Chapter 3

Results
Earlier studies have indicated that the *M. tuberculosis* heat shock protein 60 (Mtbhsp60) inhibits purified protein derivative (PPD)-induced IL-12 p40 and skews the anti-PPD T cell response towards the Th2 type in T cells isolated from PBMCs of active TB patients (Khan *et al*., 2008). It was further observed that Mtbhsp60 targets the TLR2-dependent signaling cascades to inhibit nuclear translocation of c-rel and consequently IL-12 p40 transcription (Khan *et al*., 2008). One of the possible mechanisms by which Mtbhsp60 blocks IL-12 p40 induction could be through induction of anti-inflammatory mediators like IL-10. IL-10 is known to inhibit IL-12 p40 induction in macrophages (Moore *et al*., 2001, Turner *et al*., 2002, O'Garra *et al*., 2004) primarily by targeting the c-rel transcription factor (Rahim *et al*., 2005). Therefore, it may be possible that Mtbhsp60 predominantly activates IL-10 production through its interaction with TLR2 which subsequently inhibits c-rel *vis-à-vis* IL-12 p40 in activated macrophages (Khan *et al*., 2008). Therefore, it was first examined whether Mtbhsp60 triggered IL-10 production upon its interaction with TLR2.

### 3.1. Mtbhsp60 activates IL-10 in macrophages

The SDS-PAGE analysis of the purified Mtbhsp60 protein harboring the poly-His tag revealed it to be essentially a homogenous preparation of Mtbhsp60 protein of approximately 60 kDa (Fig. 3.1). To analyze the ability of Mtbhsp60 to activate IL-10 induction in macrophages, PMA-differentiated THP-1 macrophages were treated with titrating concentrations of Mtbhsp60 and the macrophages were cultured for a period of 48 h to measure the amount of IL-10 secreted in the culture supernatants by EIA. The recombinant Mtbhsp60 protein was found to activate IL-10 production in THP-1
macrophages in a dose-dependent manner (Fig. 3.2). To rule out the observed effects due to endotoxin contamination in the recombinant Mtbhsp60 preparation, the protein was treated with polymyxin B, a specific inhibitor of bacterial lipopolysaccharide (LPS) (Khan et al., 2007) and the endotoxin content of the Mtbhsp60 protein preparation was found to be very low (less than 0.01 EU/ml) by Limulus Amebocyte lysate assay. Further, treatment of Mtbhsp60 with proteinase K (PK) was shown to significantly abrogate its ability to induce IL-10 and the induction level was comparable to that of medium alone (Fig. 3.2, compare lane 5 with lane 1) indicating that the observed effect was specific to Mtbhsp60.

Next PMA-treated THP-1 macrophages were treated with a fixed concentration of 3 μg/ml Mtbhsp60 and macrophages were harvested after 1, 2 and 5 h to analyse IL-10 mRNA expression by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). It was observed that Mtbhsp60 could induce IL-10 transcript as early as 1 h post-treatment at 3 μg/ml concentration (Fig. 3.3).
Figure 3.1. Purification of recombinant Mtbhsp60. Mtbhsp60 cloned in pET3a vector was transformed into BL21(DE3)pLysS cells and liquid broth culture was prepared from a single transformed colony. The culture was induced with 1 mM IPTG. The induced culture was harvested after 4 h and cell lysate was prepared. Protein was purified from lysate using Ni-NTA agarose. Different elution fractions (lane 1 to lane 9) were collected and purity of eluted fractions was checked on a Coomassie Blue stained SDS-polyacrylamide gel.
Figure 3.2. Mtbhsp60 induces IL-10 production in THP-1 macrophages. PMA-differentiated THP-1 macrophages were treated with titrating concentrations of purified Mtbhsp60 or a fixed concentration (3 µg/ml) of Mtbhsp60 treated with proteinase K (PK). IL-10 level was estimated by EIA in culture supernatants harvested after 48 h. Results shown are representative of mean ± SD of three independent experiments.
Figure 3.3. Mtbhsp60 induces IL-10 production in THP-1 macrophages as analysed at transcript level. PMA-differentiated THP-1 macrophages were treated with 3 µg/ml of Mtbhsp60 at different time points. Total RNA was extracted from various groups and IL-10 mRNA expression was measured by semi-quantitative RT-PCR. Results shown are representative of three independent experiments.
3.2. Interaction of Mtbhsp60 with TLR2 receptors is important for induction of IL-10 in macrophages

