In vitro infection of human cells with *Mycobacterium tuberculosis*

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S U M M A R Y

It has been estimated that approximately 50% of individuals exposed to Mycobacterium tuberculosis never become tuberculin skin test positive, which may indicate that successful human immunological responses are able not only to inhibit mycobacterial growth, but also to kill the bacteria. Nevertheless, it has been extremely difficult to reproduce this effect *in vitro* and the ability of human phagocytes to eliminate the bacteria is controversial. This is one of the reasons why we do not fully understand either tuberculosis resistance or susceptibility. Nowadays there is a pressing need in tuberculosis vaccine research to find biomarkers of successful responses to tuberculosis and the use of *in vitro* models may allow the identification of the immunological mechanisms responsible for the mycobacterial killing that could be tested in clinical settings. Besides biosafety concerns, the manipulation of mycobacteria is technically very demanding, and the optimization of *in vitro* infection protocols have been difficult. As a result, there are a large number of variations in the methodology that make arduous the comparison of results obtained in different research groups. In this review an overview of the mycobacterial and human cellular models most frequently studied are presented, together with a description of several of the modifications tried in infection protocols, from the preparation of the inoculum to the quantification of surviving mycobacteria after infection. A comment about statistical methods that may improve the detection of relevant biological effects is also included.

Keywords:

Mycobacterium tuberculosis killing

Macrophage

Infection protocols

Design of experiments

1. Introduction

At the middle of the twentieth century the eradication of tuberculosis was considered feasible. Although several problems were anticipated, it was thought possible to strengthen the control programs of the disease to the point of its total elimination.[1] The failure of these predictions became obvious when in 1986 the downward trend of the incidence rate of tuberculosis reversed. The reasons are several and complex, including the arrival and spread of HIV infection, the immigration of people from high-prevalence countries and the deterioration of tuberculosis control.[2] Nowadays, the goal to eliminate tuberculosis has been set for 2050, but it will not be met with present strategies and instruments. Besides actions like scale-up of early diagnosis and proper treatment or development of bold health-system policies, we will need to increase the promotion and intensification of research towards innovations.[3]

It was very soon recognized a wide range of susceptibility to tuberculosis, which was tragically apparent in the Lubeck disaster (1929), in which a virulent strain of *M. tuberculosis* was inadvertently inoculated to more than two hundred newborns instead of the BCG vaccine. The results were dramatic and 29% of the babies died. Nevertheless, the fact that 54% of the infected babies that suffered the disease recovered[4], provided evidence that strongly supported the influence of genetics on the tuberculosis susceptibility. In addition, nearly 50% of individuals exposed to *Mycobacterium tuberculosis* never become tuberculin skin test positive[5], which may indicate that the bacterium is removed by the innate immunity. We have accumulated a large body of knowledge about the immune response to *M. tuberculosis*, that largely depends on the IL-12-IFNγ axis[6], but we do not know whether there are immunological mechanisms able to completely eliminate the microorganism and if they exist, which their nature are. There are several candidate processes that may determine eradication or mycobacterial growth restriction, such as reactive oxygen or nitrogen species production[7,8,9], antimicrobial peptides production[10,11,12], hydrolytic enzymes and pH regulation[13,11], apoptosis[14] or autophagy[15]. If current *in vitro* infection models do not mimic successful physiological immune responses may be a result of the inadequate tuning of these and other effectors mechanisms. As a consequence, we do not have appropriate or validated biomarkers of protection immunity to tuberculosis which may help us in the development of new vaccines or treatment regimens[16]. Knowledge obtained by *in vitro* infection of human cells has not provided so far the desired

answers. In contrast with other pathogens, no consensus about the capacity of human phagocytes to display antimycobacterial activity has been reached[17]. For this reason the number of publications dealing with *in vitro* infection of human cells has decreased since the nineties of the past century. More encouraging results have been obtained in animal models, which have provided invaluable information about the development of the disease[18]. Nevertheless, *in vitro* studies with phagocytes may differ from the human model. It was initially described that, unlike human macrophages, mouse macrophages were able to control *M. tuberculosis* growth[19,20], and following studies confirmed, in general, these results[20]. Nonetheless, large variations in the intracellular growth rate of mycobacteria in either mouse or human macrophages have been reported and it is difficult to arrive at definite conclusions. Furthermore, several studies have shown that the antimycobacterial activity in the mouse depends on nitric oxide (NO) production[8]. The importance of NO in the antimycobacterial activity of human cells is less clear but several lines of evidence support the participation of this defence mechanism[9]. In this review I will focus on the analysis of *in vitro* models of infection that have been devised to study antimycobacterial activity in human cells.

