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Full Length Research Paper

Mitogen activated protein kinase Influence the immune response of a prevalent *Mycobacterium tuberculosis* clinical strain of the Indo-Oceanic Clade

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Different strains of *Mycobacterium tuberculosis (M. tuberculosis*) are known to modify the host immune response in a strain-specific manner. However, studies linking *M. tuberculosis* strain-specific impact upon the regulation of mitogen activated protein kinase (MAPK)-induced monocyte activation are limited. We have studied the immunomodulation, induced by a prevalent Indo-Oceanic Clade clinical strain S7, isolated from the *Mycobacterium bovis* bacillus Calmette–Guerin (BCG) trial area of Thiruvallur district, South India, in comparison with a low prevalent Beijing strain. It was shown that p38, stress-activated protein kinase/c-jun N-terminal kinases (SAPK/JNK) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) of the MAPK pathways were not activated via cluster of differentiation 14 (CD14) and Human Leukocyte Antigen-DR (HLA-DR) receptors, upon infection of human monocytic cell line (THP-1) with clinical strain S7. The levels of proinflammatory cytokines, surface expression of CD44 and CD25 receptors, and the production of anti-apoptotic protein B-cell lymphoma 2 (Bcl-2), varied between the strains. Inhibition experiments showed that the clinical strains use different signaling pathways to induce interleukin-6 (IL-6) secretion and surface expression of CD44. In addition, contrasting patterns were observed between strain-induced tumour necrosis alpha (TNF-α) and Bcl-2 levels. These data provided a precedent for effects of *M. tuberculosis* strain-specific factors upon the mechanisms involved in MAPK-mediated immune activation.

Key words: *Mycobacterium tuberculosis*, clinical isolate S7, Beijing strain, mitogen-activated protein kinases.

INTRODUCTION

Accumulating evidence showed that different clinical strains/different genotypes of *Mycobacterium tuberculosis* (*M. tuberculosis*) differ in their transmission potential, have distinct interaction with the host, and induce differential immune response (Petrelli et al., 2004; Theus et al., 2005; McEvoy et al., 2007). In the BCG trial area of Thiruvallur district, south India, our earlier molecular epidemiologic studies based on spoligotyping, IS 6110 restriction fragment length polymorphism (RFLP) and deletion polymerase chain reaction, it has been found that Indo-Oceanic Clade strains are the most predominant ones in this region and 40% of them harbour only a single copy of the repeat element, IS 6110. Thus, these strains are involved in the active transmission of the disease in the community (Das et al., 1995; Narayanan et al., 2002, 2007). The insertion of this mobile genetic element occurs at different sites in the genome and may influence the phenotype of the strains, which is of importance in studying their virulence and role in immunity (Sampson et al., 1999; Gillespie, 2007). It has also been found that there has been a low prevalence (≤ 3%) of the Beijing spoligotype in this region (Narayanan et al., 2002, 2007).

Among the highly prevalent clinical strains in this region, strain S7 has been studied extensively in our centre. S7 strain was shown to; (a) bind more, show slower growth rate, cause less burden and minimal apoptosis in infected macrophages (Rajavelu and Das, 2007), (b) induce altered migratory and chemokine secretory patterns of human monocyte-derived DCs (MoDC) (Rajashree et al., 2008), (c) delay the maturation of MoDC (Rajashree

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and Das, 2008), (d) be less efficient in inducing T-cell proliferation and chemoattraction of $CD4^+$ T cells (Rajavelu and Das, 2003; Rajashree et al., 2008), (f) induce a predominant Th2 response in healthy subjects as sonicate antigens (Rajavelu and Das, 2005), and (g) alter the expression of co-stimulatory molecules in macrophages (Rajavelu and Das, 2008).

Pathogenic *mycobacteria* have been shown to modify several host signaling pathways, namely, phosphatidylinositol 3-kinase (PI3K), protein kinase C and mitogen-activated protein kinase (MAPK) cascades, to enable them survive inside host cells. Several reports have documented the link between MAPK activation in monocytes/macrophages and the production of various effector molecules following a mycobacterial infection (Roach and Schorey, 2002; Blumenthal et al., 2002; Tse et al., 2002; Schorey and Cooper, 2003; Koul et al., 2004; Jo et al., 2007). The ubiquitously expressed MAPKs represent highly conserved serine-threonine kinases that are activated by upstream MAPK kinases [MKK or MAPKK or MEK (eg., mitogen-activated protein kinase kinase-1)] through a Th-XXX-Tyr phosphorylation motif. In mammals, there are three subfamilies of MAP kinases that can be activated independently and simultaneously: p46 and p54 c-Jun-NH2-terminal kinases or stress-activated protein kinases (JNK/SAPK), p42 and p44 extracellular signal-regulated kinases 1 and 2 (ERK1/2), and p38 MAP kinase (Cano and Mahadevan, 1995). MAP kinases mediate cellular responses to a variety of extracellular stimuli, such as physical stress (e.g osmotic changes), inflammatory cytokines, growth factors, and bacterial components [e.g lipopolysaccharide (LPS)] (Lewis et al., 1998; Kyriakis and Avruch, 2001; Cowan and Storey, 2003).

Song et al. (2003) has demonstrated that both extracellular signal regulated kinase (ERK) and p38 MAPKs were essential for *M. tuberculosis* H37Rv-induced tumour necrosis factor - α (TNF-α) production, whereas activation of the p38 MAPK pathway alone was essential for *M. tuberculosis* H37Rv-induced Interleukin (IL) -10 production. Hasan et al. (2003) has documented the relation between mycobacterial virulence, MAP kinase signaling and reduced TNF-α secretion in human monocytes. Furthermore, Bluementhal et al. (2002) has established the role of MAPKs in controlling *M. avium* replication in human macrophages. However, data about the influence of different *M. tuberculosis* lineages on MAPK-mediated immune response is limited. So, we have chosen to study the modulation of MAPK-mediated immune activation induced by two clinical strains isolated in South India, namely, Indo-oceanic clade clinical strain S7 (prevalent strain) and the Beijing strain (less prevalent strain).

As markers of monocytic response to infection, we have evaluated proinflammatory cytokines TNF-α, IL-6 antiapoptotic marker, B-cell lymphoma 2 (Bcl-2) and surface markers namely; CD25, CD44 etc. TNF-α and IL-6 were chosen for their proven role in mycobacterial pathogenesis (Beltan et al., 2000; Roach et al., 2002; Nagabhushanam et al., 2003) and for their relation with MAPK pathway in mycobacterial infection (Song et al., 2003; Yadav et al., 2004; Bhatnagar and Schorey, 2006). CD44 was selected for its role in migration of leukocytes (DeGrendele et al., 1997), protective role in tuberculosis (Leemans et al., 2003; Waters et al., 2009), and MAPK-mediated sensitivity to TNF-α in monocytes. (Gee et al., 2002). Bcl-2 being an essential antiapoptotic intracellular regulatory molecule was included in the study because it has been shown; (a) that Bcl-2 or its family members can resist apoptosis of activated monocytes (Perera and Waldmann, 1998) or infected macrophages (Zhang et al., 1995), (b) to be differentially regulated by virulent and avirulent strains of *mycobacteria (*Zhang et al., 1995), and (c) to be regulated by TNF-α in macrophages (Rojas et al., 1999; Spira et al., 2003). On the other hand, CD25 being an activation marker of monocytes (Farina et al., 2004; Weber et al., 2004), was measured in the infected cells for its observed sensitivity to bacterial cell wall components/TNF-α (Farina et al., 2004). We hypothesize that the signaling pathways involved in S7-mediated immune activation will be different from that utilized by the Beijing strain.

MATERIALS AND METHODS

Reagents

Antibodies (Ab) against total and phosphorylated forms of MAPKs (p-MAPKs) were purchased from Cell Signaling Technology (Beverly, MA). PD98059 (MEK1 inhibitor), SB203580 (p38 MAPK inhibitor), dexamethasone (JNK inhibitor), genistein (tyrosine kinase inhibitor), wortmannin (PI3-K inhibitor) and Bay 11-7082 [Nuclear Factor-κB (NF- κB) inhibitor] were purchased from Calbiochem Biosciences (San Diego, CA). Rabbit polyclonal anti-human Bcl-2 Ab was obtained from BD Biosciences (San Jose, CA). Horseradish peroxidase-linked secondary antibodies and polyvinylidene difluoride membrane were from Amersham Biosciences (Piscataway, NJ). Supersignal western pico chemiluminiscent substrate was purchased from Pierce laboratories (Rockford, USA). Histopaque-1077 and dimethylsulfoxide (DMSO) were purchased 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 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), albumin–dextrose–catalase supplement, antibiotics and phosphate-buffered saline (PBS) pH 7.2 were from Invitrogen Corporation (Carlsbad, CA). The IL-6 and TNF-α enzyme-linked immunosorbent assay (ELISA) kit were from BD Biosciences (San Jose, CA). Human anti-CD 14 Ab, anti-HLA DR Ab, appropriate IgG1 and IgG2a isotype control Abs and all fluorochrome conjugated surface Abs were from BD PharMingen (Heidelberg, Germany).

Mycobacterial strains

The *M. tuberculosis* strains, H37Rv (standard laboratory virulent strain) and two clinical isolates, namely, S7 and Beijing strain. S7 strain; a prevalent Indo-Oceanic Clade clinical strain isolated from the BCG trial area of Thiruvallur district, South India. Beijing strain;

a low prevalent clinical strain isolated from the same area.

