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ORIGINAL PAPER

Lipoarabinomannan as a Regulator of the Monocyte Apoptotic Response to *Mycobacterium bovis* BCG Danish Strain 1331 Infection

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Abstract

The mannosylated lipoarabinomannan (ManLAM) from mycobacterial species possesses strong immunomodulatory effects. Here we examined the ability of *Mycobacterium tuberculosis* ManLAM to interfere with the apoptotic response of mouse monocyte cell line, RAW 264.7 infected with *Mycobacterium bovis* BCG Danish strain. Incubation of BCG-infected monocytes with ManLAM decreased production of NO and the numbers of apoptotic cells which synergized with the polarization of mitochondrial membrane. Activities of caspase-1, -3, -8 and 9 followed pattern of apoptosis suppression by ManLAM, except for caspase-1, which showed no significant change in activity. ManLAM also stabilized anti-apoptotic ratio of bcl-2/bax expression in BCG-infected cells and blocked activation of Fas/FasL-induced pathway of apoptosis. Thus, ManLAM, apart from blocking mitochondrial pathway of apoptosis, may induce several other pathways regulating apoptotic response in BCG-infected mouse monocytes.

Key words: *Mycobacterium bovis* BCG, apoptosis, Bcl-2, Fas, lipoarabinomannan

Introduction

Tuberculosis (TB) is increasing in prevalence in many countries and is now the leading infectious cause of death worldwide, being responsible for three million deaths annually (Raviglione and Uplekar, 2006; Squire *et al*., 2006). Such a dramatic situation is due, at least in part, to the ability of the airborne bacillus to resist killing by, and to parasitize host alveolar macrophages (Flynn and Chan, 2001; Flynn and Chan, 2003). The mycobacterial cell wall plays an important role in modulation of immune response to mycobacterial infections by specialised molecules such as lipoarabinomannan (LAM) – the predominant antigenic lipoglycan of mycobacterial surface (Strohmeier and Fenton, 1999). LAM is a branched form of phosphatidylinositol mannoside, the characteristic cell wall mannophosphoinositide of mycobacteria. LAM is expressed in a variety of distinct structures, which are generally grouped into two categories: mannose-capped (ManLAM) and uncapped or arabinofuranosyl-terminated, LAM (AraLAM). ManLAM is abundant in slow-growing, virulent mycobacteria, whereas AraLAM is abundant in fast-growing, avirulent mycobacteria (such as, for example, *M. smegmatis*) (Briken *et al*., 2004; Nigou *et al*., 2004; Nigou *et al*., 2003). ManLAMs have been shown to inhibit production of proinflammantory cytokines (IL-12, TNF-α) by human dendritic and macrophage cells, in contrast to AraLAM (Nigou *et al*., 2001; Yoshida and Koide, 1997). Furthermore, ManLAM induces production of the anti-inflammatory cytokine IL-10. IL-10 treated DCs are not only less efficient in stimulating T-cell responses but also induce a state of antigen-specific tolerance leading to T cell anergy (Geijtenbeek *et al*., 2003). Purified ManLAM has been shown to be a potent

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chemotactic factor for both CD4+ and CD8+ lymphocytes *in vitro* and an inhibitor of cytokines production by T-cells (Barnes *et al*., 1992).

It has been shown *in vitro* that attenuated mycobacterial strains, such as *M. bovis* BCG or *M. tuberculosis* H37Ra, induced pronounced apoptosis of human and mouse macrophages while fully virulent strain, such as *M. tuberculosis* H37Rv, were suppressing apoptosis (Nigou *et al*., 2002; Rojas *et al*., 1999). Furthermore, the infection of macrophages with attenuated mycobacterial strains resulted in only limited intracellular growth while virulent strains survived longer and allowed for unlimited multiplication of bacilli (Nigou *et al*., 2002). These observations suggest that apoptosis of infected host cells might constitute an important innate protective mechanism which limits bacterial load in the tissues early in the course of infection. Although specific bacterial factors responsible for induction/evasion of apoptosis are unknown, several reports suggested that in certain systems a 19-kDa mycobacterial lipoprotein may act as apoptotic inducer (Ciaramella *et al*., 2000) and ManLAM of mycobacterial cell wall may function as inhibitor of apoptosis of macrophages *in vitro* (Maiti *et al*., 2001; Rojas *et al*., 2000). However, the exact mechanism of LAM action upon macrophages remains to be elucidated.

