Mitogen-activated protein kinases mediate *Mycobacterium tuberculosis*–induced CD44 surface expression in monocytes

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CD44, an adhesion molecule, has been reported to be a binding site for *Mycobacterium tuberculosis* (*M. tuberculosis*) in macrophages and it also mediates mycobacterial phagocytosis, macrophage recruitment and protective immunity against pulmonary tuberculosis *in vivo*. However, the signalling pathways that are involved in *M. tuberculosis*–induced CD44 surface expression in monocytic cells are currently unknown. Exposure of THP-1 human monocytes to *M. tuberculosis* H37Rv and H37Ra induced distinct, time-dependent, phosphorylation of mitogen-activated protein kinase kinase-1, extracellular signal regulated kinase 1/2, mitogen-activated protein kinase kinase 3/6, p38 mitogen-activated protein kinase and c-jun N-terminal kinases. The strains also differed in their usage of CD14 and human leukocyte antigen-DR (HLA-DR) receptors in mediating mitogen-activated protein kinase activation. *M. tuberculosis* H37Rv strain induced lower CD44 surface expression and tumour necrosis factor-alpha levels, whereas H37Ra the reverse. Using highly specific inhibitors of mitogen-activated protein kinase kinase-1, p38 mitogen-activated protein kinase and c-jun N-terminal kinase, we report that inhibition of extracellular signal regulated kinase 1/2 and c-jun N-terminal kinases increases, but that inhibition of p38 mitogen-activated protein kinase decreases *M. tuberculosis*–induced CD44 surface expression in THP-1 human monocytes.

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1. Introduction

Resistance against tuberculosis depends on effective phagocytosis, timely cell migration and well-organized granuloma formation, processes which in turn are critically dependent on cytoskeletal rearrangements, cell–cell and cell–matrix adhesion (Kaufmann and Hess 2000; Flynn and Chan 2001; Pure and Cuff 2001). CD44 is a member of the hyaluronate receptor family of cell adhesion molecules, which has been linked to cytoskeletal elements (Goodison *et al.* 1999) and shown to play a selective role in controlling lymphocyte migration, extravasation of activated T cells into inflammatory sites (DeGrendele *et al.* 1997), macrophage fusion (Cui *et al.* 2006) and aggregation of macrophages and lymphocytes (Underhill and Dorfman 1978; Green *et al.* 1988). Interaction of CD44 with its ligand, hyaluronan (HA), in monocytic cells

Keywords. CD44 surface expression; MAPK; *M. tuberculosis*

Abbreviations used: Abs, antibodies; ANOVA, analysis of variance; AP-1, activator protein -1; BCG, *M. bovis* bacillus Calmette-Guérin; CaMK-II, calmodulin-dependent protein kinase II; CR3, complement receptor 3; dexamethasone, Dex–JNK inhibitor; DMSO, dimethylsulfoxide; DTT, dithiothreitol; EBP50, ezrin/radixin/moesin family binding protein 50; ECM, extracellular matrix; Egr-1, early growth response protein-1; ELISA, enzyme linked immunosorbent assay; ERK, extracellular signal regulated kinase; Ets/Elk, DNA binding nuclear phosphoprotein/Ets-domain transcription factor; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; Genistein, Gen–pan tyrosine kinase inhibitor; HA, hyaluronan; HLA-DR, human leukocyte antigen-DR; H37Ra, *M. tuberculosis* H37Ra; H37Rv, *M. tuberculosis* H37Rv; iNOS, inducible nitric oxide synthase; JNK, c-jun N-terminal kinases; LPS, lipopolysacharide; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase-1; MKK or MAPKK or MEK, MAPK kinases; MKP-1, MAPK phosphatase-1; MMP-9, matrix metalloproteinase-9 expression; MOI, multiplicity of infection; NF-κB, nuclear factor-kappa B; PBS, phosphate-buffered saline; PD98059, PD–MEK inhibitor; PE, phycoerythrin; PIM2, phosphatidyl-myo-inositol dimannosides; PI3K, phosphatidylinositol 3-kinase; SAPK, stress-activated protein kinase; SB203580, SB–p38 inhibitor; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLRs, Toll-like receptors; TNF-α, tumour necrosis factor-alpha; Wortmannin, Wort–PI3K inhibitor

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plays a critical role in cell migration, inflammation and immune responses (Siegelman *et al.* 1999; Pure and Cuff 2001). A potential role for CD44 in the immune response to *M. tuberculosis* was suggested by the observation that $CD44^{high}$-expressing T cells (memory T cells) accumulate in the lungs of mice during infection with this pathogen (Lyadova *et al.* 1998; Feng *et al.* 1999, 2000). Subsequently, in a CD44-deficient mice model, it was reported that CD44 mediates resistance against mycobacterial infection, presumably by promoting binding and phagocytosis of *M. tuberculosis* by macrophages, and migration of macrophages/lymphocytes to the site of infection (Leemans *et al.* 2003). Numerous other studies based on stimulation with several mycobacterial antigens (Ags) (Waters *et al.* 2003, 2009; Li *et al.* 2008; Giri *et al.* 2009) also support the role of CD44 in protective immunity against tuberculosis. However, the signalling pathways that mediate *M. tuberculosis*–induced CD44 surface expression in monocytic cells have not yet been reported.

Recent studies have identified the signalling cascades that are induced by mycobacterial strains in monocytes/macrophages, namely, phosphatidylinositol 3-kinase (PI3K), protein kinase C and mitogen-activated protein kinase (MAPK) cascades. MAPK activation in macrophages/monocytes appears to play an important role in the production of various effector molecules and also in the control of intracellular bacterial replication, following a mycobacterial infection (Blumenthal *et al.* 2002; Schorey and Cooper 2003; Koul *et al.* 2004). MAPKs represent highly conserved serine-threonine kinases that are activated by upstream MAPK kinases [MKK or MAPKK or MEK (e.g. mitogen-activated protein kinase kinase-1 or MEK1)] through a Th-XXX-Tyr phosphorylation motif and are critical for cell proliferation, differentiation and death, as well as for inflammatory responses. The MAPKs comprise three distinct subfamilies with multiple subisoforms: p38 MAPK with α, β, γ and δ isoforms; c-jun N-terminal kinases or stress-activated protein kinase (JNK or SAPK), with p46 and p54 as the main isoforms; and extracellular signal regulated kinase (ERK), which has p44 (ERK1) and p42 (ERK2) isoforms. The c-jun N-terminal kinase and p38 MAPK are primarily induced in response to cellular stress, osmolarity, heat shock, UV irradiation and also to inflammatory cytokines. ERK is mainly activated by growth factors and phorbol esters (Lewis *et al.* 1998; Cowan and Storey 2003).

The activation of some MAPK family members by *M. tuberculosis* H37Rv in human monocytes has already been reported. Song *et al.* (2003) demonstrated that both ERK and p38 MAPKs were essential for *M. tuberculosis* H37Rv–induced TNF-α production, whereas activation of the p38 MAPK pathway alone was essential for *M. tuberculosis* H37Rv–induced IL-10 production. Hasan *et al.* (2003) has documented the relation between mycobacterial virulence and MAP kinase signalling in human monocytes. But whether MAPKs mediate the surface expression of CD44 in *M. tuberculosis*–infected monocytes is not known. Among the signalling kinases, JNK (Gee *et al.* 2002), p38 MAPK (Gee *et al.* 2003) and PI3K (Guha and Mackman 2002) have been implicated in regulating lipopolysaccharide (LPS)-induced expression of CD44/inflammatory mediators in monocytes. So the aim of the present study is to elucidate the relation between activation of MAPKs and CD44 surface expression in *M. tuberculosis*–infected THP-1 monocytes. The data obtained by us, using MAPK-specific inhibitors, demonstrated that MAPKs modulate the surface expression of CD44 in *M. tuberculosis*–infected THP-1 monocytes.

