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Gene Expression Profiling of Lipoarabinomannan-treated Mouse Macrophage Cultures Infected with *Mycobacterium bovis* BCG

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Abstract

The mannosylated lipoarabinomanan (ManLAM) from mycobacterial species possesses strong anti-apoptotic action. Here we examined the ability of ManLAM isolated from *Mycobacterium tuberculosis* H37Rv to alter expression profiles of apoptosis-related genes in mouse macrophages infected with *Mycobacterium bovis* BCG Danish strain. ManLAM suppressed BCG-induced apoptosis and activities of caspase-1, -3, -8 and 9. Mouse Apoptosis Gene Array showed that ManLAM significantly down-regulated pro-apoptotic and pro-inflammatory genes: caspase-1, -3, -7, -8 and -9, TNF-α/TNFSF2, Fas/TNFRSF6, Bax-α, as well as IL-12 p35 and iNOS simultaneously up-regulating anti-apoptotic genes such as Bcl-2 and Mcl-1. The effect of ManLAM was contrary to BCG-induced up-regulation of pro-apoptotic and pro-inflammatory genes and consistent with the functional data.

K e y w o r d s: *Mycobacterium bovis* BCG, apoptosis, DNA microarray, lipoarabinomannan

Introduction

Apoptosis, or programmed cell death is essential for the homeostatic regulation of cells, restriction of intracellular pathogens and also for stimulation of the host adoptive immune response. It has been shown *in vitro* that attenuated mycobacterial strains, such as *Mycobacterium bovis* BCG or *Mycobacterium 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). Virulent mycobacteria modulate host cell apoptosis to create a protected niche within cells (Keane *et al*., 1997; Klingler *et al*., 1997; Perskvist *et al*., 2002), and downregulate protective host cytokines such as TNFα and IL-12 (Roach *et al*., 2002; Rojas *et al*., 1999) to reduce effector T cell and macrophage responses to the pathogen. (Nigou *et al*., 2002). The series of events that allow pathogenic mycobacteria to alter the normal progression of events within mononuclear phagocytes is poorly understood, but probably involves multiple factors, including the specialised molecules of mycobacterial cell wall such as lipoarabinomannan (LAM) – the predominant antigenic lipoglycan of mycobacterial surface (Strohmeier and Fenton, 1999). LAM is a branched form of phosphatidyinolinositol 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 (Man LAM) and uncapped or arabinofuranosyl-terminated, LAM (AraLAM). (Briken *et al*., 2004; Nigou *et al*., 2004; Nigou *et al*., 2003). ManLAM is abundant in slow-growing, virulent

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mycobacteria and in the attenuated *M. bovis* BCG, whereas AraLAM is abundant in fast-growing, avirulent mycobacteria (such as, for example, *M. smegmatis*) (Monsarrat, 1999; Nigou *et al*., 2003). ManLAMs have been shown to inhibit production of proinflammantory cytokines (IL-12, TNF-α), by human dendritic cells and macrophages (Nigou *et al*., 2002; Yoshida and Koide, 1997). ManLAM is also responsible for suppression of apoptosis induced by mycobacterial infection both through mitochondrial-dependent and -independent mechanisms (Nigou *et al*., 2002). The mechanism by which ManLAM isolated from pathogenic mycobacteria alters the normal progression of events within mononuclear phagocytes is poorly understood, but involves multiple factors, including the alteration of phagocyte gene expression patterns. We hypothesized that ManLAM isolated from pathogenic *M. tuberculosis* H37Rv strain may alter gene expression profile of mononuclear phagocytes infected with attenuated *M. bovis* BCG towards expression of apoptosis-suppressing genes – both directly and indirectly involved in apoptosis (kinases, pro-inflammatory cytokines).