Experimental

Materials and Methods

Macrophage cell line. The murinemacrophage-like cell line RAW 264.7 (ATCC – TIB-71) was supplied by LGC Promochem. The macrophages were cultured in RPMI-1640 supplemented with 10% foetal bovine serum (FBS, Invitrogen), antibiotics and L-glutamine (Sigma-Aldrich). Following three washes with phosphate-buffered saline (PBS) they were cultured for 24 hours before each experiment.

***Mycobacterium bovis* BCG and ManLAM.** Non-pathogenic *M. bovis* BCG Danish strain 1331 was provided by Statens Serum Institute, Copenhagen, Denmark. The bacteria were grown in Middlebrook 7H9 broth (BD Biosciences) supplemented with OADC supplement (BD Biosciences). After 4 weeks of incubation bacteria were washed by centrifugation and suspended in the growth medium supplemented with 10% glycerol (Sigma-Aldrich) and frozen in 1 ml aliquots in –20°C. Before each experiment bacteria were thawed and dispersed using glass beads until no bacterial clumps were present. The number of bacteria (CFU) per ml was checked by plating on Middlebrook 7H9 agar. ManLAM from highly virulent *M. tuberculosis* H37Rv was obtained in the Department of Bacteriology, Swedish Institute for Infectious Disease Control using a previously described protocol (Hamasur *et al.*, 1999) complemented with a concanavalin A-sepharose and phenyl-sepharose chromatography (Hamasur, unpublished). This procedure yielded a ManLAM preparation which was free of contaminating protein, migrated as single broad band in SDS-PAGE, showed carbohydrate composition with arabinose: mannose ratio characteristic for ManLAM, and reacted with LAM-specific monoclonal antibodies.

Infection assay. RAW 264.7 cells were cultured in 24-well plates at 2×10^4 cells per well overnight and the infection experiment was performed by incubation with the bacilli suspension at multiplicity of infection (moi) of 5 in RAW-1640 medium without FBS. After 2 hours, cells were washed three times with PBS and kept in RPMI-1640 supplemented with 10% FBS without antibiotics for 3 days. For each experiment, moi was checked by acid-fast staining using TB colour set (Merck). In all experiments ManLAM was added at 7 μg/ml subsequently after washing of unbounded bacilli.

Annexin V-FITC/propidium iodide cell death detection. To assess cell viability, a cell death detection assay was used which detects both apoptosis and necrosis. The BD Biosciences annexin V-FITC apoptosis detection kit was used according to the manufacturer's directions. Fluorescent green staining of the plasma membrane indicates apoptosis by binding of annexin V to the outer layer of the plasma membrane. Red staining of DNA with propidium iodide, in conjunction with green annexin V staining, indicates a loss of plasma membrane integrity typical of necrosis. Cells were analysed in FACScan using CellQuest programme (BD Biosciences).