2. Materials and methods

2.1 *Reagents*

Antibodies against total and phosphorylated forms of MAPKs were purchased from Cell Signaling Technology (Beverly, MA). PD98059 (Calbiochem, San Diego, CA), 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 JNK 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. Dexamethasone (Calbiochem) was used as an inhibitor of JNK activity. Genistein (tyrosine kinase inhibitor) and wortmannin [phosphatidylinositol 3 kinase (PI3K) inhibitor] were also purchased from Calbiochem Biosciences (San Diego, CA). Horseradish peroxidase-linked secondary antibodies, polyvinylidene difluoride membrane and Supersignal western pico chemiluminiscent substrate were from Pierce Laboratories (Rockford). Histopaque-1077 and dimethylsulfoxide (DMSO) were from Sigma Chemicals (St Louis, MO). Middlebrook 7H9 medium was from Difco laboratories (Sparks, MD). 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 (Carlsbad, CA). The TNF-α enzyme-linked immunosorbent assay (ELISA) kit were from BD Biosciences (San Jose, CA). Human anti-CD 14 Ab, anti-human leukocyte antigen-DR (HLA DR) Ab, appropriate IgG isotype control Abs and fluorochrome conjugated surface Abs were from BD PharMingen (Heidelberg, Germany).

2.2 *Infection studies*

Standard laboratory strains *M. tuberculosis* H37Rv and H37Ra have been 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 (2007). Briefly, the strains were grown to mid-log phase in 7H9 medium with albumin–dextrose complex. The bacterial suspension was washed and re-suspended in RPMI containing 10% FCS. Bacterial clumps were disaggregated by vortexing and passing them 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. A multiplicity of infection (MOI) of 10 was chosen because our standardization experiments and previous reports (Lee *et al.* 2006) showed increased cytolysis within 24 h of infection with MOIs greater than 10. Additionally, it has been already reported that high MOI-related cell death does not require TNF-α (Spira *et al.* 2003; Lee *et al.* 2006) and low MOI infection model does not mimic *in vivo* immune response (Silver *et al.* 1998; Lee and Kornfeld 2010; Welina *et al.* 2011). For receptor blocking studies, the cells were preincubated with anti-CD14 Abs or anti-HLA DR Abs, or isotype control Abs (5 μg/mL) and subsequently stimulated with *M. tuberculosis* strains (ratio of 10:1). These cultures were terminated at time points during which peak phosphorylation of MAPKs was present for individual strains in serial kinetic studies. Around 0.5 million cells were lysed with 100 μL of 2X sample buffer [125 mmol Tris (pH 6.8), 4% SDS, 20% glycerol, 100 mmol dithiothreitol (DTT), and 0.05% bromophenol blue], and denatured at 95°C for 5 min. The cellular proteins were analysed through 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, for the presence of p-MEK1/2, p-ERK1/2, p-MKK3/6, p-p38 or p-JNK. 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. Blots were analysed using GS 700 Imaging Densitometer to confirm the differences in the intensity of the blots (Bio-Rad Laboratories, Hercules, CA). To show that the stimulatory capacity of mycobacteria was not the result of contamination with LPS, we added the specific LPS-inhibiting oligopeptide polymyxin B (10 μg/mL) before mycobacterial stimulation.

2.3 *Flow cytometry and surface receptor expression*

THP-1 cells (1×10^6/mL) were incubated with 40 μmol PD98059, 30 μmol SB203580, 50 nmol dexamethasone, 25 μmol genistein, 100 nmol wortmannin or with vehicle 0.1% DMSO for 60 min before infection with *M. tuberculosis* H37Rv or H37Ra (MOI, 10:1) for 12 or 24 h. The concentrations of inhibitors chosen above were based on various published reports (Gee *et al.* 2002; Tse *et al.* 2002; Guha and Mackman 2002; Hasan *et al.* 2003), and on also optimization experiments performed by us. The cells from each well were pelleted in Falcon 2054 tubes and were double-stained with phycoerythrin (PE)-labelled mouse anti-human CD14 antibodies (Abs) and with fluorescein isothiocyanate (FITC)-labelled mouse anti-human CD44 Abs. Cells were processed to remove excess unbound antibodies, fixed in 500 μL of 4% paraformaldehyde and acquired for fluorescence using flow cytometer (BD FACS Calibur). Autofluorescence and isotype matched control Abs (BD Biosciences) were also included. The gates were set in accordance with gates obtained with the isotype-matched control Abs. Twenty-thousand events were collected and CD44 expression on $CD14^+$ THP-1 cells was analysed using Cell Quest/Flow Jo software tool. The results were expressed as percentage of double-stained cells among the total cells acquired.

2.4 *Cytokine measurement in culture supernatants*

In experiments involving serial kinetic measurements of TNF-α, the THP-1 cells (1×10^6/mL) were challenged with *M. tuberculosis* strains [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 (1×10^6/mL) were left untreated or first treated with 40 μmol PD98059 (MEK1 inhibitor), or 30 μmol SB203580 (p38 MAPK 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 μmol PD98059 or 30 μmol SB203580 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 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.

2.5 *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 *post hoc* Tukey's test, and the *a priori* level of significance at 95% confidence level was considered at $P<0.05$.

3. Results

3.1 *M. tuberculosis H37Rv and H37Ra infection leads to differential activation of MAPKs in THP-1 human monocytes*

Interference with intracellular signalling is central to the virulence strategy of other pathogens such as Leishmania and Yersinia (Ruckdeschel *et al.* 1997). To study the phosphorylation profile of the MAPKs, we challenged THP-1 human monocytes with *M. tuberculosis* H37Rv and H37Ra at an MOI of 10:1. Time-dependent phosphorylation of ERK1/2 and its upstream activator MEK1/2, p38 and its upstream activator MKK3/6, and JNK was observed. The peak activation of p-MAPKs occurred at different time points with *M. tuberculosis* H37Rv and H37Ra (figures 1 and 2). Total MEK, ERK, p38, MKK3 and JNK levels remained consistent throughout the infections (figures 1 and 2, bottom lanes), indicating that phosphorylation was specific to the external stimuli by the mycobacteria.

3.2 *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 analysed MAPK phosphorylation (ERK1/2, p38 and JNK) 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 (figure 3). 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 (figure 4a–c). Total ERK, p38 or JNK levels remained consistent throughout the infections, indicating that phosphorylation was specific to the external stimuli by the mycobacteria (figure 3).