Study subjects

Heparinised blood was obtained from healthy volunteers. All healthy controls gave consent before being enrolled, and the study was approved by the Tuberculosis Research Centre (TRC) – Institutional Ethics Committee review board. They had no previous history of clinical TB, all were negative for HIV and had received the *M. bovis* BCG vaccinations at birth. They have not been previously treated for tuberculosis.

Infection studies

Propagation of *mycobacterial* strains, maintenance of THP-1 cell culture, isolation and culture of human primary monocytes from peripheral blood, infection and preparation of cell lysates, inhibition of MAPKs, measurements of cytokines (IL-6 and TNF-α), determination of MAP kinase phosphorylation and Bcl-2 through western immunoblotting, densitometric analysis of the blots, etc. were done as per protocols of Natarajan and Narayanan, (2007). For receptor blocking studies, the cells were preincubated with anti-CD14 Abs or anti-HLA DR Abs, or isotype control Abs (5µg/ml) and subsequently stimulated with *M. tuberculosis* strains [multiplicity of infection (MOI) of 10:1]. These cultures were terminated at time points during which peak phosphorylation of MAPKs was presented for individual strains in our serial kinetic studies. These lysates were subjected to immunoblotting for p-MAPKs.

Flow cytometry and surface receptor expression

THP-1 monocytes (1×10^6/ml) were incubated without or with 40 µM PD98059, or 30 µM SB203580, or 50 nM dexamethasone (Dex), or 25 µM genistein (Gen), or 100 nM wortmannin (Wort), or 0.1% DMSO for 60 min before infection and once infected with *M. tuberculosis* strains (MOI – 10:1), the cultures were left for 12 or 24 h. THP-1 monocytes were double stained by combining fluorochrome-phycoerythrin (PE) conjugated anti-CD14 along with fluorescein isothiocyanate (FITC) conjugated anti-CD25 or CD44 (BD Biosciences), and acquired. The results were expressed as percentage of total cells acquired. Samples were analyzed on a FACS Caliber flow cytometer (Becton Dickinson) using Cell Quest software.

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 (SPSS software „14.0"), and the *a priori* level of significance at 95% confidence level was considered at $P < 0.05$. Log transformation was used wherever necessary, to stabilize variance before performing the test.

RESULTS

Infection of THP-1 monocytes by *M. tuberculosis* H37Rv, S7 and Beijing strains leads to differential activation of MAP kinases

When the kinetics of the MAP kinases was compared, the activation status and the time of activation were strain-dependent. Densitometric readings showed that the peak activation of phosphorylated forms of MEK1/2, ERK1/2, MKK3/6, p38 and JNK MAPKs occurred at 45", 45", 60", 45" and 15" min for H37Rv; 60", 60", 45", 45"; and 30" for S7 and at 120", 120", 30", 45"and 60" for Beijing strain; respectively. S7 strain showed rapid and higher activation of many MAPKs when compared to Beijing strain which showed delayed activation (Figures 1, 2a and b).

CD14 and HLA-DR may not be critical for MAP kinase phosphorylation by clinical strain S7 in THP-1 monocytes

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). To assess the receptor that mediated MAPK activation by *M. tuberculosis* strains, we analyzed 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 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 these *M. tuberculosis* strains (Figures 1, 2a and b). Hence, the cells were lysed during those peak time points in this experiment for assessing phosphorylation of MAPKs through the receptors. 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. Total ERK or p38 or JNK levels remained consistent throughout the infections, indicating that phosphorylation was specific to the external stimuli by the mycobacteria (Figure 3B, D and F).

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 (Figure 3A). In contrast, H37Rv-induced p38 and JNK phosphorylation was greatly reduced by blocking either of the receptors; the reduction was 32 and 73% with anti-CD14 Ab and 66 and 75% with anti-HLA-DR Ab, respectively (Figure 3C and E). The activation status of p-ERK, p38 and p-JNK induced by clinical strain S7 was unaltered after blocking CD14 and HLA-DR receptors. Preincubation with anti-CD14 Ab resulted in 2-fold increase of Beijing-induced p38 phosphorylation (Figure 3C). However, Beijing-induced p-JNK increased by 2.3 and 2-fold in the presence of anti-CD14 and anti-HLA-DR Abs, respec-tively (Figure 3E). An IgG1 isotype control Ab used at the same concentration did not affect strain-induced MAPK phosphorylation (Figure 3A to F). Following this,

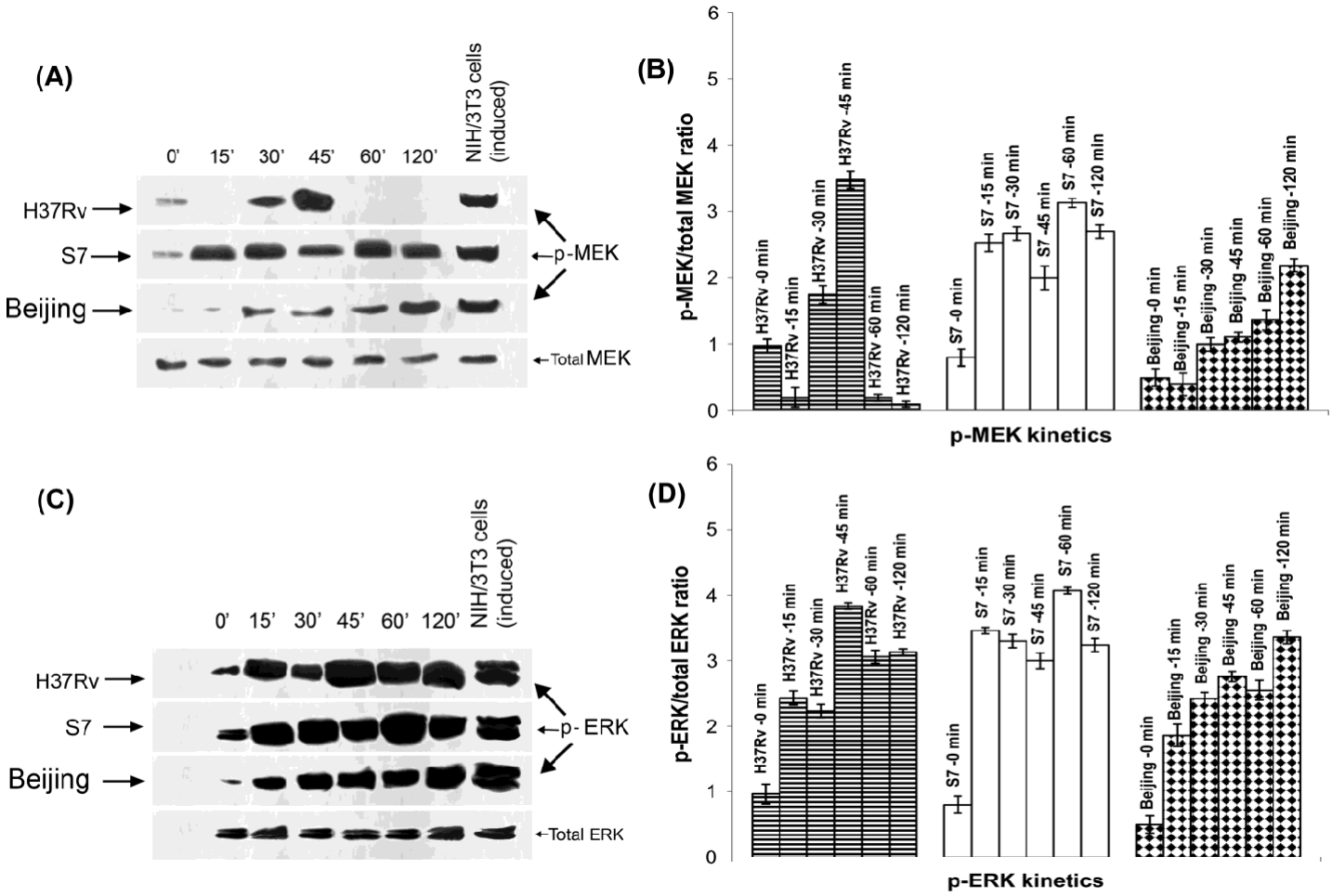

Figure 1. Activation of MEK1/2 and ERK1/2 in THP-1 human monocytes in response to infection with *M. tuberculosis* strains. THP-1 human monocytes were treated with *M. tuberculosis* strains (H37Rv or S7 or Beijing strain) (bacteria : host cell, 10:1) for various lengths of time (indicated in minutes 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 reprobed with antibodies against total MEK or ERK (bottom rows in A & 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 phosphoantibodies. Data are from five independent experiments performed in duplicate; bars represent mean ± SD. Induced NIH/3 T3 cell extracts were used as a positive control.

M. tuberculosis-induced phosphorylation of MAPKs (ERK, p38, JNK etc.,) was shown to be significantly reduced by inhibitors namely PD98059, SB203580 and dexamethasone etc. (data not shown).

Kinetics of *M. tuberculosis*-induced IL-6 and TNF-α

We learned from our serial kinetic studies that the induction of MAPKs by different *M. tuberculosis* strains was different (Figures 1, 2a and b). So, to determine whether different strains of *M. tuberculosis* were inducing different levels of proinflammatory cytokines following infection, the production of TNF-*α* and IL-6 were measured at 12 and 24 h in THP-1 monocytes after infection with different strains of *M. tuberculosis*.

The kinetics of IL-6 secretion by the strains showed that there was always a significant increase in the secretion from 12 to 24 h. The induction by S7 strain was higher than Beijing and *M. tuberculosis* H37Rv strains. However, the induction by Beijing strain was lower or comparable to *M. tuberculosis* H37Rv (Figure 4A). The kinetics of TNF-α secretion by the strains showed a significant decrease in the secretion from 12 to 24 h. Clinical strain S7 induced higher levels than Beijing and *M. tuberculosis* H37Rv strains (Figure 5A).