2. The mycobacteria

The more important pathogen of the *Mycobacterium* genus is *M. tuberculosis*. Nevertheless, other mycobacteria from either the *Mycobacterium tuberculosis* complex or from the nontuberculous group cause disease.[21] Published *in vitro* experiments are conducted with both the laboratory strains, as well as clinical isolates of *M. tuberculosis*. The more widespread laboratory strains are H37Ra, H37Rv ('a' stands for avirulent and 'v' for virulent), Erdman, the clinical strains CDC1551 and HN878, and the vaccine strain *M. bovis* BCG. A wildtype behaviour may be better observed in clinical isolates, but they are more difficult to standardize[22]. From the phylogenetic point of view, a useful classification has divided all *M. tuberculosis* strains between 'ancient' and 'modern', depending on the presence or absence of an *M. tuberculosis* specific deletion (TbD1).[23] Posterior phylogenetic studies have classified the *M. tuberculosis* strains in six lineages, based on the comparative analyses of a large set of coding sequences from a global collection of strains.[24] Lineages 1, 5 and 6 would correspond to 'ancient' strains and lineages 3, 2 (that includes strain HN878) and 4 (that includes strains H37Rv/Ra, CDC1551 and Erdman) to 'modern' strains. It has been

suggested that 'ancient' strains promote a stronger proinflammatory response that frequently controls the infection, while progression to active tuberculosis is more characteristic of 'modern' strains.[22,25]

Regarding the laboratory strains, there is agreement with respect to the capacity of H37Rv to grow intracellularly faster than H37Ra, but different studies show disparate results. Some reports indicate that H37Ra does not multiply in human macrophages[26,27], others that it grows but slower than H37Rv[28,29] and even that it is killed[30]. Similar differences are described for other strains. CDC1551 is reported to grow faster[31] or slower[32] than H37Rv, and the Erdman strain has been reported to grow faster than CDC1551 but slower than H37Rv[32]. The inconsistent results should not be surprising because diverse infection protocols were used, which may have a major influence on the outcome of the experiments. Also, laboratory strains are grown for years in different laboratories in which they will suffer various mutations, as has been demonstrated for H37Rv[33], that may induce important phenotypic changes.

Clinical strains also behave in a variable way. The growth rate of *M. tuberculosis* within isolated human macrophages *in vitro* has been correlated to the ability of the strain to spread and infect multiple individuals within a community[34], as well as the ability of the strain to establish extrapulmonary disease[35]. The *in vitro* growth rate is also associated with the phylogenetic lineage[32], their isolation from tuberculosis meningitis[30] or the epidemiological evidence of their virulence[36]. In contrast, there is also a report that indicates that regardless of the strains origin (large, small or unique clusters in the community), they did not exhibit differences in intracellular growth.[37] From these studies it may be concluded that the strain selected for *in vitro* infection experiments will determine the kind of results obtained.

3. Human cellular models

Undoubtedly, the monocyte isolated from peripheral blood is the more important model for primary cell infection. It is easily obtained and may be differentiated *in vitro* to macrophages, the main cell target of *M. tuberculosis*. In initial *in vitro* infection experiments the mononuclear cells were obtained from animals like rabbits[38], guinea pigs[39] or mice[40]. Human macrophages were first infected with *M. leprae*,[41] and afterwards Crowle and May infected monocytes with *M. tuberculosis*.[42] Difficulties in obtaining any other type of human tissue macrophages have hindered their widespread

use. Nevertheless, another very important model is the alveolar macrophage,[43] believed to be among the first cells to phagocyte the bacilli in the lung.[44] Occasionally, other human macrophage models, like microglia[45], are investigated. The other important phagocyte, the neutrophil, has also been considered as a cellular model for *M. tuberculosis* infection[46]. Other immune cells, like lymphocytes, have been described in cocultures with infected macrophages.[42] Finally, a very interesting model is whole blood[47], which contains many components of the immune system that may cooperate in the elimination of the bacilli but is, on the other hand, very difficult to analyze given the complexity of the cellular and humoral interactions that take place in this tissue.