Experimental

Materials and Methods

Primary cultures of mouse macrophages. Bone marrow derived macrophage primary cultures were prepared by culturing bone marrow cells with M-CSF. Briefly, bone marrow cells isolated from femoral and crural bones of BALB/c mice were suspended in a culture medium consisting of D-MEM (Invitrogen) supplemented with 25 ng/ml M-CSF (Sigma-Aldrich), 4500 mg/l glucose, antibiotics (penicillin and streptomycin), L-glutamine, 10% fetal bovine serum (Invitrogen) and cultured for 5 days. At day 5 cells were washed and the purity of culture was determined by staining with anti-CD11b and anti-F4/80 antibody and further analysed by flow cytometry. Typically, 95% of cells were expressing both CD11b and F4/80 markers. The viability of cultures was determined by trypan blue exclusion test before the start of each experiment.

Bacterial strains. *Mycobacterium bovis* BCG and ManLAM. *M. bovis* BCG Danish strain 1331 was provided by the 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 same 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 as previously described (Hamasur *et al*., 2003), using a previously described protocol (Hamasur *et al*., 1999), complemented with a concanavalin A-sepharose and phenyl-sepharose chromatography (Hamasur B, unpublished).

Infection assay. Primary cultures of mouse macrophages were cultured in 24-well plates at 10^5 cells/well overnight and the infection experiment was performed by incubation with the bacilli suspension at MOI (multiplicity of infection) of 10 in D-MEM medium without antibiotics (*i.e*. 10^6 bacilli per well). After 2 hours, cells were washed and kept in D-MEM without antibiotics for 5 days. For each experiment MOI was checked by acid-fast staining using TB colour set (Merck). In all experiments ManLAM was added at 10 mg/ml subsequently after washing of unbound bacilli (final concentration).

Measurement of apoptosis. (i) Annexin V-FITC/propidium iodide staining. Cell death was assessed by dual colour flow cytometry after staining with annexin-V-FITC (BD Biosciences) and propidium iodide (PI)(Sigma-Aldrich). (ii) Caspase-1, -3, -8 and -9 activity assays. Caspase-1, -3, -7 and -8 activities were measured according to the producer manuals and as described previously (Krzyżowska *et al*., 2002). Briefly, 1×10^7 cells were centrifuged, washed with PBS and lysed with 1 ml of lysis buffer (10 mM Tris-HCl, 10 mM $NaH_2PO_4/NaHPO_4$, 130 mM NaCl, 1% Triton-X-100, 10 mM NaPPi; pH 7.5). 50 ml of each extract were added in duplicate to 100 ml of HEPES buffer supplemented with appropriate substrate to a final concentration of 1 mg/ml. 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 and -7; Ac-IETD-AFC[N-acetyl-Val-Glu-Ile-Asp-AFC (7-amino-4-trifluormethylcoumarin] substrate for caspase-8, (BD Biosciences). For caspase-9 activity we used "Caspase-9 fluorometric assay" (Oncogene) with LEHD-AMC [N-acetylo-Leu-Glu-His-Asp-AMC (7-amino-4-methylcoumarin] as caspase-9 substrate. Results for this assay are expressed as relative fluorescence unit/50 mg protein/h (RFU) calculated from triplicate numerical data acquired from test and control samples on Fluoroskan Neonate fluorometer by Transmit Software (Labsystems).

RNA extraction. Total RNA was isolated at day 3 from primary macrophage cultures subjected to (i) *M. bovis* BCG infection, (ii) *M. bovis* BCG and ManLAM treatment at 10 μg/ml, (iii) ManLAM treatment at 10 μg/ml, or (iv) were untreated controls.

TRIZOL® reagent (Invitrogen) was used for all extractions according to the manufacturer protocol.

