

The fate of *Mycobacterium tuberculosis* in activated human macrophages

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Human peripheral blood monocytes, that are unstimulated *in vitro*, permit free multiplication of intracellular *Mycobacterium tuberculosis* after 72 h in culture. There was no killing of bacilli in the intracellular environment even after *in vitro* activation of monocytes with a cocktail of lipopolysaccharide, phorbol myristate acetate, interferon gamma and tumour necrosis factor-alpha. We also tested the ability of adenosine triphosphate (ATP) in reducing the intracellular viability of mycobacteria. Infected monocytes upon ATP treatment underwent cell death, but no loss in the intracellular viability of *M. tuberculosis* or *M. smegmatis* could be observed.

TUBERCULOSIS remains an important global health problem with approximately one billion people presently infected with the disease¹. During hundred years since the discovery of the tubercle bacillus, the increased understanding of bacteriological and pathological characters has resulted in important public health measures such as pasteurization of milk and BCG vaccination. However, it was chemotherapy that was introduced during the end of the last century which brought about tremendous decline in the mortality rates². But the increasing number of multidrug resistant *M. tuberculosis* isolates from both AIDS and non-AIDS patients is an ominous trend that threatens the tuberculosis eradication programme³. Tuberculosis is actually on the rise again. The resurgence of tuberculosis appears to have its roots in the AIDS epidemic, although declining control programmes, increased levels of homelessness and drug addiction are also contributing factors².

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The major phagocytic cell involved in protection against *M. tuberculosis* infection is probably the activated macrophages. Activation of macrophages is brought about by the cytokines released by the antigen sensitized specific T lymphocytes. The mechanisms mediating growth inhibition of mycobacteria by activated macrophages has not been elucidated till date.

In order to study the host parasite relationship in tuberculosis, we infected the human peripheral blood monocytes *in vitro* with *M. tuberculosis* and the fate of *M. tuberculosis* inside the unstimulated and cytokine stimulated human monocytes was studied. We also attempted to study the intracellular fate of *M. tuberculosis* and *M. smegmatis*, following treatment of monocytes with adenosine triphosphate (ATP).

Interferon gamma (IFN- γ) and tumour necrosis factor-alpha (TNF- α) were purchased from Genzyme (Cambridge, MA). ATP, lipopolysaccharide (LPS), phorbol myristate acetate (PMA) and RPMI 1640 were purchased from Sigma (St. Louis, MO). OADC was purchased from Difco (Detroit, MI) and lymphocyte separation medium was purchased from Flow Laboratories (Scotland, UK).

Blood (30-35 ml) was collected from healthy volunteers in a heparinized container (10 U/ml). The mononuclear cells were separated by centrifugation at 400 x g for 30 min on Ficoll hypaque. These cells were washed three times with Hanks balanced salt solution (HBSS) at 300 x g for 10 min. After the final wash the cells were suspended in Roswell Park Memorial Institute (RPMI 1640) medium containing pooled heat inactivated normal human serum (PHINHS). Total number of viable mononuclear cells in the suspension was ascertained by trypan blue dye exclusion. One million mononuclear cells were added per well in a 96 well flat bottomed plate and incubated for 4.5 min at 37°C, 5% CO₂ in a humidified atmosphere for adherence. The non-adherent cells were removed by gentle washing. The adherent monocytes were cultured in RPMI 1640 containing 5% PHINHS. Monocytes were maintained in culture for 24 h prior to infection with *M. tuberculosis*.

M. tuberculosis (H37Rv) was grown in Dubos broth containing 1% Tween 80. Single cell suspension of *M. tuberculosis*, at peak log phase of growth was used for infection. At the time of infection, the bacterial cell suspension was washed three times in HBSS to remove Tween 80. The bacterial cell suspension was subjected to mild sonication using Fisher sonic dismembrator (Model 300) for 30 s twice to disaggregate the clumps. Total number of *M. tuberculosis* in the suspension was ascertained by counts in a Thoma counting chamber. There was no loss in viability which was checked by trypan blue exclusion method.

For every single monocyte, approximately ten bacilli were added and incubated at 37°C for 1 h, after which

the extracellular organisms were removed by gentle washing. Infected monocytes were maintained in RPMI containing 5% PHINHS. Cell free supernatant from *M. tuberculosis* infected monocyte culture was plated on 7H₁₁ agar, at all the time points to ascertain the extracellular *M. tuberculosis* growth.

Along with every experiment a cell-free control was included, where *M. tuberculosis* was maintained in 200 μ l of RPMI containing 5% PHINHS, in order to ascertain the growth pattern of *M. tuberculosis* in RPMI (Figure 1).