Human cell lines has also been used for a long time as infection models.[48] Lines that may be differentiated to monocyte and macrophage-like cells, like THP-1[49], HL60[50] and U937[51], have been successfully analyzed for intracellular multiplication of *M. tuberculosis*. Furthermore, *M. tuberculosis* have been described to invade and grow within other non-phagocytic cell lines, like HeLa[48] or A549[52], allowing the study of the participation of non-immune cells in tuberculosis.

4. Infection protocols

An infection experiment with *M. tuberculosis* is a multistep process that demands careful planning and much experience. The most frequently used methods are presented, but each of them has numerous small variations that will not be detailed. For the analysis of antimycobacterial activity the protocol may be divided as follows:

4.1. M. tuberculosis growth and individualization

The manipulation of mycobacteria is very difficult because they are highly hydrophobic. The main consequence is that bacilli remain together after division, forming very large clumps of bacteria that may be difficult to phagocyte. Therefore, we need to disperse them in such a way that bacterium are isolated from each other. Several means have been tried to accomplish this goal. The main one is the addition of detergents to liquid cultures (e.g. 7H9), like Tween-80[53] or the nonhydrolyzable tyloxapol, or the incubation in roller bottles in the presence of glass beads (around 3-mm diameter) that allows a dramatic, but not complete, inhibition of bacterial aggregation. Further steps are required to obtain individualized bacteria, including vortexing with glass beads, passing several times through a 25-gauge needle or

sonication, either in water baths or with tip sonicators, at a setting that allows bacilli dispersion but does not compromise viability. A usual protocol also includes the centrifugation at low speed (100 ×g) to remove the remaining microscopic clumps, before recovering the supernatant that contains the isolated bacteria. Many researchers consider acceptable the presence of a low number of small bacterial aggregations (< 5 bacilli) among predominantly individualized bacteria. Growth in liquid media may, however, have some disadvantages. It requires additional manipulation for the removal of the bacterial nutrients and suspension in cellular medium, like RPMI 1640. Furthermore, growth of mycobacteria in the presence of detergents might be detrimental for the virulence of the bacilli as has been reported for the experimental mouse. On the other hand, when using a detergent-free synthetic medium bacilli have been described to be more virulent[54]. The reasons for this feature are not known, but it may be related to the presence of a mycobacterial capsule that is almost eliminated by the addition of small amounts of detergents to the growth medium. Moreover, surface lipids such as phthiocerol dimycocerosates are extracted if Tween-80 is added at a concentration of 0.05%[55]. Changes in the *M. tuberculosis* cell envelope may have a major influence in the interaction with the host cells. To circumvent these problems the mycobacteria may be grown on solid media (usually 7H10 or 7H11), and directly suspended in cellular medium, before manipulation to disperse the bacterial clumps. Individualized bacteria may be then frozen preferably at -70 ºC or in liquid nitrogen. A convenient strategy is freezing a large stock of single use aliquots, a method that provides homogeneous inoculums to grant high reproducibility in the number of bacteria inoculated.

Quantification of mycobacteria is not a simple task either. The measure of turbidity (e.g. 600 nm), the comparison with a McFarland standard or direct quantification in counter chambers are sometimes used, but the viability of the bacteria is not assessed and, in our experience, reproducibility is not good. More frequently, inoculation of serial dilutions in solid media and estimation of colony forming units (CFU) is preferred. Before freezing, viability of the individualized mycobacteria may be analyzed with the Baclight kit (Molecular Probes, Invitrogen) that distinguishes between live (green) and dead (red) bacteria. After trying the described methods, at our laboratory we favour the growth in solid media, the individualization of bacteria by using a sonicator with a microtip, followed by low speed centrifugation, and CFU quantification in solid media.