cDNA macroarray. For cDNA synthesis equal amount of RNA extracted from five different experiments for each experimental setting were pooled. To confirm the validity of the assay, cDNA synthesis was performed in duplicate. The cDNA labelling reactions were performed in two steps according to the manufacturer's protocol. Detail of labelled cDNA synthesis and hybridization were described in (Malewski *et al*., 2005). Briefly Nylon array (Panorama Mouse Apoptosis Gene Arrays, Sigma-Genosys, The Woodlands, TX) were hybridised with labelled probes at 65°C overnight. After hybridisation, the nylon membranes were washed with 0.5×SSPE+1% sodium dodecyl sulphate (SDS) and 0.1×SSPE+1% SDS, and then exposed to Phosphorscreen (Kodak, Japan) for 24 hours. The screens were scanned by BioRad FX Scanner. Results from three independent hybridizations were obtained for each probe. Images were analysed by Quantity One (BioRad) software. Each image was overlaid with grids so that signal intensities of individual spots could be assessed. Local background for each membrane was calculated on the basis of 10 positions with no DNA spotted area. Expression levels of individual genes are represented in arbitrary units of density after subtracting background. Intensity-based global normalization was then performed using intensities obtained for GADPH gene in each sample. After correction for the background, averaging the replicates and transforming to base 1, each gene was assigned a relative expression value when compared in pairs (control – BCG-infected; control – ManLAM treated and BCG-infected – BCG-infected and Man LAM treated). Only genes that were shown to be differentially expressed by at least twofold in two independent experiments were included. This was calculated on the base of non-parametric Mann-Whitney U test for whole data.

Real-time quantitative RT-PCR. First-strand complementary DNA (cDNA) was synthesized with oligo$(dT)_{12–18}$ as primers, using SuperScript First-Strand Synthesis System according to manufacturer's protocol (Invitrogen). The sequences of primers are given in Table I. Reactions were performed in a 20-µl volume and Taq ReadyMix™ for Quantitative PCR (Sigma-Aldrich) using LightCycler 2.0 (Roche). Average threshold cycle (C_T) values from the triplicate PCR reactions for the particular gene were normalized against the average C_T values for β-actin from the same cDNA sample and shown as $2D^{CT}$ were $DC_T = C_{T\ \beta\text{-actin}} - C_{T\ gene}$.

Statistics. The non-parametric Mann-Whitney U test was carried out for pair-wise comparison of samples using SPSS software. The results represent mean triplicate numerical data acquired for each time point (day of infection – dpi).

Table I
Primers used for real-time PCR

Gene	Sequence (5'-3')
β-actin	forward GTGGGCCGCTCTAGGCACCAA
	reverse CTCTTTGATGTCACGCACGATTTC
iNOS	forward CCTTCCGAAGTTTCTGGCAGCAGCGGC
	reverse GGCTGTCAGAGCCTCGTGGCTTTGG
TNF-α	forward CCTTGTTGCCTCCTCTTTTGC
	reverse CAGTGTGATGTGATGTAGCGACAGCCTG
IL-10	forward TGTGAAAATAAGAGCAAGGCAGTG
	reverse CATTCATGGCCTTGTAGACACC
IL-12	forward GGAACGCACCTTTCTGGTTACA
	reverse CCTTGTTGCCTCCTCTTTTGC
Mcl-2	forward GCTCCGGAAACTGGACATTA
	reverse CCCAGTTTGTTACGCCATCT
Bcl-2	forward ACT TCG CAG AGA TGT CCAGTCA
	reverse TGG CAA AGC GTC CCC TC
Bax	forward GGA GCA GCT TGG GAG CG
	reverse AAA AGG CCC CTG TCT TCA TGA

Results

ManLAM ameliorates BCG-induced macrophage death. The primary mouse macrophage cultures obtained from the bone marrow of BALB/c mice were infected with *M. bovis* BCG Danish strain and cultured for 5 days in the presence of 10 µg/ml of ManLAM or left without ManLAM treatment. Viability was determined by annexin V/propidium iodide staining at 1, 3 and 5 day after infection. BCG-infected mouse macrophage cultures showed significant increase of apoptotic cell numbers already at 24 hours of infection ($p = 0.01$) and significant apoptosis induction was observed at all tested time points ($p \leq 0.01$) (Fig. 1). Addition of ManLAM to BCG-infected cultures significantly decreased apoptosis at day 1 and 3 ($p \leq 0.05$), but not at day 5 (Fig. 1). The cultures were observed for apoptosis only up to 5 dpi due to the increase of spontaneous apoptosis in control, uninfected cells, already observed at 5 dpi.