Next, it was checked whether Mtbhsp60 binds to both TLR2 and TLR4 receptors efficiently. Therefore, THP-1 macrophages were treated with 10 µg/ml of either neutralizing anti-TLR2 mAb to allow binding of Mtbhsp60 mostly to TLR4 or neutralizing anti-TLR4 mAb to allow binding of Mtbhsp60 mainly to TLR2 receptors. These cells were then treated with biotin labeled Mtbhsp60 (3 µg/ml) followed by incubation with streptavidin-FITC conjugate and binding of Mtbhsp60 to TLR2 and TLR4 in THP-1 macrophages was examined by flow cytometry. It was observed that Mtbhsp60 could bind strongly to both TLR2 and TLR4 receptors (Fig. 3.4).
Figure 3.4. Mtbhsp60 binds efficiently to both TLR2 and TLR4 receptors. PMA-differentiated THP-1 macrophages were pre-treated with either 10 µg/ml of anti-TLR2 mAb or anti-TLR4 mAb or isotype-matched control antibody for 1 h and then incubated with 10 µg/ml of biotin labeled Mtbhsp60 at 4°C for 5 min or 30 min followed by incubation with streptavidin-FITC. The fluorescence was measured by flow cytometry. Results shown are representative of three individual experiments.
Since, Mtbhsp60 was found to bind strongly to both TLR2 and TLR4 receptors, it was next examined which TLR receptor was predominantly involved in IL-10 induction by Mtbhsp60. Therefore, specific TLR receptors were blocked by pre-incubating the THP-1 macrophages with either 10 µg/ml of neutralizing anti-TLR2 mAb or anti-TLR4 mAb or isotype-matched control antibody and subsequently treated with Mtbhsp60 and the level of IL-10 expression in these macrophages was measured by RT-PCR at 2 h or by EIA at 48 h time point. It was observed that compared to the group treated with medium alone, groups pre-treated with either anti-TLR4 mAb or isotype-matched control antibody had significant increase in IL-10 mRNA (Fig. 3.5). In contrast, pre-treatment with anti-TLR2 mAb had little effect on IL-10 gene expression in the presence of Mtbhsp60 (Fig. 3.5). When cells were treated with both anti-TLR2 and anti-TLR4 mAb, IL-10 level was comparable to that of medium alone as measured by RT-PCR (Fig 3.6). The RT-PCR data was further confirmed by EIA and similar results were obtained (Fig. 3.7, compare bars 5 and 7 with bar 3; p < 0.001 in both the cases). Experiments using peritoneal macrophages from C57Bl/6 mice once again indicated that blocking of TLR2 but not TLR4 receptors using specific antibodies resulted in decreased production of IL-10 (Fig. 3.8, compare bars 5 and 7 with bar 3; p < 0.001 in both the cases). All these experiments suggest that Mtbhsp60-mediated induction of IL-10 in macrophages is predominantly dependent on its interaction with TLR2.
Figure 3.5. Semi-quantitative RT-PCR data indicated that IL-10 activation by Mtbhsp60 is TLR2 dependent. PMA-differentiated THP-1 macrophages were pretreated with 10 µg/ml of either anti-TLR2 mAb or anti-TLR4 mAb or isotype-matched control antibody for 1 h and then cultured for another 2 h in the presence of 3 µg/ml of Mtbhsp60. Total RNA was extracted and (A) IL-10 levels were measured by semi-quantitative RT-PCR and (B) quantification of the IL-10 mRNA was performed by densitometric analysis using AlphaEaseFC software and the Spot Denso tool (Version 7.0.1; Alpha Innotech) and data are expressed as mean ± SD of three independent experiments.
Figure 3.6. IL-10 induction by Mtbhsp60 is inhibited in THP-1 macrophages when both TLR2 and TLR4 receptors are blocked. (A) PMA-differentiated THP-1 macrophages were pre-treated with either 10 µg/ml of isotype-matched control antibody or both anti-TLR2 mAb and anti-TLR4 mAb for 1 h and then incubated with 3 µg/ml of Mtbhsp60 for another 2 h. Total RNA was extracted and IL-10 levels were measured by semi-quantitative RT-PCR. (B) Densitometric analyses were performed using the software AlphaEaseFC software and the Spot Denso tool (Version 7.0.1; Alpha Innotech), normalized for β-actin and reported as arbitrary densitometric units. Values are means ± SD of the densitometric analysis of three independent experiments.
Figure 3.7. Anti-TLR2 mAb inhibits Mtbhsp60-induced IL-10 production in THP-1 macrophages. PMA-differentiated THP-1 macrophages were pre-treated with neutralizing mAb to either TLR2 or TLR4 or isotype-matched control antibody or with both anti-TLR2 mAb and anti-TLR4 mAb in the absence or presence of Mtbhsp60 (3 μg/ml). After 48 h, IL-10 cytokine levels in culture supernatants from various groups were measured by EIA. Results are representative of mean ± SD of three individual experiments.
Figure 3.8. Mtbhsp60 induces IL-10 in peritoneal macrophages during its interaction with TLR2. The IL-10 levels were measured by EIA in C57Bl/6 peritoneal macrophages treated with 10 µg/ml of either rabbit anti-TLR2 or rabbit anti-TLR4 or control antibody (normal rabbit serum) in the absence or presence of Mtbhsp60 (3 µg/ml). Results shown are representative of mean ± SD of three independent experiments.
Next to rule out the cytotoxic effect of Mtbhsp60 and neutralizing antibodies, the THP-1 macrophages were pre-treated with anti-TLR2 or anti-TLR4 or isotype control antibody (10 µg/ml) for 1 h before the addition of Mtbhsp60 at 3 µg/ml concentration. After 24 h, cell cytotoxicity was measured by MTT assay. No significant differences were observed in the cell viability in antibody and Mtbhsp60 co-treated groups with that of the control group that was treated with medium alone (Fig. 3.9).

