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Mycobacterium tuberculosis heat shock protein 60 modulates immune response to PPD by manipulating the surface expression of TLR2 on macrophages

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Summary

The T-helper (Th) 1 T-cell response to purified protein derivative (PPD) is known to be suppressed in tuberculosis patients which favours intracellular survival of the bacilli. We demonstrate that the *Mycobacterium tuberculosis* heat shock protein 60 (Mtbhsp60) plays an important role to skew the anti-PPD T-cell response towards the Th2 type when macrophages were used as antigen presenting cells. We found that the PPD-induced IL-12 p40 was downregulated in macrophages by Mtbhsp60. The Mtbhsp60 preferentially induced Toll-like receptor (TLR) 2 without affecting TLR4 expression on macrophages. Interaction of Mtbhsp60 with TLR2 resulted in significant suppression of nuclear c-rel and consequently IL-12 p40 levels in PPD-activated macrophages. Our findings reveal a unique role of the Mtbhsp60 favouring development of Th2 type response by upregulating surface expression of TLR2 on macrophages which could be a survival strategy adopted by the bacilli.

Introduction

Interaction between the pathogen and the host is a dynamic confrontation where the microbe's strategy of survival by implementing various devices that challenges the formidable defences of the host immune system. A complex interplay between the 'host's defence mechanisms' and 'attempts to circumvent these defences by *Mycobacterium tuberculosis*' plays a key role in determining the outcome of tuberculosis (TB) infection.

Various studies reveal that *M. tuberculosis* promotes an environment characterized by T-helper (Th) 2 cytokines during infection (Hernandez-Pando *et al*., 1996; Rook *et al*., 2005). The observation that the anti-purified protein derivative (PPD) Th1 T-cell response is far less pronounced in TB patient (Baliko *et al*., 1998; Wilsher *et al*., 1999) suggests the possibility that an active TB infection is associated with a decrease in Th1 response. Therefore, it seems that failure to resolve infection in susceptible individuals could be a consequence of strategies used by the *M. tuberculosis* to suppress Th1 response. The interleukin (IL)-12 cytokine secreted by the antigen presenting cells (APCs) during the innate immune response (D'Andrea *et al*., 1992; Trinchieri, 1997) plays an important role in stimulating IFN-γ production and a Th1 T-cell response (Hsieh *et al*., 1993; Trinchieri, 1994; Magram *et al*., 1996; Mukhopadhyay *et al*., 1999). The bacilli being the macrophage-resident (Fenton, 1998; Schnappinger *et al*., 2003) are likely to suppress IL-12 induction in macrophages (Cooper *et al*., 1997; Fenton, 1998; Flynn and Chan, 2003) to create a favourable Th2 environment for it to thrive.

Heat shock proteins (hsps) were initially identified as prominent antigens in a range of infectious diseases and autoimmune disorders (Young *et al*., 1988; Zugel and Kaufmann, 1999). In addition to immune recognition of the protein themselves, the major functions of hsps are to act as molecular chaperones to assist in folding and assembly of polypeptides within the cell (Zugel and Kaufmann, 1999). Various studies reveal that the biochemical features of *M. tuberculosis* hsp60 (Mtbhsp60) deviate significantly from the characteristic properties of the *Escherichia coli* hsp60 (Gaston, 2002; Qamra *et al*., 2004). Interestingly, an attempt to monitor gene expression patterns of mycobacteria residing within macrophages revealed upregulation of Mtbhsp60 protein (Young and Garbe, 1991; Zugel and Kaufmann, 1999; Monahan *et al*., 2001), suggesting that the protein might offer a survival advantage to mycobacteria within the host (Shinnick, 1991).

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The hsp60 is known to interact with Toll-like receptors (TLRs), namely the TLR2 and the TLR4 (Ohashi *et al*., 2000; Vabulas *et al*., 2001). These receptors are involved in triggering different signalling pathways (Mean *et al*., 2001; Bulut *et al*., 2005; Basu *et al*., 2007; Jo *et al*., 2007) and have been shown to play important roles in *M. tuberculosis* pathogenesis (Sugawara *et al*., 2003; Branger *et al*., 2004; Drennan *et al*., 2004; Jang *et al*., 2004; Pompei *et al*., 2007). Therefore, it is possible that Mtbhsp60 can manipulate the TLR-dependent signalling events to modulate macrophage IL-12 induction and the subsequent T-cell immune responses to favour its own survival in the host. In this study, we report that Mtbhsp60 inhibits PPD-induced IL-12 p40 by manipulating the TLR2 signalling in macrophages.

Results

IL-12 p40 induction is poorer in PPD-activated macrophages treated with Mtbhsp60

As IL-12 cytokine plays a crucial role in promoting IFN-γ production and a Th1 immune response (Hsieh *et al*., 1993; Magram *et al*., 1996), downregulation of IL-12 induction in macrophages can lead to a Th2-dominant immune environment (Rook *et al*., 2005; Mukhopadhyay *et al*., 2007). We therefore examined whether Mtbhsp60 inhibited IL-12 induction in macrophages treated with PPD.

It is known that the IL-12 is a heterodimeric protein of 70 kDa composed of two subunits, IL-12 p35 and IL-12 p40 (Trinchieri, 2003), and the regulation of biologically active IL-12 p70 depends upon transcriptional regulation of the gene encoding the IL-12 p40 subunit mainly (Ma and Trinchieri, 2001). Therefore, the PPD-induced IL-12 p40 levels were measured in the groups treated with Mtbhsp60. The PMA-differentiated THP-1 macrophages were stimulated with PPD (10 μg ml^{-1}) in the presence of various concentrations of recombinant Mtbhsp60 (Fig. S1) or control glutathione *S*-transferase (GST) protein, and after 48 h culture supernatants were harvested and assayed for the levels of IL-12 p40 by enzyme immunoassay (EIA). It could be seen that IL-12 p40 induction is downregulated in a dose-dependent manner in macrophages cotreated with Mtbhsp60 and PPD but not with GST and PPD (Fig. 1A). The MTT viability test confirmed that the inhibition was not due to any direct cytotoxic effect of Mtbhsp60 (Fig. S2), indicating that the protein affected the signalling events important for IL-12 p40 induction. The immunofluorescence microscopic evaluation of the intracellular level of IL-12 p40 again indicates that IL-12 p40 induction in PPD-activated macrophages was downregulated in the presence of Mtbhsp60 (Fig. 1B). Similar observations were made in peripheral blood mononuclear cell (PBMC)-derived

