Human phagocytes lack the ability to kill *Mycobacterium gordonae*, a non-pathogenic mycobacteria

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Abstract

Non-pathogenic mycobacteria, like *Mycobacterium gordonae,* are rarely associated to disease. The analysis of the mechanisms which are successful against them in the human host may provide useful information to understand why they fail against the pathogenic *M. tuberculosis*. We have developed an infection model in human phagocytes to test their ability to kill two strains of *M. gordonae*, HL184G and an attenuated variety, HL184Gat. As controls we included a strain of *M. tuberculosis* (HL186T) and another of *L. pneumophila* (ATCC13151). We observed that human phagocytes lack the intrinsic ability to eliminate either *M. gordonae* or *M. tuberculosis,* but they kill the attenuated strain. We could observe a relationship between pathogenicity and the pattern of cytokine production. Thus, both the pathogenic *M. tuberculosis* and *L. pneumophila,* but not the non-pathogenic *M. gordonae,* induced the production of significantly different levels of IL-1β, IL-6 and TNF-α in monocytes and IL-8 in neutrophils. Although both monocytes and neutrophils killed HL184Gat, but not HL184G, the patterns of cytokine production induced by either strain were identical. Addition of INF-γ and/or TNF-α did not improve the antimycobacterial activity of phagocytes.

Keywords: *Mycobacterium gordonae*; *Mycobacterium tuberculosis*; neutrophils; monocytes; cytokines

1. Introduction

The current economic and social importance of tuberculosis is enormous [1], which contrasts with our still poor knowledge of its immunopathogenesis. In spite of intense research, it has not been possible to establish a reproducible model of in vitro infection in which human phagocytes kill pathogenic mycobacteria, especially from the tuberculosis complex [2]. A possible consequence of this failure has been a decrease in the number of articles published in this area in the last few years.

An interesting question to address is whether there is any kind of relationship between the pathogenicity of the mycobacteria and the ability of human phagocytes to kill them. If non-pathogenic mycobacteria were more susceptible to their antimicrobial activity, they would be a useful model to compare with, which may allow us to find the mechanisms used by pathogenic mycobacteria to survive intracellularly, that are absent in the non-pathogenic. It is assumed that alveolar macrophages in the lung are the target cells for *M. tuberculosis*. When they do not control the multiplication of the bacteria, neutrophils arrive to the infection focus, but their role in the immune response has not been fully established [3].

Interferon-γ (IFN-γ), a cytokine which activates monocytes and macrophages against bacteria, is critical in the immune response to *M. tuberculosis*, because inactivating mutations in its receptor increase the susceptibility to mycobacteriosis [4]. IFN-γ successfully activates macrophages in vitro against *M. tuberculosis* in the murine model, although it is not effective in human monocytes [5]. An unexpected finding was the discovery that *M. phlei*, a non-pathogenic

mycobacteria, is not eliminated by human monocytes even when they are activated with IFN-γ [6]. This cytokine has also been described to activate neutrophils against another non-pathogenic mycobacteria, *M. fortuitum* [7]. It fails, however, to induce the killing of *M. tuberculosis*, which may be instead accomplished by Tumor Necrosis Factor α (TNF-α) [8]. It is noticeable that both *M. fortuitum* and *M. phlei* are fast growing mycobacteria, and comparisons with *M. tuberculosis* may not be straightforward. Other cytokines have been studied in relationship with *M. tuberculosis*. Among the proinflammatory cytokines is interleukin 12 (IL-12), which is induced following phagocytosis of *M. tuberculosis* [9]. Others, like IL-6 and IL-1β, have an unclear role; IL-6 -/- and IL-1R -/- mice seem to be more susceptible to tuberculosis [2,10]. It has also been described the production of IL-8 in monocytes infected with *M. tuberculosis* [11]. The inhibitory cytokines IL-4, IL-10 and TGF-β1 have also being analysed. It has been suggested that progressive tuberculosis might be due to a TH2 response, involving IL-4 and IL-4δ2 [12]; and both IL-10 and TGF-β1 are present in lung bronchoalveolar lavage of tuberculosis patients [13,14].

