation was obtained after some months. Nobody has demonstrated whether these bacilli are able to build any special resistance structure like a spine (i.e. like in the genera *Bacillus*), or a cyst (i.e. like *Toxoplasma*).

This is the reason why dissemination of infected foamy macrophages is a very interesting hypothesis. It could explain what happens in humans at the very beginning of the infection, before granulomas are very well confined to a fibrotic structure, where the possibility to «smuggle» is almost impossible. In this regard, it must be stated that humans trigger a higher inflammatory response against *M. tuberculosis* causing, for instance, the intragranulomatous necrosis (which is not usually seen in mice) at the beginning of the infection, before induction of the immune response(21). In this case, we must also consider the presence of an extracellular bacillary population. In particular, we can find a percentage of bacilli that has resisted to the inflammatory phenomenon, which causes necrosis, as well as the macrophage intracellular ones, which must behave like those bacilli in mice. Probably, foamy macrophages have also this role of smuggling bacilli towards the healthy parenchyma allowing another lesion to settle and so on.

These mechanisms can help us to understand that the presence of latent bacilli inside fibrotic lesions may be an evasion mechanism for *M. tuberculosis* rather than a latency mechanism. The bacilli may play this evasion role in new scenarios *ad libitum* until finding a favourable anatomic lesion (like the pulmonary apex) and being able to grow to the point of generating a huge lesion like the cavitary one. This point of view questions the general theory by which once *M. tuberculosis* has infected the host, tuberculosis may be able to reactivate along his life. In fact, it is not clear how bacilli are able to survive inside a necrotic tissue for years, or how a fibrotic lesion can become a bigger granuloma, which in turn becomes a «tuberculoma» and then a «cavitary» lesion as it is described by classical authors(18). In this regard, it should be stated that the terms «infection» or «not treated disease» have been misleadingly used. The latter includes patients from the pre-chemotherapy era in which the presence of huge lesions (related euphemistically as «a shadow in the lungs» or «pleurisy») were treated by collapse therapy or hygienic measures, for instance. In these patients, the bacilli have a superior probability to reactivate, as well as to begin disseminating from a non-fibrotic area.

These patients should be studied apart from those infected without any lesion in their lungs. In this case, it would be appropriated to consider that they could acquire the disease in a period of 5 years, at most. In fact, it has been very well defined that the maximum risk to develop a disease happens in the first year after infection(22). But these episodes are usually limited to children, teenagers or immunodepressed patients.

Our point questions the typical consideration of the apical lesion as just «post primary» one, as a result of a lesion in an immunocompetent patient, where a reactivation of an old granuloma exists. It could as well be the consequence of a 1 year infection in which the «smuggler» bacilli reach the alveolar spaces in the apical zone of the lung and grow a lot as a consequence of the privileged high concentration of oxygen that stimulates bacillary growth and depresses local immunity.

Considering the bronchoalveolar spreading of the bacilli through foamy macrophages a way to keep the latency by *M. tuberculosis*, it is clear that once the «first generation» of lesions, the bigger ones because of the lack of immunity, have been surrounded by fibrosis, the chance to «smuggle» for the bacilli will be almost impossible. Then, the only way to keep the chronicity would be to generate another granuloma, a «second generation» one, and to «smuggle» more bacilli from there. In this case, the growth of bacilli will be limited, because the host will have been immunised. So again, it is easy to imagine that the third, fourth or fifth generation of lesions will have less and less chances to develop. Thus, the time of latency and the time to suffer this «reactivation» will be shorter as time goes by. We believe that in human the term «reactivation» as well as the presence of «post primary» lesions should be reconsidered. These would only be the consequence of the chance of foamy macrophages to reach the immunosupressed zone in the apex of the lungs, and start to grow, generate a huge inflammatory response and an extraordinary lesion with a large necrosis inside, which finally liquefies.

In conclusion, this review wants to highlight the need to revisit the murine model of tuberculosis. First, we should reconsider the limited resistance triggered against *M. tuberculosis* by mice. Second, we should study better the
phenomenon of bronchial dissemination through foamy macrophages in mice and humans, not only in new cases but also in old descriptions and pathological material. Finally, we would like to challenge a new discussion on the bases of latency and the objective mechanisms by which _M. tuberculosis_ is able to reactivate from old lesions to induce post primary tuberculosis.

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REFERENCES