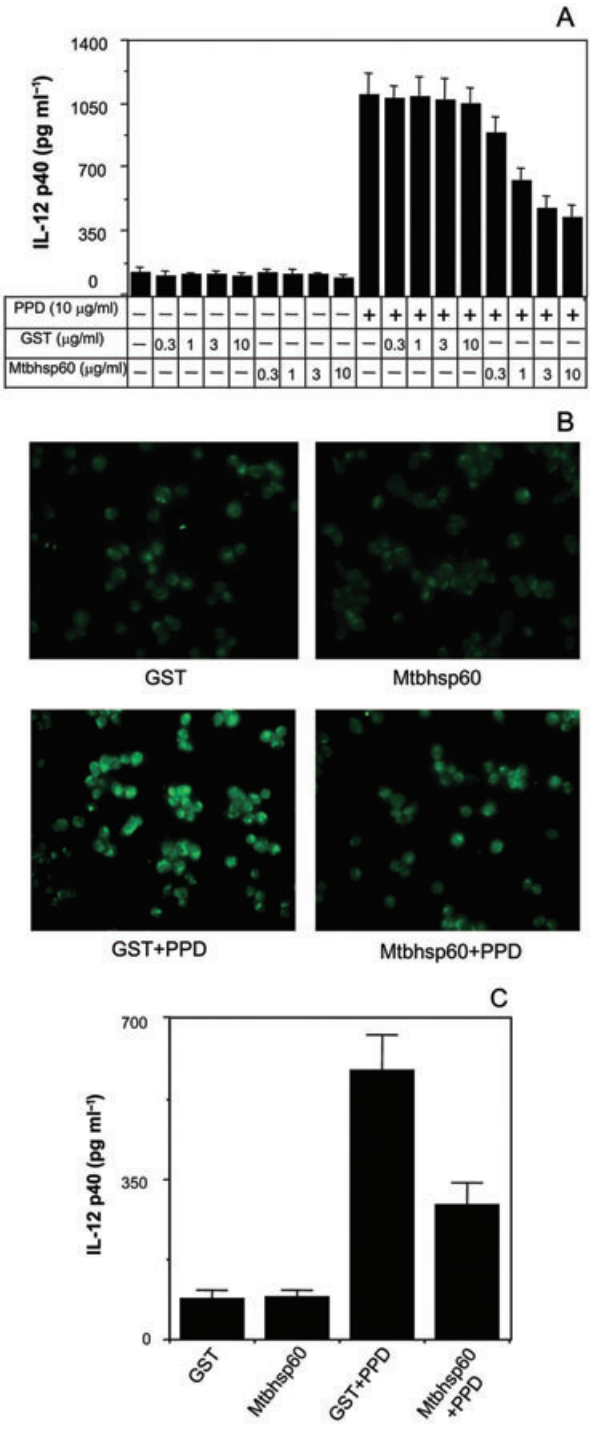

Fig. 1. The Mtbhsp60 protein inhibits IL-12 p40 induction in macrophages activated with PPD. The PMA-differentiated THP-1 macrophages were either left untreated or treated with various concentrations of Mtbhsp60 or GST protein for 2 h and then stimulated with 10 μg ml^{-1} of PPD for 48 h. Culture supernatants were harvested and the level of IL-12 p40 cytokine was measured by EIA (A). The intracellular IL-12 p40 level was measured by immunofluorescence assay (B). IL-12 p40 induction was measured in PBMC-derived macrophages that were stimulated with PPD in the presence of Mtbhsp60/GST (C). Results shown are representative of 3–4 independent experiments.