Figure 3.9. The anti-TLR2 or the anti-TLR4 neutralizing mAb or the isotype control antibody is not cytotoxic to THP-1 macrophages. PMA-differentiated THP-1 macrophages were seeded at 2 x 10^5/100 µl/well into a 96-well microplate and pre-treated with either anti-TLR2 mAb or anti-TLR4 mAb or isotype control antibody (10 µg/ml) for 1 h before the addition of Mtbhsp60 at 3 µg/ml concentration. The cell cytotoxicity was measured by MTT assay 24 h after Mtbhsp60 treatment. Results shown are representative of mean ± SD of three individual experiments.
To further confirm the role of TLR2 in Mtbhsp60-mediated IL-10 induction, the amount of IL-10 produced was measured in macrophages that are deficient in either TLR2 or TLR4 receptors. For this, siRNA-mediated gene silencing was carried out in THP-1 macrophages using TLR2- and TLR4-specific siRNAs. Decrease in the levels of surface expression of TLR2 or TLR4 was confirmed by flow cytometry using antibody specific to TLR2 and TLR4 receptors respectively. The results indicated reduction of these surface receptors when cells were treated with TLR2/TLR4-specific siRNAs (Fig. 3.10). The transfected cells were then incubated with Mtbhsp60 (3 µg/ml) and IL-10 levels were quantified after 48 h by EIA. Consistent with the previous observations with neutralizing antibodies (Fig. 3.5 and Fig. 3.7), Mtbhsp60-mediated IL-10 production was found to be higher in TLR4-deficient cells when compared to that of TLR2-deficient cells (Fig. 3.11, compare bar 6 with bar 5; p < 0.001). Collectively these data suggest that IL-10 induction by Mtbhsp60 is predominantly mediated through TLR2-induced signaling.
Figure 3.10. siRNA mediated gene silencing of TLR2 and TLR4 receptors. Transfection of PMA-differentiated THP-1 macrophages was carried out using lipofectamine 2000. Macrophages were seeded at a density of $2 \times 10^6$ cells per well in a 12-well plate and were transfected with 100 nM siRNAs. After 6 h, culture medium was replaced and the cells were kept in culture for an additional 24 h. Repression of surface expression of TLR2 and TLR4 in the transfected cells were confirmed by flow cytometry using specific antibodies against TLR2 and TLR4 receptors. Results shown are representative of three individual experiments.
Figure 3.11. Silencing of TLR2 expression by siRNA down-regulates Mtbhsp60-induced IL-10 in THP-1 macrophages. PMA-differentiated THP-1 macrophages were transfected with negative control siRNA or TLR2-specific siRNA or TLR4-specific siRNA. After 24 h, cells were incubated with Mtbhsp60 (3 µg/ml) for another 48 h and levels of IL-10 were measured by EIA in the culture supernatants. Results shown are representative of mean ± SD of three independent experiments.
3.3. Mtbhsp60 undergoes endocytosis to induce IL-10

Next, the probable mechanism involved in the TLR2-dependent activation of IL-10 by Mtbhsp60 was investigated. Earlier studies on human and chlamydial Hsp60s suggest that activation of macrophage innate-effector responses by heat shock proteins is dependent to a great extent on the TLR-mediated, clathrin-dependent endocytosis (Vabulas et al., 2001) and clathrin-dependent endocytosis is known to be a major pathway for internalization of transmembrane receptors (Schmid, 1997). The receptor-mediated endocytosis of Mtbhsp60 was confirmed by checking its localization in endosome. For this, PMA-differentiated THP-1 macrophages were treated with FITC-labeled Mtbhsp60 at 37°C for 15 min. Cells were fixed, washed and permeabilized with Triton-X 100. Cells were then incubated with antibody to Early Endosome Antigen 1 (EEA1, an early endosome-specific marker) followed by staining with Alexa 594 labeled secondary antibody (anti-rabbit Alexa 594). The endocytosis analysis was performed by confocal laser scanning microscopy and the results indicated that Mtbhsp60 protein was colocalized with EEA1 (Fig. 3.12). Therefore, next it was investigated whether receptor-mediated endocytosis of Mtbhsp60 was a prerequisite for activation of IL-10. PMA-differentiated THP-1 macrophages were therefore, incubated with FITC-labeled Mtbhsp60 (Mtbhsp60-FITC) in the absence or presence of monodansylcadaverine (MDC), a selective cytoskeleton microtubule inhibitor that is known to block clathrin-dependent endocytosis (Davies et al., 1980) and examined the cellular distribution of Mtbhsp60 associated fluorescence using confocal microscopy. It was observed that THP-1 macrophages treated with MDC had poorly internalized Mtbhsp60-FITC resulting in increased cell surface accumulation of Mtbhsp60 (Fig. 3.13). To further confirm accumulation of Mtbhsp60 on THP-1 cell surface in the presence of MDC, PMA-
differentiated THP-1 macrophages were pre-treated with MDC for 30 min and subsequently incubated with 10 μg/ml of biotin labeled Mtbhsp60 for 15 min at 37°C followed by incubation with streptavidin-FITC and the surface bound fluorescence was measured by flow cytometry. An increase in cell surface bound fluorescence was observed in the cells treated with MDC as compared to the cells treated with medium alone (Fig. 3.14) suggesting that the Mtbhsp60 protein undergoes receptor-mediated endocytosis. Concurrently, Mtbhsp60-mediated IL-10 induction was also significantly compromised in THP-1 macrophages when endocytosis of this protein was blocked using MDC (Fig. 3.15, compare bar 4 and bar 5 with bar 3; p < 0.01 and p < 0.001 respectively). MDC had no significant effect on cell viability at both 50 μM and 100 μM concentrations used in these experiments as measured by MTT assay (Fig 3.16). Thus these results suggest that receptor-mediated clathrin-dependent endocytosis of Mtbhsp60 is required for IL-10 induction in macrophages.
Figure 3.12. Mtbhsp60 colocalizes with Early Endosome Antigen 1 (EEA1) in endosomes. PMA-differentiated THP-1 macrophages in a chamber slide were treated with FITC-labeled Mtbhsp60 (10 μg/ml) at 37°C for 15 min. The cells were fixed, washed and permeabilized with Triton-X 100. After blocking, cells were incubated with antibody to EEA1 for 1 h at room temperature followed by staining with Alexa 594 labeled secondary antibody (anti-rabbit Alexa 594) for 1 h at room temperature. Cells were then washed and the endocytosis analysis was performed by confocal laser scanning microscopy. Results shown are representative of three individual experiments.
Figure 3.13. Monodansylcadaverine (MDC) inhibits endocytosis of Mtbhsp60 in THP-1 macrophages. PMA-differentiated macrophages in a chamber slide were either left untreated or treated with MDC (100 μM) for 30 min followed by incubation with 10 μg/ml of Mtbhsp60-FITC at 37°C for 15 min. Cells were fixed, washed and the cell bound fluorescence was analyzed by confocal laser scanning microscopy. Results shown are representative of three independent experiments.
Figure 3.14. MDC increased cell-surface accumulation of Mtbhsp60. PMA-differentiated THP-1 macrophages were either left untreated or pre-treated with 100 μM concentration of MDC for 30 min and subsequently incubated with 10 μg/ml of biotin labeled Mtbhsp60 for 15 min at 37°C followed by incubation with streptavidin-FITC. The fluorescence was measured by flow cytometry. The result shown is representative of at least three independent experiments.
Figure 3.15. Monodansylcadaverine (MDC) inhibits Mtbhsp60-mediated induction of IL-10 in THP-1 macrophages. PMA-differentiated THP-1 macrophages were either left untreated or pre-treated with 50 µM or 100 µM MDC and subsequently incubated with 3 µg/ml of Mtbhsp60. IL-10 levels were estimated by EIA in various culture supernatants harvested after 48 h. Results shown are representative of mean ± SD of three independent experiments.
Figure 3.16. MDC does not affect viability of cells treated with Mtbhsp60. PMA-differentiated THP-1 macrophages were seeded at $3 \times 10^5/100 \mu l/well$ into a 96-well microplate and either left untreated or pre-treated with different concentrations of MDC for 30 min and then incubated with Mtbhsp60 (3 µg/ml). The cell cytotoxicity was measured by MTT assay after 48 h. Results shown are representative of mean ± SD of three individual experiments.
3.4. IL-10 activation by Mtbhsp60 is dependent on the endocytosis of Mtbhsp60 via TLR2 receptors