Freshly drawn human peripheral blood monocytes were infected with *M. tuberculosis* (Monocyte: *M. tuberculosis* ratio is 1 : 10). 1 h later, the extracellular organisms were removed. Infected monocytes were cultured in culture medium containing LPS (from *E. coli* serotype No. 0127 B8) at three different concentrations 5 ng/ml, 5 μ g/ml and 5 fg/ml.

Freshly drawn human monocytes were cultured in RPMI containing TNF- α and IFN- γ at two concentrations 100 U/ml and 1000 U/ml each. 24 h later, the cytokine treated monocytes were infected with *M. tuberculosis* (Monocyte: *M. tuberculosis* ratio is 1 : 10). 1 h later the extracellular organisms were removed. Infected monocytes were then cultured in medium containing IFN- γ and TNF- α (at 1000 U/ml or 100 U/ml concentrations). Along with these two cytokines LPS at a concentration of 5 ng/ml or PMA at a concentration of 10 ng/ml or LPS and PMA together were also added to the infected monocytes, in separate experiments.

Infected monocytes (either untreated or cytokine treated) were terminated at various time points, viz. 1 h, 24 h, 48 h, 72 h and one week after infection. During the termination process, the infected monocytes were lysed with sterile distilled water. The total volume of the lysate was adjusted to 500 μ l. The stock was diluted 10 and 100 times. The diluted lysate was plated on 7H₁₁ containing oleic acid albumin dextrose complex

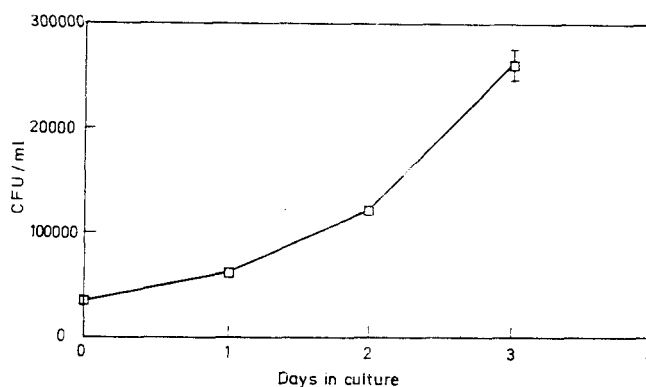


Figure 1. Cell free control: *M. tuberculosis* was cultured in 200 μ l of RPMI containing 5% PHINHS. The cultures were terminated at the following time points, 1 h, 24 h, 48 h, 72 h and 1 week in order to study the growth pattern of *M. tuberculosis* in RPMI.

(OADC). Inoculated plates were incubated for 15 days at 37°C to observe *M. tuberculosis* growth.

M. tuberculosis H37Rv strain and *M. smegmatis* were processed for infection as described previously. Freshly drawn human peripheral blood monocytes were infected *in vitro* with *M. tuberculosis* at 1 : 10 and 1 : 1 ratio. Unphagocytosed bacilli were removed by gentle washing, when the macrophage : mycobacteria ratio was adjusted to 1 : 10. Monocyte : mycobacteria ratio was adjusted to 10 : 1, when *M. smegmatis* was used for infection and extracellular bacilli were removed after phagocytosis. *M. tuberculosis* infected monocytes were maintained in culture for seven days. Seven days after infection, monocytes were treated with 1 mM, ATP and 6 h later, the cultures were terminated. *M. smegmatis* infected human monocytes were maintained in culture for 3 days after infection and then treated with 1 mM ATP. *M. smegmatis* infected cultures were terminated after three days, because *M. smegmatis* has a short generation time as compared to *M. tuberculosis*. Cultures were terminated six hours after ATP treatment.

The termination procedure is very similar to what has been described previously.

As seen in Figure 1 the generation time of *M. tuberculosis* in RPMI containing PHINHS (cell free control) is between 20-24 h. Human peripheral blood monocytes infected with *M. tuberculosis in vitro*, inhibit the growth and multiplication of *M. tuberculosis* till 72 h, after which it was able to multiply within the intracellular environment (Figure 2). On day 8 there was almost one log difference in colony forming units (CFU) values as compared to day 0. Activation of human monocytes with LPS at concentrations such as 5 ng/ml, 5 pg/ml and 5 fg/ml, did not enhance the antimycobacterial nature of human monocytes *in vitro*.