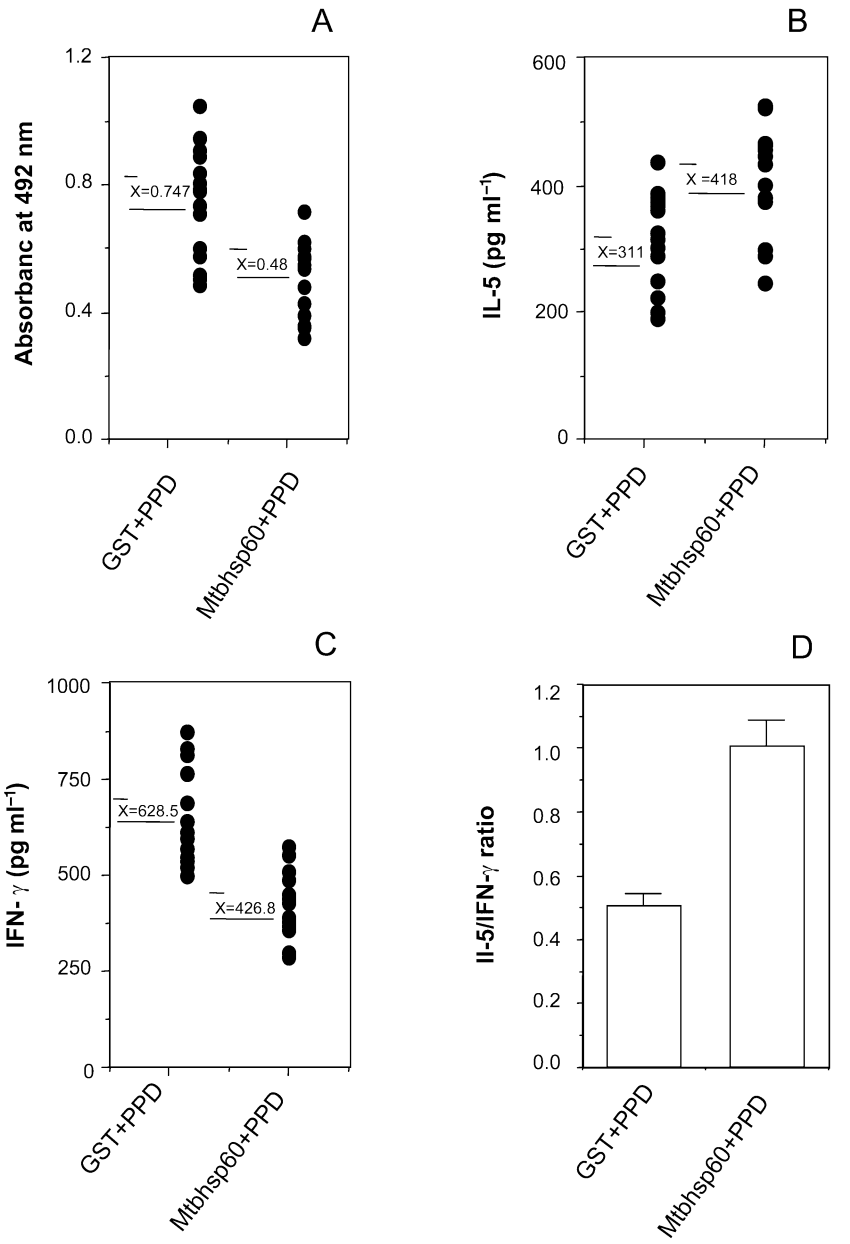

Fig. 2. The anti-PPD T-cell responses are skewed to Th2 type when the PBMC-derived macrophages are used as APCs in T-cell proliferation responses. The T-cells from PBMC from TB patients ($n = 15$) were purified by nylon wool and were cultured using Mtbhsp60/GST-treated autologous macrophages as APCs with 10 μg ml^{-1} of PPD. After 4 days, T-cell proliferation (A), IL-5 (B) and IFN-γ (C) production were measured. The Th2/Th1 balance represented by IL-5/IFN-γ ratios (mean ± SEM) is shown for various patient samples (D).

macrophages (Fig. 1C), indicating that inhibition of PPD-induced IL-12 p40 by Mtbhsp60 was signal specific and not restricted only to the transformed macrophage cell line (THP-1).

The anti-PPD T-cell response is biased to Th2 type by Mtbhsp60

We found that the PPD-induced IL-12 in macrophages was inhibited by Mtbhsp60. As IL-12 activates Th1 immune response (Hsieh *et al.*, 1993; Magram *et al.*, 1996), downregulation of IL-12 induction by Mtbhsp60 can repress the development of Th1-type response. Therefore, we next examined whether Mtbhsp60 is involved in suppressing the anti-PPD Th1 immune response (Baliko *et al.*, 1998; Wilsher *et al.*, 1999) by modulating the macrophage function(s). The macrophages were purified from individual patient and incubated for 2 h with 3 μg ml^{-1} of Mtbhsp60 or GST protein. Cells were washed to remove any external Mtbhsp60 or GST protein and used as APCs. The purified T-cells were cultured with autologous macrophages (as APCs) pretreated with Mtbhsp60 or GST and PPD (10 μg ml^{-1}) as recall antigen. After 96 h, the culture supernatants were tested for the levels of IL-5 and IFN-γ cytokines. The data revealed that the anti-PPD T-cell response was downregulated (Fig. 2A; $P < 0.0001$) and

biased to Th2 type (Fig. 2D; $P < 0.0001$) with higher production of IL-5 (Fig. 2B; $P < 0.0001$) and decreased production of IFN-γ (Fig. 2C; $P < 0.0001$) in the group that received Mtbhsp60-treated macrophages as APCs, as compared with the group receiving GST-treated macrophages as APCs. This study indicates that Mtbhsp60 can suppress the Th1 response to PPD.

Mtbhsp60 protein affects the rel signalling in PPD-activated macrophages

The IL-12 p40 is dominantly regulated by the rel transcription factors, p50, p65 and c-rel (Murphy *et al*., 1995; Plevy *et al*., 1997; Sanjabi *et al*., 2000; Trinchieri, 2003; Rahim *et al*., 2005). We therefore examined whether Mtbhsp60 modulates the rel signalling to affect IL-12 p40 induction in macrophages. The nuclear extracts were prepared from PMA-differentiated THP-1 macrophages pretreated for 2 h with 3 μg ml^{-1} of Mtbhsp60 or GST and then stimulated for 1 h with 10 μg ml^{-1} of PPD. These were then subjected to Western blotting for examining the expression profile of p50, p65 and c-rel. While control macrophages (incubated with GST) show very little of nuclear p50 and p65 NF-κB proteins, PPD-activated macrophages show significant induction of these proteins (Fig. 3A, second and third panels; compare lane 2 with lane 1). Higher expression of these proteins could be observed in the macrophages cotreated with Mtbhsp60 and PPD (Fig. 3A, third and second panels, compare lane 4 with lane 2).

As c-rel plays a dominant role over the p65 NF-κB in IL-12 p40 transcription (Sanjabi *et al*., 2000; Rahim *et al*., 2005), it is possible that Mtbhsp60 downregulates mainly the c-rel transcription factor to inhibit IL-12 p40 transcription. When expression profile of the c-rel was examined in the same nuclear extracts that were used for studying the p50 and the p65 NF-κB level, it could be seen that the nuclear c-rel level in PPD-activated macrophages (Fig. 3A, first panel, compare lane 4 with lane 2) was downregulated in the presence of Mtbhsp60.

To confirm a role of c-rel in the Mtbhsp60-mediated downregulation of IL-12 p40, the PMA-differentiated THP-1 macrophages were next transfected transiently with the c-rel overexpressing plasmid construct. The control group received the backbone vector (PRC/CMV). After 24 h, macrophages were washed and stimulated with PPD in the presence of Mtbhsp60 or GST protein and IL-12 p40 production was measured at 48 h post stimulation. The results shown in Fig. 3B reveal that IL-12 p40 production is decreased by Mtbhsp60 in the group transfected with the PRC/CMV vector alone (Fig. 3B, compare bar 3 with bar 2), which is expected. However, transfection with c-rel plasmid (PRC/CMV-c-rel) results in significant upregulation of IL-12 p40 generation in PPD-activated macrophages even when treated with Mtbhsp60