Assays for caspase-1, -3, -8 and -9 activities. Caspase-1, -3, -8 and -9 activities were measured according to the producer's instruction as described elsewhere (Krzyzowska *et al*., 2005). Substrates were as follows: Ac-YVAD-AMC [N-acetyl-Tyr-Val-Ala-Asp-AMC (7-amino-4- methylcoumarin] substrate for caspase-1; Ac-DEVD-AMC [N-acetyl-Asp-Glu-Val-Asp-AMC(7-amino-4-methylcoumarin)] substrate for caspase-3; Ac-IETD-AFC [N-acetyl-Val-Glu-Ile-Asp-AFC (7-amino-4-trifluormethylcoumarin] substrate for caspase-8 (BD Biosciences) and LEHD-AMC [N-acetylo-Leu-Glu-His-Asp-AMC (7-amino-4-methylcoumarin] for caspase-9 (Sigma). Results for this assay are expressed as relative fluorescence unit/50 μg protein/h (RFU) calculated from triplicate numerical data acquired from test and control samples on Fluoroskan Neonate fluorometer by Transmit Software (Labsystems Oy, Finland).

JC-1 staining. Following experimental treatments, cells seeded in a 24-well dish were stained with the cationic dye, 5,5',6,6'-tetrachloro1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1; Sigma) as previously described (Salvioli *et al*., 1997) in order to determine the state of mitochondrial membrane

potential. JC-1 is a potentiometric dye which exhibits a membrane potential dependent loss as J-aggregates (polarized mitochondria) transition to JC-1 monomers (depolarized mitochondria) as indicated by fluorescence emission shift from red to green (Salvioli *et al*., 1997). Therefore, mitochondrial depolarization is indicated by an increase in the green/red fluorescence intensity ratio. Briefly, cell monolayers were incubated with RPMI-1640 containing 10% FBS and 5 μg/ml JC-1 at 37°C for 15 min. Following the incubation, cells were detached from the wells and washed two times in PBS and analysed in FACScan using CellQuest programme (BD Biosciences).

Measurement of nitric oxide. The production of reactive nitrogen radicals (RNI) was determined by measuring the amount of nitrite, a metabolic product of NO. Diluted samples (250 μl) were incubated with 2,3-diaminonaphthalene (DAN, 633 mM in 0.67 N HCl, Sigma-Aldrich) at room temperature in plastic containers in the dark for 10 min (Marzinzig *et al*., 1997). The mixture was adjusted to pH 11.5–12.0 with 1 N NaOH. The fluorescence was measured with a microtiter Fluoroskan Neonate fluorometer with an excitation of 365 nm and an emission of 405 nm. The nitrite amount was calculated from a $NaNO_2$ standard curve (Marzinzig *et al*., 1997).

Measurement of Fas, FasL, Bcl-2 and Bax expression. For detection of extracellular Fas and FasL expression, cells were stained with polyclonal rabbit anti-FasL (M-20, Santa Cruz Biotechnology) antibody and mouse monoclonal anti-Fas (clone 13) (BD Biosciences) antibody, followed by FITC-conjugated bovine anti-rabbit polyclonal antibody (Santa Cruz Biotechnology) and goat anti-mouse IgG_1 PE-conjugated (BD Biosciences) polyclonal antibodies. For double staining of Bcl-2 and Bax, cells were stained with monoclonal mouse anti-Bcl-2 (clone 7) antibody (BD Biosciences) and polyclonal rabbit anti-mouse bax antibody (BD Biosciences) using BD Cytofix/Cytoperm™ kit. In the second step rat FITC-conjugated anti-mouse IgG_1 polyclonal antibody (BD Biosciences) and PE-conjugated bovine anti-rabbit polyclonal antibody (Santa Cruz Biotechnology) were used, as described above. Cells were analyzed in FACScan (BD Biosciences) by comparing mean fluorescence for Bax and Bcl-2 and percentage of positive cells for Fas and FasL.

Statistics. The non-parametric Mann-Whitney U test was carried out for pair-wise comparison of samples using SPSS software.

Results

RAW 264.7 cells infected with *M. bovis* BCG showed a statistically significant increase in NO production throughout the course of infection, whereas BCG-infected, ManLAM treated-cells also showed an increase in NO production, albeit this increase was statistically lower ($p \leq 0.05$) in 1 and 2 d.p.i. (day post infection) in comparison to NO_2^- concentration in supernatants from BCG-infected cells (Table I).