It was observed that preincubation with anti-CD14 Ab and anti-HLA-DR Abs did not show considerable effect over *M. tuberculosis* H37Rv–induced ERK1/2 phosphorylation. In contrast, *M. tuberculosis* H37Rv–induced p38 and JNK phosphorylation was greatly reduced by blocking either of the receptors – the reduction was 32% and 73% with anti-CD14 Abs; 66% and 75% with anti-HLA-DR Abs, respectively. *M. 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 by H37Ra did not change considerably with anti-CD14 and anti-HLA-DR Abs, respectively. H37Ra-induced JNK phosphorylation increased by 2.5-fold with anti-HLA-DR Ab, but did not change considerably with anti-CD14 Ab. An IgG1 isotype control Ab used at the same concentration did not affect either H37Rv- or H37Ra-induced phosphorylation (figure 4a–c).

3.3 *M. tuberculosis H37Rv and H37Ra infection leads to differential CD44 surface expression in THP-1 human monocytes*

The induction of CD44 surface expression is a key event in the migration of monocytic cells to sites of inflammation or injury (Pure and Cuff 2001; Siegelman *et al.* 1999). When THP-1 cells were infected with *M. tuberculosis* H37Rv and H37Ra for 12 and 24 h, there was higher expression of CD44 by H37Ra when compared with H37Rv at both time points. The peak expression levels for both strains were observed at 12 h time point (figure 5).

3.4 *Kinetics of M. tuberculosis–induced TNF-α in THP-1 cells*

Among several cytokines induced by *M. tuberculosis*, the proinflammatory cytokine TNF-α plays an important role in the apoptosis of infected macrophages, structural maintenance of granuloma and control of infection (Ciaramella *et al.* 2002; Stenger 2005). It has been already reported that *M. tuberculosis* H37Rv induces less TNF-α than H37Ra in murine peritoneal macrophages (Falcone *et al.* 1994). Since we observed differences in the cell surface expression of CD44 between *M. tuberculosis* H37Rv and H37Ra, and also TNF-α has been linked to CD44 surface expression in monocytes (Levesque and Haynes 1997; Gee *et al.* 2003), we set out to determine the kinetics of TNF-α production at 12 and 24 h posinfection in THP-1 cells. 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 (table 1). So, the kinetic patterns of strain-induced CD44 surface expression and TNF-α production are similar (figure 5 and table 1).

3.5 *M. tuberculosis–induced phosphorylation of ERK1/2, p38 and JNK was inhibited by MAPK-specific inhibitors PD98059, SB203580 and dexamethasone*

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 MEK1/2, ERK1/2, JNK, MKK3/6, p38 MAPKs occured at 45′, 45′, 15′, 60′ and

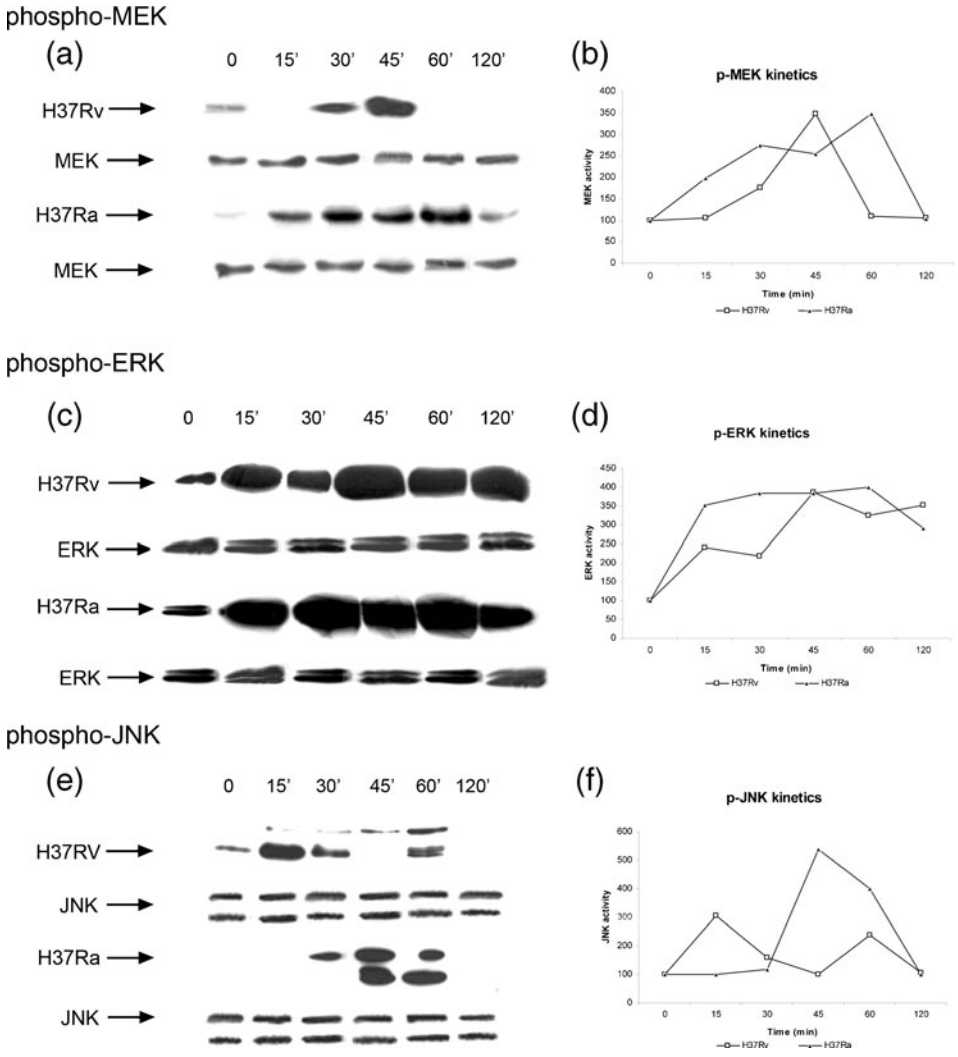

Figure 1. MEK1/2, ERK1/2 and JNK activation in THP-1 human monocytes in response to infection with *M. tuberculosis* strains. THP-1 human monocytes were left untreated ('0' min) or treated with *M. tuberculosis* H37Rv and H37Ra (bacteria:host cell, 10:1) for various lengths of time (indicated in minutes at top). Cellular extracts were analysed by western blotting for the presence of p-MEK1/2 (**a** and **b**), or p-ERK1/2 (**c** and **d**), or p-JNK (**e** and **f**). Each of the blots was also probed with antibodies against total MEK or ERK or JNK (bottom lanes, **a**–**f**), to ensure equal loading of protein in all the lanes. Right panel shows the corresponding densitometric analyses of blots probed with corresponding phosphoantibodies. Data shown are the mean±SD of three independent experiments performed in triplicate.

45′ for H37Rv, and at 60′, 60′, 45′, 30′ and 60′ for H37Ra, respectively (figures 1 and 2). To determine whether MAPK-specific inhibitors inhibit *M. tuberculosis*-induced phosphorylation of MAPKs, THP-1 cells were infected with *M. tuberculosis* H37Rv or H37Ra in the presence of specific inhibitors, and immunoblotting of p-ERK1/2, p-p38 and p-JNK was performed after terminating the cultures at the peak time points indicated above. It was observed that both *M. tuberculosis* H37Rv– and *M. tuberculosis* H37Ra–induced phosphorylation of ERK1/2, p38 and JNK was reduced by PD98059, SB203580 and dexamethasone, respectively. The observed inhibition was not due to DMSO, as DMSO alone did not exhibit any inhibitory effects at this concentration (0.1%) (figure 6).