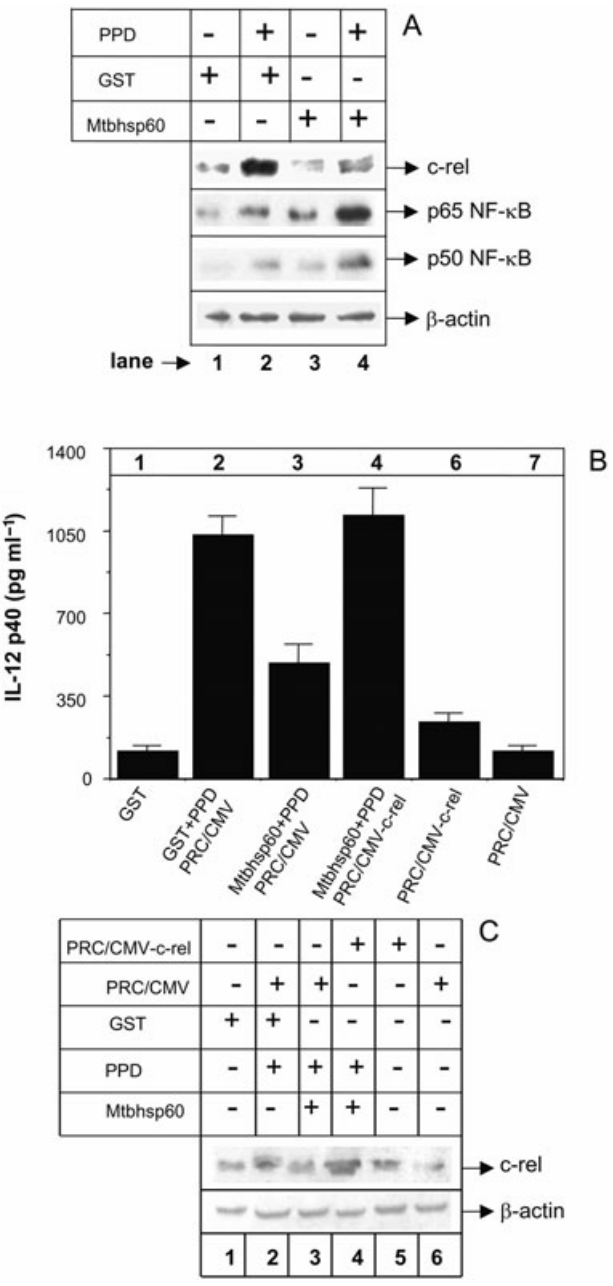

Fig. 3. The Mtbhsp60 protein targets the c-rel transcription factor to inhibit IL-12 p40 induction in PPD-stimulated THP-1 macrophages. The PMA-differentiated macrophages were pretreated with 3 μg ml^{-1} of Mtbhsp60 or GST protein for 2 h and further stimulated for 1 h with 10 μg ml^{-1} of PPD. Cells were harvested, washed and nuclear extracts were prepared. The p50, p65 and c-rel levels in the nuclear extracts were measured by Western blotting using p50- or p65- or c-rel-specific antibody (A). In another experiment, PMA-treated macrophages were transfected with either the vector control (PRC/CMV) or the c-rel overexpression plasmid construct (PRC/CMV-c-rel) using lipofectamine 2000. After 24 h, these cells were stimulated with 10 μg ml^{-1} of PPD in the presence of Mtbhsp60 or GST protein and the amounts of IL-12 p40 secreted in the culture supernatants were estimated by EIA (B). The overexpression of c-rel in the nuclear extracts is shown by Western blot analysis (C). β-actin immunoblot was used to show that equal amounts of total proteins were loaded. Data shown are representative of 3–4 different experiments.

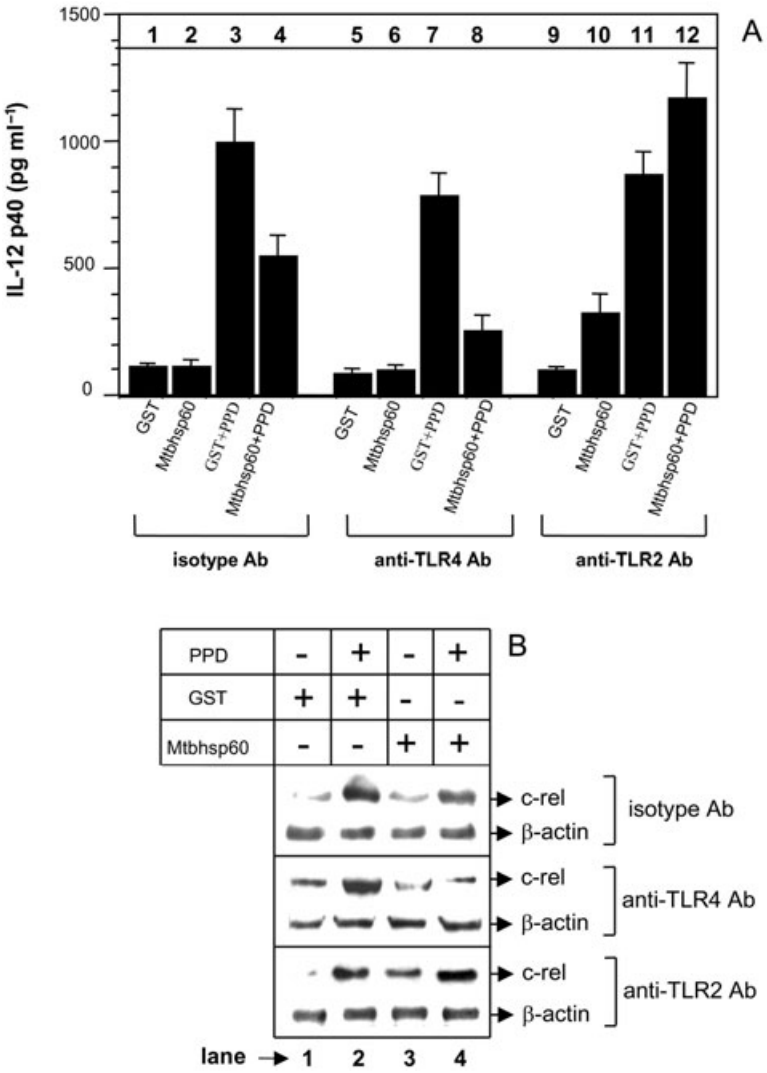

Fig. 4. The regulation of IL-12 p40 by Mtbhsp60 is TLR dependent. The PMA-differentiated THP-1 macrophages were either left untreated or pretreated with 10 μg ml^{-1} of anti-TLR2 mAb or anti-TLR4 mAb or isotype-matched control Ab for 1 h. These cells were then activated with PPD in the presence of 3 μg ml^{-1} of Mtbhsp60 or GST. Cells were either cultured for 48 h for estimating IL-12 p40 cytokine by EIA (A) or for 1 h to measure c-rel levels in the nuclear extracts by Western blot analysis (B). Results shown are representative of three different experiments.

(Fig. 3B, compare bar 4 with bar 3; $P < 0.001$). These results indicate a role of the c-rel in the Mtbhsp60-mediated downregulation of IL-12 p40. In the transfected macrophages, overexpression of c-rel was confirmed by Western blotting (Fig. 3C).

