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Mitogen-Activated Protein Kinases Mediate the Production of B-Cell Lymphoma 2 Protein by *Mycobacterium tuberculosis* in Monocytes

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Abstract—Changes in the levels of antiapoptotic protein B-cell lymphoma 2 (Bcl-2) protein has been reported in murine and human tuberculosis. We investigated the role of mitogen-activated protein kinase pathways in the production of Bcl-2 protein in THP-1 human monocytes infected with *Mycobacterium tuberculosis* H37Rv and H37Ra. Analysis of phosphorylation profiles of mitogen-activated protein kinase kinase-1, extracellular-signal regulated kinase 1/2, mitogen-activated protein kinase kinase 3/6, and p38 mitogen-activated protein kinase; B-cell lymphoma 2 kinetics; and tumor necrosis factor-α (TNF-α) secretion levels showed variation between the two strains. *Mycobacterium tuberculosis* H37Rv induced higher Bcl-2 and lower TNF-α levels, whereas H37Ra the reverse. The strains also differed in their usage of CD14 and human leukocyte antigen-DR receptors in mediating extracellular-signal regulated kinase 1/2 and p38 mitogen-activated protein kinase activation. *Mycobacterium tuberculosis* H37Rv- and H37Ra-induced Bcl-2 production was reduced by specific inhibitors of mitogen-activated protein kinase kinase-1 (PD98059) and p38 (SB203580), but increased by nuclear factor κB (NF-κB) inhibitor (BAY 11-7082). TNF-α production by both strains was reduced in the presence of specific inhibitors of mitogen-activated protein kinase kinase-1 (PD98059), p38 (SB203580), and NF-κB (BAY 11-7082). Furthermore, inhibition of NF-κB was accompanied by an increase in strain-induced extracellular-signal regulated kinase 1/2 phosphorylation. Collectively, these results indicate for the first time that the production of Bcl-2 and TNF-α by *M. tuberculosis* H37Rv/H37Ra-infected THP-1 human monocytes is mediated through mitogen-activated protein kinases and NF-κB.

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Key words: *M. tuberculosis*, ERK, p38 MAPK, Bcl-2 production, NF-κB

Monocytes/macrophages are the key target cells for a variety of pathogens, including mycobacteria, which invade macrophages and replicate intracellularly [1]. Despite the potential role of the macrophage in the eradication of microbes, pathogenic mycobacteria have survived down the ages as some of the most successful in evading macrophage surveillance mechanisms in a manner that ensures their survival and replication inside the macrophage. The mechanisms that contribute to immune evasion and survival of *Mycobacterium tuberculosis* within macrophages are (a) the inhibition of phagosome-lysosome fusion; (b) the inhibition of phagosome acidification; (c) the recruitment and retention of tryptophan-aspartate containing coat protein on phagosomes to prevent their delivery to lysosomes; (d) the expression of members of the host-induced repetitive glycine-rich pro-

Abbreviations: ANOVA, analysis of variance; AP-1, activator protein-1; BCG, bacillus Calmette–Guerin; CR, complement receptor; CREB, cAMP-response element-binding protein; DMSO, dimethylsulfoxide; ELISA, enzyme linked immunosorbent assay; ERK, extracellular signal regulated kinase; FCS, fetal calf serum; H37Ra, *M. tuberculosis* H37Ra; H37Rv, *M. tuberculosis* H37Rv; JNK or SAPK, c-jun N-terminal kinases or stress-activated protein kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MKK or MAPKK or MEK, MAPK kinases; MKP-1, MAPK phosphatase-1, NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PD98059, MEK inhibitor; PI3K, phosphatidylinositol 3-kinase; SB203580, p38 inhibitor; SRF, serum response factor; TLRs, Toll-like receptors; TNF-α, tumor necrosis factor-α.

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tein family of proteins; (e) modification of host proteins by phosphatases and kinases of the bacteria [2], and (f) the evasion of host cell apoptosis [3, 4]. An effective strategy adopted specifically by virulent mycobacteria for survival in the host is modulation of apoptotic signaling in macrophages because apoptosis has been associated with increased bacterial killing [5-7] and cross priming of antigens to $CD8^+$ T cells [8].

Previous reports show that *M. tuberculosis* H37Rv persist and replicate within macrophages by inducing less apoptosis than avirulent strain *M. tuberculosis* H37Ra [5, 9-13]. In general, virulent mycobacteria have been shown to induce many antiapoptotic genes, namely *bcl-2*, *bcl-xL*, *bfl-1*, and *mcl-1* in order to create a protective niche within infected cells [14-18]. Compared to *M. tuberculosis* H37Ra, H37Rv has been shown to induce lower level of apoptosis by upregulating antiapoptotic protein B-cell lymphoma 2 (Bcl-2) in murine macrophages [17] or bfl-1A1 in THP-1 cells [19, 20], and this has been associated with higher intracellular growth of H37Rv.

Among several cytokines induced by *M. tuberculosis*, tumor necrosis factor-α (TNF-α) plays an important role in the apoptosis of infected macrophages, structural maintenance of granuloma, and control of infection [12, 21]. Falcone et al. [22] showed that *M. tuberculosis* H37Rv infection in murine peritoneal macrophages is associated with induction of minimal TNF-α and increased intracellular multiplication when compared to avirulent strain *M. tuberculosis* H37Ra. It has also been suggested that inhibition of TNF-α-mediated macrophage apoptosis is a virulence strategy of *M. tuberculosis*. Avirulent H37Ra induced TNF-α-dependent macrophage apoptosis, while virulent H37Rv released soluble TNFR2 that reduced TNF-α activity and subsequent apoptosis of macrophages [3]. Even though the role of Bcl-2 and TNF-α in apoptosis of *M. tuberculosis*-infected macrophages/monocytes has been studied in detail, studies describing the signaling pathways, which mediate the induction of these effector molecules by *M. tuberculosis* H37Rv and H37Ra, are scarce. Maiti et al. [11] have demonstrated the phosphatidylinositol 3-kinase (PI-3K)-dependent phosphorylation of proapoptotic protein Bad by man-LAM from the virulent Erdman strain of *M. tuberculosis* in THP-1 cells. Song et al. [23] have shown that both extracellular-signal regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinases (MAPKs) are essential for *M. tuberculosis* H37Rv-induced TNF-α production in primary human monocytes.

Therefore, we have investigated the role of the MAPK signaling pathway in the production of Bcl-2 and TNF-α by *M. tuberculosis* H37Rv and H37Ra-infected THP-1 cells. We have chosen the MAPK pathway because this is one of the evolutionarily conserved phosphorylation-regulated protein kinase cascades, which is involved in controlling the decision of cell survival or cell death. The p38 MAPK with α, β, γ, and δ isoforms is primarily induced in response to cellular stress, osmolarity, heat shock, UV irradiation, and inflammatory cytokines. It plays a major role in stress-induced apoptosis [24, 25]. ERK, which has p44 (ERK1) and p42 (ERK2) isoforms, is mainly activated by growth factors and phorbol esters. It plays an important role in cell survival and differentiation [25, 26]. Moreover, *M. avium* has been shown to mediate caspase 8 activation and macrophage apoptosis through p38 MAPK [27]. Our data show that ERK, p38 MAPK, and nuclear factor κB (NF-κB) are essential for Bcl-2 and TNF-α production induced by *M. tuberculosis* H37Rv and H37Ra.

MATERIALS AND METHODS

Reagents. Antibodies against total and phosphorylated forms of MAPKs were purchased from Cell Signaling Technology (USA). PD98059 (Calbiochem Biosciences, USA), an inhibitor of mitogen-activated protein kinase kinase-1 (MKK1 or MEK1), selectively blocks the activity of ERK MAPK and has no effect on the activity of other serine threonine protein kinases including Raf-1, p38, and c-jun N-terminal kinases or stress-activated protein kinase (JNK or SAPK) MAPKs, or protein kinase C and protein kinase A. The pyridinyl imidazole SB203580 (Calbiochem), a potent inhibitor of p38 MAPK, has no significant effect on the activity of the ERK or JNK MAPK subgroups. BAY 11-7082 (NF-κB inhibitor) was also purchased from Calbiochem Biosciences. Rabbit polyclonal anti-human Bcl-2 Ab was from BD Biosciences (USA). Horseradish peroxidase-linked secondary antibodies and polyvinylidene difluoride membrane were from Amersham Biosciences (USA). SuperSignal West Pico chemiluminescent substrate was from Pierce (USA). Dimethylsulfoxide (DMSO) was from Sigma Chemicals (USA). Middlebrook 7H9 medium was from Difco (USA). Endotoxin-free fetal calf serum (FCS), RPMI 1640 (with glutamine and HEPES), albumin-dextrose-catalase supplement, antibiotics, and phosphate-buffered saline (PBS), pH 7.2, were from Invitrogen Corporation (USA). Human anti-CD 14 Ab, anti-(Human Leukocyte Antigen) HLA-DR Ab, and appropriate IgG1 isotype control Ab were from BD PharMingen (Germany).