Effect of pathway inhibitors over *M. tuberculosis*-induced IL-6 and TNF-α

In order to decipher the signaling pathways involved, the secretion of these cytokines was assessed in the presence or in the absence of ERK, p38 and NF-κB

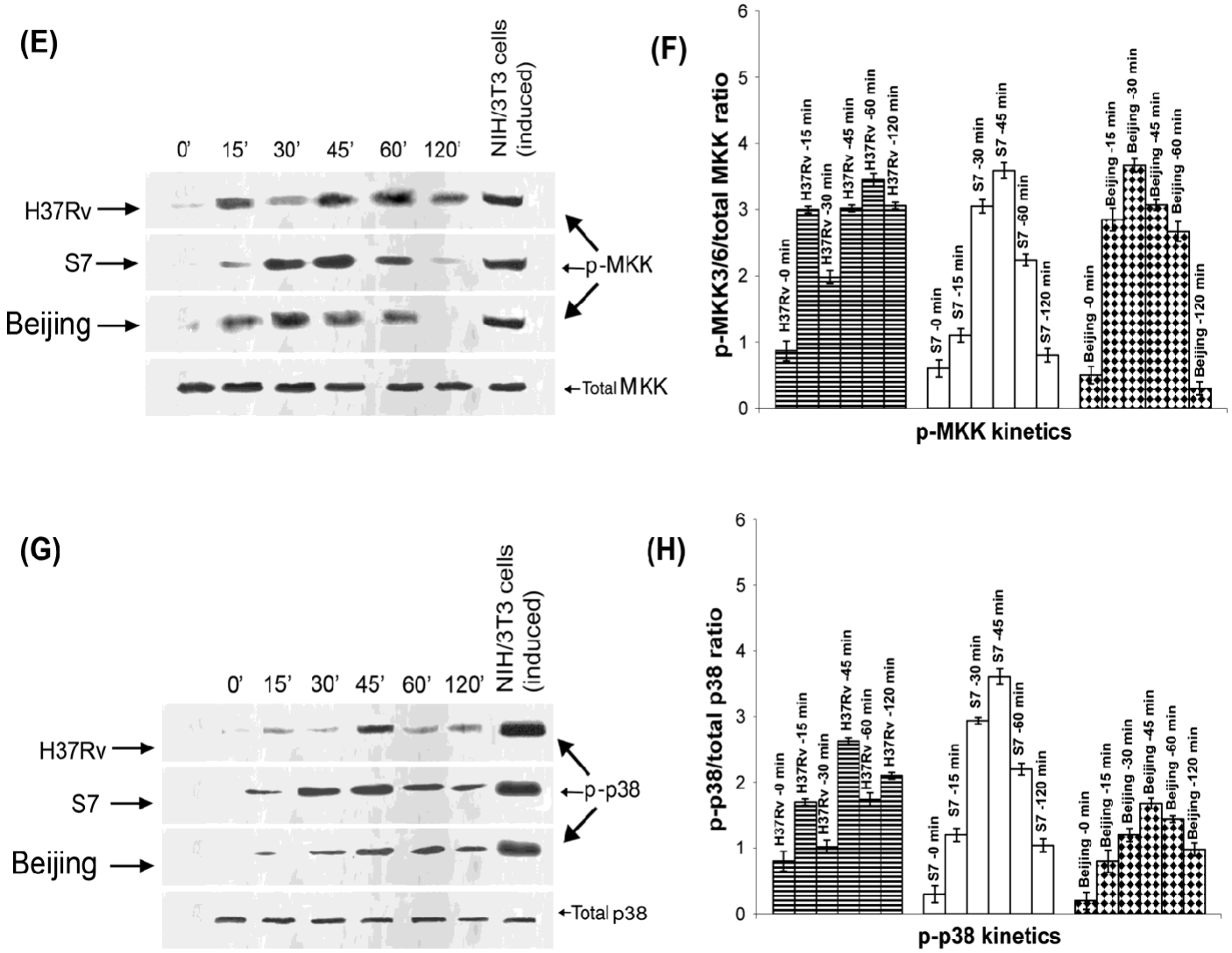

Figure 2a. Activation of MKK3/6 and p38 in THP-1 human monocytes in response to infection with *M. tuberculosis* strains. THP-1 human monocytes were treated with *M. tuberculosis* strains (bacteria : host cell, 10:1) for various lengths of time (indicated in minutes at top). Cellular extracts were analyzed by western blotting for the presence of p-MKK3/6 (E), or p-p38 (G). Each of the blots was also stripped and reprobed with antibodies against total MKK or p38 (bottom rows in E & G), to ensure equal loading of protein in all the lanes. Right panels (F & H) show the corresponding densitometric analyses of blots probed with corresponding phosphoantibodies. Data are from five independent experiments performed in duplicate; bars represent mean ± SD. Induced NIH/3T3 cell extracts were used as a positive control.

inhibitors, in THP-1 monocytes, at 24 h (IL-6) or 12 h (TNF-α). It was observed that the induction of IL-6 by *M. tuberculosis* H37Rv and S7 strains were mediated significantly through ERK, p38 and NF-κB in THP-1 monocytes (Figures 4B and C). In contrast, Beijing-induced IL-6 was reduced significantly only with p38 inhibitor in THP-1 monocytes (Figure 4D). All the strains were found to induce TNF-α significantly through ERK, p38 and NF-κB in THP-1 monocytes (Figure 5B and D), with maximum contribution from ERK and p38 MAPKs. DMSO or inhibitors alone did not exhibit any inhibitory effect over strain-induced cytokine secretion (Figures 4 and 5). Similar results were obtained with primary human monocytes for both cytokines (data not shown).

Bcl-2 production by different strains of *M. tuberculosis* was time-dependent

Kinetics of Bcl-2 production induced by all the strains showed that there was a tendency to increase from 12 to 24 h, a pattern which was in contrast to the pattern observed with TNF-α kinetics (Figures 5 and 6A). Densitometry analysis of the blots showed that the induction caused by strain S7 remained lower than control cultures at both 12 and 24 h. The induction by

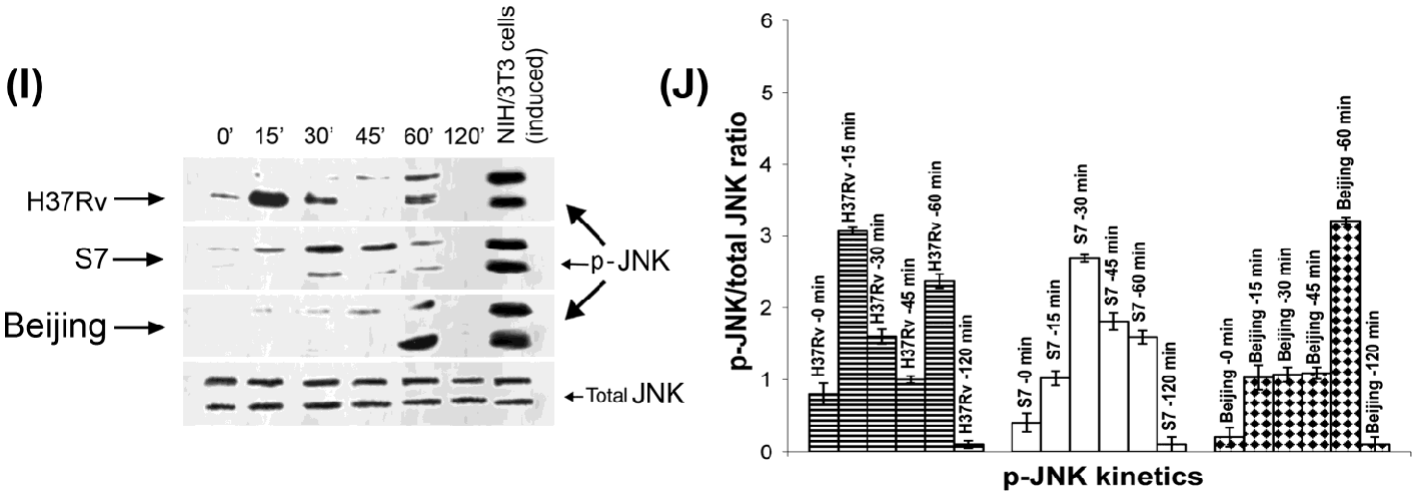

Figure 2b. Activation of JNK in THP-1 human monocytes in response to infection with *M. tuberculosis* strains. THP-1 human monocytes were treated with *M. tuberculosis* strains (bacteria : host cell, 10:1) for various lengths of time (indicated in minutes at top). Cellular extr acts were analyzed by western blotting for the presence of p-JNK (I). Each of the blots was also stripped and reprobed with antibody against total JNK (bottom row in I), to ensure equal loading of protein in all the lanes. Right panel (J) show the corresp onding densitometric analyses of blots probed with corresponding phosphoantibody. Data are from five independent experiments performed in duplicate; bars represent mean ± SD. Induced NIH/3T3 cell extracts were used as a positive control.