Caspases play a central role in the execution phase of apoptosis and are responsible for biochemical and morphological features associated with this form of cell death. In order to elucidate the apoptotic pathways during BCG infection and the influence of ManLAM upon the outcome of apoptosis, the activity of caspase-1, -3, -8 and 9 was measured. The activity of caspase-1, classified as pro-inflammatory caspase and involved in maturation of IL-1, was insignificant throughout the whole tested period (Fig. 2), except for BCG-infected and BCG-infected, ManLAM-treated cells at day 3 ($p \leq 0.0$) (Fig. 2). The caspase-3 activity significantly increased only at day 3 in BCG-infected macrophages and ManLAM-treated cells showed significant decrease of its activity ($p \leq 0.05$) (Fig. 2). Caspase-3 is an effector caspase,

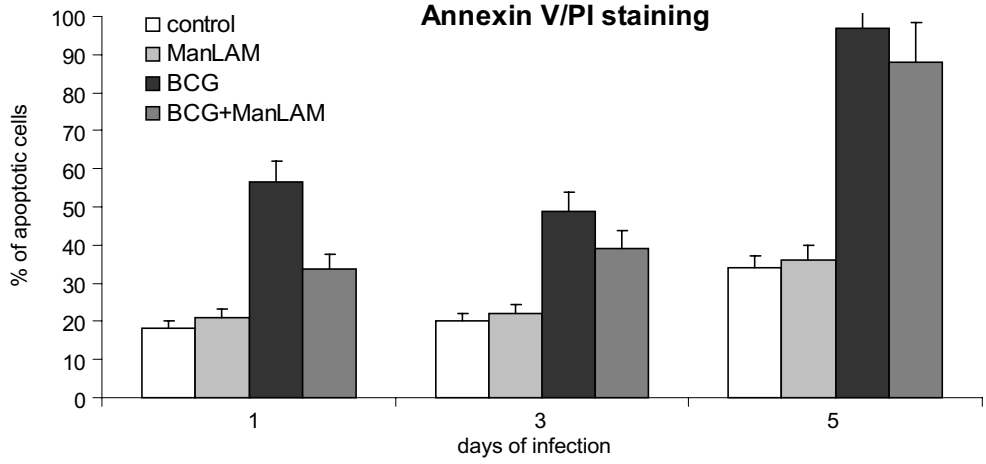

Fig. 1. Identification of apoptotic cells by annexin-V staining in mouse macrophage cultures treated with 10 μg/ml of ManLAM or infected with *M. bovis* BCG and treated or not with 10 μg/ml of ManLAM.

Control cells are the cells treated with similar amounts of PBS. Results are expressed as the percentage of annexin V-positive, PI-negative cells and expressed as mean value of percentage from three separate experiments ± SEM.

and its activity is dependent upon activation by upstream, initiator caspases, such as caspase-9 for the mitochondrial pathway and caspase-8 for the receptor pathway of apoptosis. In our experiments activity of caspase-8 significantly increased only at day 3 in BCG-infected cells ($p \leq 0.05$) and ManLAM-treated infected cells showed some decrease of its activity, although not significant (Fig. 2). 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 ($p \leq 0.05$) (Fig. 2), but again, only at day 3. In all experiments, no significant differences for all tested caspases were observed between control cells and the cells treated only with ManLAM.