Infection studies. Standard laboratory strains *M. tuberculosis* H37Rv (virulent) and H37Ra (avirulent) were included in the study. Processing of mycobacterial strains, maintenance of THP-1 cell culture, infection and preparation of cell lysates, determination of MAP kinase phosphorylation through western immunoblotting, etc. were done as described in Natarajan and Narayanan [28]. Briefly, the strains were grown to mid-log phase in 7H9 medium with albumin-dextrose complex. The bacterial suspension was washed and resuspended in RPMI containing 10% FCS. Bacterial clumps were disaggregated by

vortexing and passing through a 27 gauge needle. The total number of bacilli per milliliter of suspension was ascertained by assessing colony forming units on agar plates and simultaneously counting in a Thoma counting chamber.

THP-1 human monocytes grown in RPMI containing 10% FCS were left untreated or treated with *M. tuberculosis* H37Rv and H37Ra (bacteria/host cell 10 : 1) for various lengths of time in the presence or absence of pathway inhibitors. The inhibitors were added to the cell cultures at a final DMSO concentration of 0.1%. About 0.5 million cells were lysed with 100 μl of 2× sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 100 mM DTT, and 0.05% bromophenol blue) and denatured at 95°C for 5 min.

The cellular proteins were analyzed through 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting for the presence of phospho-MEK1/2 (p-MEK1/2), or phospho-ERK1/2 (p-ERK1/2), or phospho-mitogen-activated protein kinase kinase 3/6 (p-MKK3/6), or phospho-p38 (p-p38). Each of the blots was also probed with antibodies against their corresponding non-phosphorylated forms to ensure equal loading of protein in all the lanes. In experiments involving kinetics of Bcl-2 protein, the infected cultures were treated without inhibitors for 12 and 24 h and with 40 μM PD98059 (MEK1 inhibitor), or 30 μM SB203580 (p38 MAPK inhibitor), or 5 μM BAY 11-7082 (NF-κB inhibitor) for 24 h. These lysates were subsequently probed with anti-human Bcl-2 Ab. All the blots were analyzed using GS 700 Imaging Densitometer to confirm the differences in the intensity of the blots (Bio-Rad, USA). To show that the stimulatory capacity of mycobacteria was not the result of contamination with lipopolysaccharide (LPS), we added the specific LPS-inhibiting oligopeptide polymyxin B (10 μg/ml) before mycobacterial stimulation. Viability of THP-1 cells as assessed by trypan blue dye exclusion test was always >99%.

Cytokine measurement in culture supernatants. In experiments involving serial kinetic measurements of TNF-α, the THP-1 cells ($1 \cdot 10^6$/ml) were challenged with *M. tuberculosis* strains (multiplicity of infection (MOI) of 10 : 1) for 12 or 24 h. The cell-free supernatants were stored for cytokine estimation. In inhibition experiments, the THP-1 cells ($1 \cdot 10^6$/ml) were left untreated or first treated with 40 μM PD98059 (MEK1 inhibitor), or 30 μM SB203580 (p38 MAPK inhibitor), or 5 μM BAY 11-7082 (NF-κB inhibitor) for 60 min before infection, and once infected with *M. tuberculosis* strains the cultures were left for 12 h. To serve as vehicle control, the volume of the diluent DMSO (0.1% v/v) contained in 40 μM PD98059, or 30 μM SB203580, or 5 μM BAY 11-7082 (NF-κB inhibitor) was added to the control cultures. The viability of the infected monolayers versus an uninfected control was monitored by the trypan blue dye exclusion method and found to be unaffected in all of the experiments described. The cell-free supernatants were removed and assayed for TNF-α by enzyme linked immunosorbent assay using the human BD OPTEIA cytokine assay kits according to the manufacturer's protocol. The lower limit of detection was 4.7 pg/ml.

Statistical analysis and data presentation. The data from independent experiments are presented as mean ± SD. Statistical evaluation of the difference in mean separation was performed by one-way analysis of variance (ANOVA) followed by the *post hoc* Tukey's test, and the *a priori* level of significance at 95% confidence level was considered at $P < 0.05$.

RESULTS

***Mycobacterium tuberculosis* H37Rv and H37Ra infection leads to differential activation of MAPKs in THP-1 human monocytes.** To study the phosphorylation profile of the MAPKs, we challenged THP-1 human monocytes with *M. tuberculosis* H37Rv and H37Ra at MOI of 10 : 1. Time-dependent phosphorylation of ERK1/2 and its upstream activator MEK1/2, p38 and its upstream activator MKK3/6 was observed. We found that the peak activation of phosphorylated forms of MEK1/2, ERK1/2, MKK3/6, and p38 MAPKs occurs at 45, 45, 60, and 45 min for H37Rv; and at 60, 60, 30, and 60 min for H37Ra, respectively (Figs. 1, 2A, and 2B). So, the time point at which peak activation occurs for corresponding p-MAPKs is different between *M. tuberculosis* H37Rv and H37Ra. Total MEK, ERK, p38, and MKK3 levels remained consistent throughout the infections (bottom rows of (a) and (c) in Figs. 2A and 2B), indicating that phosphorylation was specific to the external stimuli by the mycobacteria.

Role of CD14 and HLA-DR receptors in *M. tuberculosis*-induced MAP kinase phosphorylation in THP-1 human monocytes. To determine which receptor mediates MAPK activation by *M. tuberculosis* strains, we analyzed MAPK phosphorylation (ERK1/2 and p38) in the presence or the absence of inhibitory Abs against CD14 or against the HLA-DR receptors in THP-1 human monocytes. An IgG1 isotype control Ab was used as a negative control at the same concentration as anti-CD14 Abs. In serial kinetic studies it was observed that the peak phosphorylation signal of MAP kinases occurred at different time points for *M. tuberculosis* H37Rv and H37Ra. Hence the cells were lysed during those peak time points in this experiment for assessing phosphorylation of MAPKs through the receptors (Figs. 2A and 2B). Densitometric analysis of the blots was done to confirm the degree of alteration in the intensity of phosphorylation both in the presence or absence of receptor blocking antibodies (data not shown). Total ERK or p38 levels remained consistent throughout the infections, indicating that phosphorylation was specific to the external stimuli by the mycobac-

teria (Fig. 3, b and d). It was observed that preincubation with anti-CD14 Ab resulted in a reduction (~10%) of the *M. tuberculosis* H37Rv-induced ERK1/2 phosphorylation, whereas anti-HLA-DR Ab did not show a significant effect. In contrast, *M. tuberculosis* H37Rv-induced p38 phosphorylation was greatly reduced by blocking either of the receptors – the reduction was 32 and 66% with anti-CD14 Ab and anti-HLA-DR Ab, respectively. *Mycobacterium tuberculosis* H37Ra-induced ERK1/2 activation was decreased to a great extent by 79 and 74% with anti-CD14 and anti-HLA-DR Abs, respectively. The phosphorylation of p38 also decreased by 10% with anti-HLA-DR Ab, but did not change with anti-CD14. An IgG1 isotype control Ab used at the same concentration did not affect either H37Rv or H37Ra-induced ERK/p38 phosphorylation (Fig. 3, a and c).

Production of Bcl-2 protein by different strains of *M. tuberculosis* was time-dependent. Bcl-2 is an essential anti-apoptotic intracellular regulatory molecule that rescues cell from apoptosis and prolongs cell survival. Zhang et al. [17] has already shown differential regulation of Bcl-2 and apoptosis between *M. tuberculosis* H37Rv and H37Ra-infected macrophages. Our studies on kinetics of Bcl-2 production showed that the induction caused by strain *M. tuberculosis* H37Rv and H37Ra remained closer to control values till 12 h. From 12 to 24 h, the induction by *M. tuberculosis* H37Rv raised twofold higher than control values, but the induction by *M. tuberculosis* H37Ra decreased by 50% compared to control values (Fig. 4, a and b).