3.6 *SB203580 decreases, but PD98059 and dexamethasone increase M. tuberculosis–induced CD44 expression*

It was already reported that JNK plays a predominant role in LPS-induced CD44 expression in monocytic cells (Gee *et al.* 2002). Therefore, it was of interest to identify the members of

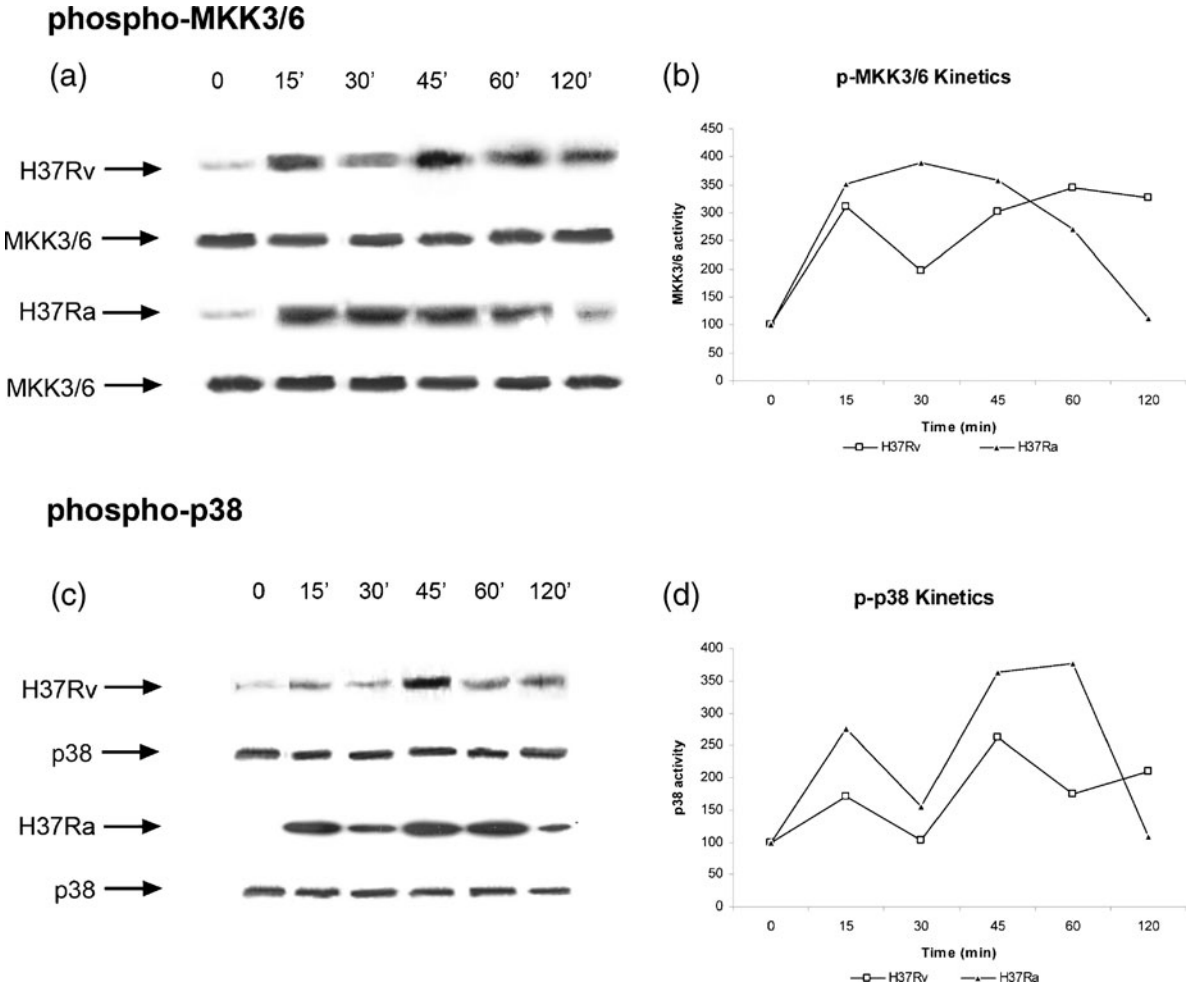

Figure 2. MKK and p38 MAPK activation in THP-1 human monocytes in response to infection with *M. tuberculosis* strains. THP-1 human monocytes were left untreated ('0' min) or treated with *M. tuberculosis* H37Rv and H37Ra (bacteria : host cell, 10:1) for various lengths of time (indicated in minutes at top). Cellular extracts were analysed by western blotting for the presence of p-MKK3/6 (**a** and **b**), or p-p38 (**c** and **d**). Each of the blots was also probed with antibodies against total MKK3 or p38 (bottom lanes, **a**–**d**), to ensure equal loading of protein in all the lanes. Right panel shows the corresponding densitometric analyses of blots probed with corresponding phosphoantibodies. Data shown are the mean±SD of three independent experiments performed in triplicate.

the MAPK family that are involved in *M. tuberculosis*–induced CD44 surface expression in monocytic cells. The optimal time period of 12 h post infection was chosen for this inhibition studies because both strains induced higher CD44 surface expression only at 12 h in CD44 surface expression kinetic studies (figure 5). When CD44 expression was analysed in infected cells in the presence of pathway inhibitors, it was observed that both *M. tuberculosis* H37Rv– and *M. tuberculosis* H37Ra–induced CD44 surface expression was reduced by SB203580, and increased by PD98059 and dexamethasone (MAPK-specific inhibitors). It was also increased by non-specific inhibitors like genistein and wortmannin (figure 7).

3.7 *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 (figures 1 and 2; table 1), and also strain-induced TNF-α secretion was observed to peak at 12 h time point similar to strain-induced CD44 kinectics (table 1 and figure 5), we were interested to know whether the MAPKs involved in mediating strain-induced CD44 (ERK and p38) (figure 7) are also involved in mediating strain-induced TNF-α secretion. The secretion of TNF-α was assessed both in the presence or the absence of PD98059 and SB203580, in

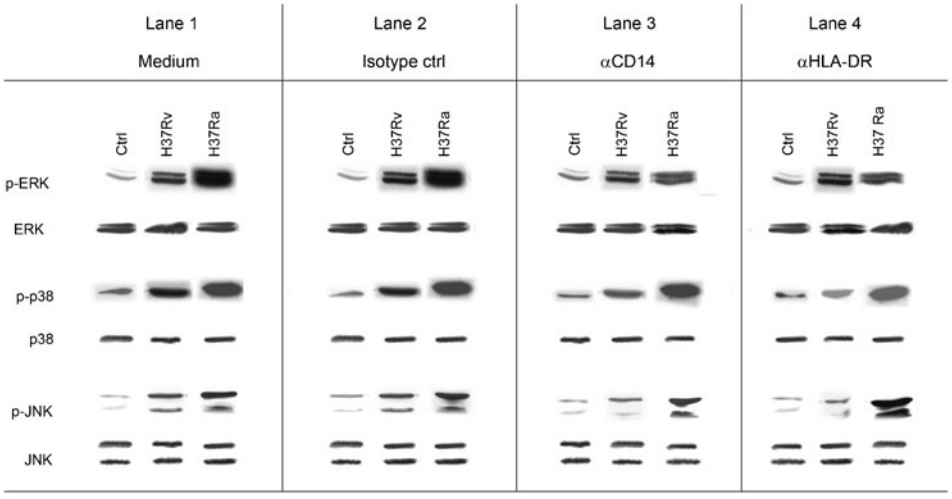

Figure 3. Activation of MAP kinases by *M. tuberculosis* strains through CD14 and HLA-DR receptors. THP-1 cells were preincubated with isotype control Abs (lane 2) or anti-CD14 monoclonal (lane 3) or anti-HLA-DR monoclonal (lane 4) (each 5 μg/mL) for 60 min. Subsequently, *M. tuberculosis* strains were added for various time points (lane 1–4). Cells were lysed and subjected to immunoblotting with specific anti-phospho antibodies. As controls, the total amounts of ERK, p38 and JNK, as detected by anti-ERK, anti-p38 and anti-JNK Abs, are shown. Similar data were obtained in three independent experiments.

