Mycobacterium bovis BCG but not *Mycobacterium leprae* Induces TNF-α Secretion in Human Monocytic THP-1 Cells

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In this study, we compared the level of TNF-α secretion induced in monocytic THP-1 cells after phagocytosis of Mycobacterium leprae, *the causative agent of leprosy, and* M. bovis *BCG, an attenuated strain used as a vaccine against leprosy and tuberculosis. The presence of* M. leprae *and BCG was observed in more than 80% of the cells after 24 h of exposure. However, BCG but not* M. leprae *was able to induce TNF-α secretion in these cells. Moreover, THP-1 cells treated simultaneously with BCG and* M. leprae *secreted lower levels of TNF-α compared to cells incubated with BCG alone.* M. leprae *was able, however, to induce TNF-α secretion both in blood-derived monocytes as well as in THP-1 cells pretreated with phorbol myristate acetate. The inclusion of streptomycin in our cultures, together with the fact that the use of both gamma-irradiated* M. leprae *and heat-killed BCG gave similar results, indicate that the differences observed were not due to differences in viability but in intrinsic properties between* M. leprae *and BCG. These data suggest that the capacity of* M. leprae *to induce TNF-α is dependent on the stage of cell maturation and emphasize the potential of this model to explore differences in the effects triggered by vaccine strain versus pathogenic species of mycobacteria on the host cell physiology and metabolism.*

Key words: *Mycobacterium leprae* - BCG - THP-1 - TNF-α

Mononuclear phagocytes are target cells for pathogenic mycobacteria that generally require an intracellular environment in which they survive and replicate. Although these cells can offer a quite hostile environment to the entering pathogen, they can also provide unique advantages to the infectious organism. They are long-living cells and thus provide a potential long-term habitat for the bacterial invader. In addition, macrophages play a pivotal role in host defense against infection, primarily due to the vast array of mediators that these cells can secrete, offering in this way to the invader organism an opportunity to manipulate the immune system to its own advantage (Russel 1995). Immunity to intracellular pathogens depends mainly upon the activation of IFN-γ producing $CD4^+$ T cells, the so called Th1 response, which increases the microbicidal capacity of macrophages (Kaufmann 1995). In this context, microorganisms that supress or avoid, early during infection, infected macrophages to produce cytokines such as TNF-α and IL-12, cricial for the emergency of a Th1-like response (Trinchieri 1993, Flesch et al. 1995), will favor the development of a susceptible phenotype.

The early molecular events that occur during the interaction between monocytic cells and mycobacteria remain poorly defined. Experiments comparing attenuated versus virulent strains and live versus dead bacteria have revealed critical differences in their effects on the host cell suggesting the importance of these events in mycobacterial pathogenesis. To gain a better understanding of the mycobacteria-host cell interaction, we have employed an in vitro model of infection using THP-1 cells, a human immature monocytic cell line. As a first approach, we compared the level of TNF-α secretion induced in these cells after phagocytosis of *Mycobacterium leprae*, the causative agent of leprosy, and *M. bovis* BCG, an attenuated strain used as a vaccine against leprosy and tuberculosis. Blood mononuclear cells of both normal individuals or leprosy patients secrete TNF-α in response to *M. leprae* and BCG (Santos et al. 1993, Suzuki et al. 1993, Lima et al. 2000). Besides its role in the differentiation of Th1 cells, TNF-α is involved in the formation of granulomas that play an essential role in preventing the extension and dissemination of mycobacterial infections (Kindler et al. 1989). The protective function of TNF-α has been

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emphasized in a report that describes the deleterious effects of deletion in TNF-α gene on mycobacterial infections (Flynn et al. 1995). Our results demonstrate that BCG but not *M. leprae* induces TNF-α secretion in THP-1 cells, emphasizing the potential of this model to explore differences in signaling pathways triggered by vaccine versus pathogenic species of mycobacteria.

MATERIALS AND METHODS

Source of reagents - RPMI 1640 medium, penicillin/streptomycin, L-glutamine were obtained from Gibco BRL (Gaithersburg, MD, USA); phorbol-12-myristate-13-acetate (PMA) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA).