Table I
Nitric oxide production in RAW 264.7 cells treated with 7 μg/ml of ManLAM or infected with *M. bovis* BCG and treated or not with 7 μg/ml of ManLAM

	day 1	day 2	day 3
control	24.32±2.34	49±4.5	59.59±3.87
ManLAM	26.01±1.72	43.34±3.1	52.43±2.78
BCG	35.8±4.65*	115.1±9.1**	78.24±1.99*
BCG+ManLAM	24.45±1.82	69.85±4.32*	71.22±2.67*

* $p \leq 0.05$, ** $p \leq 0.01$ in comparison to control, uninfected cells. Results are expressed as mg NO_2^-/ml. Each value represents mean value obtained from 3 separate experiments ± SEM.

Since high levels of NO production may lead to disruption of mitochondrial functioning, the cells were tested for mitochondrial potential. At 2 d.p.i. RAW 264.7 cells infected with BCG showed statistically significant higher number of cells with depolarised mitochondria (increase in green/red fluorescence ratio from JC-1 stain), thus indicating the decrease in mitochondrial potential. At the same time, BCG-infected, ManLAM treated samples showed increased, but statistically unimportant number of cells with depolarised mitochondria. At day 3 of infection the number of cells with green fluorescence was almost equally high in both BCG-infected and BCG-infected, ManLAM treated cells ($p \leq 0.005$, Fig. 1). Furthermore, the mitochondrial potential decrease was well reflected by the number of the apoptotic cells – at day 2 the percentage of annexin V-positive cells was significantly lower in BCG-infected, ManLAM-treated cells in comparison to BCG-infected RAW 264.7 cells, while at day 3 no significant difference was observed (results not shown).

In order to elucidate the apoptotic pathways during BCG infection and the influence of ManLAM upon the outcome of apoptosis, the activities of caspase-1, -3, -8 and 9 were measured. The activity of caspase-1, classified as a pro-inflammatory caspase and involved in maturation of IL-1, was insignificant throughout the whole tested period (Table II), while the activity of caspase-3 followed apoptosis pattern showed by JC-1 and annexin V staining (Table II). The activity of caspase-3 significantly increased at day 1, 2 and 3 in BCG-infected samples and at day 2 in BCG-infected, ManLAM-treated cells ($p \leq 0.05$). However, at day 2 and 3, the activity of caspase-3 in BCG-infected, ManLAM-treated cells was significantly

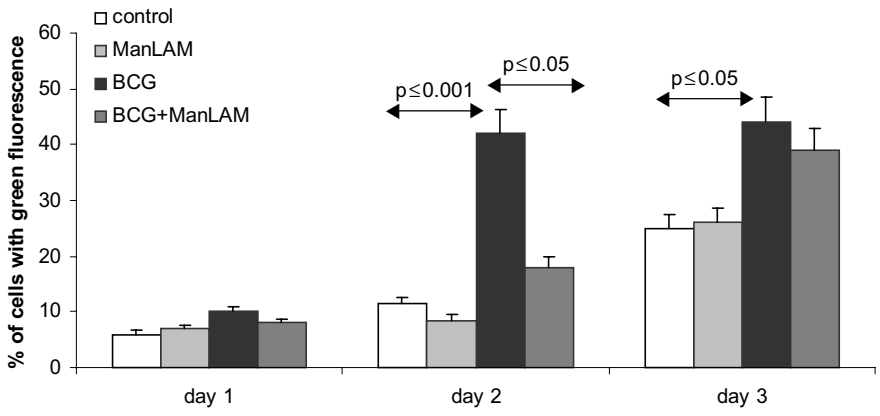

Fig. 1. Decrease of mitochondrial potential (Δψ) in RAW 264.7 cells treated with 7 μg/ml of ManLAM or infected with *M. bovis* BCG and treated or not with 7 μg/ml of ManLAM.