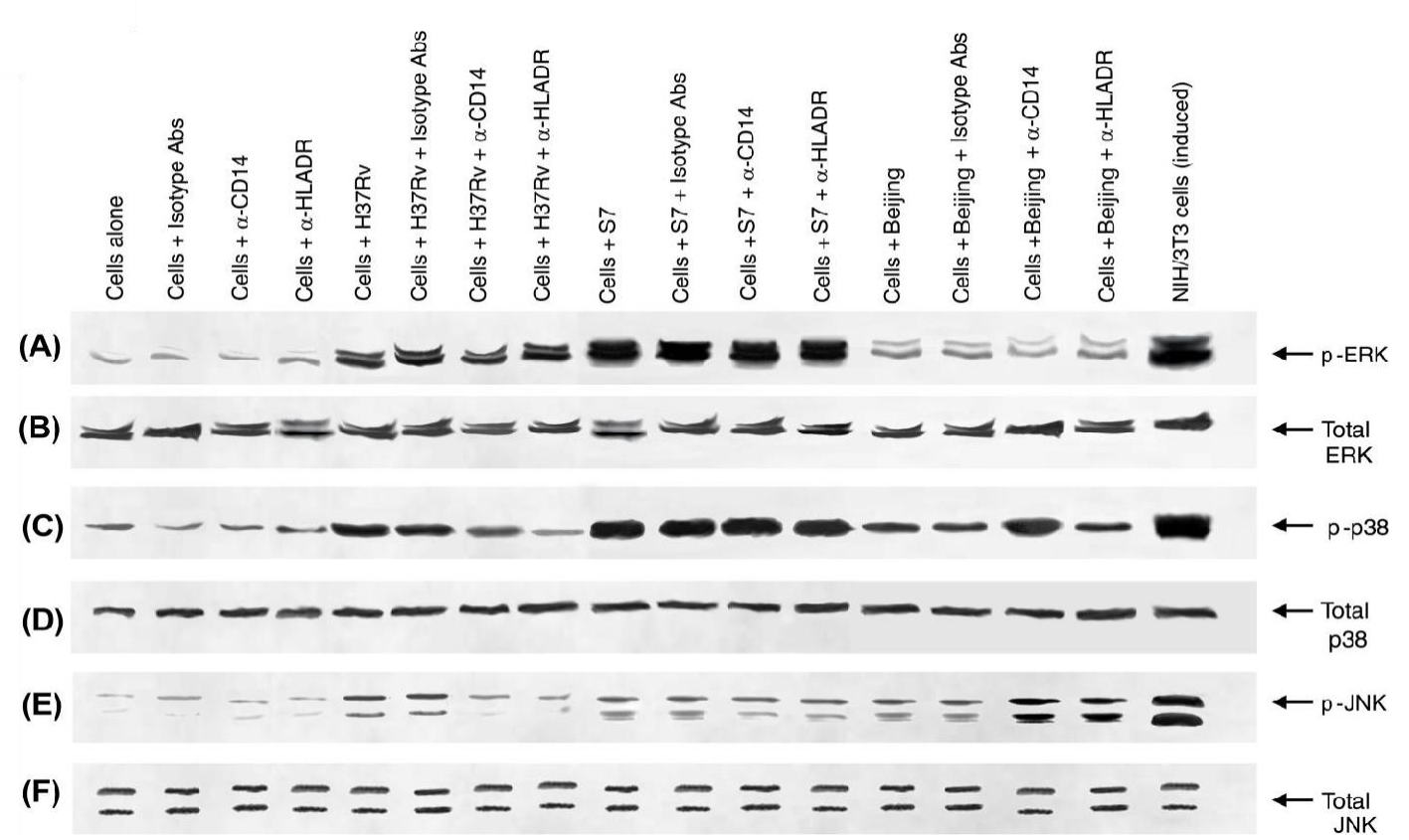

Figure 3. Activation of ERK1/2, p38 and JNK by *M. tuberculosis* strains through CD14 and HLA-DR receptors. THP-1 cells were preincubated with, medium alone, or isotype control Abs, or anti-CD14 monoclonal Abs, or anti-HLA-DR monoclonal Abs (each 5µg/ml) for 60 min. Subsequently, *M. tuberculosis* strains were added and the cells were lysed during those time points at which peak phosphorylation signals of ERK1/2, p38 and JNK MAPKs occurred with *M. tuberculosis* strains in our serial kinetic studies (Figure 1, 2a and 2b). The lysates were subjected to immunoblotting with specific antiphospho antibodies (A, C & E). The blots were stripped an d reprobed with antibodies against total ERK or p38 or JNK (B, D & F), 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.

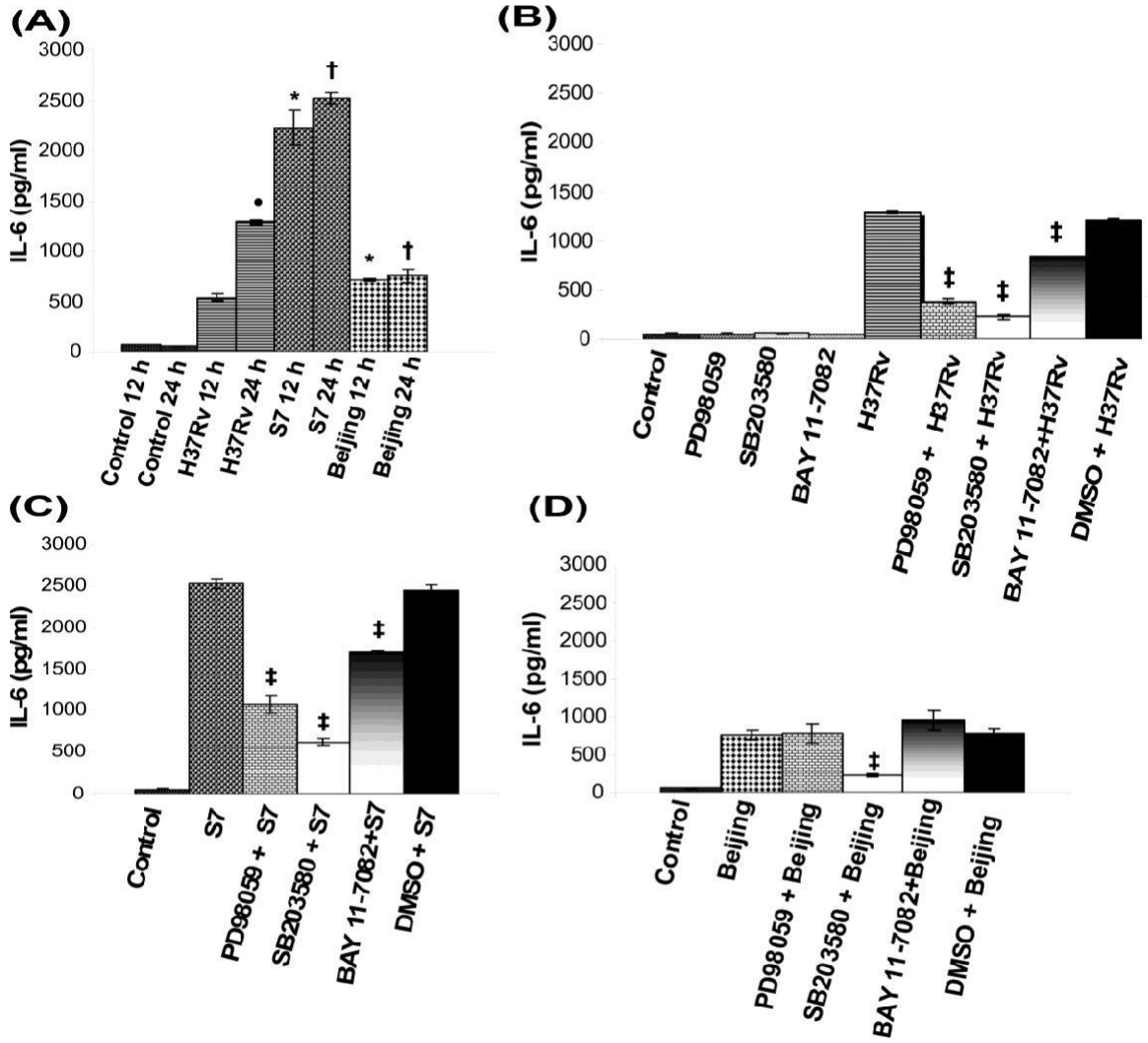

Figure 4. Effect of pharmacological inhibitors of ERK, p38 MAPK and NF-kB over *M. tuberculosis*-induced IL-6 production. THP-1 cells were left untreated or treated with *M. tuberculosis* strains (bacteria : host cell, 10:1) for 12 & 24 h (A). Cells were also separately treated with 40 µM PD98059 or 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 S7 (C) or Beijing strain (D) for 24 h. Supernatants were harvested and IL-6 formation was measured by ELISA. Data are from five independent experiments performed in triplicate; bars represent mean ± SD. IL-6 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 IL-6 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 24 h time point.

M. tuberculosis H37Rv and Beijing strain raised 2 fold and 3 fold above control cultures at 24 h, respectively (Figure 6B).

ERK and p38 pathways are essential for *M. tuberculosis*-induced Bcl-2 production

Immunoblotting of Bcl-2 was performed after 24 h of infection in the presence of pathway inhibitors. Densitometric analysis of the Bcl-2 immunoblots showed that the production of Bcl-2 caused by *M. tuberculosis* strains decreased after inhibition of ERK1/2 or p38 pathways. With H37Rv, the decrease in Bcl-2 activation was 78 and 53% by PD98059 and SB203580, correspondingly. S7 strain showed a decrease of 70 and 85%, respectively. Beijing strain-induced activation was decreased by 60 and 68%, correspondingly (Figure 7A).