The activities of both human monocytes and neutrophils against non-pathogenic mycobacteria have already been addressed [6,7,15,16] . The comparison of results between reports is, however, very difficult because of the application of various methods of purification of cells, protocols of infections, and models of bacteria. This concern prompted us to use a non-pathogenic bacteria, *M. gordonae*, which have not been tested yet, and infect both monocytes and neutrophils from the same volunteer, in the same conditions and at the same time, to accurately determine the antimicrobial activities of these phagocytes. *M. gordonae*, which is a slow growing mycobacteria with duplication times

comparable to *M. tuberculosis*. It is often isolated at hospitals, although it is usually considered as a contaminant, and very rarely has been associated to disease [17]. We have studied the capacity of human phagocytes to eliminate two strains of *M. gordonae*, one of them attenuated by serial passage in the laboratory, and the pattern of cytokine production induced by the infection.

2. Materials and methods

2.1. Microorganisms.

Mycobacterium tuberculosis HL186T and *Mycobacterium gordonae* HL184G were isolated at the Hospital of León (Microbiology Service) and kindly provided by Julio Blanco and Manuela Caño. They were grown on 7H11 agar supplemented with 0.2% glycerol and 10% Middlebrook enrichment OADC (Becton Dickinson Microbiology Systems). After passage *M. gordonae* in the laboratory for more than a year we realized that it had lost virulence in monocytes, and we designated it HL184Gat (for attenuated). We kept the HL184G strain frozen, which remained with its original properties. *Legionella pneumophila* Philadelphia, ATCC 13151, generously provided by Carmen Pelaz, was grown on buffered charcoal yeast extract agar plates. Bacteria from fresh culture in agar plates were suspended in X-VIVO 15 medium (Cambrex Bio Science). To obtain isolated mycobacteria, they were sonicated using an S-450 digital ultrasonic cell disruptor (Branson). Pulses of 10 s were applied with a microtip at an amplitude of 10% (2 Watts), and sonicated bacteria were centrifuged at $100 \times g$ for 1 m at room temperature. After recovering the supernatants, sonications were repeated as many times as necessary to obtain individualized bacteria, usually three or four rounds. At the end most bacteria were alive and very few groups remained, with ≤ 5 bacteria per group, as determined by the LIVE/DEAD Baclight bacterial kit

(Molecular Probes, Invitrogen). This treatment was not necessary for *Legionella*. After addition of glycerol to 20%, single use aliquots were frozen at – 80ºC.

2.2. Isolation of monocytes and neutrophils.

Each experiment was performed with blood from a different healthy volunteer, and monocytes and neutrophils were obtained simultaneously. Peripheral blood mononuclear cells were isolated by Ficoll-Paque Plus density gradient sedimentation (GE Healthcare, Life Sciences), and CD14$^+$ cells (monocytes) were purified by magnetic cell separation (Myltenyi Biotec).. Neutrophils were isolated from blood by dextran sedimentation and subjected to hypotonic lysis in cold distilled water to remove erythrocytes. Mononuclear cells and neutrophils were separated by Ficoll-Paque Plus density gradient sedimentation. After removal of the supernatant, neutrophils were recovered from the bottom of the tube. The number of both monocytes and neutrophils was calculated by counting in a Neubauer chamber and were cultivated, within 4 hours from blood collection, in serum free media X-VIVO 15 without antibiotics (Cambrex Bio Science), at 37ºC in 95% air/5% CO_2. We ascertained the purity of cells by flow cytometry with the use of appropriate labeled antibodies (Becton Dickinson). > 94 % of cells in the monocyte preparation were CD14$^+$, and > 99 % of cells in the neutrophil preparation were CD66b$^+$.

2.3. Monocyte and neutrophil infection.

Experiments were made in 96-well plates, always in a total volume of 100 µl. 10^5 cells were infected with 10^3 bacteria (Multiplicity of Infection, MOI = 0.01) in X-VIVO 15. For opsonization, infections with *L. pneumophila* were made in the presence of 10% autologous unheated serum. This treatment was not required for mycobacteria. When indicated, 25 ng/ml was added of either interferon