Results are expressed as percentage of cells with decreased Δψ (depolarised mitochondria), *i.e.* cells with the increase of green/red fluorescence ratio. Results are expressed as mean from three separate experiments ± SEM.

lower then in BCG-infected cells ($p \leq 0.05$) (Table II). Caspase-3 is an effector caspase, and its activity is dependent upon the activation by up-stream, initiator caspases, such as caspase-9 for the mitochondrial pathway and caspase-8 for the receptor pathway of apoptosis. In our experiments the activity of caspase-8 significantly increased only in cells infected with BCG ($p \leq 0.05$), whereas ManLAM significantly influenced its activity in the BCG-infected cells ($p \leq 0.05$) (Table II). Since the mitochondrial potential was decreased in BCG-infected cells at all tested time points and it was also decreased in BCG-infected ManLAM-treated cells, we expected that this decrease should have been reflected by the activity of caspase-9, unless any anti-apoptotic mitochondrial proteins were involved in its suppression. Indeed, the results of caspase-9 activity tests showed that caspase-9 activity was significantly increased only in BCG-infected cells and not in ManLAM treated control or infected cells ($p \leq 0.05$) (Table II). ManLAM also had some influence upon lowering caspase-9 activity in ManLAM treated control cells, however this decrease was statistically insignificant ($p \leq 0.06$) (Table II).

Table II

Caspase 1, 3, 8 and 9 activity assays in protein lysates of RAW 264.7 treated with 7 μg/ml of ManLAM or infected with *M.bovis* BCG and treated or not with 7 μg/ml of ManLAM

Caspase -1 activity			
Day	1	2	3
Control	15.53±0.99	34.13±3.22	26.79±1.99
ManLAM	16.33±1.89	36.70±3.01	30.73±3.03
BCG	12.45±1.98	32.61±2.09	26.73±2.06
BCG+ManLAM	12.61±2.02	25.89±2.14	27.26±1.7
Caspase-3 activity			
Day	1	2	3
Control	87.85±7.02	240.33±11.69	232.53±12.4
ManLAM	90.36±5.01	234.26±13.04	245.36±11.8
BCG	103.90±7.19*	299.6±15.07**	289.63±10.2*
BCG+ManLAM	86.88±6.22	207.76±10.34*	227.53±13.89
Caspase-8 activity			
Day	1	2	3
Control	132.55±11.99	391 9.92	278.86±7.78
ManLAM	146.45±8.1	359.56±9.12	295.33±9.34
BCG	155.7±9.38	440.8±21.22**	324.5±9.67*
BCG+ManLAM	136.85±9.78	345.5±18.97	281.76±10.23
Caspase-9 activity			
Day	**1**	**2**	**3**
Control	113.37±6.11	243.23±10.94	222.53±12
ManLAM	119.15±10.56	250.03±12.66	179.66±11.1
BCG	119.50±9.28	289.46±11.89*	255±10.11*
BCG+ManLAM	119.85±9.93	246.23±10.23	209.8±10.12

Each value represents mean value obtained from 3 separate experiments ± SEM. Results are expressed as relative fluorescence units. For details – see materials and methods.

* indicate $p \leq 0.05$, ** $p \leq 0.01$ in comparison to control cells.

The activity of caspase-9 is dependent upon the formation of apoptosome, which in turn depends on the action of specific proteins belonging to Bcl-2 family of mitochondrial proteins. The family of Bcl-2 proteins consists of many proteins exerting both pro-apoptotic (for example Bax) and anti-apoptotic (Bcl-2, among many others) action. Since the mitochondrial potential and caspase-9 activity was decreased in BCG-infected, ManLAM treated cells we decided to test expression of Bax and Bcl-2 proteins. Flow cytometry analysis of bcl-2 and bax expression in BCG-infected RAW 264.7 cells showed a significant increase in both antigens expression at day 1 of infection with BCG ($p \leq 0.05$) with no change of expression for other groups (Fig. 2A). However, at day 2 bax expression was significantly increased in BCG-infected cells, exceeding significantly ($p \leq 0.001$) the level of bcl-2