ManLAM influences pattern of pro-apoptotic and inflammatory-related gene expression in mouse macrophages infected with BCG. As detailed in materials and methods, the cultures of control, ManLAM-treated, BCG-infected, BCG-infected and ManLAM-treated mouse macrophages were at day 3 of experiment harvested, subjected to RNA isolation and further cDNA synthesis and hybridization with Panorama Mouse Apoptosis Gene Arrays representing 250 mouse apoptosis-related genes. Through analysis we were able to identify a set of genes that was consistently over- or underexpressed, on average, in comparison between infected and uninfected cells, ManLAM-treated and non-treated cells. This was reflected by a significant ≥2-fold mean change ($p \leq 0.05$)

Table II
Gene names, gene bank accession numbers and popular descriptions Genes diffor the genes included in this analysis in Panorama Mouse Apoptosis Gene Arrays, Sigma-Genosys

Gene Name	Gene Bank Accession Number	Gene Description
ASK1/MAP3K5	NM_008580	Mus musculus mitogen activated protein kinase kinase kinase 5 (Map3k5)
Bax-α	NM_007527	Bcl2-associated X protein (Bax)
Bcl-2	NM_009741	B-cell leukemia/lymphoma 2 (Bcl2)
Bcl-w	NM_007537	Bcl2-like 2 (Bcl2l2)
Caspase-1	NM_009807	caspase 1
Caspase-3	NM_009810	caspase 3
Caspase-7	NM_007611	caspase 7
Caspase-8	AJ007749	caspase-8
Caspase-9	NM_015733	caspase-9
IL-12 p35	M86672	interleukin 12 p35 subunit
iNOS	NM_010927	nitric oxide synthase 2, inducible
Mcl-1	NM_008562	myeloid cell leukemia sequence 1 (Mcl1)
TNF-α/TNFSF2	NM_013693	tumor necrosis factor (TNF)