gamma (IFN-γ, > 2 × 10^7 units/mg) or Tumor Necrosis factor alpha (TNF-α, > 2 × 10^7 units/mg, Peprotech). In neutralization studies 2 μg of either mouse IgG1 anti-TNF-α, mouse IgG1 anti-IL-1β, or rat IgG1 anti-IL-6 (functional grade, eBioscience) were added. Antibodies of the same isotypes were used as controls. No washes were done at any moment. Neutrophils were lysed after 24 h and monocytes after four days by sonication with a microtip (Branson), at an amplitude of 10% (2 Watts) for 3 s, to release bacteria. At this setting ultrasounds were able to lyse cells without affecting the bacterial viability. Decimal dilutions of the sonicates were inoculated and incubated at 37ºC in either BCYE agar plates (*Legionella*) for four days or 7H9 broth supplemented with 0.2% glycerol and 10% Middlebrook enrichment ADC (mycobacteria) for ten days. CFU were determined for mycobacteria under an inverted microscope at × 100 magnification [18]. To determine the number of remaining extracellular *M. tuberculosis* one day after the infection, we collected the supernatant and washed once the culture with phosphate buffered saline. We put together both fractions (extracellular bacteria), added fresh phosphate buffered saline to the wells and lysed the cells (intracellular bacteria). We quantified the microorganisms as described above. The number of extracellular bacteria was approximately 29% in the monocytes infections and 10% in the neutrophils infections.

2.4. Quantification of cytokines in supernatants.

To remove bacteria from supernatants, samples were centrifuged for 3 minutes at 8000 × g at room temperature in ultrafree-MC filter units (Millipore) of 0.45 μm and frozen at –80ºC. Cytokine determination was done by flow cytometry with the BD Cytometric Bead Array system (Becton Dickinson), using the Human Inflammation kit, which measure the amounts of IL-12 p70, TNF-α, IL-10, IL-6,

IL-1 β and IL-8. 50 μl of each supernatant were processed following manufacturer's instructions and analysed in a FACSCanto instrument (Becton Dickinson). IL-4 and TGF-β1 were determined by ELISA using IL-4 BD OptEIA ELISA (Becton Dickinson) and TGF-β1 E_{max} Immunoassay System (Promega), respectively. Quantities were expressed as pg/ml. Supernatants of the negative controls (non-infected cells) had medians < 20 pg/ml, with the exception of IL-6 in supernatant of monocytes (161.9 pg/ml) and IL-8 in supernatant of both monocytes (21594.0 pg/ml) and neutrophils (2494.2 pg/ml). TGF-β1 was present in the serum used for opsonization in *Legionella* infections (3184.5 pg/ml). The median of each cytokine in the negative controls was subtracted from the quantities calculated from the samples. Amounts after subtraction ≤ 0 were given a value of 0.0 pg/ml.

2.5. Statistical analysis.

We determined that a log transformation of the variable CFU followed a normal distribution. When comparing two samples, log CFU was analyzed using Student's t-test. When more than two groups were compared, we performed a one-way analysis of variance and made pairwise comparisons by the Tukey's procedure. When variances were unequal, we applied the post-hoc Tamhane test. Cytokines distributions were not normal, and we analysed them by the non-parametric Kruskal-Wallis method. We used Dunn's test for pairwise comparisons. In all cases, a p value < 0.05 was considered significant. Analysis was performed with SPSS v. 14.0 and G-Stat v. 2.0 (Dunn's test).

3. Results

3.1. Antimycobacterial activity of human phagocytes.

There has been several reports that indicate variable activities of phagocytes against non-pathogenic mycobacteria [6,7,15,16] . The bacteria usually analysed,

however, are fast growing like *M. fortuitum* or *M. smegmatis*. To test the ability of phagocytes to kill a non-pathogenic mycobacteria with a generation time similar to *M. tuberculosis*, we infected both monocytes and neutrophils with *M. gordonae* (HL184G). As controls we included three other bacteria: a strain of the pathogenic *M. tuberculosis* (HL186T) isolated from a patient; another pulmonary intracellular bacteria, *L. pneumophila* (ATCC 13151) and an attenuated strain of *M. gordonae* (HL184Gat), obtained by serial passage of the original strain. We infected 10^5 monocytes with 10^3 bacteria, but in our culture conditions little, although consistent, growth of *M. tuberculosis* was observed (p = 0.07, Fig. 1, panel A). In contrast, more than 10^6 *L. pneumophila* were recovered after four days of incubation, an evidence of the good condition of the cells. We were able to detect significant levels of multiplication in the case of *M. gordonae*, demonstrating that although this mycobacteria is largely recognized as non-pathogenic [17], monocytes do not have the capacity to kill them. On the contrary, when we infected monocytes with the attenuated strain, very significant levels of killing were detected.