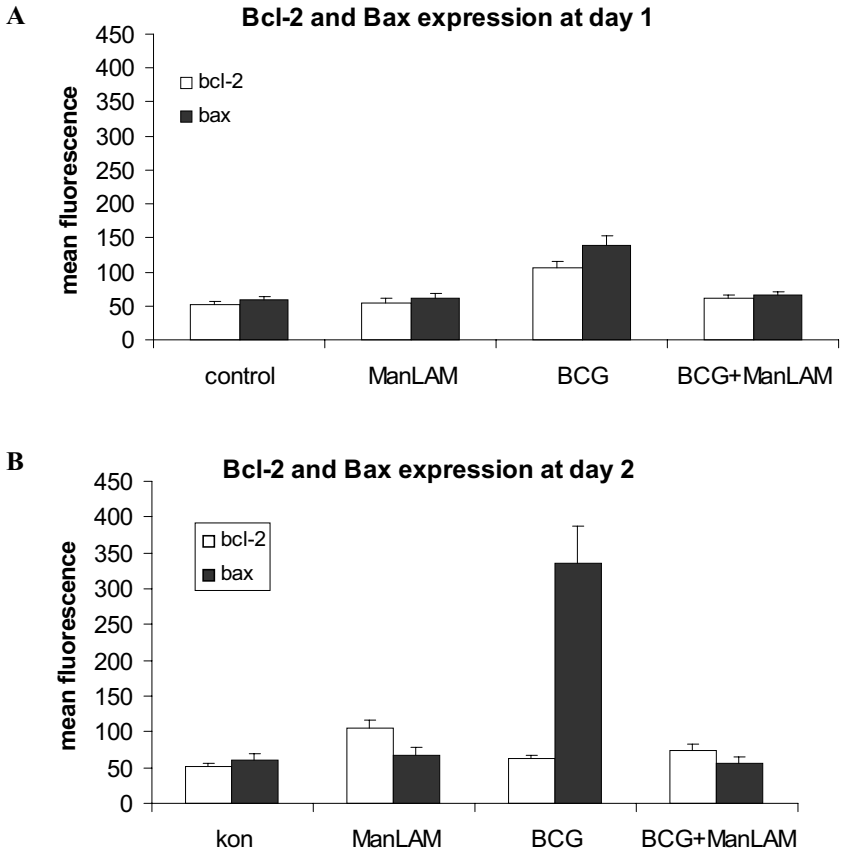

Fig. 2. Flow cytometry analysis of Bcl-2 and Bax expression in RAW 264.7 treated with 7 µg/ml of ManLAM or infected with *M. bovis* BCG and treated or not with 7 µg/ml of ManLAM at day 1 (2A) and at day 2 (2B). Results are expressed as mean value of mean fluorescence from three separate experiments ± SEM.

expression (Fig. 2B). Addition of ManLAM had a significant influence upon Bax expression at day 1 and 2 in BCG-infected cells (Fig. 2A, B), decreasing it to the level observed in control and ManLAM-treated cells ($p \leq 0.05$). ManLAM alone caused up-regulation of Bcl-2 expression at day 2 in uninfected RAW 264.7 cells ($p \leq 0.05$) (Fig. 2B).