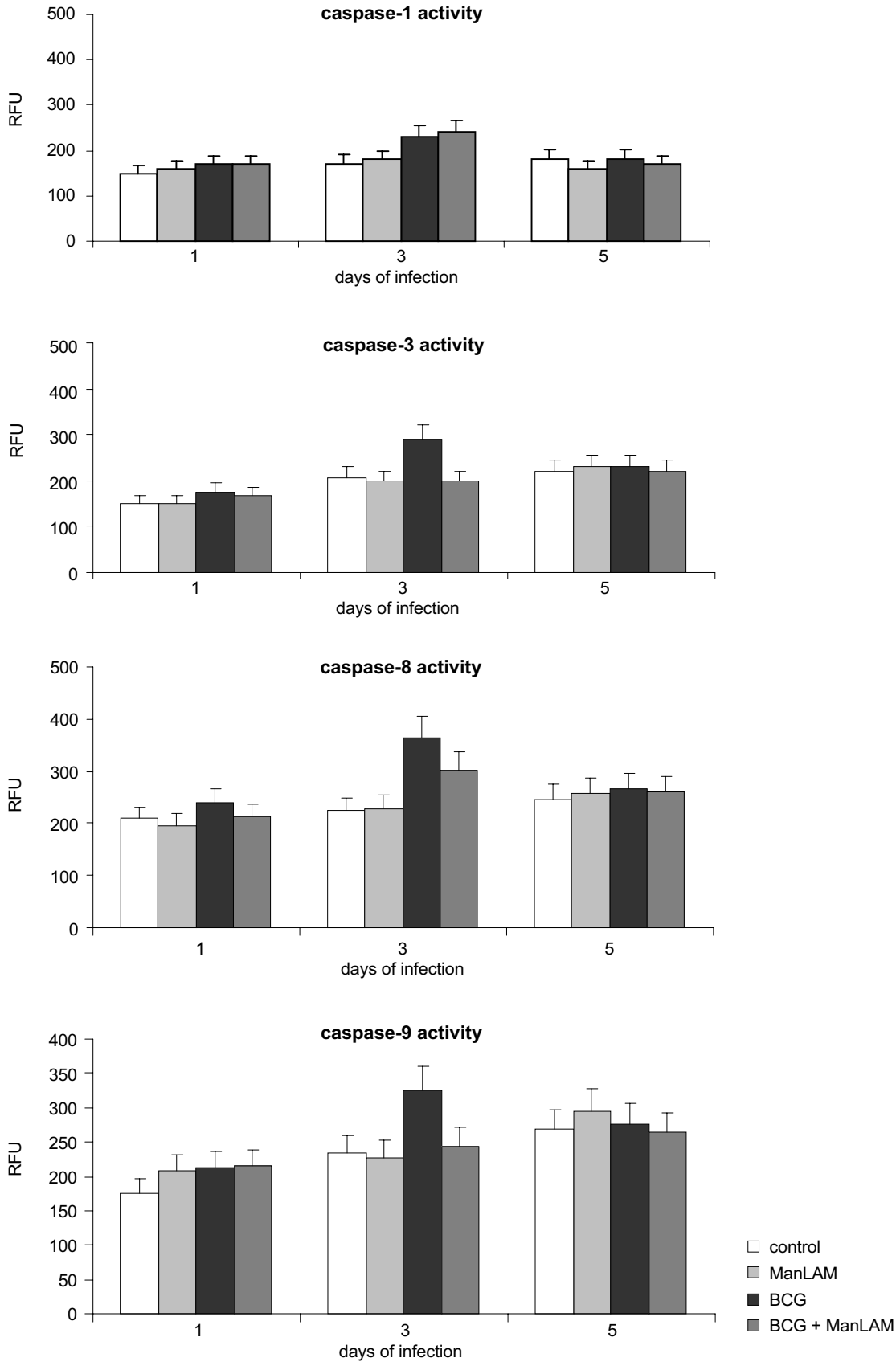

Fig. 2. Caspase 1, 3, 8 and 9 activity assays in protein lysates of mouse macrophages treated with 10 μg/ml of ManLAM or infected with *M. bovis* BCG and treated or not with 10 μg/ml of ManLAM at 3 day of the experiment.

Control cells are the cells treated with similar amounts of PBS. Each value represents mean value obtained from 3 separate experiments SEM. Results for this assay are expressed as relative fluorescence unit/50 mg protein per h (RFU) calculated from triplicate numerical data.

Table III
Genes differentially expressed in mouse macrophages compared in pairs

Gene Name	Ratio		
	Control-LAM*	BCG-BCG+LAM	Control-BCG
Mcl-1	0.77	3.46	4.65
ASK1/MAP3K5	2.13	2	nd.
Bcl-2	2	2.15	2.52
iNOS	2.33	0.94	nd.
Caspase-3	2.33	0.75	nd.
IL-12 p35	4.84	0.41	nd.
Caspase-1	8.69	0.29	nd.
Caspase-8	4.84	0.2	3.19
Bax-a	5	0.16	nd.
Caspase-9	5.3	0.1	nd.
TNF-α/TNFSF2	6.9	0.08	nd.
Bcl-w	2	0.05	nd.

* control – treated with ManLAM; infected with BCG or infected with BCG and ManLAM-treated
n.d. – not detected

and only genes with 2 fold change were shown in Tables II–IV. Eight pro-apoptotic genes were up-regulated in BCG-infected cells, as compared to uninfected, control cells (Table III). These genes were caspase-1, -3, -7, -8 and -9, TNF-α/TNFSF2, Fas/TNFRSF6, Bax-α, ASK1/MAP3K5 while anti-apoptotic genes such as Bcl-2, Bcl-w showed only 2-fold induction. IL-12 p35 and iNOS, inflammation-related genes were also up-regulated more then 2-folds in comparison to uninfected cells (Table III). Down-regulated genes represented anti-apoptotic Mcl-1 and TGF-β (Table III).

Addition of ManLAM to BCG-infected cells caused changes in the pattern of apoptosis-related genes expression at day 3 of infection: pro-apoptotic genes such as caspase-1, -3, -7, -8 and -9, TNF-α/TNFSF2, Fas/TNFRSF6, Bax-α, as well as IL-12 p35 and iNOS were significantly down-regulated. Anti-apoptotic genes such as Bcl-2 and Mcl-1 were more then 2-fold up-regulated (Table III). Surprisingly, although anti-apoptotic Bcl-w gene was up-regulated in BCG infected cells, addition of ManLAM caused its strong down-regulation (Table III).