4.2. Cellular purification

As already indicated, the more important cellular models are monocyte-derived-macrophages and neutrophils. The latter are easily isolated by consecutive steps of dextran sedimentation, density centrifugation in Ficoll-Paque and lysis of contaminating red blood cells.[56] The purity of the cell preparation is often above 98%.

The more widespread method for monocyte purification is the isolation of mononuclear cells by Ficoll-Paque[57], and depletion of lymphocytes using the adherence method, taking advantage of the fact that monocytes adhere to plastic and lymphocytes may be removed by extensive washing. Nevertheless, the proportion of monocytes is variable among donor, and the addition of equal number of mononuclear cells does not guarantee similar number of monocytes. Moreover, in our experience, it is difficult to perform a homogeneous wash in every well, and there is some variation in the number of cells that remain adhered. Finally, there might be a selection against weakly attached monocytes. Despite these disadvantages this method is popular because is easy to perform, reliable and inexpensive. The cellular adherence attribute is also useful for the purification of alveolar macrophages from bronchoalveolar lavage. Counting the number of adherent monocytes is complicated by the fact that they can be difficult to detach from the wells. The preferred method of quantification is based on the lysis of cells with detergents and subsequent quantification of nuclei stained with naphtol blue black.[58] The other two purification methods that are often used by researchers in the tuberculosis field is elutriation, which requires specific instrumentation, and positive immunomagnetic separation using a monoclonal anti-CD14 antibody labelled with superparamagnetic particles. Immunomagnetic separation is our method of choice because it allows the quantification in a counting chamber and, consequently, a uniform number of cells in every well, but it is expensive. In all cases, purity of monocytes is usually above 95%, and after a few days they differentiate to macrophages. In the first reports of antimycobacterial activity assays sometimes monocytes from several donors were mixed to increase the number of cells available for experimentation. Nowadays, this practice is not usual because monocytes from each volunteer allow different levels of intracellular multiplication[59,37,32], and this variability information is lost in pooled monocytes.

4.3. Cellular infection

This step is likely to be the one that has the most important influence on the final outcome of experiments dealing with analysis of antimycobacterial activity. Besides the selection of the bacterial strain and the cellular model, two main factors, multiplicity of infection (MOI) and incubation media, may change depending on the experimental design.

Large MOI (5-100 bacteria per cell) induce necrosis and apoptosis[60,61], which will greatly interfere with the intracellular multiplication of *M. tuberculosis*. For this reason, infected cells are incubated for a short period of time (1 hour to overnight) and non-phagocyted bacteria are removed by extensive washing. Again, the washing procedure will increase the manipulation of the samples and introduce technical variability, because is difficult to ensure that every well is washed in the same manner. An alternative is the use of low (1) or very low MOI (<0.1). In this case, many or most cells are not infected, as it is supposed to happen at the beginning of the tuberculosis infection, and no washes are needed. Although most bacteria are assumed to be phagocytosed, some will remain free and may multiply extracellularly, creating artifacts that need to be taken into account with appropriate controls. For assays other than the analysis of antimycobacterial activity it is usually necessary to perform *in vitro* infections with high MOI in order to detect any effect of interest, like cytokine or reactive oxygen species production.

There are several incubation media appropriate for *in vitro* infection. In primary cultures the standard medium is RPMI 1640 supplemented with foetal bovine, heterologous or autologous serum. The use of a large stock of foetal bovine or heterologous serum allows more homogeneous experimental conditions, but the most preferred supplement is autologous serum because not only takes into account biological variations in cellular characteristics but also in serum factors. Serum free medium has only recently become available, allowing an increased uniformity in the assays[62]. Serum-free media may be used because *M. tuberculosis* does not strictly need opsonisation to be phagocytosed.[63]