Since, in the previous experiments, a direct role of TLR2 in the Mtbhsp60-mediated activation of IL-10 was observed (Fig. 3.5, Fig. 3.7 and Fig. 3.11), next it was studied whether internalization of Mtbhsp60 was actually mediated through the TLR2 receptor. Therefore, PMA-differentiated THP-1 macrophages were pre-treated with either 10 µg/ml of neutralizing anti-TLR2 mAb or anti-TLR4 mAb to block the TLR2 or TLR4 receptors respectively and then incubated with Mtbhsp60-FITC (10 µg/ml) for 15 min at 37°C and the intracellular distribution of Mtbhsp60 was examined. The control group received isotype-matched antibody. The THP-1 macrophages pre-treated with anti-TLR2 mAb showed higher cell-surface-associated fluorescence as compared to the macrophages pre-treated with either anti-TLR4 mAb or isotype control antibody (Fig. 3.17) indicating impaired endocytosis in the group treated with anti-TLR2 mAb. In contrast, endocytosis of Mtbhsp60 was observed in cells treated with anti-TLR4 mAb or isotype control antibody (Fig. 3.17). These results suggest that Mtbhsp60 is internalized upon interaction with TLR2 whereas its interaction with TLR4 stalls this protein predominantly at the cell surface (Fig. 3.17). The confocal analysis further reveals that upon internalization, Mtbhsp60 was indeed colocalized along with TLR2 (Fig. 3.18). For the Mtbhsp60-TLR2 colocalization studies, PMA-differentiated THP-1 macrophages were treated with biotin-labeled Mtbhsp60 (10 µg/ml) at 37°C for 15 min. Cells were fixed, washed and permeabilized and after blocking, cells were incubated with anti-TLR2 mAb for 1 h followed by staining with Alexa 594 labeled secondary antibody and streptavidin-FITC for 1 h.
In the next experiment, it was checked whether TLR2-mediated endocytosis of Mtbhsp60 was required for induction of IL-10. The THP-1 macrophages were therefore, pre-treated with anti-TLR4 mAb to allow Mtbhsp60 to interact predominantly with TLR2 and the TLR2-mediated endocytosis was blocked by MDC and the levels of IL-10 were measured in these cells. As expected, IL-10 induction was strongly inhibited when endocytosis of Mtbhsp60 through TLR2 was inhibited by MDC (Fig. 3.19A, compare bar 4 with bar 3; p < 0.01). Similar results were obtained using peritoneal macrophages from C57Bl/6 mice (Fig. 3.19B, compare bar 4 with bar 3; p < 0.001). These data suggest that endocytosis of Mtbhsp60 is required for induction of IL-10 post binding to the TLR2 receptors.
Figure 3.17. Mtbhsp60 undergoes receptor-mediated endocytosis through interaction with TLR2. PMA-differentiated THP-1 macrophages were pre-treated with 10 µg/ml of either anti-TLR2 mAb or anti-TLR4 mAb or isotype-matched control antibody for 1 h and further incubated for 15 min at 37°C in the presence of Mtbhsp60-FITC (10 µg/ml). Cells were fixed, washed and the endocytosis was examined by confocal laser scanning microscope. Results are representative of three independent experiments.
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Results