Involvement of the Toll-like receptors in the regulation of IL-12 p40 by Mtbhsp60

The hsp60 is known to interact strongly with the TLRs, namely the TLR2 and the TLR4 (Ohashi *et al*., 2000; Vabulas *et al*., 2001). Several studies indicate that induction of specific cytokine profile upon recognition of microbial pathogens depends, to a great extent, on specific TLRs being triggered (Brightbill *et al*., 1999; Means *et al*., 2001; Noss *et al*., 2001; Abel *et al*., 2002; Barton and Medzhitov, 2003). We therefore hypothesized that the IL-12 p40 regulation by Mtbhsp60 could be TLR dependent. The THP-1 macrophages which express higher levels of both TLR2 and TLR4 upon PMA activation as observed by us (data not shown) and others (Kokkinopoulos *et al*., 2005) were selected. These macrophages were pretreated with either neutralizing anti-human TLR2 monoclonal antibody (mAb) or neutralizing anti-human TLR4 mAb to block TLR2/TLR4 receptors and then activated with PPD in the presence of Mtbhsp60 or GST. The control group received the isotype-matched Ab (IgG2a). Results reveal that PPD-induced IL-12 p40 (Fig. 4A) and nuclear c-rel (Fig. 4B, panel 1) were suppressed by Mtbhsp60 in the group treated with isotype-matched Ab, which was expected. When anti-TLR4 mAb was used, both the IL-12 p40 (Fig. 4A) and the nuclear c-rel levels (Fig. 4B, panel 2) were downregulated. In contrast, when macrophages were pretreated with anti-TLR2 mAb, Mtbhsp60 augmented IL-12 p40 induction (Fig. 4A, compare lane 10 with lane 2, $P < 0.001$ and lane 12 with lane 4, $P < 0.001$). The increase in IL-12 p40 in the anti-TLR2 mAb-treated macrophages was correlated well with

an increase in the nuclear c-rel level (Fig. 4B, panel 3). This increase was not due to any lipopolysaccharide (LPS; a TLR4 ligand, Bulut *et al*., 2005) contamination in the protein preparation as proteolysis of Mtbhsp60 by proteinase K (200 ng ml^{-1}) blocked the ability of Mtbhsp60 to induce IL-12 p40 in the anti-TLR2 mAb-treated macrophages (Fig. S3). These results (Fig. 4) confirm that Mtbhsp60-mediated downregulation of IL-12 is dependent predominantly on TLR2 population present on the macrophage surface via c-rel signalling downstream of TLR2.

Mtbhsp60 increases TLR2 expression on macrophages

As TLR4 positively regulates IL-12 secretion, and we observed a net negative effect of Mtbhsp60 in PPD-stimulated macrophages, it is likely that TLR2 is the predominant molecule by which IL-12 expression is downregulated. The net negative effect on IL-12 expression could be brought upon either by preferential signalling by Mtbhsp60 through TLR2 or by modulating TLR2 expression in such a way that surface TLR2 population is much higher than that of TLR4. As Mtbhsp60 interacts with both the TLR2 and TLR4 (Ohashi *et al*., 2000; Vabulas *et al*., 2001), it is likely that Mtbhsp60 increases TLR2 expression in the macrophages. To test this hypothesis, the THP-1 macrophages were either left untreated or activated with PMA and used for examining surface expression profiles of TLR2 and TLR4 upon stimulation with Mtbhsp60. Interestingly, we observed that Mtbhsp60 upregulated surface expression of TLR2 in both PMA-differentiated (Fig. 5A) and undifferentiated THP-1 macrophages (Fig. 5B). However, there were no significant changes in the surface expression of TLR4 in both the cases (Fig. 5A and B). When Mtbhsp60 was added to human blood-derived monocyte-macrophages, again an upregulation of TLR2 but not TLR4 was noticed (data not shown).

To categorically document that Mtbhsp60-mediated suppression of IL-12 p40 was indeed TLR2 dependent, we next silenced the TLR2 by siRNA approach. When compared with the negative control siRNA-transfected THP-1, the group containing TLR2 siRNA did not exhibit substantially increased levels of TLR2 upon treatment with Mtbhsp60 (Fig. S4). The TLR2 deficiency in the TLR2 siRNA group led to an increase in IL-12 p40 induction during treatment with Mtbhsp60 (Fig. 5C, compare lane 8 with lane 4, $P < 0.001$). These results demonstrate that Mtbhsp60-mediated downregulation of IL-12 p40 in activated macrophages is dependent predominantly on the TLR2 population.

Discussion

The T-cell proliferation and effector commitment in response to pathogenic stimulation largely depends upon the signals generated from the cytokines induced in the activated APCs (D'Andrea *et al*., 1993; Magram *et al*., 1996). Thus, modulation of induction of various cytokines in macrophages during *M. tuberculosis* infection appears to be one of the major causes of immunoregulation for diversion of protective and relevant antimycobacterial T-cell responses. Regulation of induction of these cytokines thus becomes an issue of interest in understanding how T-cell responses are controlled during infection in TB (Rook *et al*., 2001; Mukhopadhyay *et al*., 2007).

It has been reported that Mtbhsp60 protein concentration can increase from 1% to 10% or more during infection (Young and Garbe, 1991), suggesting that it may play an important role in bacterial virulence (Lewthwaite *et al*., 1998). Several reports indicate that hsp60 has important roles in immune regulation (Lamb *et al*., 1989; Lewthwaite *et al*., 1998; Srivastava, 2003). In this study (the first time to the best of our knowledge) we demonstrate that Mtbhsp60 can modulate IL-12 p40 expression either negatively or positively depending on the relative ratio of TLR2/TLR4 population present in the macrophages. Mtbhsp60 was found to activate IL-12 p40 when predominantly interacts with the TLR4, but in contrast, it could significantly downregulate IL-12 p40 production when TLR2 is the predominant population in PPD-stimulated macrophages. The ability of Mtbhsp60 to either decrease (please see Fig. 1) or increase (Lewthwaite *et al*., 2001) IL-12 production depends largely on its specific interaction with the TLR2 or the TLR4 receptors and ultimately the c-rel signalling cascade (Fig. 4). In our study, we observed that the Mtbhsp60 alone could increase nuclear p50 and p65 NF-κB to a moderate level, however, a higher expression of these proteins were found in the group cotreated with Mtbhsp60 and PPD. Although the p50/p65 NF-κB proteins levels were increased, the c-rel level in response to PPD was downregulated by Mtbhsp60. Thus, the downregulation of c-rel possibly resulted in decreased IL-12 p40 induction in macrophages stimulated with PPD. Again, overexpression of c-rel caused significant upregulation of IL-12 p40 in PPD-activated macrophages even in the presence of Mtbhsp60.