Inhibition of NF-κB augments *M. tuberculosis*-induced ERK and Bcl-2 activation in THP-1 monocytes

To assess whether the induction of Bcl-2 by *M. tuberculosis* strains is mediated through NF-κB, we

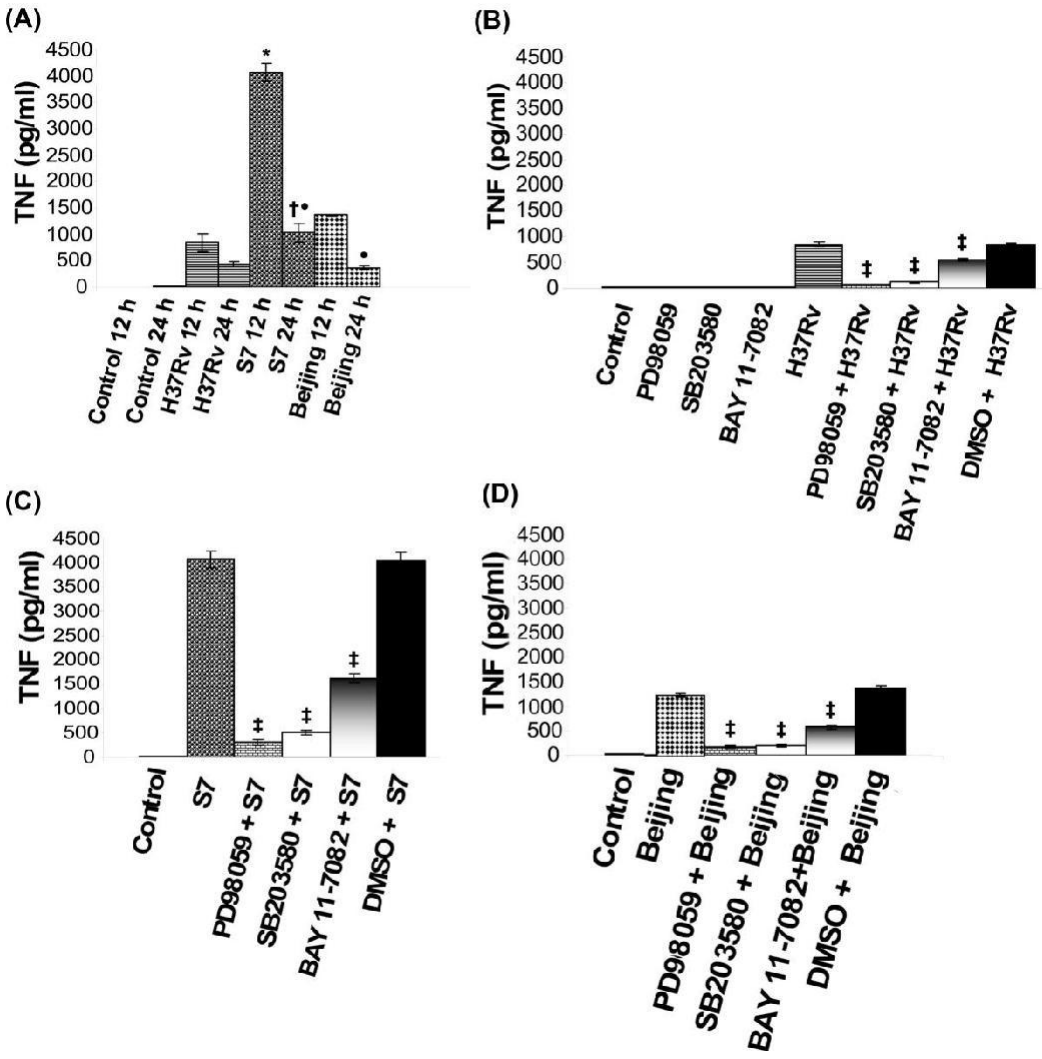

Figure 5. Effect of pharmacological inhibitors of ERK, p38 MAPK and NF-kB over *M. tuberculosis*-induced TNF-α production. THP-1 cells were left untreated or treated with *M. tuberculosis* strains (bacteria : host cell, 10:1) for 12 & 24 h (A). Cells were also separately treated with 40 µM PD98059 or 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 S7 (C) or Beijing strain (D) for 12 h. Supernatants were harvested and TNF-α formation was measured by ELISA. Data are from five independent experiments performed in triplicate; bars represent mean ± SD. 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 & 24 h values**,** ‡*P*<0.05 when the induction of strains was compared with the inhibitor-treated cultures at 12 h time point.

analyzed Bcl-2 production in the presence or in the absence of 5 µM NF-κB inhibitor (BAY 11-7082) for 24 h in THP-1 monocytes. To find out the crosstalk if any, ERK1/2 phosphorylation was also evaluated in the same cellular lysates. Densitometric analysis of the blots revealed that the induction of both Bcl-2 (Figure 8C) and p-ERK1/2 (Figure 8B) 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* S7 strain, the presence of inhibitor led to an increase of p-ERK1/2 and Bcl-2 by < 1.5-fold. Beijing strain showed a maximum rise of p-ERK1/2 and Bcl-2 by >3 fold, respectively (Figure 8A to C).

Expression kinetics of surface receptors CD44 and CD25 in THP-1 monocytes is strain-specific

When we challenged THP-1 monocytes with different

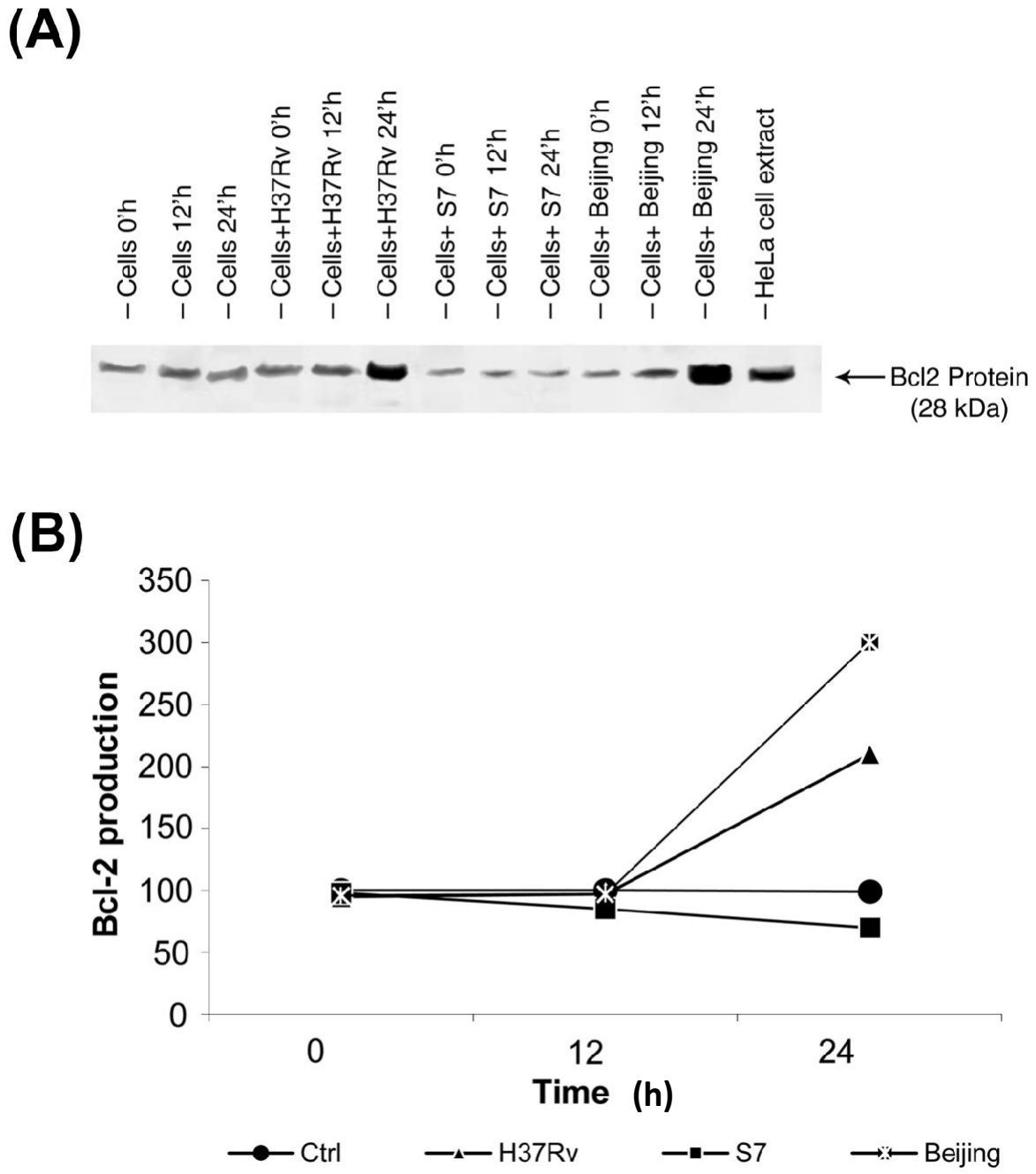

Figure 6. Analysis of Bcl-2 kinetics by western blotting. THP-1 cells were left untreated or treated with *M. tuberculosis* strains (bacteria: host cell, 10:1) for 12 and 24 h. The harvested cellular lysates were subjected to western blot analysis with anti-human Bcl-2 antibody. (A) Bottom panel shows the corresponding densitometric analyses of the blots. (B) Data shown are mean of five independent experiments performed in duplicate. HeLa cell extracts were also blotted.

M. tuberculosis strains for 12 and 24 h, it was observed that the clinical strain S7 induced the highest expression levels of CD44 and CD25, followed by *M. tuberculosis* H37Rv and Beijing strain. When S7 and Beijing strains were compared with *M. tuberculosis* H37Rv, the expression of both CD44 and CD25 were significantly different between the strains at 24 h (Figure 9A and D).