4.4. Cell lysis and quantification of mycobacterial growth

Experiments designed to determine the survival of mycobacteria within host cells require that a method be established for the quantification of surviving bacteria. The first attempts to analyse the level of intracellular multiplication were the staining of intracellular mycobacteria, and express the quantification under the microscope as the

mean number of bacilli per macrophage.[42] Soon after, the need to know the total number of bacteria prompted the development of a method to release the intracellular bacteria for direct quantification. This was accomplished by developing cellular lysis procedures that did not affect bacterial viability, based on the method reported by Biroum-Noerjasin for listerial *in vitro* infection[64], with the use of sodium dodecyl sulphate (SDS) and neutralization of the detergent with bovine serum albumin.[17] This method is still widely used, sometimes with minor variations like the change of the detergent to Triton-X100 or digitonin. Released bacteria are then serially diluted and plated, usually onto 7H10 or 7H11 solid media. After more than 15 days the number of colonies is counted as CFU. This length of time may be drastically reduced by growing the released bacteria in liquid media (usually in 7H9 medium) where, because of their hydrophobic nature, they will form microcolonies that can be counted under an inverted microscope.[65] An additional advantage of this method is that samples may be aliquoted in 96 wells plates, with important savings in media consumption and storage space in the incubators. An inconvenience of the detergent lysis method is that SDS lysis affects the growth in liquid of mycobacteria like *M. gordonae* (unpublished results). As an alternative method, bacteria may be released by ultrasonication, using a sonicator with a microtip.[66] We have chosen this approach because it requires minimal manipulation; it does not add reagents to the culture and it does not inhibit the growth of any tested mycobacteria.

Whatever the method utilized for CFU quantification, many days are needed to allow the bacteria to form colonies. Faster indirect methods have been developed for mycobacterial growth quantification. The first one was the [H^3]-thymidine incorporation to the nucleic acids from intracellularly growing mycobacteria.[67] Later, other techniques were devised, including quantification by use of the BACTEC system (Becton Dickinson)[47] or by using a mycobacterial reporter strain (usually BCG) transformed with *luc*, that encodes the enzyme luciferase.[68] Nevertheless, determination of CFU remains the method of choice for most researchers.

5. Antimycobacterial activity

The first reports of human phagocyte infections with mycobacteria described an unrestricted intracellular growth of *M. tuberculosis*[42]. Since then, many attempts to activate human cells against *M. tuberculosis* have been tried. The more extensively

studied molecules for this purpose are the cytokines, but also vitamins[69,70], lipids[71], nucleotides[72] and others have been investigated. A cytokine recognized as essential in tuberculosis is IFNγ because genetic deficiencies in molecules involved in IFNγ signalling predispose to mycobacterial infections by BCG or non-tuberculous mycobacteria[73]. Nevertheless, in an early report from 1985 this cytokine was found to be unable to stimulate the antimycobacterial activity of human macrophages *in vitro*[17]. Afterwards, other studies presented evidence of *M. tuberculosis* killing by either monocytes[74] or neutrophils[75]. In spite of this, the efforts of other investigators to reproduce these results were unsuccessful[76,77]. In an attempt to make sense of these disparate results, we designed an experiment with very well defined experimental conditions in which we infected simultaneously monocyte derived macrophages and neutrophils from the same donors with both *M. tuberculosis* and *M. gordonae*, a non-pathogenic mycobacteria, in the presence of IFNγ. We could not detect any killing in either cellular type for any of the microorganisms[78]. A comparable result had been previously obtained by Robertson and Andrew for *M. phlei*, another non-pathogenic mycobacteria[79].

Looking for mycobactericidal activity, some research groups have tried to stimulate human macrophages with many cytokines. Bonay et al performed infections with a mycobacterial reporter strain (*luc* transformed BCG), and activated macrophages with 19 cytokines[68], but they observed that only IL-3 and GM-CSF were able to promote some bacteriostatic activity. In another study, Vogt and Nathan also tested a large number of cytokines, and they reached several interesting conclusions regarding the experimental conditions[80]. They observed that RPMI-1640 medium supplemented with foetal calf serum failed to control BCG replication of any remaining free bacteria, whereas 40% human plasma improved cell survival and did not allow extracellular BCG replication. They analyzed several variables like the influence of cytokines, culture media or time of infection. Macrophages were able to kill *M. bovis* BCG, and severely limit the replication of *M. tuberculosis* for several weeks if differentiated in 40% human plasma under 5%-10% (physiologic) oxygen in the presence of GM-CSF and/or TNFα followed by *IFN*γ. Control was lost with fetal bovine serum, 20% oxygen, M-CSF, higher concentrations of cytokines, or premature exposure to *IFN*γ[80]. Another factor that is undoubtedly important is the interaction between cytokines in the modulation of the antimicrobial activity of macrophages. It is common to test some cytokines simultaneously, specially IFNγ and TNFα, and several groups have analyzed