Mycobacteria - Frozen aliquots of *M. bovis* BCG (Pasteur strain) had been produced by the Pasteur Institute (Paris, France), and were kindly donated by Dr Sylvio CG Costa (Oswaldo Cruz Foundation, Brazil). The mycobacteria was stored in vials at a concentration of about $5x10^8$ CFU/ml in PBS at -20°C until use. Armadillo-derived *M. leprae* was kindly donated by Dr PJ Brennan (Department of Microbiology, Colorado State University, Fort Collins, CO) through NIAID contract NO155262. For each experiment, a sample was thawed and diluted in a small volume of PBS supplemented with 0.005% Tween 80. Before addition to blood-derived macrophages or THP-1 cells, clumps of bacteria were removed by repeated passages through a 27 gauge needle and mild sonication for 10 min. Total *M. leprae* and BCG counts were estimated as described by Shepard and MacRae (1968).

Cell culture and infection

THP-1 cells - Cells from a human myelomonocytic cell line, THP-1 (American Type Culture Collection, Rockville, MD), were grown as suspension cultures in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mmol/l glutamine (RPMI complete medium) at 37°C in a humidified atmosphere of 5% CO_2 and used between 3-14 passages. For the experiments, $2x10^5$ cells/ml/well were dispensed in 24-well microtiter plates in the same medium and further incubated with or without mycobacteria (BCG and/or *M. leprae*) or PMA (0.16-160 nM) up to 48 h at 37°C in a humidified atmosphere of 5% CO_2. A fraction of the PMA- or BCG-treated THP-1 cells adhered to culture wells and for analysis of the adherent population, glass cover slips were placed in culture wells before cell addition. The number of adherent cells was estimated by subtracting the number of suspended cells from that of seeded cells. In some experiments, THP-1 cells were first cultivated in the presence of PMA for 4 h and then washed and incubated in fresh RPMI complete medium for an additional 24 h in the presence of *M. leprae*.

Human blood-derived monocytes - Normal human peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque density gradients from sterile heparinized blood. The cells from the interface were washed three times in PBS (without calcium and magnesium) supplemented with 1% AB human serum, seeded at a density of $1x10^6$ per well per ml in 24-well microtiter plates in RPMI-1640 medium supplemented with 5% AB human serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mmol/l glutamine, and allowed to settle at 37°C in a humidified atmosphere of 5% CO_2. After 24 h, the non-adherent cells were removed with three changes of warm PBS and to the adherent cells (about $2x10^5$ per well), 1 ml of complete RPMI containing BCG, *M. leprae* or no mycobacteria was added and the cells were cultured up to 48 h.

Enough mycobacteria were added to the wells to achieve bacteria/cell ratios of approximately 1:1, 10:1 and 100:1. Since most armadillo-derived *M. leprae* are likely dead, experiments were always performed in the presence of streptomycin, an antibiotic known to be effective against *M. leprae* and BCG (Winder 1982, Gelber 1987). By doing this, the differences observed in TNF-α secretion in this study would be essentially due to intrinsic differences between *M. leprae* and BCG, and not to differences in their viability. After 2, 24 and 48 h of incubation, the culture supernatants of PBMC or THP-1 cells were collected and recentrifuged at 14,000 xg to remove extracellular mycobacteria and stored at -70°C until determination of TNF-α levels by ELISA. Viability of the blood-derived monocytes or THP-1 cells was assessed by staining the cells with PBS containing etidium bromide (4 µg/ml) and fluorescein diacetate (20 µg/ml) for 10 min at room temperature. Glass slides were analyzed and viewed using a Zeiss fluorescent microscope.

Evaluation of mycobacteria phagocytosis - Duplicate wells of THP-1 cells were infected with BCG or *M. leprae* as described above and after variable times of incubation, cells were harvested by centrifugation and washed three times to remove extracellular mycobacteria. The percentage of cells containing acid-fast organisms was determined by cytospin and Ziehl-Neelsen staining, and a minimun of 300 cells in each of duplicate slides were counted.

TNF-α detection - Supernatants were diluted in PBS and TNF-α levels determined by ELISA according to the manufacturer recommendations

(Pharmingen, San Diego, CA) using specific pairs of anti-TNF-α monoclonal antibodies and human recombinant TNF-α as standard. The detection limit of TNF-α was 250 pg/ml.