We also wondered whether neutrophils, isolated from the same donors, exhibited any anti-microbial activity (Fig.1, panel B). The main difference that we could observe when comparing neutrophils with monocytes was that *L. pneumophila* was effectively eliminated by neutrophils. This result demonstrates that our conditions of infection allow neutrophils to exert a potent anti-microbial activity. Nevertheless, neutrophils showed a similar pattern to that of monocytes when they were infected by mycobacteria. Again, some growth of *M. tuberculosis* and *M. gordonae* was apparent in neutrophils after one day of infection, and a significant anti-microbial activity was exhibited against the attenuated strain of *M.*

gordonae. We considered the possibility that the difference between HL184G and HL184Gat was related to structural changes in the cell wall that may have inhibited their phagocytosis. In this case, the lower recovery of bacteria could have been caused by the extracellular death in an inappropriate medium. To rule this explanation out, we infected and stained cells with the Kinyoun technique (using the Giemsa stain for contrast). We observed no differences in the number of engulfed bacteria of both strains (data not shown). Therefore, we concluded that HL184G could be a good model to study the activity of human phagocytes against mycobacteria.

3.2 Pattern of cytokine secretion in infected phagocytes.

The next question we addressed was the relationship between the observed microbicidal activity and the pattern of cytokines produced by infected cells. We focused in two main types of cytokines: proinflammatory, which included IL-1 β, IL-6, IL-8, IL-12 and TNF-α; and the inhibitory IL-4, IL-10 and TGF-β1. Our hypothesis was that inhibitory cytokines would be preferentially secreted in cells which do not control the bacterial growth. Values included in Table 1 are the quantities above the amounts detected in the negative controls (non-infected cells), as described in Materials and methods. Some quantities have medians equal to 0 and interquartile ranges > 0 because production of cytokines was detected in only one experiment out of four. We never observed secretion of either IL-4 or IL-12, even in negative controls. Pairwise comparisons by the Dunn's test with several cytokines secreted by *L. pneumophila* presented significant differences, but they were not significant for any cytokine among the three mycobacteria. IL-1 β was produced at higher levels in monocytes infected by the pathogenic bacteria *M. tuberculosis* and *L. pneumophila*, but not in neutrophils. A similar pattern was

discovered for TNF-α and IL-6. A peculiarity of neutrophils was that IL-6 was only produced by cells infected by *L. pneumophila*. IL-8 was secreted in large quantities in both monocytes and neutrophils, even when they were not infected, but amounts above those obtained for the negative controls were only detected in neutrophils. Nevertheless, there were no significant differences among the four infecting bacteria. Regarding the inhibitory cytokines, IL-10 was more secreted by monocytes infected with *M. tuberculosis* and *L. pneumophila*, but significant differences were only found for *L. pneumophila*. TGF-β1 was detected in large quantities only in *L. pneumophila*, with significant differences in neutrophils when compared with all three mycobacteria. The reason why no statistical significance in pairwise comparison was detected by the Dunn's test for this cytokine in monocytes ($p \leq 0.06$) is that this test is conservative. In fact, the Kruskall-Wallis test for this group was significant ($p = 0.017$).

We would like to remark that no association was found between antimicrobial activity and production of any of the cytokines tested. Although the difference in the number of *M. gordonae* attenuated recovered after lysis of the cells was more than one hundred fold for monocytes and ten fold for neutrophils, both the kind and amount of cytokines produced were very similar. A relationship between the pattern of production of cytokines and pathogenicity was found, but this pattern borne no association with the antimicrobial activity exhibited by either monocytes or neutrophils. It remains the possibility that the mechanisms of resistance of both *M. tuberculosis* and *M. gordonae* are different, and that conclusions obtained from *M. gordonae* infections may not be applicable to *M. tuberculosis* infections. For this reason we wanted to know whether neutralization of cytokines had any influence in the antimicrobial activities. The more