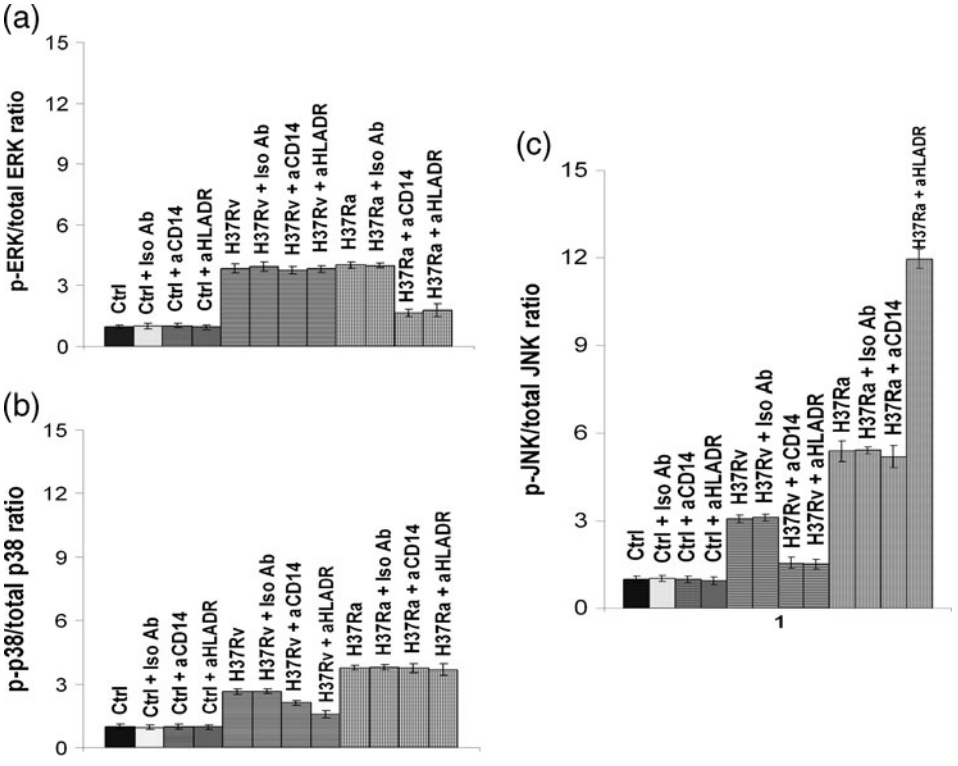

Figure 4. Densitometric analyses of blots shown in figure 3. Analysis for p-ERK (**a**), p-p38 (**b**), and p-JNK (**c**) were shown. Graphs plotted with phospho-mapk/total mapk ratio in the y-axis and different culture conditions in x-axis. Data shown are the mean±SD of three independent experiments.

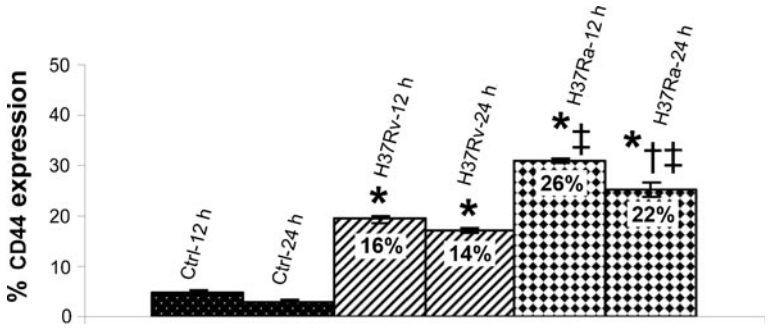

Figure 5. Kinetics of *M. tuberculosis*–induced CD44 surface expression. THP-1 cells (1×10^6 /mL) were left untreated (Ctrl) or treated with *M. tuberculosis* H37Rv and Ra (bacteria : host cell, 10:1) for 12 and 24 h. The cells were analysed for CD44 surface expression by flow cytometry as described under 'Materials and methods.' Data shown are the mean±SD of five different experiments. $^{*}P<0.05$ when *M. tuberculosis*–induced CD44 expression is compared with uninfected cells. $^{‡}P<0.05$ when *M. tuberculosis* H37Ra-induced expression is compared with that of *M. tuberculosis* H37Rv at 12 and 24 h time points. $^{†}P<0.05$ when H37Ra-induced surface expression is compared with it's expression at 12 and 24 h time points. The percentage ncrease in CD44 surface expression caused by H37Rv or H37Ra infection over untreated control cells is mentioned inside the corresponding bars.

THP-1 cells, at 12 h post infection. The optimal time period of 12 h post infection was chosen because both strains induced maximum TNF-α production only at 12 h in TNF-α kinetic studies (table 1). Inhibition experiments showed that the production of TNF-α by *M. tuberculosis* H37Rv and H37Ra was significantly reduced by the inhibitors used – 40 μM PD98059 and 30 μM SB203580 inhibited 90%, and 86%, for *M. tuberculosis* H37Rv; 90% and 87%, for *M. tuberculosis* H37Ra. 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 and p38 MAPKs are involved in the signalling of both TNF-α production and CD44 surface expression, during *M. tuberculosis* infection of THP-1 cells (table 1).

4. Discussion

Since various *in vivo* and *in vitro* studies have demonstrated the role of CD44 in protective immunity against tuberculosis (Lyadova *et al.* 1998; Feng *et al.* 1999, 2000; Leemans *et al.* 2003; Waters *et al.* 2003, 2009; Li *et al.* 2008; Giri *et al.* 2009), any data on the signalling pathways that could alter CD44 surface expression in mycobacteria-infected monocyte/macrophages might be important in the context of the effective antimycobacterial response. Since MAPKs have been implicated in LPS-induced CD44 expression (Gee *et al.* 2002), and also in the induction of immune mediators/antimycobacterial responses in mycobacteria-infected monocytes/macrophages (Tse *et al.* 2002; Schorey and Cooper 2003; Koul *et al.* 2004), we studied the involvement of MAPKs in *M. tuberculosis*–induced CD44 expression in THP-1 monocytes.