Kinetics of *M. tuberculosis*-induced TNF-α in THP-1 cells. It has been already reported that *M. tuberculosis* H37Rv induce less TNF-α than H37Ra in murine peri-

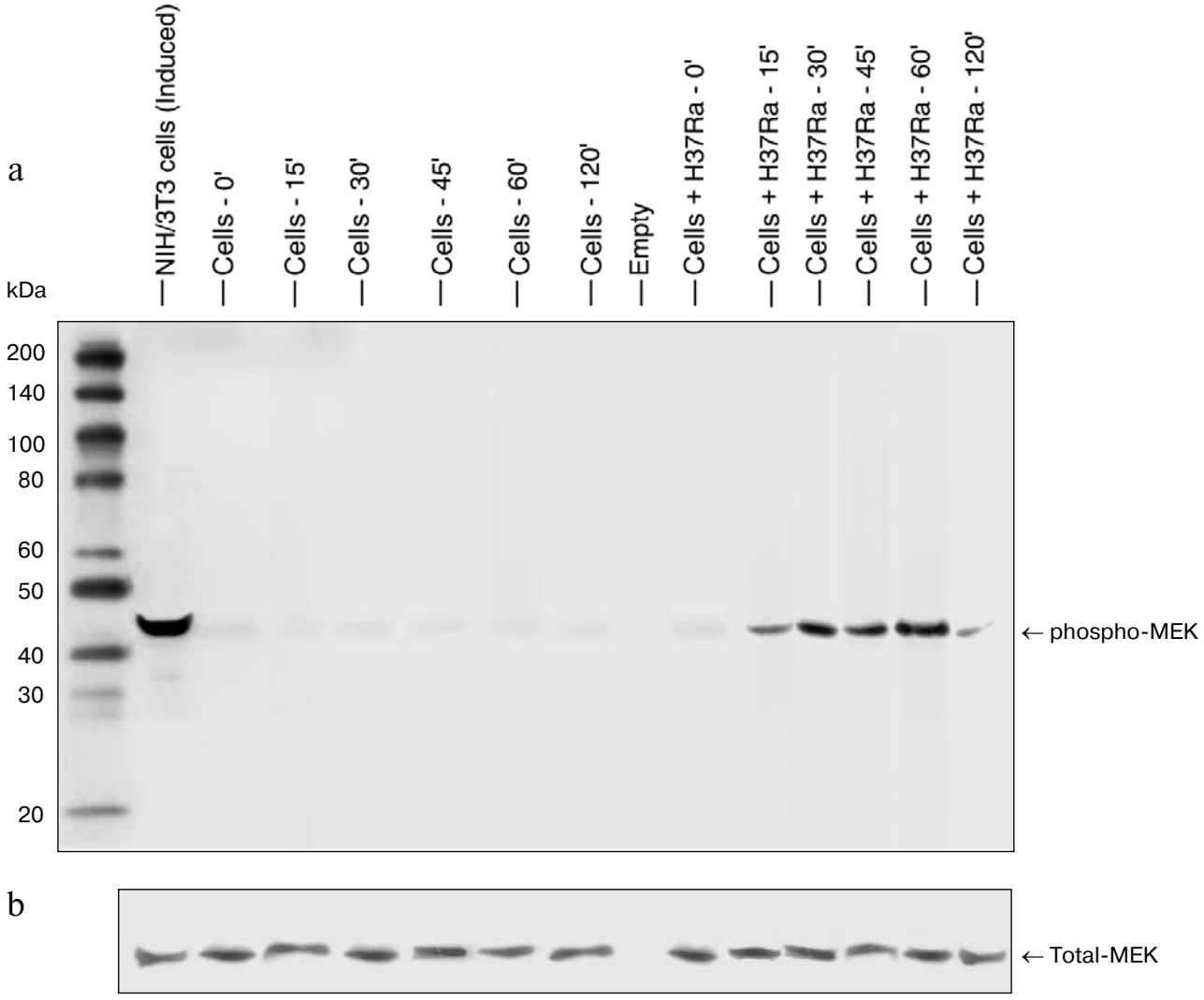

Fig. 1. Activation of MEK1/2 in THP-1 human monocytes in response to infection with *M. tuberculosis* H37Ra. THP-1 human monocytes were left untreated or treated with *M. tuberculosis* H37Ra (bacteria/host cell 10 : 1) for various lengths of time (indicated in minutes at top) (a). Cellular extracts were analyzed by western blotting for the presence of p-MEK1/2. The blot was stripped and re-probed with antibodies against total MEK (b) to ensure equal loading of protein in all the lanes. Induced NIH/3T3 cell extracts were used as a positive control.

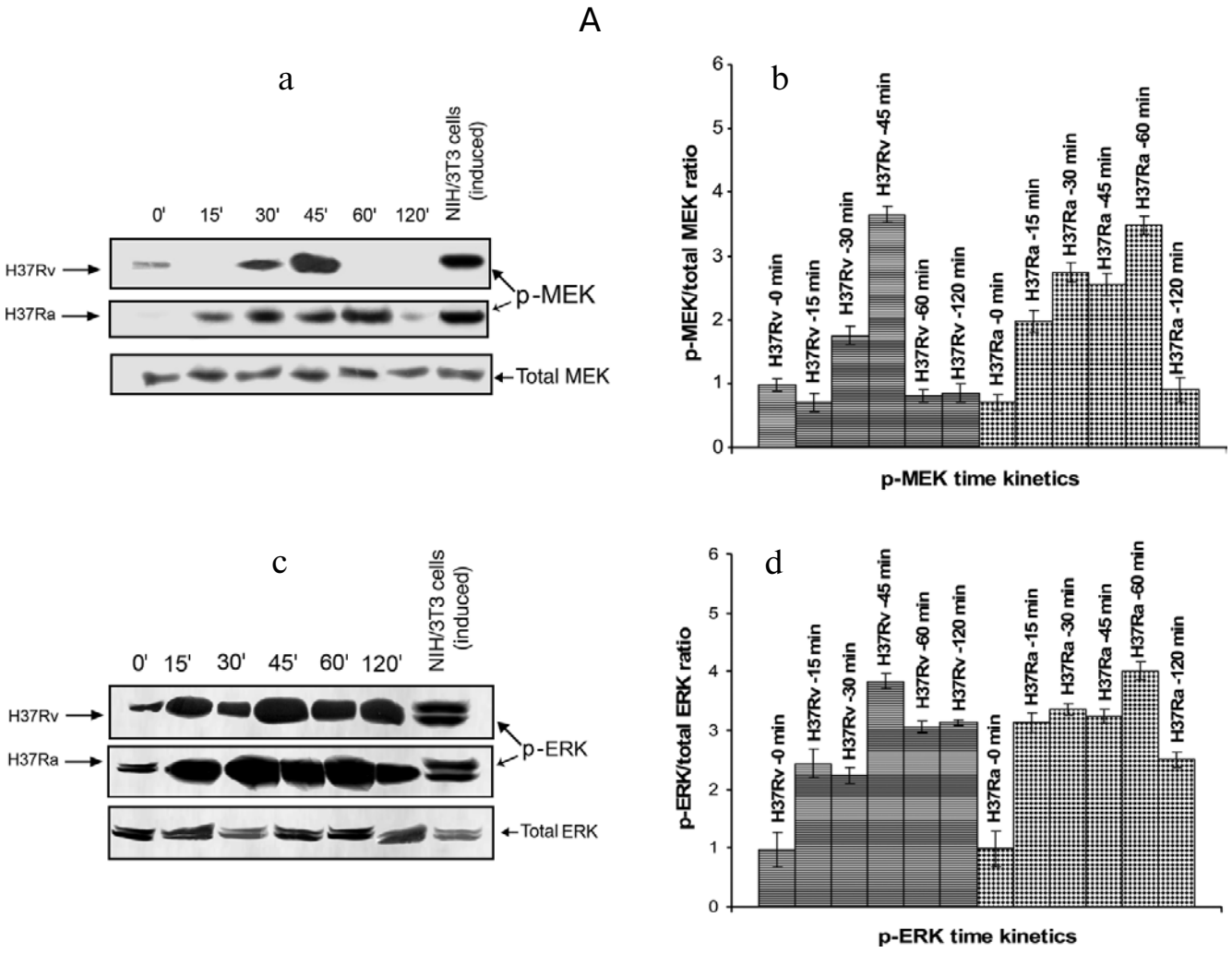

Fig. 2A. Activation of MEK1/2 and ERK1/2 in THP-1 human monocytes in response to infection with *M. tuberculosis* strains. THP-1 human monocytes were treated with *M. tuberculosis* H37Rv or H37Ra (bacteria/host cell 10 : 1) for various lengths of time (indicated in minutes at top). Cellular extracts were analyzed by western blotting for the presence of p-MEK1/2 (a) or p-ERK1/2 (c). Each of the blots was also stripped and re-probed with antibodies against total MEK or ERK (bottom rows in (a) and (c)) to ensure equal loading of protein in all the lanes. Right panels (b, d) show the corresponding densitometric analyses of blots probed with corresponding phospho-antibodies. Data shown are the mean of five independent experiments. Induced NIH/3T3 cell extracts were used as a positive control.

toneal macrophages [22]. So, to determine whether virulent and avirulent strains of *M. tuberculosis* are inducing different levels of proinflammatory cytokine following infection in THP-1 cells, the production of TNF-α was measured at 12 and 24 h postinfection. The kinetics of TNF-α secretion induced by the strains showed that the induction by H37Ra was higher than H37Rv at both time points, and there was always a significant decrease in the secretion from 12 to 24 h (see further Fig. 8a).