Activation of human monocytes with LPS at 5 ng/ml,

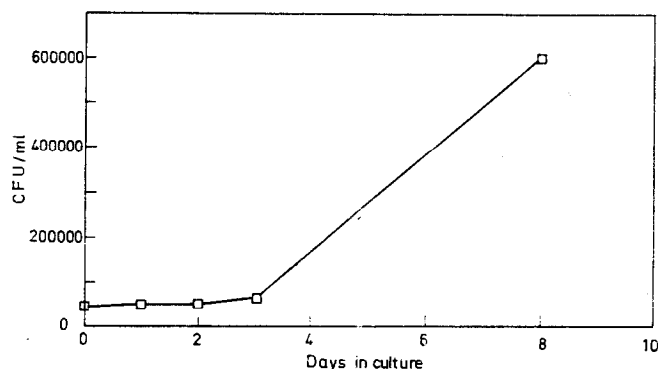


Figure 2. Fate of *M. tuberculosis* inside human monocytes *in vitro*: Human monocytes (0.1×10^6 cells/well) were infected with *M. tuberculosis*. For every single monocyte, ten bacilli were added and incubated at 37°C for 1 h. Extracellular organisms were removed by gentle washing. The infected cultures were terminated at the following time points, 1, 24, 48, 72 h and 1 week after infection.

along with IFN- γ (1000 U/ml or 100 U/ml) and TNF- α (1000 U/ml or 100 U/ml) did not alter the fate of *M. tuberculosis* inside human monocytes (Figure 3). Human monocytes treated with PMA (10 ng/ml) along with IFN- γ (1000 U/ml or 100 U/ml) and TNF- α (1000 U/ml or 100 U/ml) handled *M. tuberculosis* in a similar way as untreated monocytes (Figure 4). No changes were observed with respect to the fate of intracellular *M. tuberculosis* when human monocytes were treated with the combination of PMA (10 ng/ml), LPS (5 ng/ml) IFN- γ (1000 U/ml and 100 U/ml) and TNF- α (1000 U/ml and 100 U/ml) (Figure 5). The biological activity of the

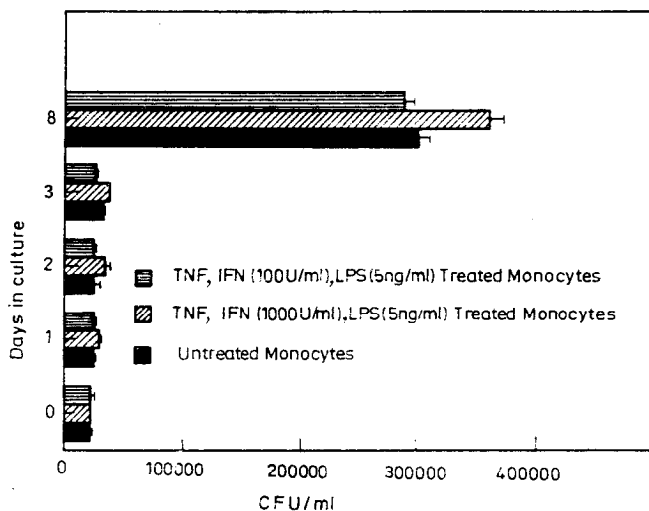


Figure 3. Fate of *M. tuberculosis* inside TNF- α , IFN- γ and LPS treated human monocytes *in vitro*: Human monocytes (0.1×10^6 cells/well) were treated with TNF- α and IFN- γ at two different concentrations (1000 U/ml of INF, 1000 U/ml of TNF- α , 5 ng of LPS and 100 U/ml of INF, 100 U/ml of TNF- α , 5 ng of LPS). 24 h later, the cultures were infected with *M. tuberculosis*. Extracellular organisms were removed by gentle washing.

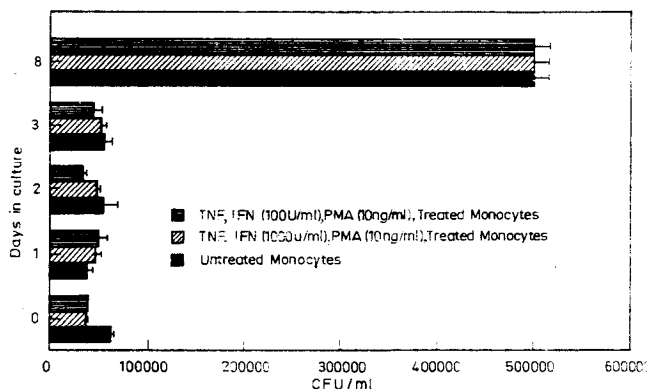


Figure 4. Fate of *M. tuberculosis* inside TNF- α , IFN- γ and PMA treated monocytes: Human monocytes (0.1×10^6 cells/well) were treated with TNF- α and IFN- γ at two different concentrations, 1000 U/ml and 100 U/ml. 24 h later, the cultures were infected with *M. tuberculosis*. The infected cultures were terminated at the following time points, 1, 24, 48, 72 h and 1 week after infection.