The results obtained for MAnLAM-treated control cells showed a different pattern of apoptosis-related genes, since ManLAM itself was able to up-regulate both anti-apoptotic genes such as Mcl-1, Bcl-2 and IL-10 and pro-apoptotic such as caspase-8 (Table III). In addition, we were able to compare the differential expression of eight genes using Real Time PCR (TNF-α, IL-12 p35, IL-10, iNOS, Bcl-2, Bax, Mcl-1). The results of these tests confirmed findings of the macroarray experiment (Table IV) except for genes strongly up-regulated such as IL-10 and iNOS. Fold changes in expression of these genes were higher then the results from the macroarray.

Discussion

Data from this DNA macroarray study support the hypothesis that the expression of certain apoptosis-related genes in mouse macrophages infected with *Mycobacterium bovis* BCG is consistently altered after addition of lipoarabinomannan isolated from virulent *Mycobacterium tuberculosis* H37Rv strain. These data confirm the results obtained in our previous studies (Krzyżowska *et al.*, 2007), by showing that lipoarabinomannan is suppressing apoptosis via down-regulation of selected pro-apoptotic genes induced by infection with avirulent mycobacteria. The results obtained for RAW 264.7 cell line were here confirmed for the primary culture of mouse macrophages infected with BCG and/or treated with ManLAM. Lipoarabinomannan suppressed apoptosis induced by BCG, as shown by annexin V/PI staining and measurement of caspase-3, -8 and -9 activity (Fig.1 and 2).

Furthermore, our data are consistent with other papers showing that mycobacteria elicit differential expression of apoptosis-related genes in a strain-specific pattern correlating with microbial virulence (McGarvey *et al.*, 2004; Spira *et al.*, 2003). Infection with the virulent *M. tuberculosis* strain H37Rv of hu-

Table IV
QRT-PCR confirmation of macroarray data

Gene name	ManLAM/control	BCG/control	BCG+ManLAM/BCG	control/ManLAM/BCG
iNOS	0.097	14.95	0.67	12.5
IL-10	18.76	0	0	0
IL-12 p35	0	5.27	0.65	1.4
Mcl-1	4.26	0.23	2.34	60.0
TNF-α/TNFSF2	1.59	6.99	0.66	4.4
Bax-α	0.66	1.66	0.04	0.06
Bcl-2	1.81	1.375	1.66	6.25

Fold changes for selected genes are shown for QRT-PCR experiments calculated from average threshold cycle (C_T) values from the triplicate PCR reactions.

man alveolar macrophages was associated with reduced expression of several proapoptotic genes and increased expression of the anti-apoptotic genes (Spira *et al.*, 2003). Suppression of apoptosis leads to survival of infected macrophages and chronic inflammatory reaction of the host. As a result of chronic inflammatory stimulation, granuloma develops and mycobacteria can further survive inside (Flynn and Chan, 2001; 2003). ManLAM has been shown to be released by infected macrophages and can be easily detected in serum of infected individuals (Hamasur *et al.*, 2001). Furthermore, purified ManLAM has been shown to be a potent chemotactic factor for both $CD4^+$ and $CD8^+$ lymphocytes *in vitro* and an inhibitor of cytokines production by T-cells (Barnes *et al*., 1992). Therefore, ManLAM can be considered as one of the virulence factors (Briken *et al.*, 2004).

There are many papers showing 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). Production of the anti-inflammatory cytokine IL-10 leads to less efficient stimulation of T-cell responses but also induce a state of antigen-specific tolerance leading to T cell anergy (Briken *et al.*, 2004). This paper confirms previous results showing that ManLAM affects the TNF-α/IL-10 balance by upregulating IL-10, which is also considered as an antiapoptotic cytokine, influencing NO production (Roach *et al.*, 2002). In our study ManLAM-treated, BCG-infected macrophages still produced NO, although its levels were significantly lower then in BCG-infected cells without ManLAM treatment (Table III). Furthermore, a number of studies have conclusively demonstrated the importance of TNF-α in defense against *Mycobacterium* sp. in animal models (Flynn *et al*., 1995; Roach *et al*., 2002) and in human disease (Tramontana *et al*., 1995), although the mechanism whereby TNF-α deficiency promotes morbidity and mortality is not well understood.