***Mycobacterium tuberculosis*-induced phosphorylation of ERK1/2 and p38 was inhibited by corresponding MAPK-specific inhibitors PD98059 and SB203580.** From the serial kinetic studies of different MAPKs carried out in *M. tuberculosis* H37Rv or H37Ra-infected THP-1 cells, we observed that the peak activation of phosphorylated forms of ERK1/2 and p38 MAPKs occurs at 45 and 45 min for H37Rv; and at 60 and 60 min for H37Ra, respectively (Figs. 2A and 2B). To determine whether MAPK-specific inhibitors inhibit *M. tuberculosis*-induced phosphorylation of MAPKs in THP-1 cells, the cells were infected with *M. tuberculosis* H37Rv or H37Ra in the presence of specific inhibitors, and immunoblotting of p-ERK1/2 (Fig. 5a) and p-p38 (Fig. 5c) was performed after terminating the cultures at the peak time points indicated above. It was observed that the phosphorylation of ERK1/2 and p38 induced by both *M. tuberculosis* H37Rv and H37Ra was reduced by PD98059 and SB203580, respectively. The observed inhibition was not due to DMSO as DMSO alone did not exhibit any inhibitory effects at this concentration (0.1%) (Fig. 5).

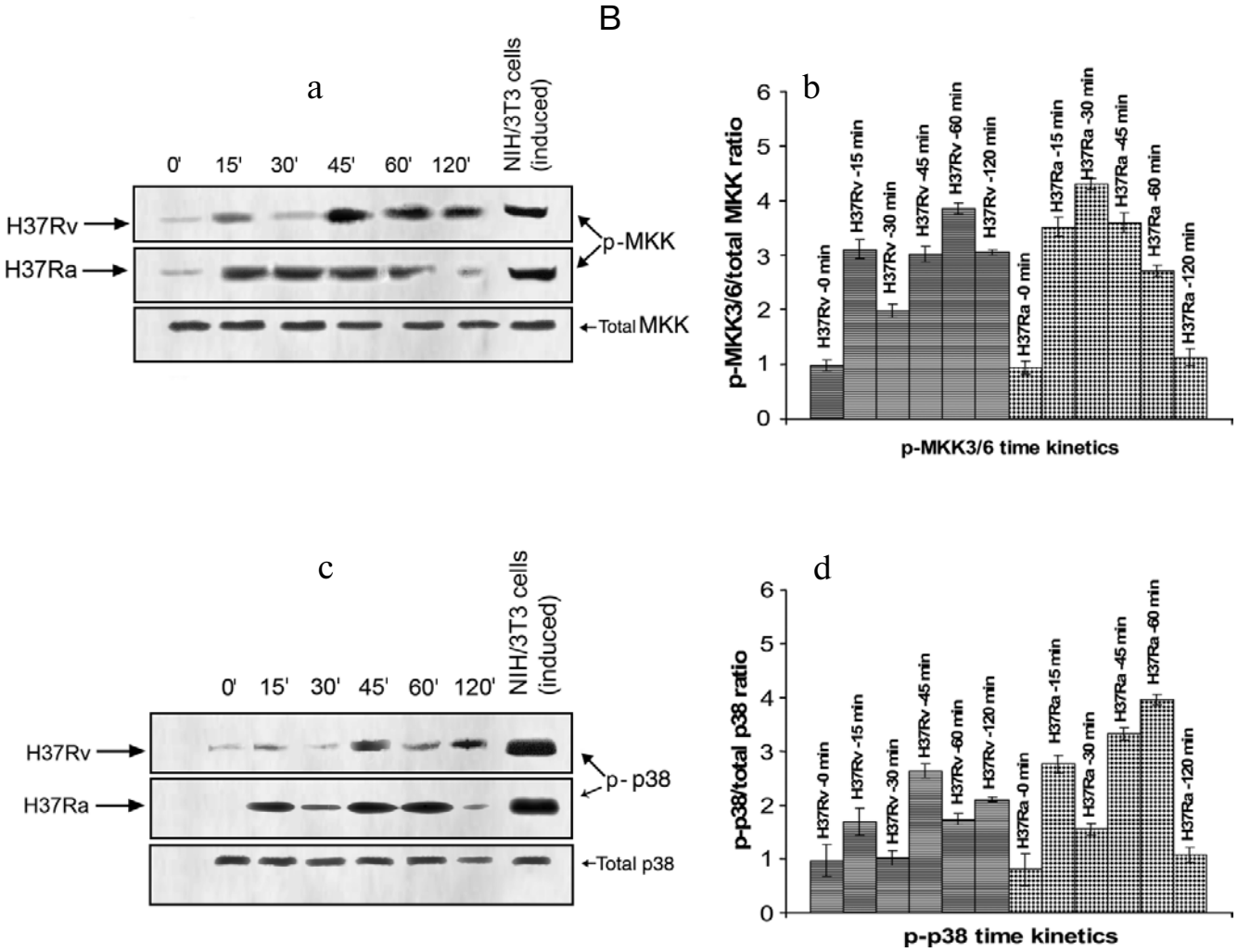

Fig. 2B. Activation of MKK3/6 and p38 in THP-1 human monocytes in response to infection with *M. tuberculosis* strains. THP-1 human monocytes were treated with *M. tuberculosis* H37Rv or H37Ra (bacteria/host cell 10 : 1) for various lengths of time (indicated in minutes at top). Cellular extracts were analyzed by western blotting for the presence of p-MKK3/6 (a) or p-p38 (c). Each of the blots was also stripped and re-probed with antibodies against total MKK or p38 (bottom rows in (a) and (c)) to ensure equal loading of protein in all the lanes. Right panels (b, d) show the corresponding densitometric analyses of blots probed with corresponding phospho-antibodies. Data shown are the mean of five independent experiments. Induced NIH/3T3 cell extracts were used as a positive control.

ERK and p38 pathways are essential for *M. tuberculosis*-induced Bcl-2 production. Since there is no report linking MAPKs and Bcl-2 in *M. tuberculosis*-infected monocytes/macrophages, we were interested in studying the role of MAPKs in the induction of Bcl-2 by *M. tuberculosis* strains in THP-1 cells. Immunoblotting of Bcl-2 was performed after 24 h of infection in the presence of pathway inhibitors. The optimal time period of 24 h postinfection was chosen because both strains induced varying Bcl-2 levels only at 24 h in Bcl-2 kinetic studies (Fig. 4). Densitometric analysis of the Bcl-2 immunoblots showed that the induction of Bcl-2 caused by the *M. tuberculosis* strains decreased after inhibition of ERK1/2 or p38 pathways. With *M. tuberculosis* H37Rv, the decrease in Bcl-2 level was 78 and 53% by PD98059 and SB203580, correspondingly (Fig. 6a). For *M. tuberculosis* H37Ra, the reduction was 45 and 45% by the respective inhibitors (Fig. 6a).