cytokines that were used in the intracellular killing experiments was confirmed by their ability to induce H₂O₂ production in monocytes (Figure 6). Hydrogen peroxide was assayed by Pick's method⁴. *M. tuberculosis* and *M. smegmatis* infected human peripheral blood monocytes upon ATP treatment underwent apoptosis, but we could observe no loss in the intracellular viability of either *M. tuberculosis* or *M. smegmatis* (Figures 7 and 8).

Attempt was made to study the fate of *M. tuberculosis* inside the human peripheral blood monocytes *in vitro*. Human monocytes inhibited the growth and multiplication of *M. tuberculosis* till 72 h, after which *M. tuberculosis* multiplied inside the intracellular environment.

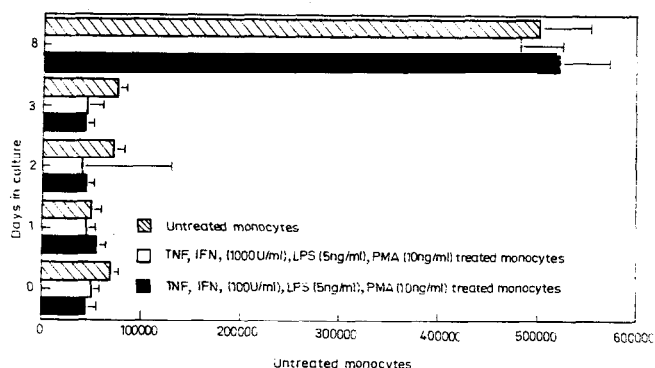


Figure 5. Fate of *M. tuberculosis* inside TNF- α , IFN- γ , LPS and PMA treated human monocytes: Human monocytes (0.1×10^6 cells/well) were treated with TNF- α , IFN- γ at two different concentrations, 1000 U/ml and 100 U/ml along with LPS at 5 ng/ml and PMA 10 ng/ml.

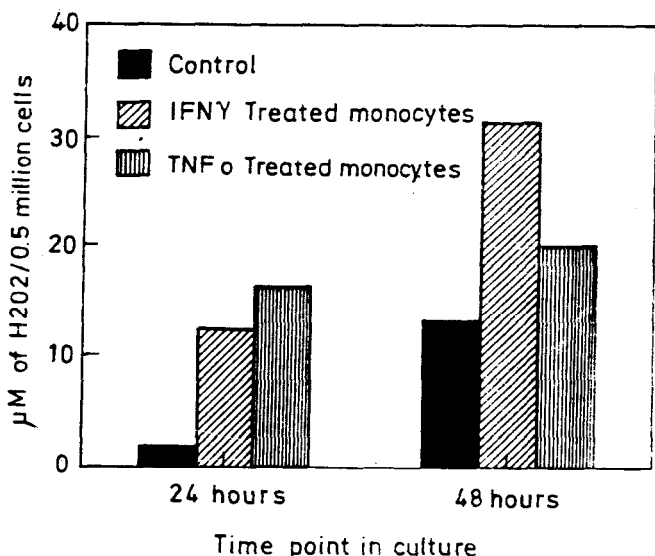


Figure 6. H₂O₂ assay: Human monocytes 0.1×10^6 /well were treated with IFN- γ or TNF- α at 1000 U/ml concentration at the start of the culture. The activity of the cytokines was tested at 24 h and 48 h on human monocytes by their ability to trigger H₂O₂ production. at the time of culture.

Based on the information available in the literature, we expect monocytes to be activated by LPS, PMA and cytokines like TNF- α and IFN- γ (refs 5-11). Intracellular *M. tuberculosis* is known to induce monocytes to synthesize and release TNF- α : (refs 12, 13). Moreover, when LPS-treated monocytes are infected with *M. tuberculosis*, TNF- α synthesis is very much enhanced¹²⁻¹⁴. TNF- α plays a vital role inducing nitric oxide. Nitric oxide metabolites play a major role in bringing about the intracellular killing of many pathogens including mycobacteria¹². Keeping these information in mind, we made an attempt to study the fate of *M. tuberculosis* inside the LPS-treated monocytes. No difference could be observed between LPS-treated monocytes and untreated monocytes with respect to handling intracellular *M. tuberculosis*. Human monocytes were treated with cytokines (TNF- α , IFN- γ) to find out if the cytokines can enhance the antimycobacterial nature of monocytes.