Upon induction by *M. tuberculosis* H37Rv and H37Ra, THP-1 cells showed distinct, time-dependent activation of MEK-1, ERK1/2, MKK3/6, p38 and JNK MAPKs. This is the first report that MAPKs, MKK1, MKK3/6 and JNK are distinctly phosphorylated by *M. tuberculosis* H37Rv and H37Ra in THP-1 cells. The fall and rise in the activation of the respective MAPKs observed in our study is consistent

Table 1. MAPK inhibitors and *M. tuberculosis*–induced TNF-α secretion in THP-1 cells

Expression	12 h	24 h	PD98059 (40 μM) (12 h)	SB203580 (30 μM) (12 h)	0.1% DMSO (12 h)
Ctrl	7±4.85	8±3.71	10.62±1.62	9±1.75	7±4.75
H37Rv	848±78	433±67.86§	80.98±7.04‡	126.6±21.5‡	840±80
H37Ra	3309±213*	1091±40†§	330±21.07‡	424±18.03‡	3312±210

THP-1 cells were left untreated (Ctrl) or treated with different *M. tuberculosis* strains (bacteria:host cell, 10:1) for 12 and 24 h. Few wells were treated with 40 μmol PD98059 or 30 μmol SB203580 or 0.1% DMSO for 60 min before stimulation with different strains (bacteria: host cell, 10:1) for 12 h. Supernatants were harvested and TNF-α levels were measured by ELISA. Data shown are the mean±SD of five independent experiments performed in triplicate. $^{*}P<0.05$ when H37Ra-induced expression is compared with H37Rv at 12 h time point, $^{†}P<0.05$ when H37Ra-induced expression is compared with 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 were compared with the inhibitor treated cultures at 12 h time point.

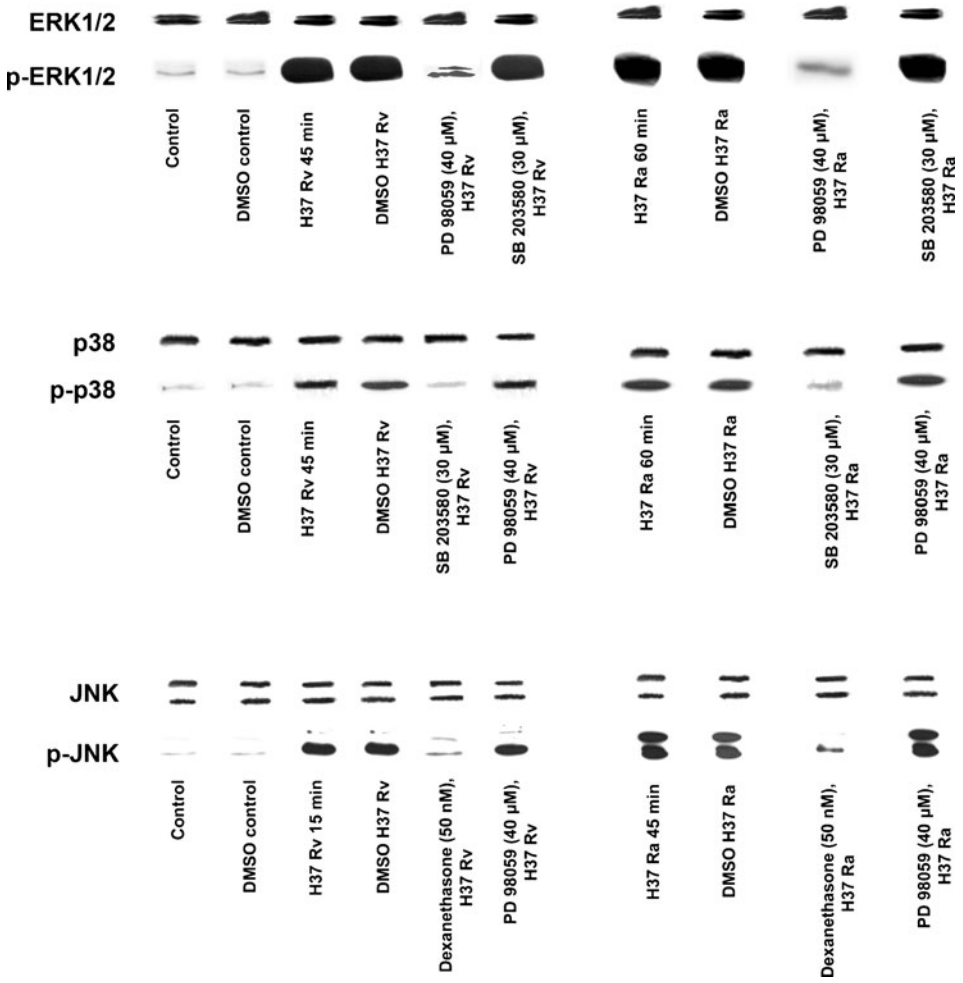

Figure 6. Influence of pathway inhibitors over *M. tuberculosis*-induced MAPK activation in THP-1 cells. THP-1 cells (1×10^6/mL) were left untreated (Ctrl) or treated with vehicle (DMSO control) or with the MAPK-specific inhibitors PD98059, SB203580 or dexamethasone, for 60 min prior to incubation with *M. tuberculosis* H37Rv or Ra (bacteria : host cell, 10:1) for various lengths of time (indicated in min.). Cellular extracts were analysed by western blotting for the presence of p-MEK1/2, p-ERK1/2 or p-JNK. Each of the blots was also probed with antibodies against total MEK or ERK or JNK, to ensure equal loading of protein in all the lanes. Similar data were obtained in four different experiments.

with p38 activation data of Song *et al.* (2003) on infection of *M. tuberculosis* H37Rv in human peripheral blood monocytes. The activation caused by H37Ra was different with respect to all the MAPKs studied. It is likely that this differential MAP kinase signalling reflects differences between virulent and avirulent *M. tuberculosis* and in the expression of components recognized by monocyte/macrophage receptors. In other words, the observed differences in the duration and intensity of signalling might be due to alterations in the activation of scaffolding proteins or phosphatases (Kolch 2005; Salojin *et al.* 2006; Cheung *et al.* 2009) by various mycobacterial cell-wall-associated virulence factors, like polymorphic PGRSdomain, lipoarabinomannan (structural difference), 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 (Davis *et al.* 2007), these differences might influence the antimycobacterial mechanisms inside the macrophages.

Since CD14 and HLA-DR receptors have already been shown to mediate activation of MAPKs in immune cells in response to external stimuli (Reiling *et al.* 2001; Meguro *et al.* 2003), we studied the usage of these receptors in mediating *M. tuberculosis* H37Rv– and *M. tuberculosis* H37Ra–induced MAPK phosphorylation. Our experiments with anti-CD14 and anti-HLADR antibodies showed for the first time, that the receptor usage in mediating MAPK activation is strain-specific. The fact that the reduction of MAPK activation

