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Tuberculosis: Efficient Methods to Study a Worldwide Human Disease

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Tuberculosis is a major global health problem, affecting one third of the world's population and leading to the death of more than two million people every year [1,2]. The causative agent of this devastating disease is *Mycobacterium (M.) tuberculosis* [3], discovered in 1882 by R. Koch [4]. For decades, extensive in vitro research on the pathogen itself has been carried out (i) on the microbiology side, to increase basic knowledge of *M. tuberculosis* and to decipher the characteristics (genome structure, culture conditions…) of this "slow growth Killer"[5,6], (ii) on the immunology side, to determine the fundamental biological aspects of the host pathogen co-evolution, such as the host immune response, and the mechanisms developed by the pathogen to elicit it [7,8] and (iii) on the pharmacology side, to test the antimycobacterial activity of some chemical substances [9]. This research has led to the discovery of new original targets from *M. tuberculosis* and the development of new drugs that are effective in vitro [10-12].

Nevertheless, some drugs which are efficient in vitro are not efficient in vivo, most likely because in vitro assays cannot mimic the complex interplay between pathogen and host that occurs under the real conditions of infection. Indeed, one of the particularities of *M tuberculosis* is its capacity to aggregate with itself. To get around this problem, detergents (like Tyloxapol, Tween-80…) have to be used in the bacterial preparation to be able to count the "real" colony forming units (CFU). The CFU method is still considered the only way to determine the killing *M. tuberculosis*. Unfortunately, it has also been shown that the use of detergents did not prevent the aggregation of *M. tuberculosis* (i.e. one observed CFU may be one bacillus or ten bacilli) [13,14]. Moreover, among all well-known pathogens, only *M. tuberculosis'* culture required such drastic treatment to prevent aggregation, suggesting that this function is fundamental to its life cycle [13]. These in vitro conditions are thus far removed from the natural growth of this pathogen. This may explain why has been so challenging to develop new drugs that will prove efficient in natura. This is also why it is essential to keep developing new tools to better understand the physiopathology of *M. tuberculosis*.

In order to achieve this goal, one approach was to use the animal model, in particular the mouse model. This in vivo experimental model has led to critical discoveries: (i) how *M. tuberculosis* is able to escape the immune response of the host, (ii) the characterization of some crucial steps of the infection (macrophage, pH acidification, O_2 level ...) [15]. One to 10 bacilli are enough to perform efficient infections in the mouse, followed by the logarithm growth phase (dissemination), followed by the static and then the latent phase. Some genes from *M. tuberculosis* have been discovered as essential for these steps, leading to a new angle for research for the development of drugs [5,11,16]. Thus the mouse model has been a fundamental part of the first steps towards understanding the disease caused by *M. tuberculosis*. Nevertheless, *M. tuberculosis* is not a natural pathogen of the mouse; rather, it is strictly a human pathogen. There are probably more scientists able to characterize a "granuloma" from a mouse than one from a patient suffering from *M. tuberculosis*. Multiple data clearly demonstrate that

this model is somehow inadequate [17]: (i) the structure of mouse "granuloma" is very different to human ones, (ii) essential genes for the murine immune response have never been transposable in humans, (iii) the latency phase of *M. tuberculosis* is difficult to evaluate (due to the 2 year life span of this model) and (iv) mice infected with *M. tuberculosis* will succumb before their natural death [18,19]. However, this research was essential in order to characterize some specific and fundamental biology and to provide gene candidates to investigate in in vitro experiments.

It has been proposed that from these imperfect in vivo experiments, sophisticated in vitro models should be developed. Indeed, mycobacteria are intracellular pathogens infecting macrophages in the human and the mouse. Macrophages are also the cells with the most powerful antimycobacterial activity. Some in vitro experiments have shown that murine monocyte-derived macrophages are able to restrain and kill *M. tuberculosis*. However, because of the high numbers that are required, most laboratories do not use murine circulating monocytes or peritoneal macrophages, but bone marrow derived-macrophages (BMM) instead. That BMM kills *M. tuberculosis* was considered a fact for the last twenty years; however, now it has been clearly established that this was in fact an artifact caused by the antibiotics used to purify BMM and other experimental conditions (washing steps, medium replacing…). Antibiotics stayed concentrated in endocytic vacuoles from the BMM, synergizing the killing of *M. tuberculosis*. Therefore, the logarithmic killing observed in the past has to be interpreted as a gross overestimate of BMM's real ability to kill *M. tuberculosis*. Stated differently, under correct culture conditions, BMM were still able to efficiently create a bacteriostatic effect, but not a clear bactericidal effect on *M. tuberculosis* [20]. Another advantage of these BMM was their ability to reproduce human antimycobacterial activity in vitro, because they also produced nitric oxide, which is considered to be the major component involved in the destruction of *M. tuberculosis,* at the difference of superoxide production in vitro [21]. Recently, it has been shown that superoxide production is essential for the control of mycobacteria as a catalyst of adapted response in-vivo, rather than a direct effector of the *M. tuberculosis* killing, at least in vitro [21,22]. Furthermore, these BMM from mice have a short life expectancy (around 2-3 weeks without being infected!), which is not compatible with a slow growth killer like *M. tuberculosis*. All together, these data

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show the power, but also the limitations, of the mouse based in vitro model for understanding *M. tuberculosis* pathogenesis.