Figure 3.18. *Mtbhsp60* colocalizes with TLR2 receptors. PMA-differentiated THP-1 macrophages in a chamber slide were treated with biotin-labeled *Mtbhsp60* (10 μg/ml) at 37°C for 15 min. Cells were then fixed, washed and permeabilized with Triton-X 100. After blocking, cells were incubated with antibody to TLR2 (raised in mouse) for 1 h at room temperature followed by staining with Alexa 594 labeled secondary antibody (anti-mouse Alexa 594) and streptavidin-FITC for 1 h at room temperature. Cells were next washed and the colocalization analysis was performed using confocal laser scanning microscopy.
Figure 3.19. IL-10 activation by Mtbhsp60 is dependent on TLR2-mediated endocytosis of Mtbhsp60 in macrophages. (A) PMA-differentiated THP-1 macrophages or (B) peritoneal macrophages from C57Bl/6 mice were pre-treated with either 10 µg/ml of anti-TLR4 or control antibody for 1 h and then incubated with 3 µg/ml of Mtbhsp60 in the absence or presence of 100 µM MDC. After 48 h of incubation, IL-10 levels were measured by EIA in various culture supernatants. Results shown are representative of mean ± SD of three independent experiments.
3.5. Mtbhsp60-induced TNF-α production is largely dependent on the signaling generated by surface-bound TLR-Mtbhsp60 immune complex

It is believed that Mtbhsp60 is also responsible for TNF-α induction as the *M. tuberculosis* mutant lacking the GroEL homologue Cpn60.1 failed to induce an inflammatory response and was unable to form granuloma in a murine experimental model (Hu *et al.*, 2008). TNF-α is known to be one of the critical factors involved in granuloma formation (Kindler *et al.*, 1989, Flynn *et al.*, 1995, Roach *et al.*, 2002, Algood *et al.*, 2005). Interestingly, in human endothelial cells, induction of TNF-α by some other mycobacterial heat shock proteins like Hsp65 and Hsp70 was found to be TLR4-dependent (Bulut *et al.*, 2005). Since, the previous experiments suggest that Mtbhsp60 interacts with both TLR2 and TLR4 receptors and TLR2 is involved in Mtbhsp60-mediated IL-10 induction, a role of TLR4 in inducing TNF-α production was speculated.

Therefore, either the TLR2 or TLR4 or both the TLR2 and TLR4 receptors on macrophage surface were blocked using respective blocking antibodies and then these cells were treated with 3 μg/ml of Mtbhsp60 for 48 h. When the culture supernatants were harvested and assayed for TNF-α produced by EIA, it was observed that inhibition of TLR4-Mtbhsp60 interaction with anti-TLR4 mAb resulted in significantly poorer TNF-α production in THP-1 macrophages in response to Mtbhsp60 when compared with that of anti-TLR2 mAb or isotype-matched control antibody-treated cells (Fig. 3.20, compare bar 4 with bar 6 and bar 2; p < 0.01 in both the cases). These results indicate that Mtbhsp60 probably targets the TLR4 to trigger induction of pro-inflammatory cytokines like TNF-α. Earlier it was observed that when Mtbhsp60 interacted with TLR2, it undergoes receptor-mediated endocytosis to induce IL-10, whereas its interaction with TLR4 left the protein mostly surface-bound (Fig. 3.17) resulting in
higher production of TNF-α. These observations led to speculate that TNF-α induction is triggered by signals generated predominantly by the surface-bound Mtbhsp60-TLR4 complexes whereas induction of IL-10 is driven by signals generated by Mtbhsp60 localized in the endosomes upon receptor-mediated endocytosis through TLR2. To test this hypothesis, next the TLR2-mediated endocytosis of Mtbhsp60 was blocked by MDC and it was examined whether, surface-bound TLR2-Mtbhsp60 complexes can also induce production of TNF-α. The macrophages were, therefore, pre-treated with either isotype control antibody or anti-TLR4 mAb and then treated with Mtbhsp60 in the absence or presence of MDC. True to the expectations, it was observed that blocking of the receptor-mediated endocytosis by MDC significantly increased the production of TNF-α by Mtbhsp60 in isotype control antibody-treated cells (Fig. 3.20, compare bar 3 with bar 2; p < 0.05). When TLR2-mediated endocytosis of Mtbhsp60 was blocked in the presence of MDC, production of TNF-α was also significantly increased (Fig. 3.20, compare bar 5 with bar 4; p < 0.01). Interestingly, in such situations, production of IL-10 was found to be significantly reduced in macrophages when TLR2-mediated endocytosis of Mtbhsp60 was inhibited in the presence of MDC (Fig. 3.19, compare bar 4 with bar 3). These data suggest that Mtbhsp60 can induce starkly opposite cytokine responses in macrophages depending on its cellular localization post binding to TLRs. Since, blocking of TLR2 and TLR4 receptors together with the respective antibodies had no significant increase in the Mtbhsp60-mediated production of TNF-α (Fig. 3.20, lane 7) or IL-10 (Fig. 3.6, lane 3 and Fig. 3.7, lane 9) over medium control, it appears that the Mtbhsp60 primarily targets the TLR2 and the TLR4 receptors to influence cytokine signaling in macrophages.
Figure 3.20. Interaction of Mtbhsp60 with TLR4 or TLR2 plus MDC triggers TNF-\(\alpha\) production. PMA-differentiated THP-1 macrophages were pre-treated with 10 µg/ml of neutralizing mAb to either TLR2 or TLR4 or isotype-matched control antibody or with both anti-TLR2 mAb and anti-TLR4 mAb for 1 h and subsequently incubated with Mtbhsp60 (3 µg/ml) in the absence or presence of MDC (100 µM) and TNF-\(\alpha\) levels were quantified after 48 h in different culture supernatants by EIA. Results shown are representative of mean ± SD of three independent experiments.
3.6. Mtbhsp60 targets TLR2 to induce IL-10 and TLR4 to activate TNF-α in macrophages harvested from TLR2 knock-out (KO) and TLR4 KO C57Bl/6 mice