pronounced differences in the pattern of cytokines induced by these bacteria were found in infected monocytes for TNF-α, IL-1β and IL-6. Because the patterns from the original and attenuated strains of *M. gordonae* were similar, we chose the attenuated bacteria against which the monocytes were effective. The addition of the neutralizing antibodies did not have any effect in either inhibiting or promoting the antimicrobial activity of monocytes (Table 2) against either *M. tuberculosis* or *M. gordonae* attenuated, because there were no significant differences between non-treated cells and cells treated with any combination of antibodies. The addition of control antibodies of the same isotypes demonstrated that unspecific antibodies did not influence the final outcome. In this set of experiments differences in the number of *M. tuberculosis* between t = 0 and not treated cells at four days reached the significance level, as compared with experiments in Fig. 1, which although not significant, were very close (p = 0.07). These results confirmed that there is no relationship between the production of these three cytokines and the antimicrobial activity exhibited by infected monocytes.

3.3. Activation of phagocytes with cytokines do not increase their antimycobacterial activity.

In the next experiment we tested whether addition of the activating cytokines IFN-γ and TNF-α to the infected cells would prompt them to control the bacterial growth. We had already realized that a modest production of TNF-α in monocytes infected with pathogenic bacteria (*M. tuberculosis* and *L. pneumophila*, Table 1) did not correlate with an apparent antimicrobial activity. But we wanted to know whether addition of larger quantities (25 ng/ml) of either, or both together, would help the cells to kill the bacteria. When we added these cytokines to monocytes at the moment of infection, no significant differences in the number of

recovered mycobacteria after lysis of the cells could be detected between activated and non treated cells (Fig. 2, panel A). Although *M. gordonae* attenuated was efficiently eliminated by monocytes, as already shown (Fig. 1, panel A), the addition of the cytokines did nor further increase this activity. In contrast, when monocytes were activated with IFN-γ, more than 80% of the control *L. pneumophila* was killed. This result demonstrate both the good condition of the cells and its capacity to be activated by IFN-γ. TNF-α, however, did not have any effect on the monocytes activity against *L. pneumophila*. These results could also be corroborated in neutrophils (Fig. 2, panel B), in which IFN-γ also induced cells to kill *L. pneumophila*, although it did not reach levels of significance. As in monocytes, cytokines did not improve the activity of neutrophils against *M. gordonae* attenuated, and did not have any effect against either *M. tuberculosis* or *M. gordonae*.

4. Discussion

We have thoroughly tested the variables that influence the outcome of in vitro infections, and have designed a strategy that, minimizing technical manipulations, benefits of several advantages: first, purification of monocytes by magnetic cell separation avoids the washes required in techniques that rely on monocytes adherence, which may select against monocytes that do not adhere strongly. Second, maintenance of cells in serum free media reduces variability, because the composition of the serum present in other media, besides being very complex, changes between lots. Third, the low MOI (0.01 bacteria/cell) resembles more closely the natural way of infection, and eliminates the need of washes to remove extracellular bacteria. We have observed that neither *M. gordonae* nor *L. pneumophila* multiply in X-VIVO 15 medium. Although *M. tuberculosis* does form

colonies in this medium, it takes it more than a week to develop (data not shown). We determined that 71 % of *M. tuberculosis* bacteria were intracellular one day after the infection, before it begins to multiply in the culture (data not shown), but we think it likely that most, if not all, of the microorganisms will have been engulfed after four days. Finally, lysis of cells to release intracellular bacteria by physical means (ultrasounds), avoid the addition of reagents (usually detergents) that increase the final volume, and that may also inhibit the growth of some mycobacteria, as is the case for *M. gordonae* (data not shown). In these conditions we have observed a lower rate of multiplication of mycobacteria as compared with the use of media with serum, but it may better reflect the natural infection in the lungs.

In our culture conditions, phagocytes activated or not with INF-γ and/or TNF-α, eliminate neither *M. tuberculosis* nor *M. gordonae*. The validity of the method is, however, corroborated by the killing of the attenuated *M. gordonae*. For monocytes, these results are in agreement with Robertson and Andrew [6] who did not find activity against *M. phlei*. Similar conclusions reached Barker *et al.*, who observed that adherent monocytes did not inhibit the growth of *M. smegmatis*, another non-pathogenic mycobacteria, although non adherent cells killed the bacteria [17]. *M. fortuitum*, a third non-pathogenic mycobacteria was, however, elimitaded by monocytes [16], and by INF-γ activated neutrophils [7]. In our experiments neutrophils did not kill *M. tuberculosis*, although it has been claimed that TNF-α succesfully activate them [8]. We do not know the reason for this discrepancy, although major differences in the infection method were used, mainly their infection with a MOI of 10 bacteria/neutrophil, and subsequent wash of extracellular bacteria. In agreement with our results with *M. gordonae,* these

reports in general show that human phagocytes do not have an intrinsic capacity to eliminate non-pathogenic mycobacteria, although they are infrequently associated with disease [19].