RESULTS

BCG but not M. leprae induced TNF-α secretion in THP-1 cells - The capacity of *M. leprae* and BCG of eliciting TNF-α secretion in THP-1 was evaluated using PMA as a positive control. After 2, 4, 24 and 48 h of continuous exposure, the TNF-α levels in culture supernatants were determined by ELISA. Light microscopic examination of acid-fast stained cultures treated with a bacteria/cell ratio of 10:1 showed that at least 80% of THP-1 cells contained *M. leprae* or BCG after 24 h treatment. Fig. 1 shows that the different stimuli evoked distinct kinetics and levels of TNF-α secretion in THP-1 cells. While PMA-induced TNF-α secretion could be observed only after 48 h of incubation in a dose-dependent manner (Fig. 1a), BCG-induced TNF-α peaked after 2 h of culture, and was continuously detected up to 48 h (Fig. 1b). In contrast, *M. leprae* induced no detectable TNF-α in the conditions examined (Fig. 1b). While BCG was able to induce TNF-α production even at bacteria/cell ratio of 1:1, no detectable levels of this cytokine were observed even when cultures were stimulated with *M. leprae* at bacteria/cell ratio of 100:1 (data not shown). Experiments performed with gamma-irradiated *M. leprae* and heat-killed BCG gave identical results (data not shown).

To ensure that the lack of detectable levels of TNF-α in *M. leprae*-stimulated THP-1 cultures were not related to the *M. leprae* sample used throughout this work, blood-derived monocytes of two healthy individuals were stimulated for up to 48 h with the same *M. leprae* sample used through out this study and with BCG (bacteria/cell ratio of 1:1 and 10:1). As can be seen in the Table, both *M. leprae* and BCG were able to induce TNF-α in blood-derived monocytes. TNF-α was detected in culture supernatants as early as 2 h of incubation and maximum values were generally observed at 24 h.

M. leprae inhibited TNF-α induced by BCG - To determine whether *M. leprae* suppressed TNF-α production by THP-1 cells or simply acted as a poor stimulus, mixtures of *M. leprae* and BCG were used to treat THP-1 cells and the TNF-α production was measured by ELISA. Mixtures containing equal amounts of BCG and *M. leprae* (both mycobacteria at bacteria/cell ratio of 10:1) elicited the production of lower levels of TNF-α when compared with BCG alone at ratios of 10:1 or 20:1 (Fig. 2), suggesting that *M. leprae* affects the ability of THP-1 cells to respond to BCG.

TABLE

TNF-α production of human monocytes infected with BCG and *Mycobacterium leprae*

Stimulus[a]	TNF-α [b] ng/ml (± SD)	
	Donor 1	Donor 2
None	0.64 (±0.20)	0.60 (± 0.09)
M. leprae	13.20 (± 1.00)	6.60 (± 1.56)
BCG	> 20.00	10.60 (± 1.20)

a: mycobacteria was added to cultures at bacteria/cell ratio of 1:1; *b*: monocytes of two healthy subjects were stimulated with BCG or *M. leprae*. Culture supernatants were harvested at 24 h and TNF-α concentration was determined by ELISA. Results are expressed as mean ± SD of duplicates.

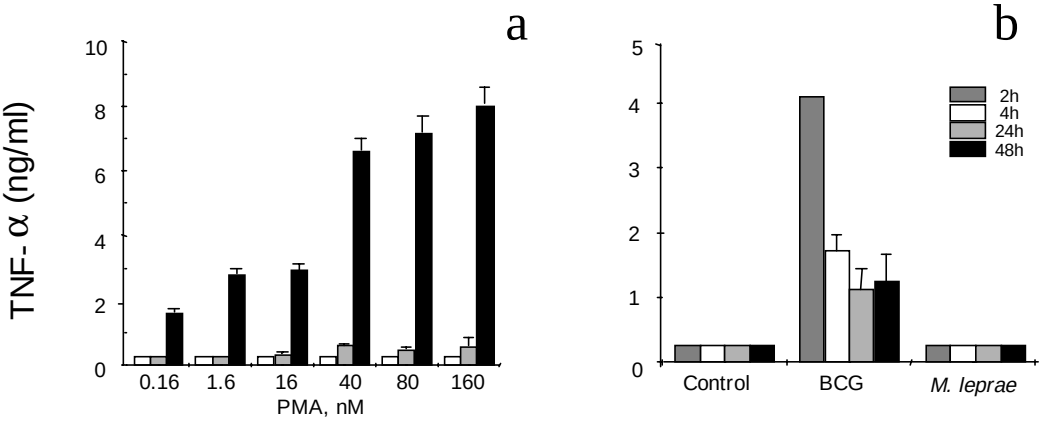

Fig. 1: kinetics of TNF-α production by THP-1 cells exposed to PMA (a), BCG (b), or *Mycobacterium leprae* (b). THP-1 cells ($2x10^5$/ml) were treated with PMA (0.16-160 nM, *M. bovis* BCG or *M. leprae* (ratio bacteria/cell of 10:1). Supernatants were harvested at the indicated times and TNF-α levels were determined by ELISA. Data are expressed as mean ± S.D. of ELISA duplicates and are representative of three independent assays.