The susceptibility of cells to apoptosis depends, among other factors, on the level of Fas and its natural ligand, FasL expression. Fas receptor is present on the surface of all cells, albeit in low number but increases upon induction of its transcription by many pro-apoptotic factors. FasL, although not normally present on the surface of cells, can also become up-regulated by many apoptotic factors. Consequently, since the activity of caspase-8 also depends on the Fas and FasL we expected that the expression of Fas and its ligand should have been elevated in BCG-infected cells. Indeed, Fas expression was significantly increased in BCG-infected cells ($p \leq 0.05$) at day 1 and 2, but also in ManLAM-treated BCG-infected cells at day 1 and 2 (Fig. 3A). Surprisingly, ManLAM alone induced significant up-regulation of Fas expression in all tested period ($p \leq 0.05$), even at day 3, when Fas expression was significantly down-regulated in BCG-infected cells ($p \leq 0.05$) (Fig. 3A). FasL expression was significantly up-regulated in both BCG-infected as well as ManLAM treated cells (Fig. 3B) at day 1 and 2. However, caspase-8 activity was increased only in BCG-infected cells at day 2 and 3. No significant increase in caspase-8 was observed upon ManLAM treatment (Table II).

Discussion

The results presented here clearly demonstrate that mouse RAW 264.7 monocytes undergo apoptosis after infection with non-virulent *M. bovis* BCG, which can be inhibited by the addition of ManLAM isolated from virulent *M. tuberculosis* H37Rv. Apoptosis was consistently demonstrated by quantification of annexin V-positive cells and disruption of mitochondrial potential. Previous reports from other laboratories, using murine and human macrophages have shown that infection with non-virulent *M. tuberculosis* leads to apoptosis of infected cells, whereas infection with low doses leads to suppression of apoptosis (Flynn and Chan, 2001; Flynn and Chan, 2003; Nigou *et al.*, 2002). There is also evidence that soluble mycobacterial

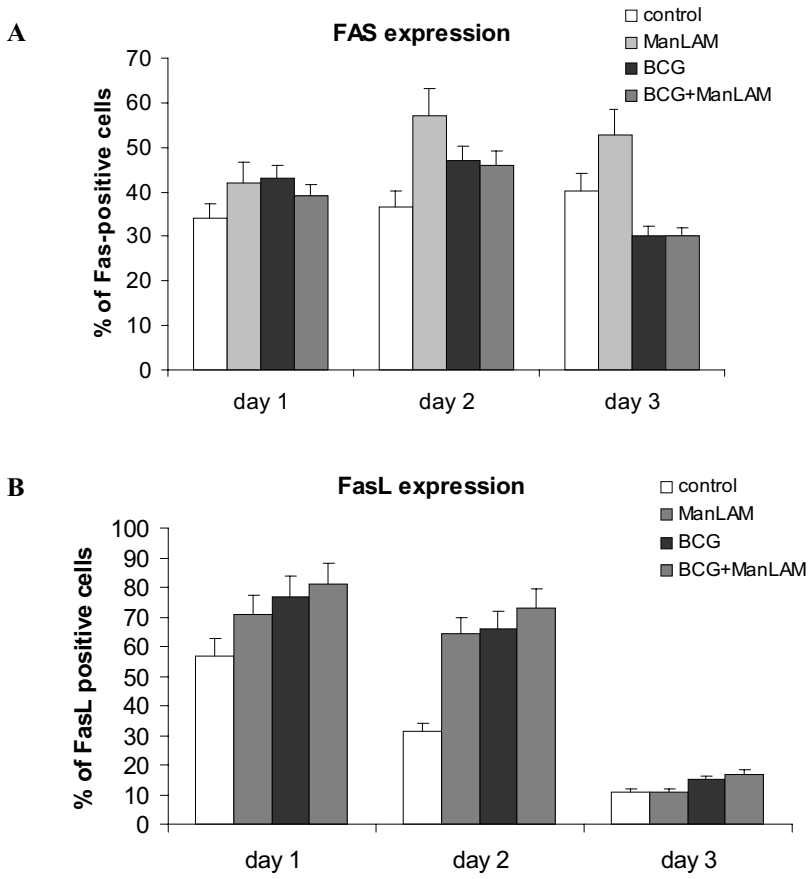

Fig. 3. Flow cytometry analysis of Fas and FasL expression in RAW 264.7 treated with 7 μg/ml of ManLAM or infected with *M. bovis* BCG and treated or not with 7 μg/ml of ManLAM at day 1 (3A) and at day 2 (3B). Results are expressed as mean value of percentage of positive cells from three separate experiments ± SEM.