Experiments carried out in the previous section clearly indicate that Mtbhsp60 primarily targets TLR2 to trigger IL-10 and TLR4 to activate TNF-α production in macrophages. However, conclusions were drawn mostly based on experiments carried out in THP-1 cell line where Mtbhsp60-TLR interaction was blocked using either the blocking antibodies or siRNA-mediated knocking down of TLR2/4 receptors. To further corroborate these findings, we next used primary macrophages harvested from TLR2 KO and TLR4 KO mice. The thioglycolate elicited peritoneal macrophages from C57Bl/6 wild-type (WT), TLR2 KO and TLR4 KO mice were left untreated or treated with Mtbhsp60 at concentration of 3 µg/ml and the levels of IL-10 and TNF-α produced by these macrophages were measured by semi-quantitative RT-PCR. The results shown in Fig. 3.21 indicate that Mtbhsp60 could increase IL-10 mRNA expression in macrophages harvested from either wild-type or TLR4 KO mice, however, near control level of IL-10 mRNA expression in response to Mtbhsp60 was observed in macrophages harvested from TLR2 KO mice (Fig. 3.21). In contrast, a strong TNF-α induction by Mtbhsp60 was observed predominantly in macrophages harvested from either wild-type or TLR2 KO mice whereas Mtbhsp60 failed to trigger TNF-α mRNA expression in macrophages harvested from TLR4 KO mice and the mRNA level was almost similar to the medium-treated control (Fig. 3.21). These results clearly demonstrate that Mtbhsp60 primarily targets TLR2 to induce IL-10 and TLR4 to activate TNF-α in macrophages.
Figure 3.21. Mtbhsp60 fails to induce IL-10 in macrophages harvested from TLR2 KO mice and TNF-α in macrophages harvested from TLR4 KO mice. Thioglycolate elicited peritoneal macrophages from C57Bl/6 WT, TLR2 KO and TLR4 KO mice were either left untreated or treated with Mtbhsp60 (3 μg/ml) for 1 h. (A) Total RNA was extracted and IL-10 and TNF-α levels were measured by semi-quantitative RT-PCR. Densitometric analyses were performed for IL-10 (B) and TNF-α (C) using the software AlphaEaseFC software and the Spot Denso tool, normalized for GAPDH and reported as arbitrary densitometric units. Values are means ± SD of the densitometric analysis of three independent experiments.
3.7. The *E. coli* heat shock protein 60 (EcoliHsp60) is retained mainly on the macrophage surface upon interaction with either TLR2 or TLR4 and triggers induction of TNF-α.

The *E. coli* homologue of Mtbhsp60, EcoliHsp60 (GroEL) is known to induce predominantly a pro-inflammatory cytokine response in macrophages and human monocytes (Retzlaff *et al.*, 1994, Marcatili *et al.*, 1997, Tabona *et al.*, 1998). To check the TLR-receptor preference of EcoliHsp60, PMA-differentiated THP-1 macrophages were pre-treated with 10 µg/ml of either anti-TLR2 mAb or anti-TLR4 mAb or isotype-matched control antibody for 1 h and then incubated with 10 µg/ml of FITC labeled EcoliHsp60 at 4°C for 30 min and the cell bound fluorescence was measured by flow cytometry. The results indicate that EcoliHsp60 can bind to both TLR2 and TLR4 receptors (Fig. 3.22). Interestingly, interaction of EcoliHsp60 with either TLR2 or TLR4 left the protein mostly stranded on the cell surface. When THP-1 macrophages were pre-treated with either isotype-matched control or anti-TLR2 mAb or anti-TLR4 mAb and subsequently incubated with EcoliHsp60-FITC and allowed it to be endocytosed at 37°C, a very strong cell surface-bound fluorescence was observed under confocal microscopy which was due to poor TLR-mediated endocytosis of the protein (Fig. 3.23). Since triggering of TNF-α production was observed by surface-bound Mtbhsp60, it was speculated that interaction of EcoliHsp60 with either TLR2 or TLR4 would trigger TNF-α production due to its inherent inability to undergo receptor-mediated endocytosis unlike interaction of Mtbhsp60 with TLR2 (Fig. 3.17). Blockage of either TLR2 or TLR4 receptors using respective blocking antibodies, resulted in production of similar levels of TNF-α in EcoliHsp60-treated THP-1 macrophages (Fig. 3.24, compare bar 3 with bar 4). In the presence of isotype-matched antibody, where both the TLR2 and
TLR4 receptors were free to interact with Ecolihsp60, the levels of TNF-α were found to be significantly higher as compared to that of TLR2 or TLR4 alone and almost summed up the levels of TNF-α produced together by these receptors (Fig. 3.24, compare bar 2 with bars 3 and 4) indicating that both TLR2 and TLR4 are responsible for predominant induction of TNF-α by Ecolihsp60 in macrophages.