several different cytokines together[78,80,81]. In a third revealing study by Jordao et al, different sources of phagocytes were analyzed, rather than treatment with various cytokines[82]. They found that infection of phagocytes from murine, human or bovine origin with BCG or *M. bovis* either allowed or inhibited mycobacterial growth. Thus, BCG was killed more efficiently by J774 than by Raw, two murine macrophagic cell lines. On the other hand, both murine bone marrow derived macrophages and bovine monocyte derived macrophages could kill BCG, but not human monocyte derived macrophages. It is complicated to make a suitable interpretation of this kind of data and more information is needed before we reach a good understanding of the mycobacteria-phagocyte interaction.

In isolated reports there are claims regarding the induction of bactericidal activity against *M. tuberculosis* in human cells. Kusner and Adams show that ATP addition induces mycobacterial killing[72]. On the other hand, the activation of TLR2 with the mycobacterial ligand 19-kD lipoprotein also promotes *M. tuberculosis* killing in both monocyte derived macrophages and alveolar macrophages[83]. Another report indicates the stimulation of mycobactericidal activity with inhibitors of Ca^{2+} and K^{+} transport[84]. These kind of findings need to be thoroughly investigated, and reproduced by other groups, which may not be easy. For example, we have not been able to confirm the TNFα activation of killing activity in human neutrophils[78] described by Kisich et al[85].

Most of the gathered evidences indicate that it is extremely difficult to induce mycobactericidal activity in purified populations of phagocytes, mainly macrophages and neutrophils. Nevertheles, the hypothesis that the innate immunity of some individuals may eliminate *M. tuberculosis* is very attractive. It is possible that human phagocytes need the assistance of other cell types or unknown humoral factors. The development of more complex cellular models may help to discover new antimycobacterial mechanisms. The simplest way is fractioning blood cellular and humoral components and putting them back together in a controlled form. Using this approach, it was found that coculture of infected monocytes with either $CD4^{+}$ T cells or NK cells induced the elimination of *M. tuberculosis* [86]. Similarly, it has been described the inhibition of intracellular growth in monocytes by coculture with either $CD4^{+}$ T cells from purified protein derivative-positive subjects[59] or primed lymphocytes and IFNγ[87]. Attempts to reproduce the microenvironment in the lung alveolus include the use of a transwell system in which is constructed a bilayer of type II alveolar epithelial

cells and endothelial cells[88,89]. Another model that does not require any manipulation is whole blood[90], which has the advantage of displaying most types of immune cells and many humoral immunological factors. We have detected mycobactericidal activity in this model depending on the donor (unpublished observations).

With the exception of primary immune deficiencies, there is general agreement that susceptibility to tuberculosis is multifactorial. There is much knowledge obtained from epidemiological studies about which conditions predispose to suffer the disease. These include malnutrition, cancer, diabetes, age, stress and others. We are acquainted with many of the cells and molecules that are affected by them and that can be tested in *M. tuberculosis* infection experiments. Cytokines are the most studied molecules but others like vitamins, hormones, adipokines, lipids or signal transduction activators or inhibitors are important candidates to study. Ideally, their activities should be effective against any *M. tuberculosis* virulent strain. Additionally, their role should be relevant to a wide variety of individuals, which make the analysis of primary cultures the preferred model of study. Frequently, results obtained with attenuated strains or immortalized cell lines may not be extrapolated to virulent strains and primary cells. Simple infection experiments with purified cells treated with single molecules, although appropriate to analyze other bacterial pathogens, have provided incomplete information regarding successful immune responses against mycobacteria. It is possible that only complex infection models that consider several cellular populations, and interaction of disparate immunomodulatory molecules, in carefully optimized and controlled experiments, will show us the immunological mechanisms that actually take place in infected resistant individuals or patients.