Signaling pathways utilized by *M. tuberculosis* strains to induce surface expression of receptors CD44 and CD25

To assess the signaling pathways involved in the surface expression of CD44 and CD25 by *M. tuberculosis* strains in THP-1 monocytes, we analyzed their surface

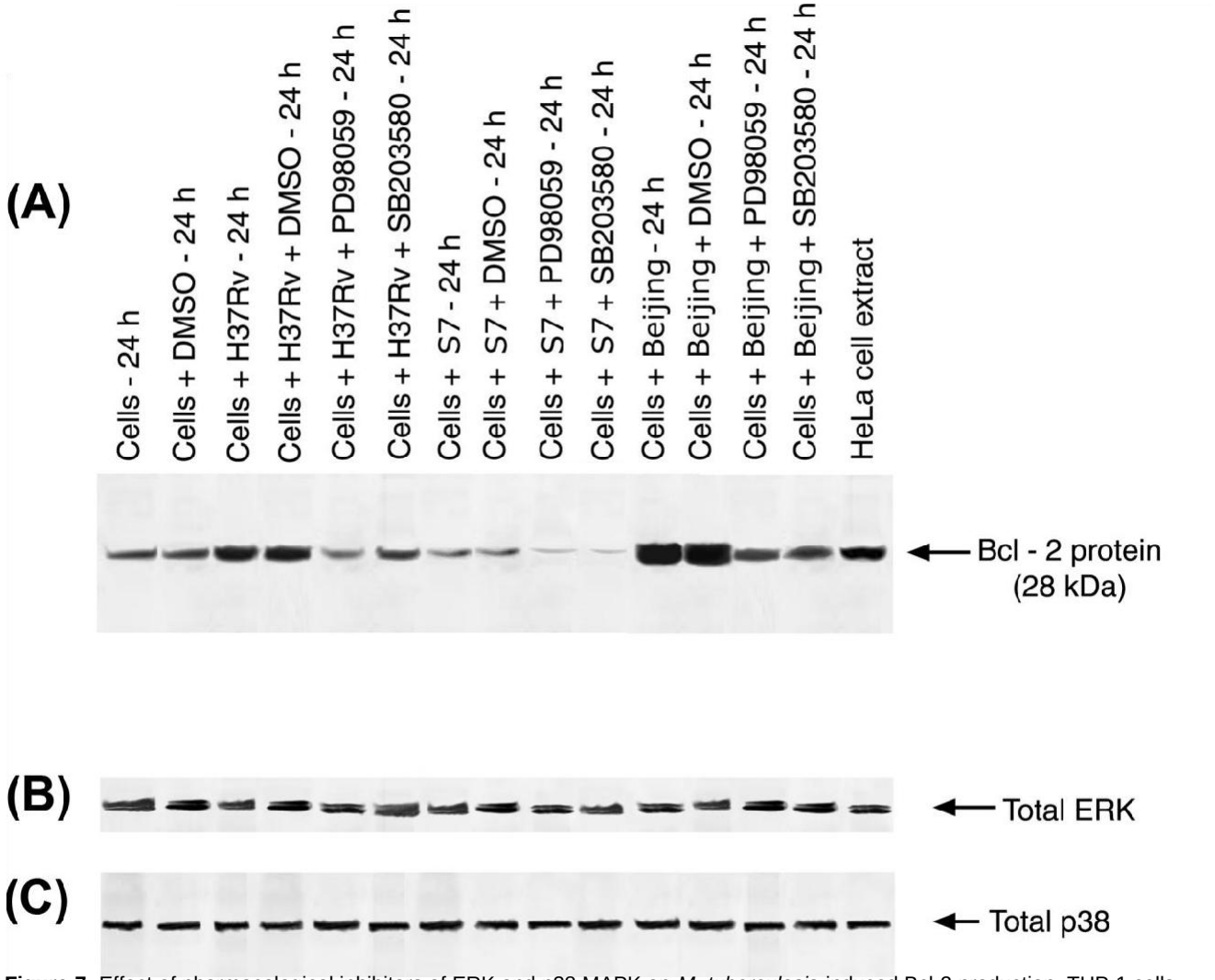

Figure 7. Effect of pharmacological inhibitors of ERK and p38 MAPK on *M. tuberculosis*-induced Bcl-2 production. THP-1 cells were untreated or treated with vehicle (0.1% DMSO) or 40 µM PD98059 or 30 µM SB203580 for 1 h prior to incubation with *M. tuberculosis* strains, and terminated at 24 h. Cellular lysates were subjected to western blot analysis with anti-human Bcl-2 antibody (A). Each of the blots was also stripped and reprobed with antibodies against total ERK (B) or p38 (C) to ensure th at these were present in equal amounts in all lanes. Similar data were obtained from five independent experiments performed in duplicate. Blots from HeLa cell extracts were also shown.

expression in the presence or in the absence of ERK, p38, JNK, protein tyrosine kinase, or PI3K inhibitors, for 24 h in THP-1 monocytes. Since Beijing strain did not induce significant surface expression than uninfected control cultures in kinetic studies (Figure 9A and D), it was not included in these inhibition experiments.

The increased surface expression of CD44 caused by inhibition of ERK1/2, JNK, protein tyrosine kinases and PI3K**,** and the decreased surface expression caused by inhibition of p38, were evident with *M. tuberculosis* H37Rv and S7 strain (Figure 9B and C). But statistical analysis revealed that the modulation caused by inhibition of ERK1/2 was not significant in the case of S7 strain (Figure 9C). Inhibition of all the kinases except ERK1/2 showed decreased CD25 expression with H37Rv (Figure 9E), whereas, inhibition of all the kinases except ERK1/2 and tyrosine kinases showed decreased CD25 surface expression with S7 strain (Figure 9F). Thus, the extent of inhibition of surface receptor expression by some inhibitors differed between the strains.

DISCUSSION

Gagneux et al. (2006) has reported that *mycobacterial* lineages are more likely to spread in sympatric than in allopatric patient populations. Recent studies are more focused in establishing the pathogenicity of clinical isolates, which are most predominant in the community and responsible for major outbreaks**.** We have studied the effect of the prevalent Indo-Oceanic Clade strain, S7, on the immune response elicited through the MAPK

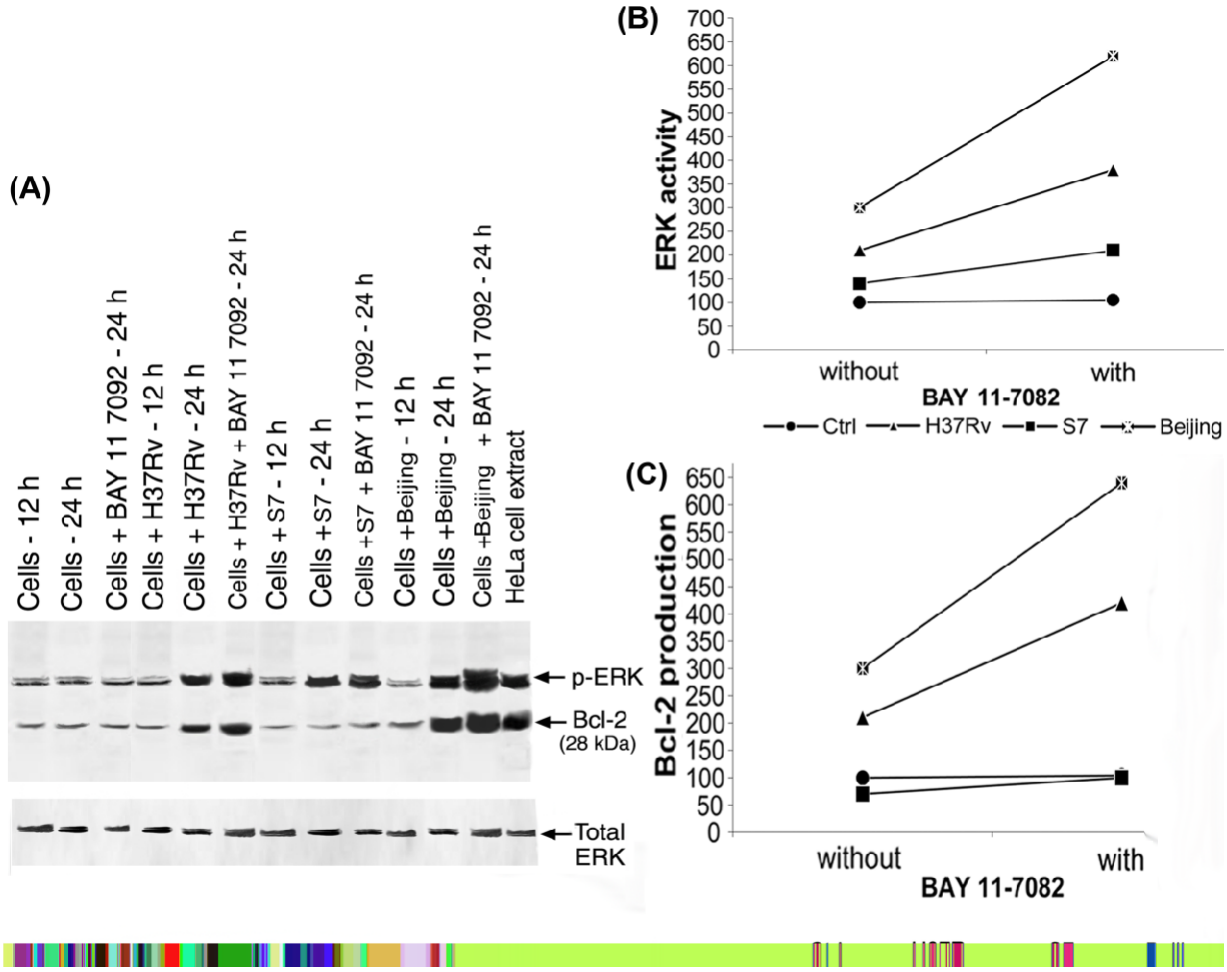

Figure 8. Effect of BAY 11-7082 on *M. tuberculosis*-induced ERK activation and Bcl-2 production. THP-1 cells were left untreated or treated with *M. tuberculosis* strains (bacteria : host cell, 10:1) for 12 and 24 h. Cells were also separately treated with 5 µM BAY 11-7082 (NF-κB inhibitor) for 60 min before stimulation with *M. tuberculosis* strains (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 reprobed with antibodies against total ERK [bottom row of (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 five independent experiments performed in duplicate. Blots from HeLa cell extracts were also shown.

pathway. Our findings demonstrated the ability of clinical strain S7 to induce differential monocytic activation in comparison with a low prevalent Beijing strain isolated from the same area.