Figure 3.22. Ecolihsp60 binds to both TLR2 and TLR4 receptors. PMA-differentiated THP-1 macrophages were pre-treated with either 10 µg/ml of anti-TLR2 mAb or anti-TLR4 mAb or isotype-matched control antibody for 1 h and further incubated with 10 µg/ml of FITC labeled Ecolihsp60 at 4°C for 30 min. The cells were then fixed, washed and the fluorescence was measured by flow cytometry. Results shown are representative of three individual experiments.
Figure 3.23. Ecolihs60 is retained at the surface of cells treated with either anti-TLR2 or anti-TLR4 mAb. PMA-differentiated THP-1 macrophages were pre-treated with 10 µg/ml of either anti-TLR2 mAb or anti-TLR4 mAb or isotype-matched control antibody for 1 h, followed by incubation with Ecolihs60-FITC (10 µg/ml) at 37°C for 15 min. Cells were fixed, washed and endocytosis of the protein was assessed by confocal laser scanning microscopy. Results shown are representative of three individual experiments.
Figure 3.24. TNF-α production by Ecolihsp60 is inhibited in THP-1 macrophages treated with either anti-TLR2 or anti-TLR4 mAb. PMA-differentiated THP-1 macrophages were pre-treated with 10 µg/ml of either anti-TLR2 mAb or anti-TLR4 mAb or isotype-matched control antibody for 1 h and then incubated with 3 µg/ml Ecolihsp60. After 48 h, TNF-α levels in different culture supernatants were quantified by EIA. Data are representative of mean ± SD of three independent experiments.
Though the heat shock proteins are highly conserved, biochemical features of Mtbhsp60 are known to be deviated significantly from its *E. coli* homologue, Ecolihsp60. Mtbhsp60 appears to exist in a lower oligomeric state as compared to its *E. coli* counterpart due to substitutions in some crucial interface residues required to stabilize its inter-subunit interactions (Qamra *et al.*, 2004). There are also significant deviations at the structural level of the monomers that might explain some of the observed functional differences between these two homologous proteins. Therefore, the 3D structure of Mtbhsp60 was determined using homology modeling (Fig. 3.25A) and superimposed with that of Ecolihsp60 (Fig. 3.25B). The overall root-mean-square deviation (RMSD) of the Mtbhsp60/Ecolihsp60 was found to be 4.89Å (Fig. 3.25C), indicating that a significant deviation exists between these two proteins at the conformational level, also.
Figure 3.25. Secondary structure comparison of Mtbhsp60 with Ecolihs6p60. (A) The secondary structure of the Mtbhsp60 protein model, predicted based on homology modeling with Modeller software is shown (green) after its energy minimization. (B) The solved crystal structure of Ecolihs6p60 obtained from Protein Data bank (chain-A of PDB-code:2EU1) is displayed (blue). (C) The superimposed structures of Mtbhsp60 and Ecolihs6p60.
3.8. The p38 MAPK and ERK 1/2 signaling play important roles in the TLR-dependent induction of IL-10 and TNF-α by Mtbhsp60

A number of TLR ligands are known to activate various MAPKs including p38 MAPK, ERK 1/2 and JNK 1/2 (O'Neill, 2006, O'Neill et al., 2007) and MAPKs play crucial roles in regulating the innate cytokine production in macrophages (Nakahara et al., 2006). Many groups have shown that p38 MAPK and ERK 1/2 signaling are crucial in regulating the anti-inflammatory and pro-inflammatory cytokines in macrophages by mycobacterial components (Blumenthal et al., 2002, Tse et al., 2002, Song et al., 2003, Yadav et al., 2004, Pathak et al., 2005, Chi et al., 2006, Souza et al., 2006, Yang et al., 2008, Nair et al., 2009). It has been reported that activation of p38 MAPK plays a critical role for IL-10 production (Song et al., 2003, Souza et al., 2006) while TNF-α secretion is predominantly dependent on ERK 1/2 activation in macrophages (Tse et al., 2002, Jo et al., 2007). Therefore, next the phosphorylation status of p38 MAPK and ERK 1/2 was compared in situations where Mtbhsp60 is endocytosed by interacting with TLR2 and thereby resulting in dominant production of IL-10 (Fig. 3.17; Fig. 3.5, Fig. 3.7 and Fig. 3.21) to situations where it is predominantly sequestered on the cell surface like interactions with TLR4 or with TLR2 in the presence of MDC resulting in increased production of TNF-α and decreased IL-10 expression (Fig. 3.19 and Fig. 3.20). Therefore, THP-1 macrophages were treated with either anti-TLR2 mAb to allow binding of Mtbhsp60 to TLR4 or with anti-TLR4 mAb to allow binding of Mtbhsp60 to TLR2 receptors respectively and the phosphorylation status of p38 MAPK and ERK 1/2 by flow cytometry and Western blotting was examined. It was observed that interaction of Mtbhsp60 with TLR2 leads to phosphorylation of p38 MAPK predominantly as early as 10 min and ERK 1/2 to a lesser extent (Fig. 3.26 and Fig. 3.27). However, engagement
of Mtbhsp60 with TLR4 predominantly phosphorylated the ERK 1/2 at the same time point as shown by both flow cytometry (Fig. 3.26) and Western blotting (Fig. 3.27). On the other hand, blocking of TLR2-mediated endocytosis of Mtbhsp60 by MDC inhibits p38 MAPK phosphorylation by Mtbhsp60, but ERK 1/2 phosphorylation was found to be increased (Fig. 3.28). Interestingly, activation of p38 MAPK has been reported to be crucial for triggering early endocytic membrane traffic (Cavalli et al., 2001, Zwang et al., 2006). Taken together these data suggest that endocytosis of Mtbhsp60 through TLR2 predominantly activates p38 MAPK leading to IL-10 induction, whereas, surface-bound Mtbhsp60 activates ERK 1/2 causing higher induction of TNF-α. Therefore, it appears that the cellular localization of Mtbhsp60 following interaction with TLRs dictates the type of MAPKs to be activated and subsequently the kind of cytokine responses to be produced in macrophages.
Figure 3.26. Comparison of phosphorylation status of p38 MAPK and ERK 1/2 mediated by Mtbhsp60 between the TLR2 receptor that undergoes endocytosis against TLR4 that does not undergo endocytosis. PMA-differentiated THP-1 macrophages were pre-treated with 10 µg/ml of either anti-TLR2 mAb or anti-TLR4 mAb or isotype-matched control antibody for 1 h and then treated with Mtbhsp60 (3 µg/ml) for 15 min. After permeabilization, macrophages were incubated with antibody to either phospho-p38 or phospho-ERK 1/2 followed by incubation with anti-rabbit IgG-FITC or anti-mouse IgG-FITC and fluorescence was analyzed by flow cytometry. Results shown are representative of three individual experiments.
Figure 3.27. Mtbhsp60 activates p38 MAPK when it interacts with TLR2 while its interaction with TLR4 predominantly induces ERK 1/2 activation. PMA differentiated THP-1 macrophages were pre-treated with 10 µg/ml of either anti-TLR2 mAb or anti-TLR4 mAb or isotype-matched control antibody followed by incubation with Mtbhsp60 (3 µg/ml) for 15 min. Cells were lysed and the levels of phosphorylated and total p38 MAPK as well as phosphorylated and total ERK 1/2 levels were measured by Western blotting. Results shown are representative of three independent experiments.
Figure 3.28. Blocking TLR2 mediated endocytosis of Mtbhsp60 results in inhibition of p38 MAPK phosphorylation but cause an increase in ERK 1/2 phosphorylation. PMA-differentiated macrophages were pre-treated with neutralizing mAb to TLR4 (10 µg/ml) for 1 h and then treated with Mtbhsp60 for 15 min in the absence or presence of MDC (100 µM). The phosphorylation status of p38 MAPK and ERK 1/2 was analyzed by flow cytometry. Results are representative of three individual experiments.
Chapter 3