Inhibition of NF-κB augments *M. tuberculosis*-induced ERK and Bcl-2 activation in THP-1 monocytes. Dhiman et al. [19] have shown that activation of NF-κB by *M. tuberculosis* H37Rv leads to upregulation of Bcl-2 family antiapoptotic member, bfl-1/A1, in THP-1 cells. To evaluate whether the production of Bcl-2 by *M. tuberculosis* strains is mediated through NF-κB, we analyzed Bcl-2 levels in the presence or the absence of 5 μM NF-κB inhibitor (BAY 11-7082) for 24 h in THP-1 cells. To ascertain the crosstalk if any between ERK1/2 and NF-κB, ERK1/2 phosphorylation was also evaluated in the same cellular lysates (Fig. 7a). Densitometric analysis of

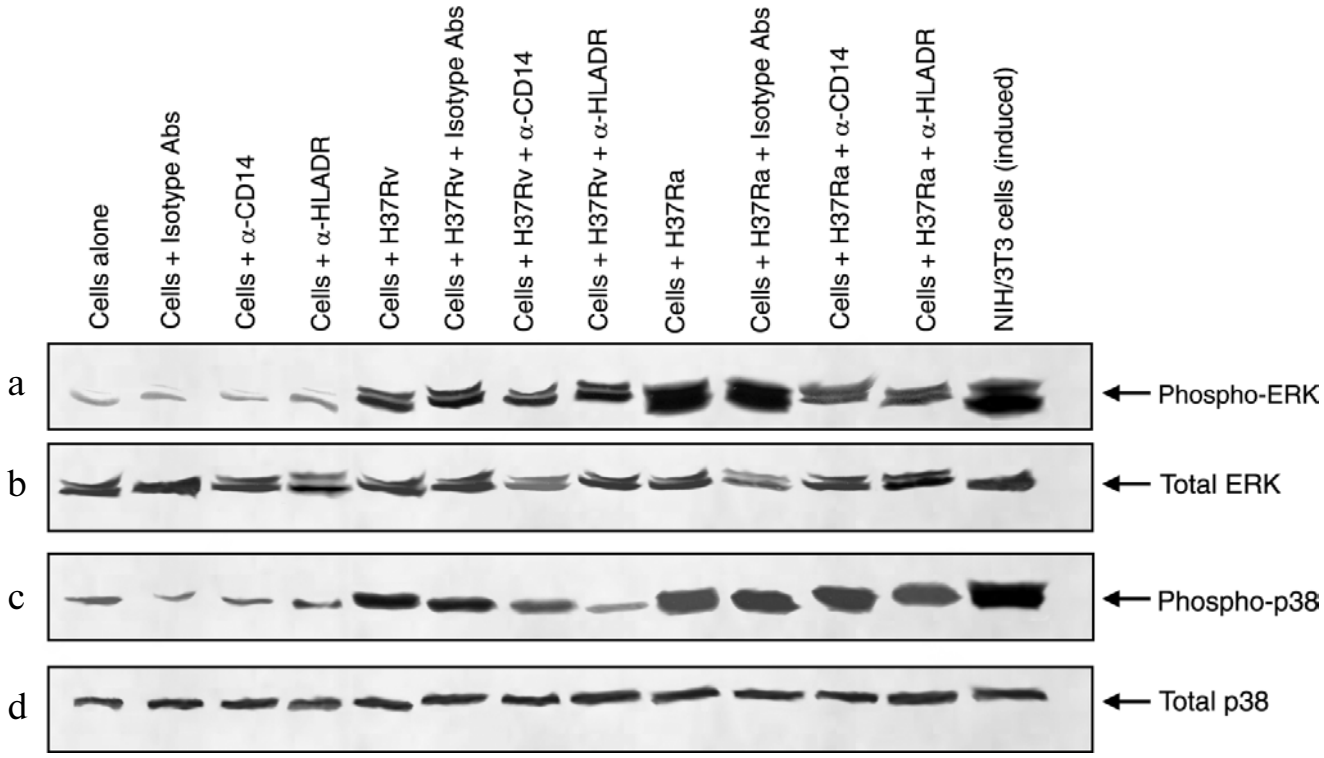

Fig. 3. Activation of ERK1/2 and p38 by *M. tuberculosis* strains through CD14 and HLA-DR receptors. THP-1 cells were preincubated with medium alone, or isotype control Abs, or anti-CD14 monoclonal Abs, or anti-HLA-DR monoclonal Abs (each 5 μg/ml) for 60 min. Subsequently, *M. tuberculosis* strains were added and the cells were lysed during those time points at which peak phosphorylation signal of ERK1/2 and p38 MAPKs occurred with *M. tuberculosis* H37Rv and H37Ra strains in our serial kinetic studies (Figs. 2A and 2B). The lysates were subjected to immunoblotting with specific anti-phospho-antibodies (a, c). The blots were stripped and re-probed with antibodies against total ERK or p38 (b, d) to ensure equal loading of protein in all the lanes. Similar data were obtained in three independent experiments performed in duplicate. Induced NIH/3T3 cell extracts were used as a positive control.

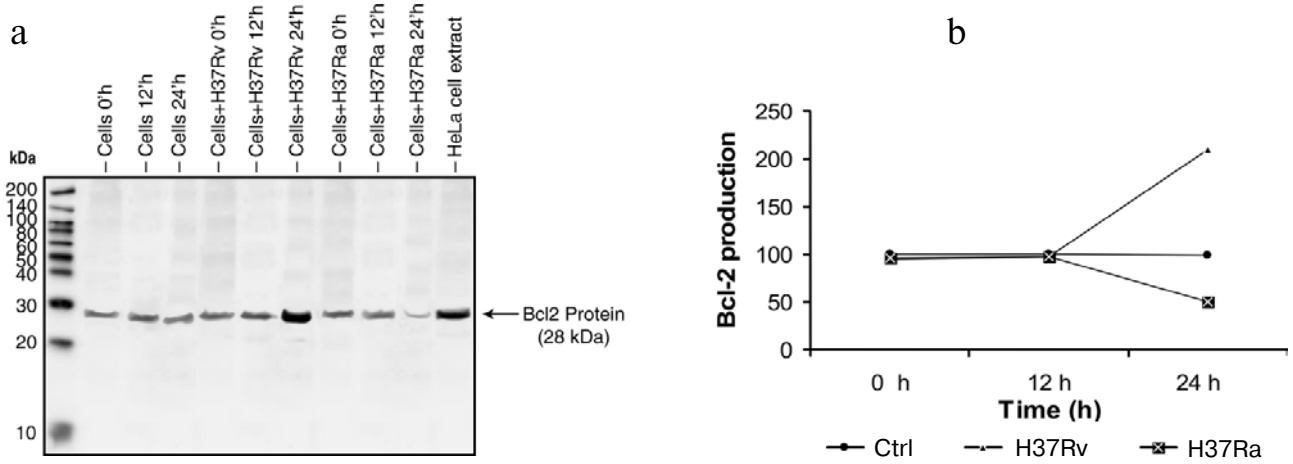

Fig. 4. Analysis of Bcl-2 kinetics by western blotting. THP-1 cells were left untreated or treated with *M. tuberculosis* H37Rv or H37Ra (bacteria/host cell 10 : 1) for 12 and 24 h. The harvested cellular lysates were subjected to western blot analysis with anti-human Bcl-2 antibody (a). Right panel shows the corresponding densitometric analyses of the blots (b). Data shown are the mean of five independent experiments. HeLa cell extracts were also blotted.

the blots revealed that the induction of both Bcl-2 and p-ERK1/2 caused by the *M. tuberculosis* strains increased after inhibition of NF-κB. While there was no change in the uninfected cultures in the presence of the NF-κB inhibitor, *M. tuberculosis* H37Rv-induced p-ERK1/2 and Bcl-2 increased by <2-fold and >2-fold, respectively. With *M. tuberculosis* H37Ra strain, the presence of inhibitor led to an increase of p-ERK1/2 and Bcl-2 by <2-fold (Fig. 7, b and c).

Effect of pathway inhibitors over *M. tuberculosis*-induced TNF-α in THP-1 cells. Since we observed differences in strain-induced MAPK kinetics and TNF-α secretion levels (Figs. 2A, 2B, and 8), and also TNF-α has been shown to be vital for induction of apoptosis/necrosis of *M. tuberculosis*-infected macrophages [17, 29, 30], we were interested in studying the MAPKs involved in strain-induced TNF-α secretion in THP-1 cells. The secretion of TNF-α was assessed both in the presence or the absence of PD98059 and SB203580 or BAY 11-7082, in THP-1 cells, at 12 h. The optimal time period of 12 h postinfection was chosen because both strains induced maximum TNF-α production only at 12 h in TNF-α kinetic studies (Fig. 8a). Inhibition experiments showed that the production of TNF-α by *M. tuberculosis* H37Rv and H37Ra was significantly reduced by all the three inhibitors used – 40 μM PD98059, 30 μM SB203580 and 5 μM BAY 11-7082 inhibited 90, 86 and 44% for *M. tuberculosis* H37Rv (Fig. 8b) and 90, 87 and 62% for *M. tuberculosis* H37Ra (Fig. 8c), respectively. The observed inhibition was not due to DMSO, as DMSO alone did not exhibit any inhibitory effects at this concentration (0.1%). These results show that ERK1/2, p38 MAPK, and NF-κB are involved in the signaling of TNF-α production during *M. tuberculosis* infection of THP-1 cells.