This also means that, for decades, there was in fact no in vitro model, mouse- or human-derived, where *M. tuberculosis* could be efficiently killed. Indeed, the mouse in vitro model looked so robust and was so convenient that nobody made any effort to develop a new protocol for the use of human cells in vitro. Indeed, the human monocyte-derived macrophages (MDM) has been an in vitro model, limited by that the fact that they are destroyed only few hours or days following the *M. tuberculosis* infection. In addition, MDM never produced detectable levels of nitric oxide (with the exception of some artifacts, misinterpretations or human cells line such as THP1) as opposed to the BMM-based model [23]. Nevertheless, the protein iNOS (inducible nitric oxide) is present inside macrophages in natura (in vivo with environmental constraint) upon infection of mycobacteria [24-27]. Human alveolar macrophages produce nitric oxide but only for an extremely short period, due to the imperfection of the in vitro cultures, and therefore were not amenable to efficient research protocol [28]. Furthermore, MDM or monocyte-differentiated dendritic cells (MDDC) are obtained by culturing monocytes with M-CSF or GM-CSF+IL4 respectively; however, they never showed any capability to restrain or efficiently kill *M. tuberculosis*. The explanation came from the deleterious impact of M-CSF or IL4 on MDM [29]. Thus the most common way to generate MDM is in fact incompatible with the exploration of efficient anti-mycobacterial activity. Furthermore, the high MOI (Multiplicity of Infection) as opposed to a low natural inoculum used leads to a high mortality of these MDM within the next few hours. These inadequate models used to study *M. tuberculosis* could explain the limited number of successful identifications of host genes involved in the control of antimycobacterial activity, even with the use of sophisticated high throughput analyses, such as genome wide RNA expression or proteomics screening.

Therefore, since both the human and mouse models are not effective, they have to be re-explored for an adequate mycobacterial activity, which is not achieved by current useful protocols. In addition, MDM, at the difference of BMM (3 weeks), can live for months in vitro and *M. tuberculosis* is strictly human pathogen. This argued in favor of developing a challenging protocol to efficiently explore mycobacterial killing with MDM. Thus, in this model, MDM have to support an infection of *M. tuberculosis* for more than few days (in natural conditions it is months/years) and optimally to produce nitric oxide derivates, as observed in natural conditions. Some microbiologists and others working in cell culture have obtained interesting results that improve some aspects of the culture conditions. However, all these works were done independently. Based on these previous works, we developed a new and original protocol to obtain a much better in vitro model for the study of mycobacteria in general and *M. tuberculosis*, in particular [29]. We successfully obtained better system conditions in which human macrophages are able to survive to the infection, kill Mycobacterium bovis BCG (Bacillus Calmette Guerin live vaccine), and severely limit the replication of *M. tuberculosis* for several weeks. The reason for our success was the fact that we used human plasma, physiologic levels of oxygen (>5%), with GM-CSF and/or TNFα followed by IFNγ. We clearly showed that the use of usual oxygen conditions in culture (20%), of M-CSF and/or IL-4 was truly deleterious in an in vitro system (again, different than in vivo, where multiple gradients of cytokines are created, avoiding to escape formally to any cytokine induction). This result has demonstrated why the control of mycobacteria was so difficult to obtain in vitro, since

the most common method of creating MDM was counterproductive to the control of *M. tuberculosis*. We hope that this model, still imperfect but definitively better that the previous protocols, allowing weeks of analysis, will help to design new antimycobacterial drugs and to decipher the genetic control that occurs during infection or latency periods with healthy MDM or mononuclear phagocyte systems (MPS) infected by *M. tuberculosis*. MPS? is probably a more appropriate term, since it includes all varieties of phagocytic cells: antigen presenting cells, not restricted to dendritic cells; phagocytic cells, not restricted to macrophages and others future functions [30]. Indeed these MPS, "macrophages" or "dendritic cells" are differentiated in different organs with a multitude of gradients of cytokines, which is impossible, for the moment, to recapitulate in an in-vitro cell system [30].

Nevertheless, *M. tuberculosis* is strictly a human pathogen and at a certain point the *M. tuberculosis* scientific community will have to switch from the pathogen point of view to the human point of view [8,19,31]. With the use of new technologies, such as whole genome sequencing rising, this is now clearly feasible. Indeed, one third of the worldwide population is infected with *M. tuberculosis* [32]. This is why it is crucial to develop projects that deal with human biological material and to develop efficient tools capable of analyzing the whole genome, in order to get closer to the disease as it happens in natural conditions of infection [3,33,34]. As proof of principle, the Bacillus Calmette Guerin (BCG) was developed nearly a century ago as an attenuated live vaccine for tuberculosis control in humans, and no major development or optimization has been shown to be more efficient since then [35]. However, some teams have already reported important, pioneering discoveries in term of genetic predisposition to mycobacterial infections. For example, a total of 8 genes implicated in the IL-12/IFNg loop have been characterized in the Mendelian Susceptibility to Mycobacteria Syndrome (MSMD) [19,32,36-38]. The study and understanding of the human model of *M. tuberculosis* infection will offer new avenues for the development of accurate molecular diagnosis and more physiological treatments based on the restoration of a partially deficient immunological pathway [2,31,32,39].

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