As Mtbhsp60 is known to interact with both TLR2 and TLR4 (Ohashi *et al*., 2000; Vabulas *et al*., 2001) with either a negative or positive effect on the IL-12 production, respectively, the final outcome, i.e. the net negative or positive regulation of IL-12 vis-a-vis Th1 or Th2 phenotype is functionally governed by the relative ratio of the TLR2/TLR4 population *per se*. This could well constitute an important point of regulation by which the TB bacilli can modulate a confronting Th1 environment to more favourable Th2. As we observed Mtbhsp60-mediated downregulation of IL-12 p40, it was likely that TLR2-mediated responses were dominated possibly by

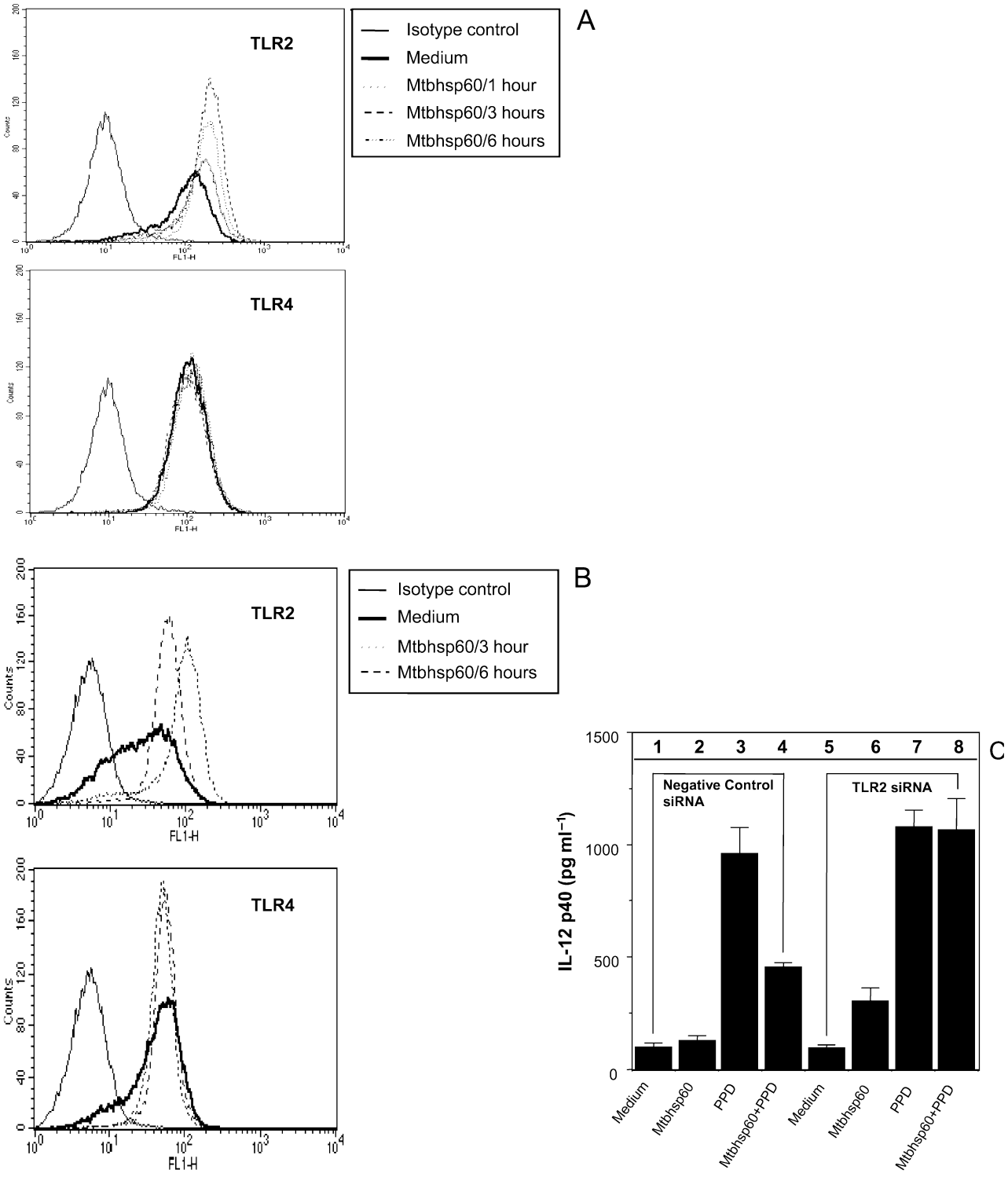

Fig. 5. The Mtbhsp60 increases surface expression of TLR2 and exploits the TLR2-triggered signalling to downregulate IL-12 p40 in activated macrophages. The THP-1 macrophages were either treated with PMA (A) or left untreated (B) and then incubated with 3 μg ml^{-1} Mtbhsp60 for various times. The cells were harvested for measuring the surface expression of TLR2 (upper panel) and TLR4 (lower panel) by FACS. In another experiment, PMA-differentiated THP-1 macrophages were transfected with negative control siRNA or TLR2 siRNA and after 24 h cells were activated with PPD in the presence of 3 μg ml^{-1} of Mtbhsp60. After 48 h, culture supernatants were harvested to estimate IL-12 p40 induction (C). Results shown are representative of 3–4 different experiments.

skewing the TLR2/TLR4 ratio by either upregulating the surface TLR2 or downregulating the surface TLR4 expression. Interestingly, we found that Mtbhsp60 increased TLR2 without significantly affecting TLR4 expression on macrophage surface. Again, silencing of the TLR2 by specific siRNA rescued the inhibitory effect of Mtbhsp60 on IL-12 p40 induction. Thus, it appears that Mtbhsp60 significantly increases TLR2 expression without affecting TLR4 population and thereby makes TLR2 to be the predominant signalling gateway to suppress IL-12 p40 production in activated macrophages. Therefore, downregulation of IL-12 production by Mtbhsp60 consequently can result into suppression of Th1 immune responses (Baliko *et al*., 1998; Wilsher *et al*., 1999). Thus, triggering of TLR2 signalling during interaction of macrophages with *M. tuberculosis* may be a critical factor to determine the magnitude and the functional polarization of adaptive immune responses and Mtbhsp60 probably plays an important role in such regulation. However, as opposed to Mtbhsp60, *E. coli* hsp60 did not have any significant effect on the TLR2 expression (N. Khan *et al.* unpubl. obs.). This indicates that Mtbhsp60 might play some role as virulent factor modulating immune responses of the host.