A great wealth of information is available on the role of IFN- γ as macrophage activating agent^{6,9,15-17}. IFN- γ is known to activate mouse macrophages to bring about

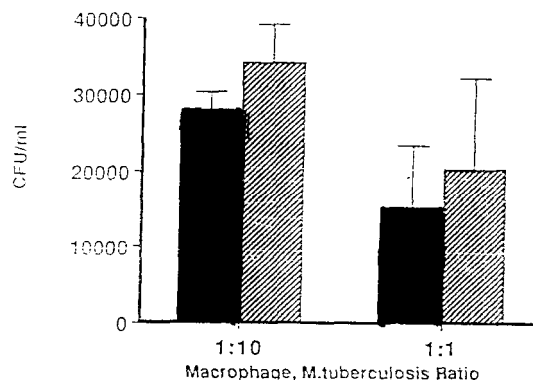


Figure 7. Fate of *M. tuberculosis* inside untreated and ATP-treated human monocytes *in vitro*. ■ Untreated human monocytes; ▨ ATP-treated monocytes.

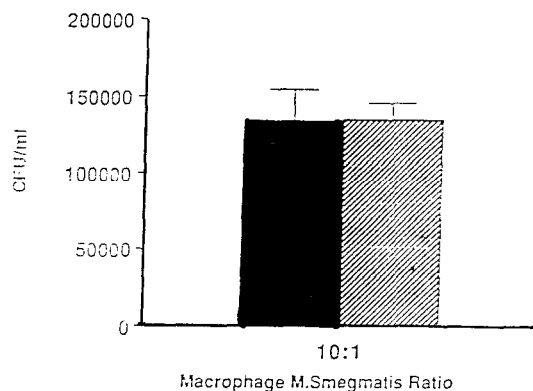


Figure 8. Fate of *M. smegmatis* inside untreated and ATP-treated human monocytes *in vitro*. ■ Untreated human monocytes; ▨ ATP-treated monocytes.

the intracellular killing of leishmania, trypanasoma and *M. bovis*^{11,12,15}. Both human monocytes and mouse macrophages when treated with IFN- γ completely eliminate *Toxoplasma gondii*⁹. Similarly TNF- α , is also known to enhance the antimycobacterial nature of macrophages^{17,18}. In our experiments, human monocytes upon treatment with TNF- α , IFN- γ , LPS and PMA together handled *M. tuberculosis* in a similar way as untreated monocytes.

Many workers have used calcitriol either alone or in association with other cocktail of cytokines to enhance the antimycobacterial nature of human monocytes *in vitro*^{10,17,19}. But there are also controversial reports about the elevated levels of calcitriol in serum samples from tuberculosis patients²⁰. Therefore we were hesitant to perform an experiment with calcitriol as one of the macrophage activating agents. Further, recently Davies *et al.*²¹ have shown that a combination of TNF- α , IFN- γ and calcitriol at optimum concentration failed to enhance the antimycobacterial nature of the human monocytes. It is generally assumed that elimination of *M. tuberculosis* from tissues of protected individuals depends on destruction of the organisms by macrophages that are activated by lymphokines derived from T lymphocytes. The right combination of cytokines that activates macrophages and the right *in vitro* condition to demonstrate the antimycobacterial nature of human monocyte has been a matter of debate for a long time.

In our observations, human monocytes inhibit the growth and multiplication of *M. tuberculosis in vitro* till 72 h, after which *M. tuberculosis* multiply inside the intracellular environment of monocytes. TNF- α , IFN- γ , LPS and PMA stimulate the intracellular respiratory burst in macrophages, but fail to inhibit the growth of intracellular mycobacteria.

Recently Molloy *et al.*²² highlighted that tuberculosis infection is controlled by granulomatous response and this is intimately associated with accumulation, activation and death of mononuclear leucocytes. Molloy *et al.* pointed out that cell death could be due to necrosis or apoptosis and provided further data to show that only during apoptosis (induced by ATP), but not necrosis, of chronically infected cells, 60-70% loss in intracellular viability of BCG resulted. They assumed that observations made on one *Mycobacterium* species are relevant to another. On the contrary, when we treated *M. tuberculosis* or *M. smegmatis* infected human monocytes with ATP, we observed loss in monocyte viability confirming apoptosis with no reduction in bacterial viability. The reason for this discrepancy is under investigation.

We conclude that there is no killing of *M. tuberculosis* by unstimulated or cytokine stimulated and ATP-treated human monocyte derived macrophages *in vitro*.

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