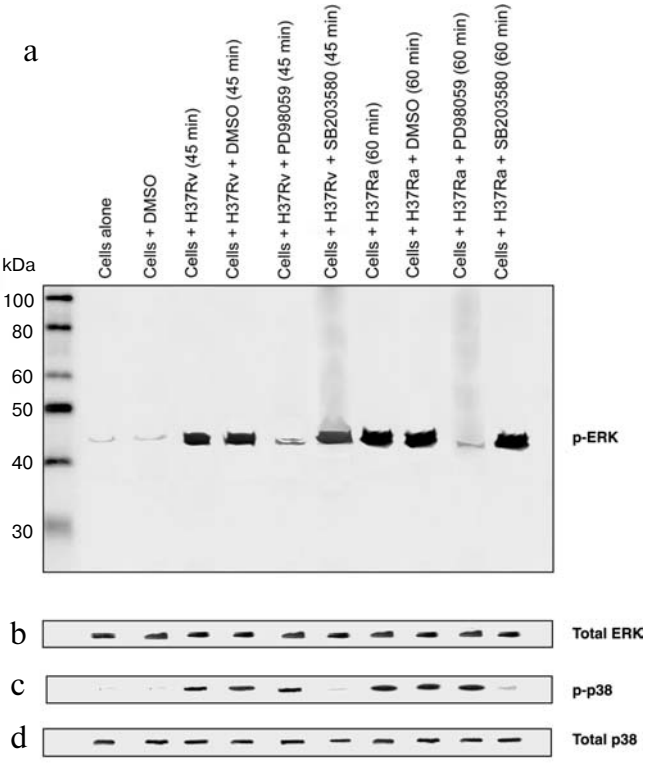

Fig. 5. Influence of pathway inhibitors over *M. tuberculosis*-induced MAPK activation in THP-1 cells. THP-1 cells ($1 \cdot 10^6$/ml) were left untreated or treated with vehicle (cells + DMSO) or with the MAPK-specific inhibitors PD98059 (40 μM) or SB203580 (30 μM) for 1 h prior to incubation with *M. tuberculosis* H37Rv or H37Ra (bacteria/host cell 10 : 1) for various lengths of time (indicated as 45 or 60 min). Cellular extracts were analyzed by western blotting for the presence of p-ERK1/2 (a) or p-p38 (c). Each of the blots was also stripped and re-probed with antibodies against total ERK (b) or p38 (d) to ensure equal loading of protein in all the lanes. Data shown are representative of four different experiments.

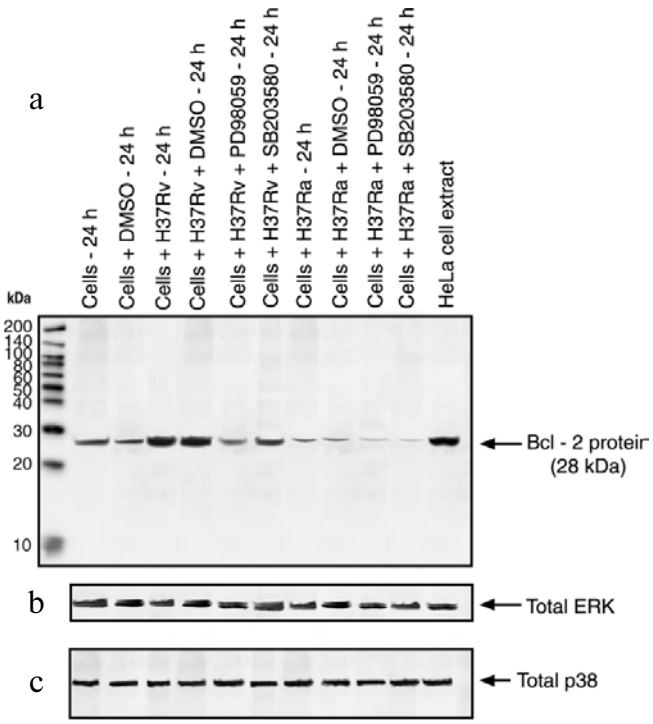

Fig. 6. Effect of pharmacological inhibitors of ERK and p38 MAPK on *M. tuberculosis* H37Rv or H37Ra-induced Bcl-2 production. THP-1 cells were untreated or treated with vehicle (0.1% DMSO), or 40 μM PD98059, or 30 μM SB203580 for 1 h prior to incubation with *M. tuberculosis* H37Rv or H37Ra, and terminated at 24 h. Cellular lysates were subjected to western blot analysis with anti-human Bcl-2 antibody (a). Each of the blots was also stripped and re-probed with antibodies against total ERK1/2 (b) or p38 (c) to ensure that these were present in equal amounts in all lanes (data from four independent experiments). Blots from HeLa cell extracts are also shown.

DISCUSSION

MAPK activation in macrophages/monocytes appears to play an important role in the production of