The observed differences in duration and intensity of signaling induced by clinical strains in our MAPK kinetic studies may be important in deciding the fate of the strain inside the macrophages (Blumenthal et al., 2002; Tse et al., 2002; Koul et al., 2004), or the fate of the host cell itself (Yoon et al., 2008). These differences could be due to alterations in the activation of scaffolding proteins or phosphatases (Kolch, 2005; Salojin et al., 2006; Cheung 2009), caused by various *mycobacterial* cell wall-associated virulence factors like polymorphic GC- rich repetitive-sequence (PGRS) domain, Lipoarabinomannan/lipomannan (LAM/LM) ratio, LAM structure etc., through various cell surface receptors. The possible influence of these differences upon antimycobacterial mechanisms has been strengthened by the findings of Davis et al. (2007), which emphasize the importance of interaction of *mycobacteria* with host scaffolding protein EBP50 (Ezrin/radixin/moesin family Binding Protein 50) in association with lowered iNOS (inducible nitric oxide synthase) recruitment to phagosome.

The differential usage of CD14 and HLA-DR receptors by *M. tuberculosis* strains in our receptor blockade experiments might explain the differential p-MAPK kinetic patterns obtained with these strains**.** To our knowledge, this is the first ever report, showing the importance of complex receptor usage in mediating differential

Fig. 9

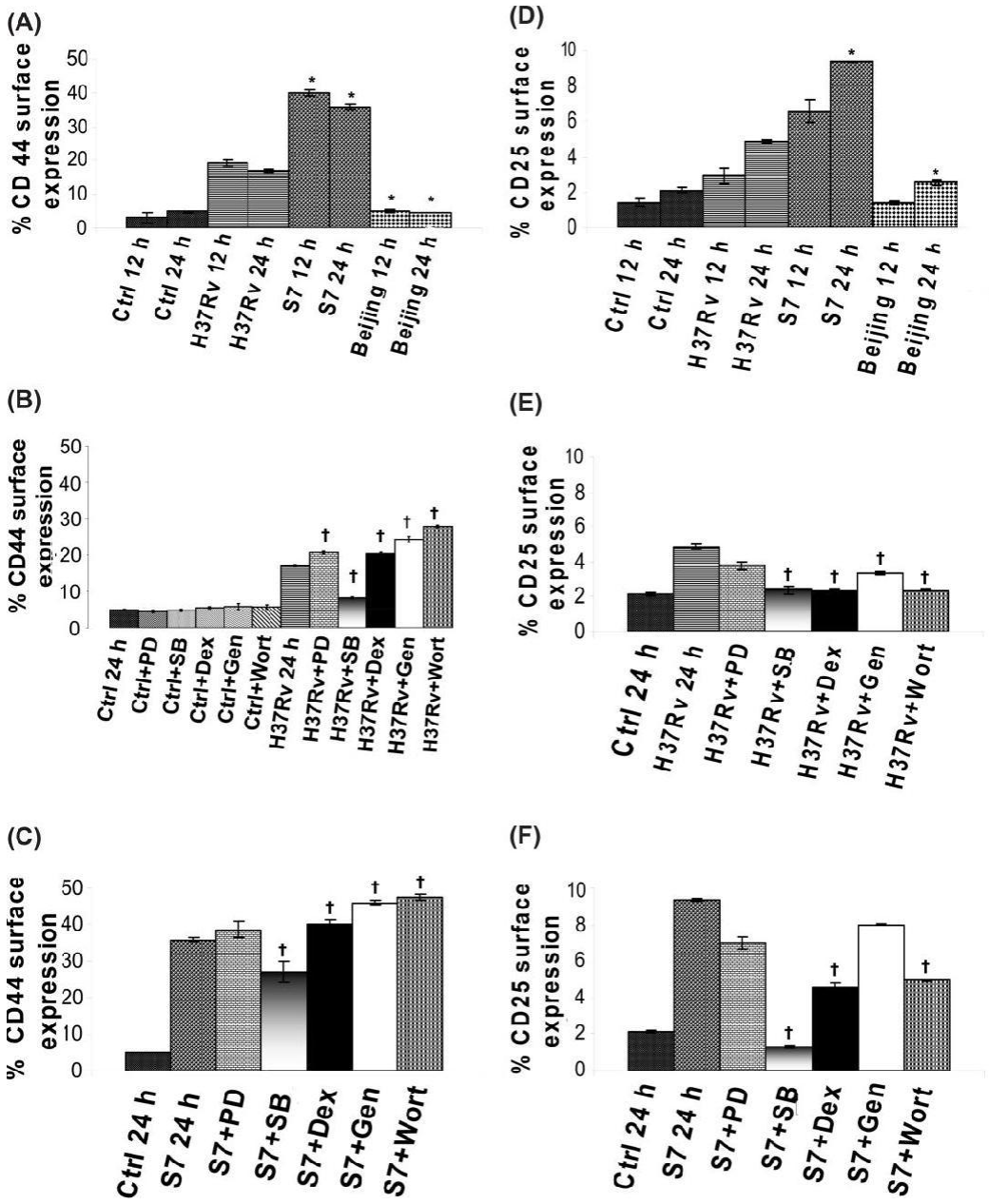

Figure 9. Kinetics and influence of pathway inhibitors over *M. tuberculosis*-induced CD44 and CD25 surface expression in THP-1 cells. THP-1 cells (1×10^6/ml) were left untreated (Ctrl) or treated with *M. tuberculosis* strains (bacteria: host cell, 10:1) for 12 and 24 h and analyzed for CD44 (A) or CD25 (D) surface expression ($CD14^+CD44^+$ or $CD14^+CD25^+$ double positive population) by flow cytometry. Cells were also separately treated with vehicle or with the MAPK-specific inhibitors PD98059 (PD), SB203580 (SB) and dexamethasone (Dex), or with non-specific inhibitors genistein (Gen) and wortmannin (Wort), for 60 min prior to incubation with *M. tuberculosis* strains (bacteria : host cell, 10:1) for 12 h. The cells were again analyzed for CD44 (B & E) and CD25 (C & F) surface expression by flow cytometry as described under "Materials and Methods." Data shown are the mean ± SD of five independent experiments performed in triplicate. *****P<0.05 when S7 or Beijing strain-induced expression is compared with that of H37Rv at 12 or 24 h time points. †P<0.05 when H37Rv or S7-induced expression is compared at 12 h time point in the presence of inhibitors.

phosphorylation of MAPKs induced by clinical isolates of *M. tuberculosis*. Also, the involvement of HLA-DR in mycobacteria-induced MAPK activation was not reported earlier. Furthermore, the observed differences in the usage of receptors between S7 and other strains might have led to differential binding and altered phagocytic

ability demonstrated for S7 strain in THP-1 cells by Rajavelu and Das (2007). This possibility is reinforced by the data of Schlesinger, (1993). The fact that there is no change in S7 strain-induced MAPK activation after blockage of CD14 or HLA-DR receptor, implies that other receptors like complement receptor 3 (CR3), mannose receptor, Toll-like receptors (TLRs), etc. might be involved in the activation of those MAPKs. Since Souza et al. (2006, 2008) has already reported the importance of TLR2-mediated (but not CD14 or CR3 receptors) p38 MAPK activation in the context of IL-10 transcription and *Mycobacterium avium* subsp. *paratuberculosis* (MAP) killing in bovine monocytes, the usage of receptors other than CD14 or HLA-DR by S7 strain might influence the activation of MAPKs and downstream antimicrobial defense mechanisms. The contrasting changes observed between H37Rv and Beijing-induced p-JNK/p-p38 signals after blocking CD14 or HLA-DR receptor, clearly distinguishes Beijing, from H37Rv and S7 strains. This observation raises the possibility of fine-tuning p38/JNK induction through more than one receptor. 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 by Sendide et al. (2005). In addition, for the reason that p38 MAPK pathway has been implicated in the arrest of phagosome maturation in *mycobacterial* infection, the differences observed in receptor-mediated activation of p38 MAPK among these strains might play a role in affecting their intracellular survival (Fratti et al., 2003).