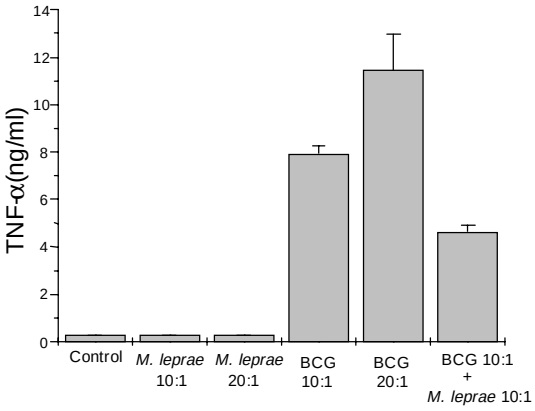

Fig. 2: effect of *Mycobacterium leprae* on TNF-α production of BCG-treated THP-1 cells. THP-1 cells ($2x10^5$/ml) were either exposed to *M. bovis* BCG (ratio of 10:1) plus *M. leprae* (ratio 10:1), to *M. leprae* alone (ratios of 10:1 and 20:1) or to BCG alone (ratios of 10:1 and 20:1). Supernatants were harvested after 2 h of incubation and TNF-α levels determined by ELISA. Data are expressed as mean ± S.D. of three independent experiments carried out in duplicates.

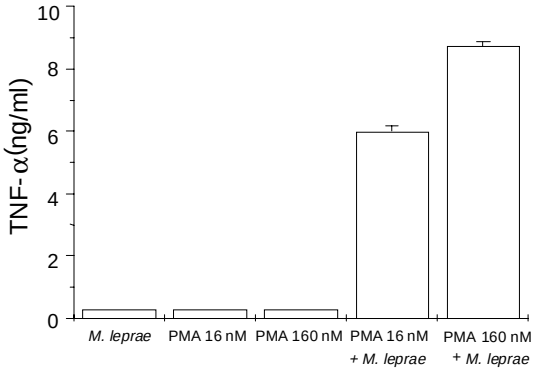

Fig. 3: TNF-α production by PMA-differentiated THP-1 cells in response to *Mycobacterium leprae*. THP-1 cells ($2x10^5$/ml) were plated and incubated in RPMI complete medium for 4 h. After this time, THP-1 cells were stimulated with PMA (16 or 160 nM) for more 4 h, then washed and exposed to *M. leprae* (MOI=10) or incubated in medium alone, as in Methods. Some cultures were continuously exposed to PMA throughout the experiment as an additional control. Supernatants were harvested after 24 h of incubation and TNF-α levels were determined by ELISA. Data are expressed as mean ± S.D. of ELISA duplicates and are representative of two independent assays.

THP-1 cells pretreated with PMA were able to produce TNF-α in response to M. leprae - Since THP-1 cells represent a relative immature monocytic cell line (Tsuchiya et al. 1980, Friedland et al. 1992), it was of interest to investigate whether more differentiated THP-1 cells could secrete TNF-α in response to *M. leprae*. Differentiation of THP-1 cells was induced by exposing cells (2×10^5 cells/ml) to 16 or 160 nM PMA, described as a potent inducer of differentiation in THP-1 cells (Tsuchiya et al. 1982). After an additional 4 h incubation, cells were washed and treated with *M. leprae* (bacteria/cell ratio of 10:1) or incubated with medium alone (control) for 24 h more. Supernatants were then collected for TNF-α measurement by ELISA. Some cultures were continuously exposed to PMA throughout the experiment as an additional control. Fig. 3 shows that while cultures continuously exposed to PMA or to *M. leprae* or exposed to PMA for 4 h and then incubated with medium alone secreted either undetectable or low levels of TNF-α, cultures pretreated with PMA and then infected with *M. leprae* secreted up to 9 ng/ml of TNF-α.

DISCUSSION

Interaction of bacterial pathogens with their host cells triggers signal transduction pathways which lead to a variety of cellular responses and ultimately favor their perpetuation in the host. Among these responses there is an extensive reorganization of the cytoskeleton resulting in morphological changes, and the secretion of cytokines into the medium. Although this interference in host cell metabolism could represent a central feature of their pathogenesis, these events are poorly understood in mononuclear phagocytes infected with mycobacteria and have only recently received attention by a few investigators (Kusner et al. 1996, Mendez-Samperio et al. 1996, Olivier et al. 1998, Malik et al. 2000). In the present study, we have employed an in vitro model to investigate the early molecular events evoked by mycobacteria after their phagocytosis by human monocytes. THP-1 cells, a human myelomonocytic cell line that lately has been extensively employed in studies of mechanisms of maturation/differentiation from monocytes to macrophages (for review see Hass 1992), were used as host cells. To our knowledge, this is the first report describing the challenge of THP-1 cells with *M. leprae*, and its effects were compared with those provoked by BCG.