Phagocytes infected with *M. tuberculosis* induce the secretion of many cytokines, some of them activators, but others inhibitors [2]. We investigated the production of five proinflammatory (IL-1β, IL-6, IL-8, IL-12 and TNF-α) and three inhibitory cytokines (IL-4, IL-10 and TGF-β1), and related it with the microbicidal activity of both monocytes and neutrophils. Our main conclusion is that there is an association between the pathogenicity of the mycobacteria and the pattern of cytokines secreted. We found that pathogenic bacteria (*M. tuberculosis* and *L. pneumophila*), but not *M. gordonae*, induced the production in monocytes of IL-1β, IL-6 and TNF-α. These results differ markedly with those obtained by Beltan *et al*. [20], who found that macrophages infected with non-pathogenic mycobacteria (*M. smegmatis* and *M. phlei*) secreted higher amounts of these cytokines than the infected with *M. tuberculosis*. In neutrophils, mycobacteria did not induce, or at very low leves, the production of any cytokine, with the exception of IL-8, which were similarly induced by all three mycobacteria. In contrast, Fäldt *et al*. found that neutrophils infected with a non-pathogenic mycobacteria (*M. smegmatis*) produced more TNF-α, IL-6 and IL-8 than those infected with *M. tuberculosis* [21]. We attribute the contradictory results to the model and method of infection. We do not use the usual RPMI with serum medium, but a serum free one (X-VIVO 15), and our MOI was very low (0.01 bacteria/cell). For example, in these conditions we do not detect production of IL-12, although it has been reported in human macrophages infected with a mycobacteria [9]. In fact, even different strains from a particular species may induce variable amounts of

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cytokines. Chacón-Salinas *et al*. have reported that the Canetti, H37Rv and Beijing strains of *M. tuberculosis* induce the secretion in monocytes of different quantities of both IL-1β, and TNF-α [22]. The disparity observed in all these results suggest that the pattern of cytokine secretion may depend on the culture conditions, the strategy of infection and the bacterial model, and that there might not be a direct correspondence with the events occurring in vivo. We are not aware of any study in which the relationship between the pattern of cytokine production and antimicrobial activity of human phagocytes is analysed. Nevertheless, phagocytes infected with both strains of *M. gordonae* (original and attenuated) did not produce almost any cytokine, with the exception of IL-8, with similar quantities in both. We conclude that the cytokines tested have no influence in the microbicidal activity of phagocytes, because the attenuated strain was significantly more susceptible than the original.

The reported results suggest that there are unknown variables which allow the immune system to control the multiplication of non-pathogenic mycobacteria in the human host, which we have not been able to reproduce in vitro, and discovering them may give us important clues about the survival strategies of the more phatogenic *M. tuberculosis*. In this regard, the susceptibility of the attenuated *M. gordonae* to human phagocytes may prove a useful tool to dissect at the molecular level successful antimicrobial mechanisms.