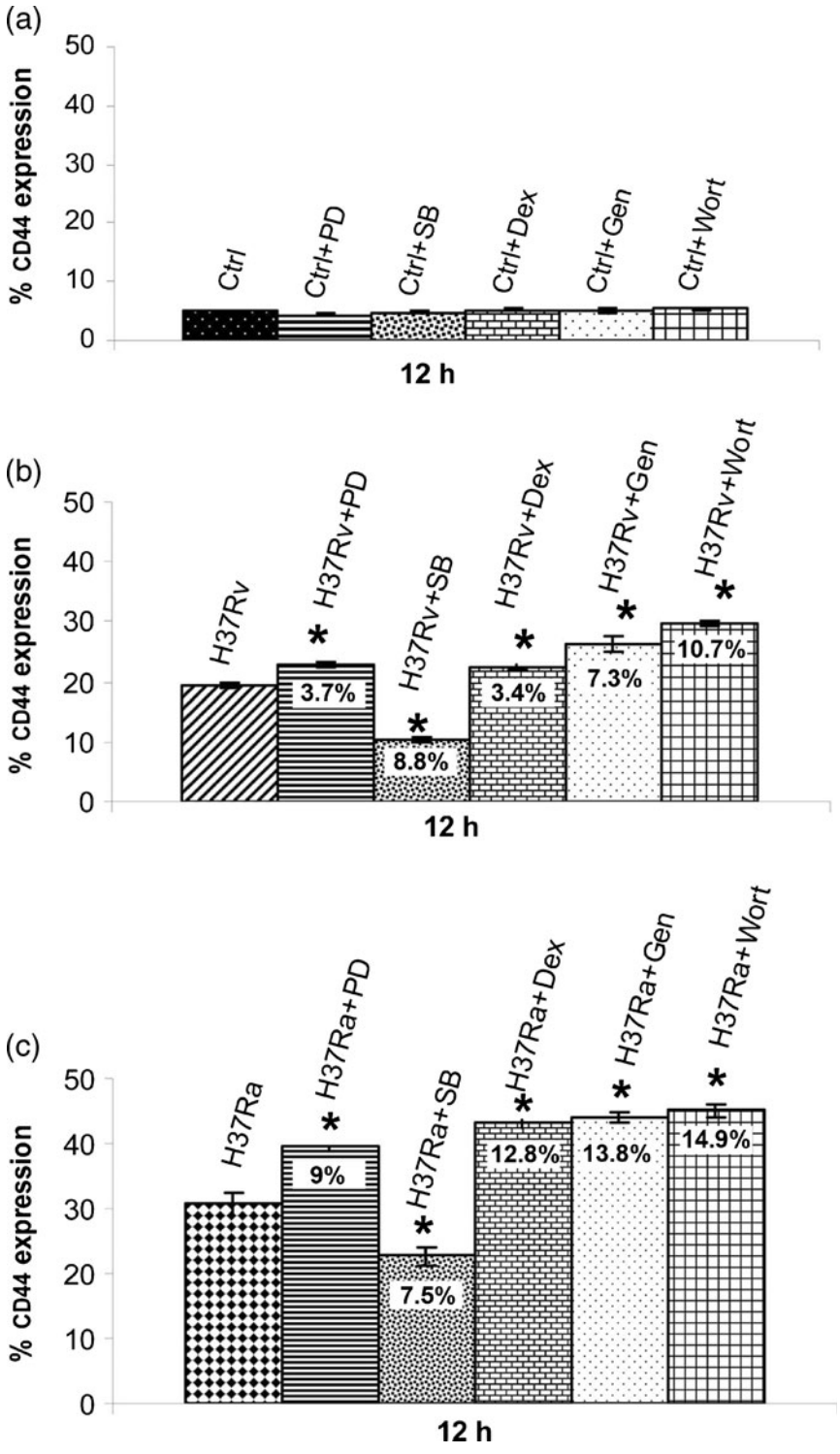

Figure 7. Influence of pathway inhibitors over *M. tuberculosis*-induced CD44 surface expression in THP-1 cells. THP-1 cells (1×10^6/mL) were left untreated (Ctrl) or treated with vehicle (DMSO control) or with the MAPK-specific inhibitors PD98059 (PD), SB203580 (SB) and dexamethasone (Dex), or with the non-specific inhibitors genistein (Gen) and wortmannin (Wort) **(a)**, for 60 min prior to incubation with *M. tuberculosis* H37Rv **(b)** or H37Ra **(c)** (bacteria : host cell, 10:1) for 12 h. The cells were analysed for CD44 surface expression by flow cytometry as described under 'Materials and methods.' Data shown are the mean±SD of five different experiments. $^*P<0.05$ when strain-induced expression is compared at 12 h time point in the presence of inhibitors. The percentage increase or decrease in CD44 surface expression caused by the effect of respective inhibitors in infected cultures (compared to uninhibited infected cultures) is mentioned inside the corresponding bars.

was not complete after blockage of a single receptor implies that other receptors like CR3, mannose receptor, TLRs *etc*., are possibly involved in the activation of those MAPKs. The cross-talk between CD14 and complement receptor 3 (CR3) receptors in the activation of PI-3 K pathway during phagocytosis of *M. bovis* bacillus Calmette-Guérin (BCG) in THP-1 cells has already been reported (Sendide *et al.* 2005). On the other hand, virulent mycobacteria have been shown to use both mannose and complement receptors for adherence and phagocytosis in contrast to attenuated strain, which uses only complement receptors (Schlesinger 1993). So, it will be interesting to investigate whether and to what extent Toll-like receptor (TLR) or complement or mannose receptor-mediated signalling contributes to MAP kinase activation, cytokine formation and bacillary killing by macrophages, in response to intact virulent and avirulent *M. tuberculosis* strains.

Our kinetic studies involving CD44 and TNF-α revealed for the first time, higher CD44 surface expression and TNF-α production by *M. tuberculosis* H37Ra compared to H37Rv in THP-1 cells. Since TNF-α has been shown to increase CD44 expression and CD44-hyaluronan (HA) binding in monocytes (Gee *et al.* 2002, 2003; Levesque and Haynes 1997), the higher TNF-α levels induced by *M. tuberculosis* H37Ra might help in higher bacterial uptake through CD44 receptors and in the initiation of an effective and protective immune response through optimum CD44-HA interaction. This might facilitate faster clearance of avirulent strain *M. tuberculosis* H37Ra.

It has been demonstrated that JNK plays a distinct role in LPS-induced CD44 expression in THP-1 cells when compared to induction by TNF-α (Gee *et al.* 2002). Our experiments showed that inhibition of p38 MAPK led to the reduction of CD44 surface expression, but inhibition of ERK1/2, JNK, tyrosine kinase and PI3K led to increases in CD44 surface expression, with both strains. However, the percentage increase or decrease in CD44 surface expression caused by various inhibitors varied between the two strains. This type of positive and negative modulation of *M. tuberculosis*–induced CD44 surface expression by various signalling kinases has not been reported earlier in monocytes. The reduction caused by p38 inhibitor alone shows that p38 might play a distinct role when compared to other kinases and mycobacteria might use this distinct role of p38 MAPK to balance the expression of CD44 during infection, in conjunction with other kinases. Evidence to this fact is supported by data of Tse *et al.* (2002) which demonstrate the importance of p38 MAPK in influencing the viability of virulent morphotype of *M. avium*, and also the existence of cross-talk between p38 MAPK and other kinases like ERK1/2, JNK, *etc*., Furthermore, the data of Jo *et al.* (2007) and Cheung *et al.* (2009) raises the feasibility of modulation of MAPK activation by *M. tuberculosis* through differential activation of TLRs and MKP-1. While this work was in progress, Bansal *et al.* (2009) demonstrated that inhibition of PI3K led to reduction in the expression of phosphatidyl-myo-inositol dimannosides (PIM2)-induced cell surface CD44, in RAW 264.7 cells. However, our PI3K inhibition experiments showed the reverse. The discrepancy seen in our data obtained with THP-1cells could be due to the use of intact mycobacteria or *M. tuberculosis* strain in our study compared with the use of an integral component of the mycobacterial envelope by Bansal *et al.* (2009). Bansal *et al.* (2009) have also established the cross-talk between PI3K and ERK1/2 kinases that contributes to changes in CD44/matrix metalloproteinase-9 expression (MMP-9). So, cross-talk between different kinases mentioned above might account for the data obtained in our inhibition experiments involving *M. tuberculosis*–induced CD44 surface expression.