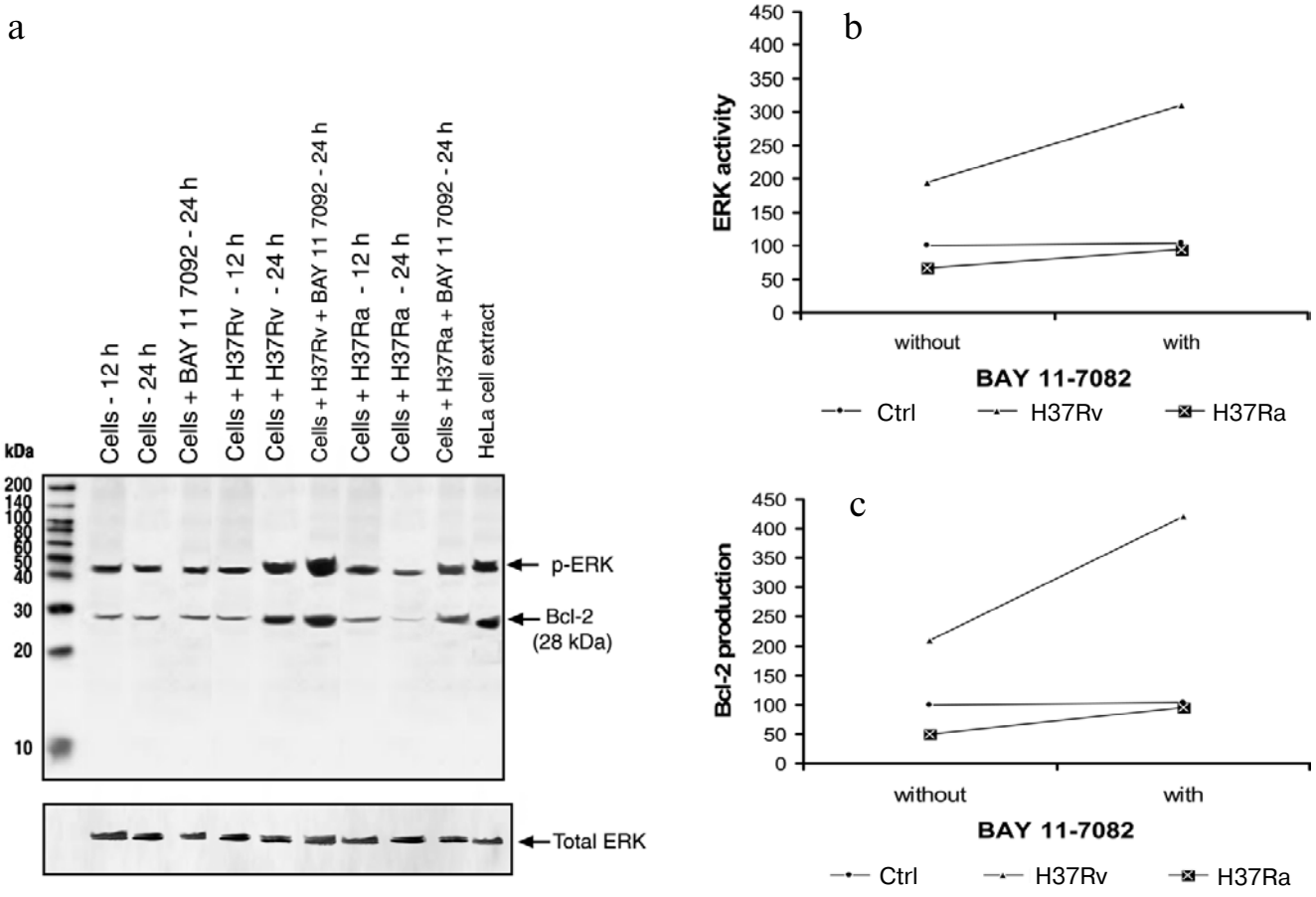

Fig. 7. Effect of BAY 11-7082 on *M. tuberculosis*-induced ERK activation and Bcl-2 production. THP-1 cells were left untreated or treated with *M. tuberculosis* H37Rv or H37Ra (bacteria/host cell 10 : 1) for 12 and 24 h. Cells were also separately treated with 5 μM BAY 11-7082 (NF-κB inhibitor) for 60 min before stimulation with *M. tuberculosis* H37Rv or H37Ra (bacteria/host cell 10 : 1) for 24 h. The harvested cellular lysates were subjected to western blot analysis with anti-human p-ERK and anti-human Bcl-2 antibodies (a). Each of the blots was stripped and re-probed with antibodies against total ERK (bottom row of panel (a)) to ensure equal loading of protein in all the lanes. The graphs on right panel show the corresponding densitometric analyses of p-ERK (b) and Bcl-2 (c) blots obtained from cultures infected for 24 h, in the absence and presence of BAY 11-7082. Data shown are the mean of three independent experiments performed in triplicate.

various effector molecules (cytokines, chemokines and reactive nitrogen intermediates) following a mycobacterial infection [31, 32]. But little is known about the role of MAPK activation during production of Bcl-2 and TNF-α by monocytes after infection with *M. tuberculosis* H37Rv and H37Ra. In the current study, we have demonstrated the following in THP-1 human monocytes: 1) *M. tuberculosis* H37Rv and H37Ra induces phosphorylation of MKK1 and MKK3/6; 2) the phosphorylation profiles of MKK1, ERK1/2, MKK3/6, and p38 MAPKs are different between H37Rv and H37Ra; 3) the contribution of CD14 and HLA-DR receptors in mediating H37Rv and H37Ra-induced ERK/p38 phosphorylation is strain-specific; 4) PD98059 and SB203580 inhibit ERK and p38 phosphorylation induced by *M. tuberculosis* H37Rv and H37Ra; 5) higher Bcl-2 and lower TNF-α levels are seen with *M. tuberculosis* H37Rv; 6) ERK, p38 MAPK, and NF-κB are involved in mediating both *M. tuberculosis* H37Rv and H37Ra-induced Bcl-2 and TNF-α production, and 7) inhibition of NF-κB led to increases in ERK phosphorylation and Bcl-2 production with both strains.

In recent years, THP-1 cells have been utilized extensively as a faithful model in the study of infection, host cell signaling, apoptosis, and intracellular survival of mycobacteria [11, 33, 34]. Upon induction by *M. tuberculosis* H37Rv and H37Ra, THP-1 cells showed distinct activation of MKK1 and MKK3/6 in a time-dependent manner. This is the first report that MAPKs, MKK1, and MKK3/6 are distinctly and rapidly phosphorylated by *M. tuberculosis* H37Rv and H37Ra in THP-1 cells. Our laboratory was also the first to demonstrate rapid phosphorylation of MKK3/6 and p38 by *M. tuberculosis* H37Rv in THP-1 cells [28]. Subsequent analysis of respective downstream kinases, namely ERK and p38, revealed that their phosphorylation profiles are also different between the strains. The fall and rise of the p38 MAPK activation observed in our study is consistent with the data of Song et al. [23] on infection of *M. tuberculosis* H37Rv in

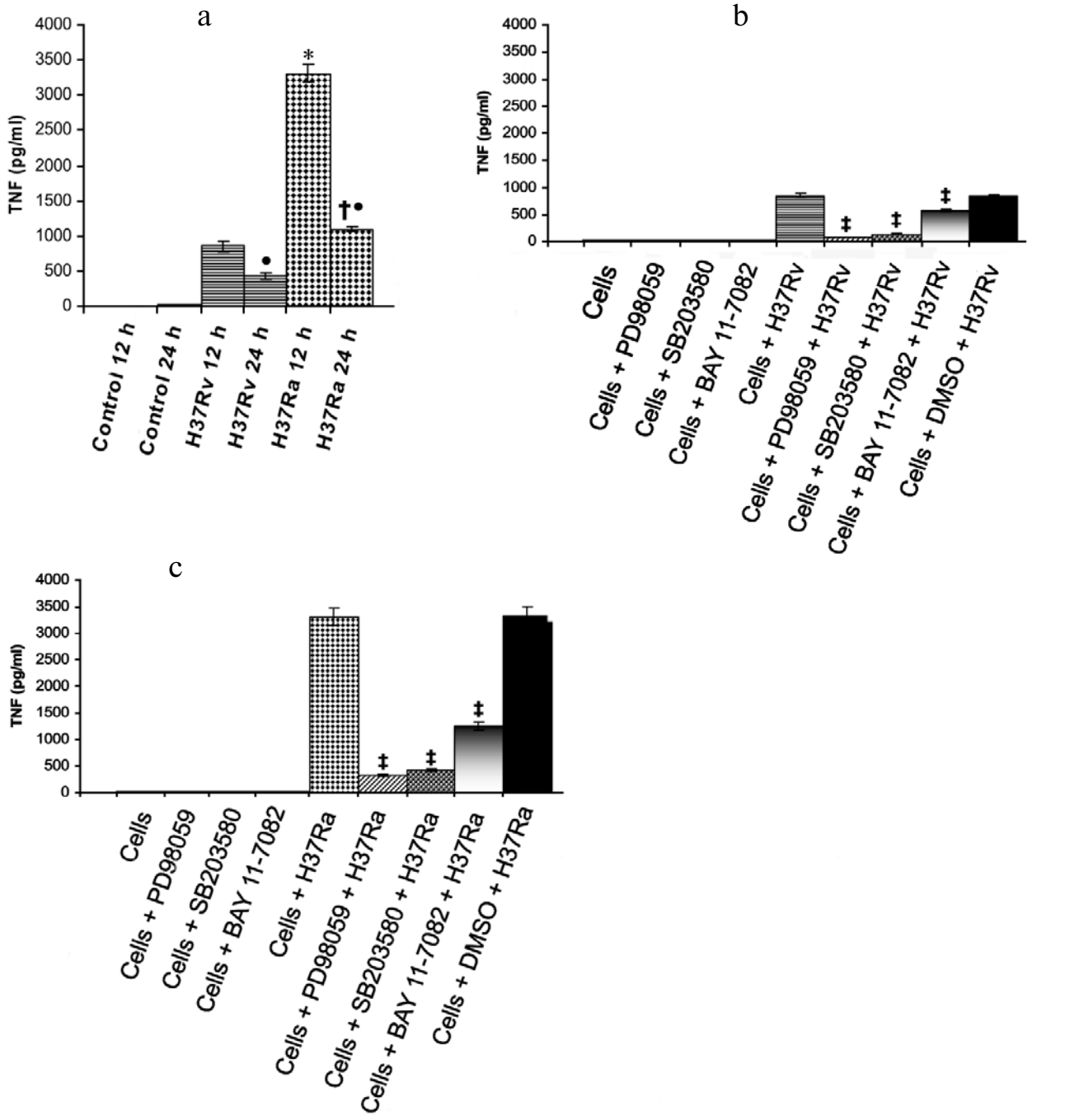

Fig. 8. Effect of pharmacological inhibitors of ERK, p38 MAPK, and NF-κB over *M. tuberculosis* H37Rv or H37Ra-induced TNF-α production. THP-1 cells were left untreated or treated with *M. tuberculosis* strains (bacteria/host cell 10 : 1) for 12 and 24 h (a). Cells were also separately treated with 40 μM PD98059, or 30 μM SB203580, or 5 μM BAY 11-7082, or 0.1% DMSO for 1 h prior to stimulation with *M. tuberculosis* H37Rv (b) or H37Ra (c) (bacteria/host cell 10 : 1) for 12 h. Supernatants were harvested and TNF-α formation was measured by ELISA. Data shown are the mean ± SD of five independent experiments performed in triplicate. TNF levels were significant as follows: * $P < 0.05$ when compared with *M. tuberculosis* H37Rv at 12 h time point; † $P < 0.05$ when compared with *M. tuberculosis* H37Rv at 24 h time point; • $P < 0.05$ when strain-induced TNF-α was compared between their corresponding 12 and 24 h values; ‡ $P < 0.05$ when the induction of strains was compared with the inhibitor-treated cultures at 12 h time point.