Several other studies indicate that *M. tuberculosis* can exploit the TLR2 signalling cascade to induce a Th2-biased immune response (Noss *et al*., 2001; Jang *et al*., 2004; Arko-Mensah *et al*., 2007; Jo *et al*., 2007). An increase in the TLR2 expression during infection of macrophages with mycobacteria is although indicated (Wang *et al*., 2000), the factors that are involved in upregulation of TLR2 as well as the signalling events triggered as a consequence of such interactions are not well understood. Our study reveals a novel role of the *M. tuberculosis* hsp60 protein to inhibit IL-12 p40 expression by increasing the TLR2 expression and hence signalling and skewing the T-cell environment towards the Th2 type as discussed earlier. Nevertheless, the mammalian hsp60 protein was also shown to inhibit Th1 cytokines and induced regulatory T-cells via interaction with TLR2. Our study of Mtbhsp60 essentially complements the findings of the mammalian hsp60 (Zanin-Zhorov *et al*., 2005; 2006). However, this group used a completely different (cell-free antigen presentation) system, suggesting that the effect of Mtbhsp60 may not be necessarily *M. tuberculosis*-specific but common to many hsp60s and that it may operate at multiple different levels. That raises the possibility of negative feedback where hsp60 inhibits both IFN-γ production by T-cells and IL-12 production by monocytes, leading to a stronger decline than would be expected from either one alone. The ability of Mtbhsp60 to downregulate IL-12 p40 and the Th1 response thus presents an important molecular target for development of therapeutic interventions against TB.

Experimental procedures

Cloning, expression and purification of M. tuberculosis *heat shock protein 60 (Mtbhsp60)*

The *M. tuberculosis* heat shock protein 60 (Mtbhsp60) was purified as described earlier (Qamra *et al*., 2004; Fig. S1). Endotoxin contamination was removed by incubating protein with 10% v/v polymyxin B-agarose (Sigma-Aldrich, St Louis, MO; binding capacity 200–500 μg ml^{-1}) as described earlier by us (Khan *et al*., 2007). We also measured the endotoxin content of the recombinant protein by using Limulus amebocyte lysate assay (E-toxate kit from Sigma-Aldrich, St Louis, MO) and the endotoxin content in the recombinant protein was found to be extremely low (< 0.0001 endotoxin unit μg^{-1}).

Expression and purification of glutathione S*-transferase protein*

The pGEX4T1 vector carrying GST coding sequence (Amersham Biosciences, Little Chalfont, UK) was transformed into *E. coli* BL21. The cells were grown at 37°C till the absorbance reached around 0.4 and induced with 1 mM IPTG for 3 h. The protein was purified using Glutathione Sepharose beads (Amersham pharmacia).

Study population

Peripheral blood was obtained from TB patients reported at the DOTS (Directly Observed Treatment – Short-course) Clinic of Mahavir Hospital and Research Centre, Hyderabad, India. The diagnosis of the TB patients was confirmed based on the results of sputum smear (presence of acid-fast bacillus), radiographic evidence, tissue biopsy and clinical symptoms. These patients were negative for HIV virus. Infected patients were mainly belonging to category 1 comprising patients who had no history of TB treatment (Choudhary *et al*., 2003). Volunteers chosen for this study were all BCG-vaccinated, healthy and negative by symptoms. The bioethics committee of Mahavir Hospital and Research Centre and CDFD approved the present study and informed consent was obtained from all the subjects.

Lymphocyte proliferation assay

Peripheral blood mononuclear cells from heparinized blood samples were isolated using gradient centrifugation in Ficoll-Hypaque (Sigma-Aldrich) solution as described (Chakhaiyar *et al*., 2004). T-cells from each sample were purified using nylon wool as described earlier (Mukhopadhyay *et al*., 1999). The macrophage population from PBMC was purified by adherence and subsequent culturing for 6 days as described (Post *et al*., 2001). The adherent cells were approximately 90% macrophages by staining. The macrophages were pretreated for 2 h with 3 μg ml^{-1} of Mtbhsp60 or GST. The cells were washed and used as APCs. Proliferation assays were performed using purified T-cells added at a concentration of 1.5×10^6 cells ml^{-1} with autologous macrophages added at 1×10^6 cells ml^{-1}. Cell suspensions (200 μl $well^{-1}$) were dispensed into 96-well, flat-bottom microtiter plates (Nunc, Roskilde, Denmark) and cultured in the presence of PPD (10 μg ml^{-1}) as recall antigen. After 4 days, culture super-

natants were harvested for the estimation of IL-5 and IFN-γ cytokines by EIA and the T-cell proliferation was determined by MTT assay. In brief, MTT (Sigma-Aldrich) was added as 5 mg ml^{-1} and incubated for 4 h. The cells were lysed overnight using 100 μl of lysis buffer (20% SDS and 50% DMF) and the absorbance determined at 550 nm.

Macrophage stimulation assay

The human monocyte/macrophage cell line THP-1 was obtained from National Centre For Cell Science (NCCS), Pune, India and maintained in RPMI-1640 medium (Invitrogen, Grand Island, NY) containing 10% FCS and antibiotics. The THP-1 macrophages were treated with 20 ng ml^{-1} phorbol myristate acetate (PMA, Sigma-Aldrich) for 24 h followed by rest for overnight. The PMA-differentiated THP-1 or PBMC-derived macrophages (3×10^6 cells ml^{-1}) were pretreated with either various concentrations or a fixed concentration of 3 μg ml^{-1} of Mtbhsp60 or GST protein for 2 h followed by stimulation with 10 μg ml^{-1} of PPD. Cells were either harvested after 1 h for examining p50 and p65 NF-κB or c-rel levels or left in the cultures for 48 h to estimate IL-12 p40 levels by EIA. In some experiments, PMA-differentiated THP-1 macrophages were pretreated with 10 μg ml^{-1} anti-human TLR2 mAb (TL2.1, mouse IgG2a) or anti-human TLR4 mAb (HTA125, mouse IgG2a) (both from Imgenex, San Diego, CA) for 1 h at 37°C and then stimulated with PPD (10 μg ml^{-1}) in the presence of 3 μg ml^{-1} of Mtbhsp60 or GST. In control groups, isotype-matched antibody of IgG2a type (BD Biosciences Pharmingen, San Diego, CA) was added at the same concentrations.