We investigated TNF-α production by THP-1 cells challenged with *M. leprae*, BCG or PMA. Besides representing a good parameter for monocyte activation/differentiation, TNF-α is known to play a critical role in mycobacterial infection, being involved both in protective as well as in pathological response (Kindler et al. 1989, Lima et al. 2000). Our data indicate that phagocytosis of armadillo-derived *M. leprae* by THP-1 cells is accompanied by undetectable levels of TNF-α secretion under the experimental conditions employed in this study. Conversely, entry of BCG in these cells, even at a very low bacteria-to-cell ratio, resulted in appreciable secretion of TNF-α. The

induction of TNF-α by BCG was not due to LPS contamination, since the same levels of the cytokine were assayed in the presence of polymyxin B (data not shown). The different behavior of THP-1 cells after challenge with BCG or *M. leprae* is also not attributed to differences in the extent of mycobacteria uptake: examination of cells by light microscopy after 24 h of exposure to mycobacteria at a ratio bacteria/cell of 10:1 revealed the presence of acid fast bacilli in more than 80% of the cells. Moreover, the inclusion of streptomycin in our cultures, together with the fact that the use of both gamma-irradiated *M. leprae* and heat-killed BCG gave similar results (data not shown), indicate that the differences observed were not due to differences in viability but to intrinsic properties between *M. leprae* and BCG.

Next, we examined the effect of *M. leprae* on TNF-α secretion using primary human monocytes instead of THP-1 as host cells. In accordance with other findings (Santos et al. 1993, Suzuki et al. 1993, Lima et al. 2000), exposure of these cells to *M. leprae* induced TNF-α secretion. Moreover, pretreatment of THP-1 cells with PMA, a well known inducer of differentiation, led these cells to produce TNF-α in response to *M. leprae*. Therefore, the incapacity of *M. leprae* to induce detectable levels of TNF-α was inherent to THP-1 cells and can perhaps related to the fact that they can be considered immature monocytes.

The employment of THP-1 as host cells is in agreement with earlier observed differences between BCG and *M. leprae* in modulating host cell metabolism previously reported by other groups. In agreement with its well known immunostimulatory effect, it has been shown that BCG is a better inducer of TNF-α, IL-1 and IL-6 from human and animal macrophages, when compared to *M. leprae* (Suzuki et al. 1993). It has also been shown that phagocytic cells (monocytes/neutrophils) produce high levels of oxygen free radicals when challenged with BCG, but not with *M. leprae* (Holzer et al. 1988). Indeed, several reports suggest that *M. leprae* has immunosupressive rather than immunostimulant effects on macrophages. In this regard, it has been shown that *M. leprae* infected macrophages became deactivated by inducing the secretion by these cells of suppressive mediators such as prostaglandins, IL-1 receptor agonist and TGF-β (Sibley & Krahenbuhl 1988, Suzuki et al. 1993, Goulart et al. 1996). Along these lines, in this study we also found that when mixtures of *M. leprae* and BCG were used to challenge THP-1, significantly lower levels of TNF-α were secreted, indicating a suppressive effect by *M. leprae*.

The formation of granuloma can be considered, the principle host defense to mycobacterial infection in mice infected with BCG. It has been demonstrated that local TNF-α production is crucial to granuloma formation (Kindler et al. 1989). In this regard, THP-1 is a relative immature human monocytic cell line, representative of cells involved in granuloma formation. The findings of the present study may then suggest that the capacity of young monocytes to respond to BCG by TNF-α secretion would tend to markedly amplify the initial inflammation and creates conditions favoring progression of granuloma.

In conclusion, the in vitro model herein employed allows the detection of differences in cell metabolism, including cytokine production, triggered by host-derived *M. leprae* and BCG. These results are in agreement with previous data that indicate THP-1 as being a useful model to investigate the nature of mycobacteria/monocyte interactions (Stokes & Doxsee 1999). The use of THP-1 as host cells have two advantages over the use of blood-derived monocytes in that (1) the variability between donors commonly encountered with primary cells is avoided; and (2) large numbers of cells can be readily cultivated. Our data suggest that THP-1 cells can be useful for a better understanding of early signal transduction pathways involved in the host response against vaccine versus pathogenic species of mycobacteria.

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