6. Statistical data analysis

The selection of an appropriate statistical test to analyze the influence of treatments on the intracellular multiplication of *M. tuberculosis* will have a large influence on the ability to detect relevant effects. The log transformation of CFU data usually allows parametric testing using the Student's *t*-test, either paired or unpaired, for two group mean comparisons or ANOVA for several group mean comparisons. Alternatively, non-parametric testing is also appropriate, using either the Mann-Whitney *U* test or the Kruskal-Wallis for comparisons between two groups or among several groups, respectively. Nevertheless, this approach very frequently qualifies mean

differences as non-significant. Slow mycobacterial growth, and the particular resistance of the intracellular pathogen makes it difficult to detect biologically meaningful effects.

In a recent report we have taken advantage of the Design of Experiments methodology (DOE) based on factorial designs[91], which have been mainly developed for engineering. Factorial designs allow the study of the effects of two or more factors, and in each complete trial or replication of the experiment all possible combinations of the levels of the factors are investigated. They are more efficient than one-factor-at-a-time experiments, and they allow the analysis of interactions between factors. Current knowledge regards the immune response to tuberculosis as multifactorial, and the analysis of interactions may uncover important biological mechanisms. The most important case of general factorial design is that of *k* factors, each at only two levels (2^k), such as two quantities of a cytokine (e.g. 0 and 25 ng/ml). When two factors are studied, 2^2 treatments need to be performed (e.g. No treatment, Cytokine 1, Cytokine 2 and Cytokine1+Cytokine 2). 3, 4 and 5 factors will require 8, 16 and 32 treatments, respectively. It is technically very difficult to handle more than 32 treatments and fractional designs may be used when 6 or more factors are studied. Usually replicates are recommended, and with 3 to 5 factors, sample sizes of 3 to 6 are frequently enough to detect differences in intracellular mycobacterial growth. These small sample sizes are accomplished with the use of blocking in the designs (equivalent to pairing in Student's *t*-test), in which each sample from a different volunteer correspond to a block, facilitating the elimination of the sample source effect on the statistical comparison among treatments. DOE has allowed us to detect mycobacterial growth inhibition induced by IFNγ[92], which we had never been able to observe with one-factor-at-a-time experiments[78], and has been very useful for identifying factors that influence mycobacterial growth, like cytokines, age or protein tyrosine phosphorylation inhibitors. Even when we are interested in only one factor, the addition of factors like IFNγ as controls will facilitate the use of DOE. A further advantage of DOE is that allows the optimization, in a series of experiments, of the amounts of each factor that will result in the best response. Nevertheless, it is very difficult to find quantities that are adequate for every individual, and possibly only a range of suitable amounts may be determined.

7. Conclusion

We need all the tools available to get the information that will allow us to implement strategies to reduce the burden of tuberculosis. *In vitro* infection studies become therefore necessary. The identification in infected cultures of cellular activities, regulating molecules, synergies or competitive interactions that result in mycobacterial inhibition will allow the analysis of their role in the susceptibility to tuberculosis in patients. It will also allow the detection of biomarkers of appropriate immune responses that are restored in patients that have overcome the disease. These biomarkers will be useful in clinical trials to shorten the time needed to determine whether a vaccine or a prevention measure is successful in tuberculosis. It may also guide the design of prevention strategies like the use of nutritional supplements (vitamins, oligoelements …) that may enhance the resistance status of individuals at risk. On the other hand, candidate immunomodulatory molecules identified in infected cell cultures, like cytokines, monoclonal antibodies, signal transduction inhibitors, or any other therapeutical approach, may be tested *in vitro* for the treatment of patients infected with *M. tuberculosis* resistant strains. Finally, from the basic research point of view, *M. tuberculosis* constitutes an excellent model to characterize the immune response to intracellular pathogens. Although discouraging, the extreme difficulties to activate human immune cells to kill *M. tuberculosis* is a testimony to the extraordinary adaptation of this pathogen to its host. Nevertheless, its enormous clinical importance should prompt us to keep testing *in vitro* the hypothesis that the human immune system is able not only to control the growth of the microorganism but also to fully eliminate it.

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