Clinical strain S7 induced higher levels of CD44, CD25 and TNF-α followed by *M. tuberculosis* H37Rv and Beijing strain. The difference in CD44 surface expression and TNF-α levels observed between S7 and Beijing strain could be due to rapid, higher activation of many MAPKs by S7 (Carter et al., 1999; Hasan et al., 2003; Dhiman et al*.,* 2007; Basler et al*.*, 2010; Damm et al., 2010), or might be because of differences in the extent of activation of CD44 and TNF-α promoters by these two strains (Lee and Schorey, 2005). Because CD44 has been shown to be important in binding of *M. tuberculosis,* phagocytosis, macrophage recruitment, and protective immunity against pulmonary tuberculosis (Leemans et al., 2003), and the role of CD25 in monocytic activation (Farina et al., 2004; Weber et al., 2004) has been well documented, this differential expression of CD25 and CD44 might influence the activation status, phagocytic efficiency, and the migratory pattern of the THP-1 monocytes infected with corresponding strains. The higher expression of CD44 seen with S7 strain might have also led to increased macrophage binding of S7 strain demonstrated by Rajavelu and Das (2007) during infection with THP-1 cells. But this did not correlate with the less efficient T cell proliferation and reduced chemoattraction of $CD4^+$-T cells shown for the S7 strain in previous studies (Rajashree et al., 2008; Rajavelu and Das, 2003). This discrepancy might be due to the altered expression of co-stimulatory molecules in macrophages (Rajavelu and Das, 2008) or altered migratory/chemokine secretory patterns exhibited by S7 strain (Rajashree et al., 2008; Rajavelu and Das, 2003).

Inhibition experiments examining CD44 and CD25 surface expression uncovered similar pattern of changes in surface expression between S7 strain and *M. tuberculosis* H37Rv. But statistical analysis revealed differences in the pathways used by these two strains to bring statistically significant changes in the level of surface expression of these receptors. This might partly explain the reason behind the differential expression of these surface receptors induced by different strains in the CD44 and CD25 kinetic studies. This is the first ever report about the involvement of signaling pathways in mediating *M. tuberculosis*-induced surface expression of CD25 and CD44 receptors. The distinct role played by p38 MAPK stands out clearly in these inhibition experiments. Bansal et al. (2009) demonstrated the cross-talk between PI3K and ERK1/2 in mediating phosphatidyl-myo-inositol dimannoside (PIM2)-induced CD44/matrix metalloproteinase-9 (MMP-9) expression in RAW 264.7 cells. Moreover, a cross-talk between p38 and other kinases has already been reported in *Mycobacterium avium* infection by Tse et al. (2002). So, a similar cross-talk between the pathways studied in our experiments might have led to the results that we observed in our inhibition experiments involving surface expression of CD44.

In spite of the fact that the kinetics of IL-6 secretion by the stimulants is same in THP-1 monocytes, the secretion levels induced by clinical strain S7 were higher than *M. tuberculosis* H37Rv and Beijng strain. Since IL-6 has been shown to induce tuberculostasis via an autocrine activation pathway in macrophages (Flesch and Kaufmann, 1990), the higher level of IL-6 associated with the clinical strain S7 might influence its rate of multiplication and prolonged survival inside the monocyte/macrophages of exposed or diseased persons. In line with this, Rajavelu and Das (2007) has shown that the clinical strain S7 grows slowly inside THP-1 cells compared to H37Rv. The higher levels of IL-6 might also help to inhibit macrophage responses to IFN-γ in the later phase of infection (Nagabhushanam et al., 2003). The distinguishing feature in inhibition experiments involving cytokine secretion is the usage of different pathways by Beijing and S7 strains to bring significant changes in IL-6 secretion. This is the first ever report on the differential usage of MAPK pathways by clinical isolates of *M. tuberculosis* to induce IL-6 in THP-1 cells.

Even though the strain-specific levels of TNF-α correlated well with their levels of CD44 and CD25 expression, contrasting patterns were observed between Bcl-2 and TNF-α expression levels. For instance, S7 showed higher activation of MAPKs in serial kinetic experiments, induced higher level of CD25, CD44 surface

expression, and TNF-α, but triggered lower levels of Bcl-2 than Beijing strain, in THP-1 monocytes. Since TNF-α has been shown to activate CD25 (Farina et al., 2004) or all three MAPKs (Hatzoglou et al., 2000), and, also shown to autoregulate CD44 expression in monocytes through p38 MAPK (Gee et al., 2002), this correlation between TNF-α and other markers might be a true autoregulatory phenomenon operating within these two clinical strains. This possibility is supported by data of Rojas et al. (1999), Spira et al. (2003), Kundu et al. (2009), and also by the study"s observation that strain-induced TNF-α and surface expression of CD44/CD25 are inhibited by p38 inhibitor, SB203580.

Although both TNF-α and Bcl-2 are positively mediated by ERK and p38 in our inhibition experiments, their expression kinetics are in contrast to each other. The differential JNK activation induced by different strains might have led to these contrasting expression patterns, because phosphorylation of JNK has been shown to decrease the expression of Bcl-2 family members and increase apoptosis in U937 monocytic leukemia cells (Hahn et al., 2005). Since TNF has been shown to cause apoptosis through activation of JNK by apoptosis signal regulating kinase (ASK-1) in fibroblasts (Tobiume et al., 2001), it is also feasible that increased TNF-α levels induced by S7 strain might have reduced Bcl-2 activation through JNK in an autoregulatory fashion in our studies.

Apart from the host-related mechanisms, the observed strain-specific patterns of proinflammatory cytokines, surface markers, and Bcl-2 might be due to strain-specific factors like: a) the sequence variations in the PPE/PGRS domain (Basu et al., 2007; Talarico et al., 2005; Daim et al., 2011), b) LAM/LM ratio, c) lipid moieties of the clinical isolates (Rocha-Ramirez et al., 2008; Mendelson et al., 2005) etc., The exclusive finding that inhibition of NF-κB further increases the strain-induced Bcl-2 activation and ERK1/2 phosphorylation could be a compensatory pathway used by the strains to tune the survival of cells. This is supported by the data of Subramanian and Shaha (2007), which highlight the link between survival of human THP-1 macrophages and estrogen-mediated upregulation of Bcl-2 through ERK phosphorylation. The extent of augmentation of Bcl-2 also differed between the strains with S7 showing the least and Beijing strain showing the maximum. The increase or the differences in strain-induced Bcl-2 levels observed with NF-κB inhibition may be due to the strain-specific modulation of potential transcription factors like activator protein-1 (AP-1) (Lee et al., 2009), serum response factor (SRF) (Schratt et al., 2004; Turjanski et al., 2007), cAMP-response element-binding protein (CREB) (Pugazhenthi et al., 1999), etc. acting downstream of ERK or, it might be due to altered regulation of factors like eukaryotic initiation factor 4E-binding protein 1 acting through ERK at the translation level (Nishioka et al., 2010). Collectively, this data also reinforces for the first time, the possible cross-talk that can exist between NF-κB and ERK in *M. tuberculosis*-infected monocytes.

In summary, MAPK-mediated immune activation induced by a prevalent Indo-Oceanic clade clinical strain S7 was different from that induced by a low prevalent Beijing strain isolated from the same region. We are currently planning to study: a) the effect of MAPK inhibitors (Bluementhal et al., 2002; Tse et al., 2002) and AKT inhibitors (Kuijl et al., 2007) over mycobacterial replication in primary human macrophages or mice-derived macrophages infected with S7, Beijing and other clinical strains, which differ in their lineage and prevalence in our region, b) the level along the pathways at which clinical isolates induce differential activation to bring changes in the levels of various effector molecules, c) the effect of these inhibitors over strain-stimulated IFN-γ production in peripheral blood mononuclear cells etc. Besides, *in vivo* experiments will be conducted in the future based on the results from these preliminary studies. All these would provide evidence of strain-related virulence.

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Abbreviation: *M. tuberculosis*, *Mycobacterium tuberculosis;* **ELISA,** Enzyme linked immunosorbent assay; **H37Rv,** *M. tuberculosis* H37Rv; **AP-1,** activator protein; **PGRS,** polymorphic *GC*- rich repetitive-sequence domain; **BCG,** *M. bovis* bacillus Calmette Guerin; **CD14 receptor,** cluster of differentiation 14 receptor; **HLADR,** human leukocyte antigen DR; **THP-1,** human monocytic cell line; **IL-6,** interleukin-6; **Bcl-2,** B-cell Lymphoma 2; **RFLP,** restriction fragment length polymorphism; **MoDC,** monocyte-derived DCs; **CREB,** cAMP-response element-binding Protein; **MAPK,** mitogen-activated protein kinase; **MKK or MAPKK or MEK,** MAPK kinases; **MEK,** MAPK kinase that phosphorylates ERK, **ERK,** extracellular signal regulated kinase; **JNK or SAPK-c,** jun N-terminal kinases or stress-activated protein kinase; **PI3K,** phosphatidylinositol 3 kinase; **p-MAPKs,** phosphorylated forms of MAPKs; **PD98059,** MEK inhibitor; **SB203580,** p38 inhibitor; **TNF,** α-tumour necrosis factor-alpha, **PI3K,** phosphatidylinositol 3-kinase; **HEPES,** 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid; **DMSO,** dimethylsulfoxide, **FCS,** fetal calf serum; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **LPS,** lipopolysacharide; **ANOVA,** analysis of variance; **CR,** complement receptor; **TLRs,** toll-like receptors; **MKP-1,** MAPK phosphatase-1; **SRF,** serum response factor; **NF-κB,** nuclear factor-KappaB; Bay 11-7082-NF-κB inhibitor, **LAM/LM,** lipoarabinomannan/

lipomannan; **EBP50,** Ezrin/radixin/moesin family Binding Protein 50; **iNOS,** inducible nitric oxide synthase; **MAP,** *Mycobacterium avium* subsp. *Paratuberculosis*; **CR3,** complement receptor 3; **TLRs,** toll-like receptors; **ASK-1,** apoptosis signal regulating kinase; **PIM2,** phosphatidyl-myo-inositol dimannoside; **MMP-9,** matrix metalloproteinase-9 expression.

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