Acknowledgments

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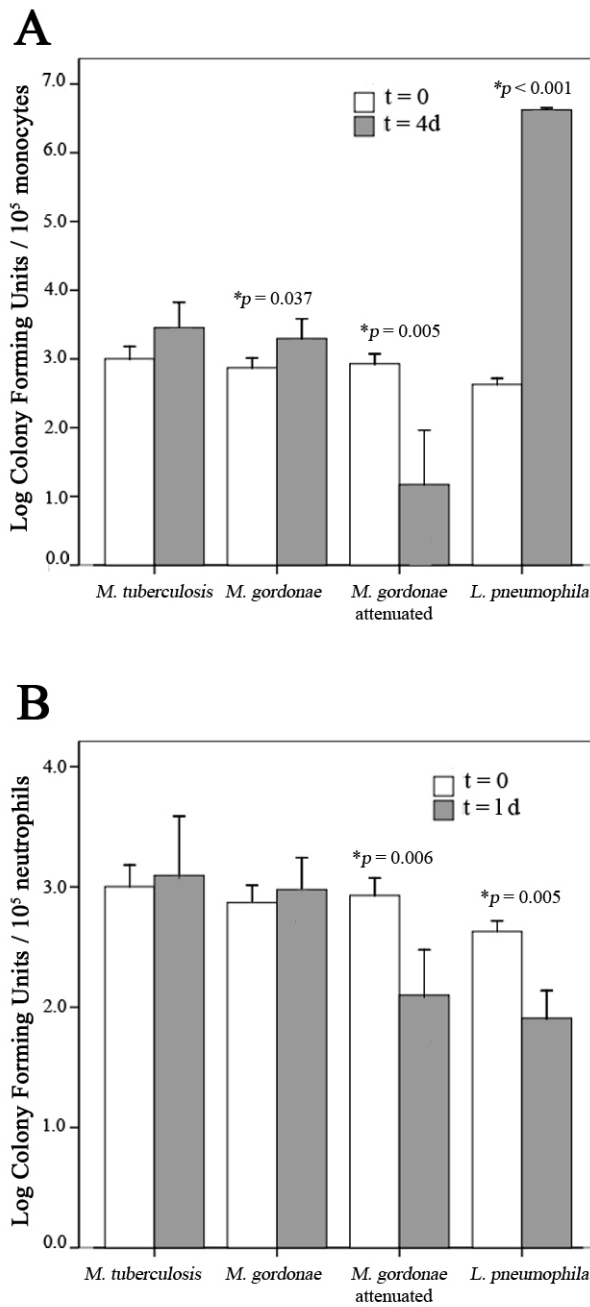*tuberculosis* genotypes. Clin Exp Immunol 2005; 140:443-9.

Figure 1. Antimicrobial activity of phagocytes. Intracellular bacteria at the moment of infection (t = 0) and at the time of cellular lysis in either (A) monocytes or (B) neutrophils. Data are the log of the colony forming units and represent the mean + SD of four independent experiments. t-Student test with *$p < 0.05$ is considered significant.

Table 1

Release to supernatants of cytokines (pg/ml) by infected phagocytes

	IL-1β	IL-6	IL-8	IL-12	TNF-α	IL-4	IL-10	TGF-1β
Monocytes								
M. tuberculosis	4.5 (507.0)	43.5 (6363.6)	0.0 (0.0)	0.0 (0.0)	20.05 (339.8)	0.0 (0.0)	0.0 (8.6)	0.0 (0.0)
M. gordonae	0.0 (2.2)	0.0* (0.0)	0.0 (0.0)	0.0 (0.0)	0.0* (0.0)	0.0 (0.0)	0.0* (0.0)	0.0 (0.0)
M. gordonae attenuated	0.0* (0.0)	4.4 (8.7)	0.0 (0.0)	0.0 (0.0)	0.0 (1.3)	0.0 (0.0)	0.0* (0.0)	0.0 (0.0)
L. pneumophila	90.5 (760.3)	6964.4 (4104.8)	0.0 (0.0)	0.0 (0.0)	62.95 (105.5)	0.0 (0.0)	34.7 (131.0)	2725.0 (1682.8)
Neutrophils								
M. tuberculosis	0.0 (0.0)	0.0 (9.0)	257.3 (3585.2)	0.0 (0.0)	0.0 (0.7)	0.0 (0.0)	0.0 (0.0)	0.0* (0.0)
M. gordonae	0.0 (0.0)	0.0* (0.0)	0.0 (2196.2)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0* (0.0)
M. gordonae attenuated	0.0 (0.0)	0.0* (0.0)	0.0 (1908.3)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0* (0.0)
L. pneumophila	0.0 (24.3)	117.1 (799.9)	4700.4 (13426.8)	0.0 (0.0)	2.3 (36.7)	0.0 (0.0)	0.0 (0.0)	2526.3 (1857.8)

Quantities are obtained by subtracting the amount of cytokines released by non-infected cells to that of infected cells. Data are median (interquartile range) of four independent experiments. Dunn's test was used for pairwise comparisons vs. *L. pneumophila*, * p < 0.05

Table 2. Antimicrobial activity of monocytes against <u>M. tuberculosis</u> and <u>M. gordonae</u> attenuated in the presence of blocking anti-cytokine antibodies.