The higher induction of CD44 surface expression and TNF-α levels by *M. tuberculosis* H37Ra seen in our experiments might be due to several possibilities including (a) the higher extent of activation of most MAPKs by H37Ra, (b) varied extent of activation of CD44 and TNF-α promoters in cells infected with Rv and Ra strains and (c) use of distinct pathways by the two strains. A number of reports favour these possibilities. The higher activation of most MAPKs by H37Ra is supported by the data of Hasan *et al.* (2003), which shows higher activation of ERK1/2 in association with higher TNF-α induction by BCG when compared with virulent *M. tuberculosis*. This is also partly supported by our observation that strain-induced CD44 surface expression and TNF-α were sensitive to inhibition of ERK1/2. The early and higher activation profile of p38 seen with *M. tuberculosis* H37Ra might lead to differential transcription of CD44 and TNF promoters, because p38 MAP kinase has been shown to regulate NF-κB-dependent transcription in part by modulating activation of basal transcription factors in THP-1 cells (Carter *et al.* 1999). This possibility is encouraged by several reports , namely, NF-kB-mediated transcriptional regulation of CD44 (Damm *et al.* 2010) and TNF-α (Dhiman *et al.* 2007), differential NF-kB activation by *M. tuberculosis* H37Rv and H37Ra in THP-1 cells (Dhiman *et al.* 2007), p38-mediated TNF-α mRNA stability by a cell wall component of *M. smegmatis* but not that of *M. avium* (Basler *et al.* 2010), and our own finding of modulation of strain-induced CD44 surface expression and TNF-α by p38 inhibitor, SB203580. Even though the signalling molecules that mediate *M. tuberculosis* H37Rv– and *M. tuberculosis* H37Ra–induced CD44 surface expression and TNF-α secretion are almost similar, the extent of activation of CD44 and TNF-α promoters by the strains may vary. Lee and Schorey (2005) have already shown increased ERK1/2 phosphorylation, increased Ets/Elk and NF-kB promoter activities, along with increased TNF-α production with *M. smegmatis*, compared to *M. avium* in macrophages. Furthermore, the chance that *M. tuberculosis* H37Rv and H37Ra might use distinct pathways and downstream transcription factors is supported by the data of Mishra

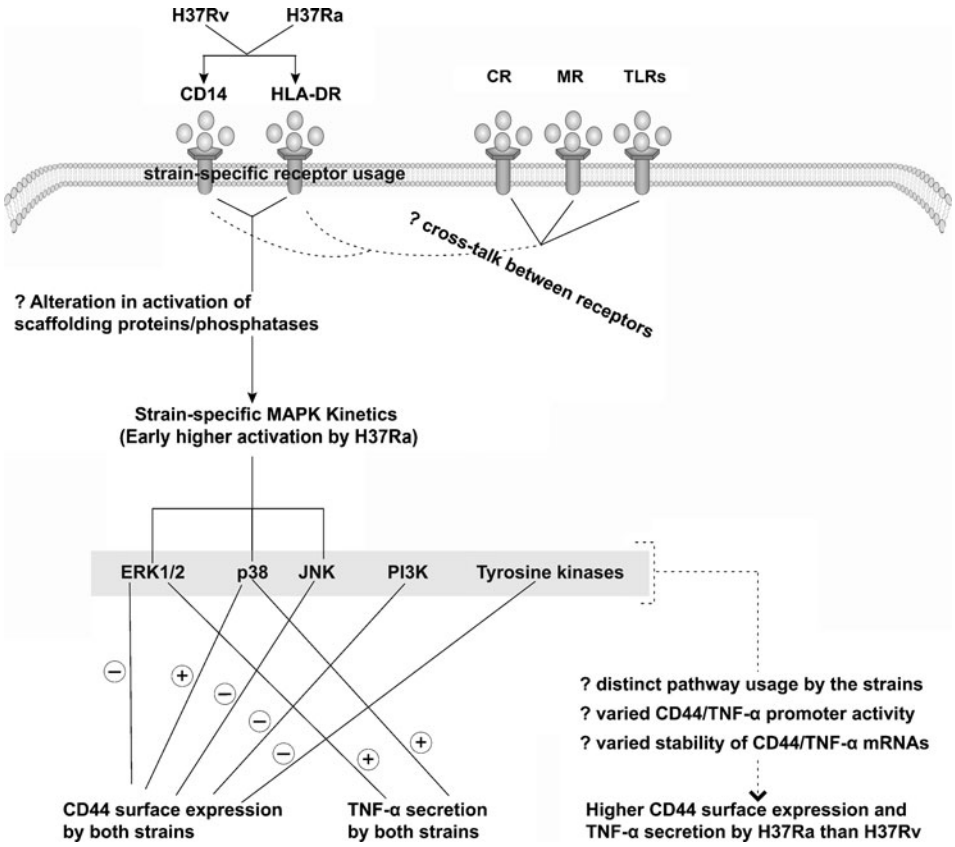

Figure 8. The proposed signal transduction model of *M. tuberculosis*–mediated CD44 surface expression and TNF-α secretion in THP-1 cells. MR, mannose receptor; CR, complement receptor; TLRs, Toll-like receptors.

et al. (2005) and Rajaram *et al.* (2010). Future studies with molecules downstream of MAPK might help in delineating the level at which *M. tuberculosis* H37Rv and H37Ra differentially induce the expression of CD44.

Overall, these results indicate that immediately after infection of monocytes, *M. tuberculosis* H37Rv and H37Ra differentially phosphorylate MEK-1, ERK1/2, MKK3/6, p38 and JNK MAPKs through CD14/HLA-DR receptors, and this was accompanied by higher CD44 surface expression and TNF-α production by *M. tuberculosis* H37Ra at 12 and 24 h post infection; the p38 MAPK promotes, whereas ERK1/2, JNK, PI3K and tyrosine kinases inhibit *M. tuberculosis*-induced surface expression of CD44. However, *M. tuberculosis*–induced TNF-α production was promoted by both p38 and ERK (figure 8). Since it has been already reported that inhibitors of MAPK (Blumenthal *et al.* 2002; Tse *et al.* 2002) and AKT (Kuijl *et al.* 2007) modulate the intracellular replication of mycobacterial strains, our future work is planned on several aspects, namely, (a) the effect of various inhibitors over mycobacterial replication during *in vivo* and *in vitro* infection with various *M. tuberculosis* clinical isolates which differ in their lineage and prevalence in our region, (b) correlation between antimycobacterial effect of inhibitors and various facets of immune response, namely, the levels of cytokines/chemokines, the phagocytic index, the expression of surface receptors including CD44, the functional CD44-HA interaction, migration of infected cells and chemoattraction of $CD4^+$ T cells, and (c) to dissect the level along the pathways at which clinical isolates induce differential activation to bring changes in the levels of various effector molecules, *etc.*, These studies will not only provide proof of strain related virulence but also might help in designing novel therapeutic strategies.

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