3.9. The NF-κB signaling pathways downstream of TLR4 play an important role in activation of TNF-α by Mtbhsp60

Since TNF-α is shown to be dominantly regulated by the NF-κB transcription factors (Baldwin, 1996), and also since TLR4 is known to activate NF-κB (Zhang et al., 2001, Fitzgerald et al., 2003), it is expected that interaction of TLR4 with Mtbhsp60 could activate NF-κB transcription factors more dominantly as compared to the condition where Mtbhsp60 was allowed to interact with TLR2. Therefore, THP-1 macrophages were treated with Mtbhsp60 in the presence of neutralizing mAb to either TLR2 or TLR4 or isotype-control antibody and nuclear extracts were used to check the specific DNA-binding activity of the NF-κB complex by EMSA using NF-κB consensus oligonucleotide probe (Wagner et al., 2002) labeled with $[^\gamma \text{P}]$-ATP. The EMSA result indeed indicated a stronger activation of NF-κB when Mtbhsp60 interacted with TLR4 (Fig. 3.29). Blocking of NF-κB activity in the anti-TLR2 antibody-treated macrophages by specific NF-κB inhibitors like BAY 11-7082 (Mori et al., 2002, Lee et al., 2012) as well as PDTC (Khan et al., 2007, Bhat et al., 2012) could inhibit TNF-α induction in these cells (Fig. 3.30) confirming a role of NF-κB downstream of TLR4 in the regulation of pro-inflammatory cytokines by Mtbhsp60. To identify which subunit of NF-κB is involved, supershift assay was performed. The PMA-differentiated THP-1 macrophages were-pretreated with 10 µg/ml of either anti-TLR2 or anti-TLR4 mAb or isotype control antibody for 1 h followed by incubation with Mtbhsp60 for another 1 h and nuclear extracts were prepared. The binding reaction was incubated with either rabbit anti-p65 or rabbit anti-p50 antibody for 30 min after addition of labeled probe and EMSA was performed. As expected, blocking of TLR4 receptor using anti-TLR4 antibody resulted
in poor induction of NF-κB in macrophages during treatment with Mtbhsp60 whereas a strong induction of NF-κB was observed in groups treated with either anti-TLR2 or isotype control antibody (Fig. 3.31). Strong supershift band with anti-p65 antibody was observed in the groups treated with either isotype control antibody or anti-TLR2 antibody where Mtbhsp60 was allowed to interact with TLR4 (Fig. 3.31). These studies indicate a predominant involvement of p65 NF-κB in the activation of TNF-α by Mtbhsp60 during its interaction with TLR4.
Figure 3.29. Mtbhsp60 activates NF-κB transcription factors mainly through TLR4. PMA-differentiated THP-1 macrophages were pre-treated with 10 µg/ml of either anti-TLR2 mAb or anti-TLR4 mAb or isotype-matched control antibody for 1 h and then incubated with 3 µg/ml of Mtbhsp60 for another 1 h. Cells were harvested and nuclear extracts were prepared. The DNA-binding activity of NF-κB complex was measured by EMSA. Results shown are representative of three independent experiments.
Figure 3.30. The NF-κB inhibitors, BAY11-7082 and PDTC prevents TNF-α induction in THP-1 macrophages by Mtbhsp60 during its interaction with TLR4. PMA-differentiated THP-1 macrophages were pre-treated with 10 µg/ml anti-TLR2 mAb for 1 h and further incubated with 3 µg/ml of Mtbhsp60 in the absence or presence of either BAY 11-7082 (10 µM) or PDTC (3 µM). After 48 h, culture supernatants were harvested and TNF-α levels were quantified by EIA. Results shown are representative of mean ± SD of three individual experiments.
Figure 3.3. DNA-binding activity of p50 and p65 NF-κB complex in THP-1 macrophages treated with Mtbhsp60 in the presence of isotype control antibody or anti-TLR2 mAb or anti-TLR4 mAb. PMA-differentiated THP-1 macrophages were pre-treated with 10 µg/ml of either isotype control antibody or anti-TLR2 mAb or anti-TLR4 mAb for 1 h followed by incubation with Mtbhsp60 for another 1 h. Cells were harvested and nuclear extracts were prepared. For supershift assay, 2 µl of either rabbit anti-p65 or rabbit anti-p50 antibody was added to the binding reaction and incubated for 30 min. The DNA-binding activity of NF-κB complex was measured by EMSA.