EIA for measuring various cytokines

The IL-12 p40 cytokine levels were quantified by two-site sandwich EIA (BD Biosciences Pharmingen) as described earlier (Mukhopadhyay *et al*., 2004; Silswal *et al*., 2005). Standard curve for the cytokine was obtained using the recombinant standard protein provided by the manufacturer.

Western blot analysis

Western blot analysis was used to detect p50 and p65 NF-κB and c-rel transcription factors in the nuclear extracts as described previously by us (Mukhopadhyay *et al*., 2002). In brief, the nuclear extracts from various experimental groups were prepared from NP-40-lysed cells as described (Dignam *et al*., 1983). Protein concentrations were estimated using the bicinchoninic acid method (Micro BCA™ Protein Assay Kit; Pierce, Rockford, USA). Equal quantities of the nuclear proteins were separated in a 10% SDS-PAGE under reducing conditions. The nuclear proteins were transferred onto the nitrocellulose membrane and incubated with rabbit antibody to either p50 or p65 or c-rel protein (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with anti-rabbit IgG-horseradish peroxidase conjugate (Sigma-Aldrich). Bound enzyme was detected by chemiluminescence following the manufacturer's protocols (ECL, Amersham Biosciences, Little Chalfont, UK). Equal loading of protein was confirmed by measuring the β-actin level by Western blotting.

Transfection with c-rel plasmid construct

The c-rel plasmid construct was a kind gift from Dr Klaus Ruckdeschel, Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, München, Germany. Transfections were conducted with 10 μg of the c-rel plasmid (PRC/CMV-c-rel) as described (Khan *et al*., 2006). Expression vector (PRC/CMV) without any insert was used as control. The plasmid construct was transfected into PMA-differentiated THP-1 macrophages using the cationic lipid suspension lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were stimulated with 10 μg ml^{-1} of PPD in the presence of 3 μg ml^{-1} of Mtbhsp60 or GST either to detect the nuclear c-rel levels by Western blotting or to estimate the amounts of IL-12 p40 secreted in the culture supernatants by EIA.

Immunofluorescence microscopy

Immunofluorescence assay for measuring the intracellular IL-12 p40 level was carried out as described earlier (Rahim *et al*., 2005). In brief, PMA-differentiated THP-1 macrophages were pretreated for 2 h with 3 μg ml^{-1} of Mtbhsp60 or GST and further stimulated with 10 μg ml^{-1} of PPD for 4 h followed by another 4 h incubation with 20 μg ml^{-1} brefeldin A (Sigma-Aldrich). The cells were fixed with 3% paraformaldehyde for half an hour, washed and permeabilized with 0.1% Triton X for 15 min. After blocking with 2% BSA (Sigma-Aldrich), cells were incubated with anti-IL-12 p40 antibody conjugated to biotin and probed with Streptavidin-FITC (Sigma-Aldrich). Cells were washed and embedded in Vectashield mounting medium (Vector Laboratories; Burlingame, CA). Microscopy was performed on a Nikon fluorescence microscope (Nikon DX1, Japan).

TLR2 siRNA

The predesigned and annealed 21-nucleotide double-stranded siRNA targeting TLR2 (5′-GCCUUGACCUGUCCAACAAtt-3′) was purchased from Ambion (Austin, TX). To demonstrate that the transfection does not induce non-specific effects on gene expression, negative control siRNA provided by the manufacturer was used. For experiments, the siRNA was re-suspended in nuclease-free water and stored at –20°C. PMA-differentiated THP-1 macrophages were transfected with the TLR2 siRNA or negative control siRNA with lipofectamine 2000 as per the manufacturer's instructions.

Flow cytometry

For TLR2 and TLR4 staining, cells were treated (30 min at 4°C) with anti-human TLR2 mAb or anti-human TLR4 mAb or isotype-matched IgG2a antibody and then incubated with FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) at 4°C for another 30 min. Cells were washed and re-suspended in sheath fluid. Cell-bound fluorescence was analyzed with FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest (Becton Dickinson) data analysis software.

Statistical analysis

Data were expressed as mean ± SD of three independent experiments performed with similar results. Student's *t*-test was used to determine statistical differences between the groups. A P-value < 0.05 was considered to be significant.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Affinity purification of *M. tuberculosis* heat shock protein 60 (Mtbhsp60). The His-tagged recombinant Mtbhsp60 protein was purified by Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography under native condition and stained with Coomassie blue after 10% SDS-PAGE. Lane 1, Protein molecular size marker, and Lane 2, Purified recombinant protein.

Fig. S2. The Mtbhsp60 does not affect the cell viability. The PMA-differentiated THP-1 macrophages were stimulated with PPD (10 μg ml^{-1}) in the presence of various concentrations of Mtbhsp60 or control GST protein and after 48 h MTT viability test was performed. In brief, MTT (Sigma-Aldrich) was added as 5 mg ml^{-1} and incubated for 4 h. The cells were lysed overnight using 100 μl of lysis buffer (20% SDS and 50% DMF) and the absorbance determined at 550 nm. This experiment is representative of three experiments performed with similar results. Results are expressed as mean + SD.

Fig. S3. The IL-12 p40 upregulation by Mtbhsp60 in anti-TLR2-treated macrophages is sensitive to proteinase K. The PMA-differentiated THP-1 macrophages were pretreated with neutralizing antibody to TLR2 and then treated with various concentrations of Mtbhsp60 either left untreated or treated with 200 ng ml^{-1} proteinase K. After 48 h, the culture supernatants were harvested and the levels of IL-12 p40 secreted in the culture supernatants were measured by EIA. This result is representative of three experiments performed with similar results.

Fig. S4. The PMA-differentiated THP-1 macrophages were transfected with negative control siRNA or TLR2 siRNA and after 24 h cells were treated with 3 μg ml^{-1} of Mtbhsp60. After 3 h, cells were harvested to examine the surface expression of TLR2 by FACS (C). Results shown are representative of three different experiments.

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