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 previous study showed that ManLAM stabilized Bcl-2 expression in RAW 264.7 cells but it also led to a decrease in Bax expression (Krzyżowska *et al.*, 2007). Here we show that BCG-infected mouse macrophages showed some induction of Bcl-2 expression, at the level comparable to ManLAM treatment of control cells (Table III). Simultaneously, Bax expression induced by BCG infection was down-regulated by ManLAM (Table III). Klingler reported that the anti-apoptotic gene bcl-2 is down-regulated and apoptosis is increased in human PMBCs after infection with *M. bovis* BCG. However, the authors did not tested simultaneously levels of Bax expression (Klingler *et al.*, 1997).

Furthermore, another member of anti-apoptotic family Mcl-1 was up-regulated upon addition of ManLAM itself (Table III and IV). Mcl-1 was already shown to be up-regulated by virulent *M. tuberculosis* H37Rv strain (Sly *et al*., 2003). Here we show that this is the mechanism elaborated by a particular component of mycobacterial cell wall, such as lipoarabinomannan.

Interestingly, our results showed that BCG infection up-regulated ASK1/MAP3K5 kinase expression, while ManLAM reduced its activity. ASK1/MAP3K5 is an ubiquitously expressed enzyme that activates JNK and p38-MAP kinase pathways by direct site-specific Ser/Thr phosphorylation of their respective up-stream activating MAP kinase kinases – MKK4/MKK7 for JNK and MKK3/MKK6 for p38-MAP kinases. JNK and p38 are MAP kinases involved in apoptosis induction in response to cell stress such as nitrosative and oxidative stress, such as, for example, NO production during BCG infection (Shen and Liu, 2006). JNK pathway is strongly activated during BCG infection of mouse macrophages (own data, unpublished). Furthermore, JNK has been shown to phosphorylate, and inactivate, Bcl-2, Bcl-xl and Mcl-1. We can therefore assume that even though Bcl-2 is up-regulated during BCG infection, it can become a substrate to JNK kinases and inactivation of this pathway leads not only to down-regulation of proinflammatory cytokines but also helps to diminish its proapoptotic properties. Another interesting finding was that BCG infection led to up-regulation of Fas expression

Although studying primary mouse macrophages response to BCG infection and ManLAM using macro- or microarray technology provides a unique opportunity to assess host-apoptotic response to mycobacteria or mycobacterial products, such as ManLAM, there are a number of important limitations to this study design. Although each sample was constructed from pooled RNA obtained from different experiments and fold change was used as a threshold to identify differentially expressed genes, there are no means to measure the statistical relevance of the results (Kaminski and Friedman, 2002). In addition, there is no consensus as to the threshold level for fold change, which typically varies between 2 and 3 but can be as low as 1.7 (Kaminski and Friedman, 2002; Reiner *et al*., 2003). Furthermore, the use of nylon macroarrays does not lead to the thresholds and sensitivity similar for the results obtained from microarrays

(Kaminski and Friedman, 2002). Therefore, we could observe differences between the fold inductions obtained in macroarray and in real-time PCR for strongly up-regulated genes (Table IV).

The third day after infection was chosen to capture transcriptional events leading to apoptosis, as this is the day when apoptosis reached its peak during BCG infection of primary mouse macrophages (Fig. 1). A multiple time point experiment would help to characterize the kinetics of the transcriptional response to BCG and ManLAM. Finally, macroarrays may be limited in their ability to characterize the regulation of apoptotic machinery in the cell, as some genes and proteins involved in the signaling and execution of apoptosis are constitutively expressed and regulated by post-translational mechanisms. Despite these limitations, the results of this study provide insight into the macrophage response to ManLAM, demonstrating changes in apoptosis related gene expression consistent with previous functional data. The biological significance of any or all of these individual genes in determining the fate of infected cells and treated with ManLAM will only be revealed by functional studies.

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