	<u>M. tuberculosis</u>	<u>M. gordonae</u> attenuated
Inoculated bacteria (t = 0)	3,24 SD 0,06 (2,66-3,82)*	2,82 SD 0,26 (0,51-5,13)*
Treatment		
No treatment	3,88 SD 0,08 (3,68-4,09)	2,27 SD 0,02 (2,21-2,33)
Anti-TNF-α	3,92 SD 0,02 (3,86-3,98)	2,38 SD 0,14 (2,02-2,73)
Anti-IL-1β	3,94 SD 0,09 (3,73-4,15)	2,29 SD 0,08 (2,08-2,50)
Anti-IL-6	3,97 SD 0,10 (3,72-4,22)	2,31 SD 0,12 (2,00-2,62)
Anti-TNF-α-IL-1β-IL-6	3,97 SD 0,07 (3,79-4,14)	2,41 SD 0,12 (2,11-2,70)
Control (rat IgG1)	3,91 SD 0,19 (2,23-5,59)	2,30 SD 0,09 (2,08-2,52)
Control (mouse IgG1)	3,90 SD 0,12 (3,60-4,20)	2,35 SD 0,04 (2,24-2,46)

Data are the average of log of the number of colonies with standard deviation, SD (95% confidence interval) from three independent experiments. Only pairwise comparisons versus t = 0 were significant, * $p < 0,05$.
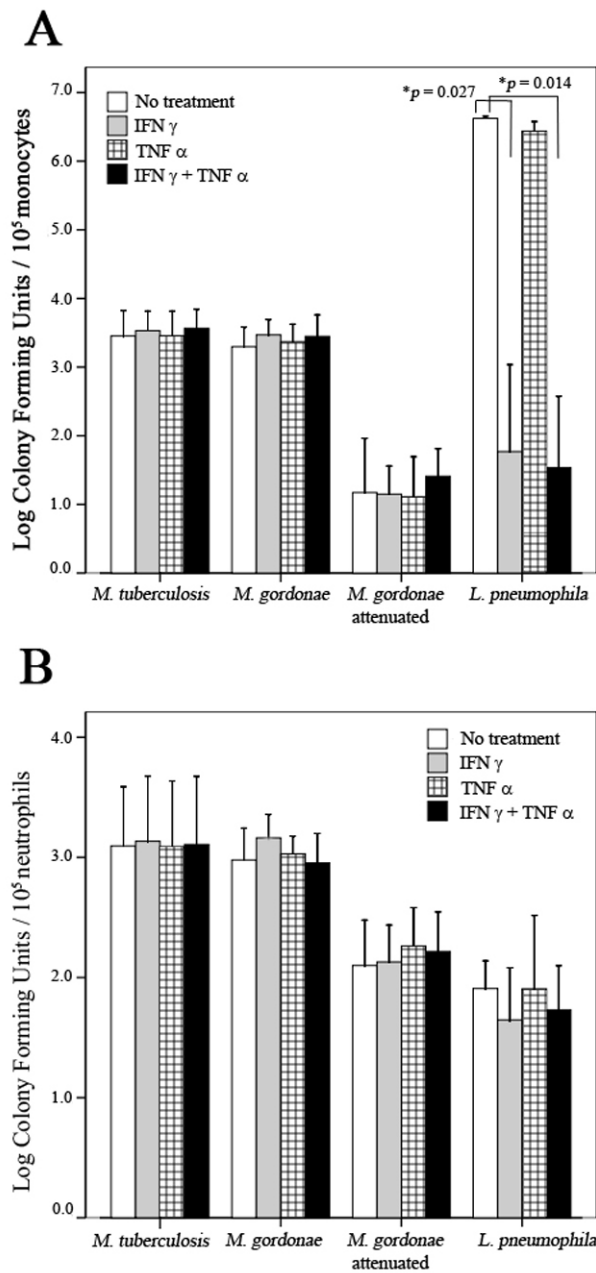
Figure 2. Activation of phagocytes with cytokines. Intracellular multiplication of bacteria in either (A) monocytes or (B) neutrophils, in the presence of 25 ng/ml of the indicated cytokines.. Data are the log of the colony forming units and represent the mean + SD of four independent experiments. Tukey's post-hoc test with $*p < 0.05$ is considered significant.