products are able to modulate apoptosis. PPD induces apoptosis of murine macrophages (Rojas *et al*., 1997) and a sonicate from *M. avium* (Rojas *et al*., 1997), as well as the 19-kDa lipoprotein of *M. tuberculosis* (Ciaramella *et al.*, 2000), have been reported to induce apoptosis of human monocytes. In addition, there are reports indicating that apoptotic macrophages may be found in bronchoalveolar lavages and pulmonary granulomas from patients with TB (Gil *et al*., 2004; Keane *et al*., 1997). On the other hand, *M. tuberculosis* has developed mechanisms to inhibit apoptosis, thus perpetuating the favorable environment for its intracellular growth. The evidence indicates that ManLAM is largely responsible for the inhibition of apoptosis in *M. tuberculosis*-infected macrophages (Maiti *et al.*, 2001, Nigou *et al.*, 2001). Many events triggered by ManLAM were proposed: (1) preferential induction of IL-10 production, which negatively regulates the production of NO and caspase activation, even in the presence of TNF-α (Rojas *et al*., 1999); (2) stabilization of Bcl-2 expression and (3) inhibition of the caspase activation cascade (Rojas *et al.*, 2000). Since Bcl-2 is a mitochondria-associated molecule and it stabilizes mitochondrial potential, the ratio of Bcl-2 protein to apoptosis-inducing mitochondrial protein Bax decides about the outcome of mitochondrial apoptotic challenge (Cory and Adams, 2005; Cory *et al*., 2003). Our study showed that ManLAM stabilized Bcl-2 expression in RAW 264.7 cells but it also led to a decrease in Bax expression (Fig. 3). Low expression of mitochondrial pore-forming Bax prevented changes in mitochondrial potential and was followed by inhibition of caspase-9 and caspase-3 activation and consequently, suppression of apoptosis (Fig. 1, 2; Table II). In human neutrophils *M. tuberculosis*-induced apoptosis is also associated with a transient increase in expression of Bax protein, and a more prominent reduction in expression of the antiapoptotic protein Bcl-x_L (Perskvist *et al*., 2002). Another possible pathway is that down-regulation of Bax expression indirectly results from the increase in NO production. NO at high concentration is toxic to mitochondria and its production leads to changes in expression of mitochondrial pro-apoptotic and anti-apoptotic proteins (Choi *et al*., 2002). ManLAM affects the TNF-α/IL-10 balance by upregulating IL-10, which is considered as an antiapoptotic cytokine, influencing NO production.

Oddo *et al*. (1998) showed that *M. tuberculosis*-infected human macrophages display a reduced susceptibility to FasL-induced apoptosis, together with reduced levels of surface Fas expression. In our study, however, we did not observed a reduction of Fas expression upon *M. bovis* BCG infection. Surprisingly, ManLAM induced up-regulation of Fas expression at day 1 and 2 of infection, while at day 3 BCG-infected cells showed a down-regulation of Fas (Fig. 4A). FasL expression was up-regulated by BCG-infection and by ManLAM (Fig. 4B). Ligation of FasL upon Fas is an important step preceding the activation of caspase-8, so up-regulation of Fas renders cells more susceptible to apoptosis. This was not observed in our study in BCG-infected, ManLAM treated cells, which indicates that ManLAM, apart from blocking mitochondrial pathway of apoptosis, may induce another pathway blocking death receptor pathway of apoptosis.

All these observations suggest that *M. tuberculosis* has evolved "double-edge sword" mechanisms influencing macrophage survival and death. The elucidation of the mechanisms that govern macrophage cell death during *M. tuberculosis* infection will open up new possibilities for understanding host-mycobacteria interactions and manipulating host immune and inflammatory responses.

Acknowledgements

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