human peripheral blood monocytes. The observed differences in the duration and intensity of signaling might be due to alterations in the activation of scaffolding proteins or phosphatases [35-37] by various mycobacterial cell wall-associated virulence factors, like polymorphic PGRS domain, lipoarabinomannan/lipomannan ratio, etc. through various receptors. Since the importance of interaction of mycobacteria with host scaffolding protein

EBP50 (Ezrin/radixin/moesin family Binding Protein 50) was well documented in association with lowered iNOS (inducible nitric oxide synthase) recruitment to phagosome [38], these differences might influence the antimycobacterial mechanisms inside the macrophages. The involvement of phosphatase is supported by the data of Cheung et al. [37], which underscores MAPK phosphatase-1 (MKP-1) in modulating p38 MAPK and ERK1/2 in mycobacterium-infected macrophages.

Since CD14 and HLA-DR receptors have already been shown to mediate activation of MAPKs in immune cells in response to external stimuli [39, 40], we studied the usage of these receptors in mediating *M. tuberculosis* H37Rv and H37Ra-induced MAPK phosphorylation. Our experiments with anti-CD14 and anti-HLADR antibodies show that the receptor usage in mediating MAPK activation is *M. tuberculosis* strain-specific. To our knowledge this is the first report, which compares the usage of CD14 or HLA-DR receptor-mediated MAPK activation between *M. tuberculosis* H37Rv and H37Ra. The fact that the reduction of MAPK activation was not complete after blockage of a single receptor implies that other receptors like complement receptor 3 (CR3), mannose receptor, Toll-like receptors (TLRs), etc. are possibly involved in the activation of those MAPKs. The cross-talk between CD14 and CR3 receptors in the activation of PI-3K pathway during phagocytosis of *M. bovis* bacillus Calmette–Guerin (BCG) in THP-1 cells has already been reported [41]. On the other hand, virulent *M. tuberculosis* strains were shown to use both mannose and complement receptors for adherence and phagocytosis in contrast to attenuated strain, which uses only complement receptors [42]. So, it will be interesting to investigate whether and to what extent TLR or complement or mannose receptor-mediated signaling contributes to MAP kinase activation and cytokine formation by macrophages in response to intact virulent and avirulent *M. tuberculosis* strains.

Our kinetic studies with strain-induced TNF-α and Bcl-2 showed that *M. tuberculosis* H37Rv induced higher Bc1-2 and lower TNF-α levels when compared to *M. tuberculosis* H37Ra. This is consistent with the data of Falcone et al. [22] who showed inverse relation between virulence and the ability of the mycobacterial strains to induce TNF-α secretion by murine peritoneal macrophages. The higher induction of Bcl-2 by *M. tuberculosis* H37Rv is also in line with the data of Zhang et al. [17] in J774 macrophages.

Recently, it was shown that estrogen-mediated upregulation of Bcl-2 through ERK phosphorylation promotes survival of human THP-1 macrophages [43], and that *M. tuberculosis* H37Rv induces TNF-α production in human peripheral blood monocytes through activation of ERK1/2 and p38 MAPKs [23]. While dissecting the signaling mechanisms underlying Bcl-2 and TNF-α production by *M. tuberculosis* H37Rv and H37Ra in THP-1 cells using specific cell permeable inhibitors, we observed that *M. tuberculosis* H37Rv and H37Ra-induced Bcl-2 and TNF-α production was sensitive to pharmacological inhibition of ERK1/2, p38 MAPK, and NF-κB. Inhibition by PD98059 led to the highest reduction of Bcl-2 production induced by *M. tuberculosis* H37Rv, but the amount of reduction of *M. tuberculosis* H37Ra-induced Bcl-2 was equal with PD98059 and SB203580. This is the first ever report linking *M. tuberculosis* strains, MAPK activation, and Bcl-2 production. In the presence of NF-κB inhibitor (BAY 11-7082) there was an increase in *M. tuberculosis* H37Rv and H37Ra-induced Bcl-2 and p-ERK. The magnitude of increase in the induction of Bcl-2 and p-ERK was higher for *M. tuberculosis* H37Rv when compared to H37Ra. Similar to the cross-talk between p38 and ERK1/2 in *M. avium*-induced TNF-α [44], we report here for the first time the likelihood of a cross-talk between NF-κB and ERK1/2 in *M. tuberculosis*-induced Bcl-2 production. This cross-talk might play a role in *M. tuberculosis* strain-specific Bcl-2 production and cell survival.

The increase in strain-induced Bcl-2 observed with NF-κB inhibition is contrary to the report of Dhiman et al. [19], which showed reduction in *M. tuberculosis*-induced Bcl-2 family protein Bfl-1Al after inhibition of NF-κB. These conflicting results might be due to existence of different mechanisms that regulate bcl-2 and bfl-1A1 expression in mycobacteria-infected macrophages.

One possibility is that mycobacteria might modulate expression of the bcl-2 gene through ERK and transcription factors other than NF-κB. The potential transcription factors linked to ERK are activator protein-1 (AP-1) [45], serum response factor (SRF) [46, 47], cAMP-response element-binding protein (CREB) [48], etc. The other possibility is that the MEK/ERK pathway might alter bcl-2 translation through regulation of factors like eukaryotic initiation factor 4E-binding protein 1, as it is seen with regulation of another antiapoptotic protein Mcl-1 in acute myelogenous leukemia cells [49]. The above mechanisms may also be responsible for the strain-specific Bcl-2 levels observed in our Bcl-2 kinetic studies in the absence of inhibitors. The differences in strain-specific Bcl-2 levels seen in our Bcl-2 kinetic studies could also be due to usage of different pathways involving different transcription factors. In relation to this, Rajaram et al. [50] have recently demonstrated the usage of different pathways involving different transcription factors in the secretion of IL-8 by *M. tuberculosis* and attenuated *Mycobacterium bovis* BCG in macrophages.

In the case of TNF-α secretion by both strains, inhibition by PD98059 led to the highest reduction followed by SB203580 and BAY 11-7082. Additionally, the reduction caused by BAY 11-7082 was greater with *M. tuberculosis* H37Ra compared to H37Rv. These data reinforce the previous finding that secretion of TNF-α by *M. tuberculosis* H37Rv is mediated by ERK and p38 [23]. The fact

that ERK, p38, and NF-κB pathways are involved in both TNF-α and Bcl-2 production raises the possibility of an autocrine regulation of Bcl-2 by TNF-α in infected cells. This is supported by two reports, namely (1) prevention of the downregulation of Bcl-2 during *M. tuberculosis* infection by TNF-α blockade [29, 51] and (2) activation of MAPK and NF-κB through TNF receptor [52, 53].

From all these findings, it is reasonable to speculate that immediately after infection of THP-1 human monocytes, *M. tuberculosis* H37Rv and H37Ra differentially phosphorylate MEK-1, ERK1/2, MKK3/6, and p38 MAPKs; ERK1/2 and p38 MAPKs in turn might interact with NF-κB and other transcription factors, finally resulting in differential production of Bcl-2 and TNF-α. The differences in the contribution of ERK1/2, p38 MAPK, and NF-κB pathways seen in our inhibition experiments, in mediating *M. tuberculosis* H37Rv and H37Ra-induced Bcl-2 and TNF-α production, might play a role in the differential production of these effector molecules by these strains. Since studies with THP-1 cells have shown differences in NF-κB activation [19, 20] and in the ratio of pro-/antiapoptotic proteins [18] between virulent and attenuated/avirulent mycobacterial strains, future studies linking activation of MAPK or other signaling pathways with activation of transcription factors and pro-/antiapoptotic proteins ratio will elucidate the mechanisms of apoptosis during infection with virulent, avirulent, and clinical isolates of *M. tuberculosis*.

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