Chapter 5

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*Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB) has been plaguing mankind for millennia, infecting an estimated one-third of the world’s population and causing 8.6 million new cases of active TB annually world-wide (WHO, 2013). Tuberculosis is still one of the most formidable diseases to treat. The bacilli have the capacity to remain viable within infected hosts for a prolonged time. The conventional control measures have had little impact on the relentless progress of the TB epidemic due to rapid emergence of multidrug resistant and extensively drug resistant strains, HIV, diabetes and other health problems associated with immunosuppression. A better understanding of the strategies employed by *M. tuberculosis* bacilli that modulate the host protective responses to survive inside host and transmit the infection is necessary to develop new generation anti-TB drugs and effective therapeutic vaccine(s).

The growth and persistence of *M. tuberculosis* is promoted by an environment characterized by Th 2 response and cytokines like IL-10 (Hernandez-Pando et al., 1996, Rook, 2007). On the other hand, a Th1 response and the effector cytokines like TNF-α, IL-12 are critical for conferring protection against the bacilli (Flynn et al., 1995, Quesniaux et al., 2010). However, mechanisms underlying this *Mycobacterium*-macrophage interaction that regulates Th1 (pro-inflammatory) and Th2 (anti-inflammatory) responses in tuberculosis are poorly understood at both the molecular and cellular levels. A better understanding of host-pathogen interactions and the signaling pathways altered by the bacteria during infection may help us in combatting this deadly pathogen. *M. tuberculosis* responds to the intracellular milieu of macrophages by overexpressing genes required for its survival and multiplication inside the host. Studies have shown that *M. tuberculosis* up-regulates its heat shock protein 60 (Mtbhsp60,
Cpn60.1, Rv3417c) during infection in macrophages (Young et al., 1991, Zugel et al., 1999b, Monahan et al., 2001). This suggests that the Mtbhsp60 protein may play important roles in modulation of Th1/Th2 cytokine balance contributing to *M. tuberculosis* pathogenesis.

Earlier studies have revealed that Mtbhsp60 can skew the anti-PPD Th1 response towards the non-protective Th2 type (Khan et al., 2008) indicating its probable role in the modulation of the T cell response. Further studies have indicated that Mtbhsp60 inhibits induction of Th1 cytokine, IL-12p40 in macrophages in response to PPD targeting TLR2. Since IL-10 is known to inhibit induction of IL-12p40, it was predicted that Mtbhsp60 probably targets TLR2 of the host to induce IL-10 and subsequently block IL-12p40 expression and the Th1 response. The results indicated that Mtbhsp60 interacts with both TLR2 and TLR4 receptors. Interaction of Mtbhsp60 with TLR2 leads to an increased production of IL-10 at both mRNA level as quantified by semi-quantitative RT-PCR and at protein level as indicated by cytokine EIA. In contrast, binding of Mtbhsp60 with TLR4 resulted in predominant induction of TNF-α indicating that IL-10 activation by Mtbhsp60 was restricted predominantly to TLR2-mediated signaling as opposed to TLR4 that induced a minute quantity of IL-10. These experiments were carried out in both PMA-differentiated THP-1 macrophages and primary macrophages from C57Bl/6 mice using either blocking antibodies against TLR2 and TLR4 or knocking down the TLR2 and TLR4 by TLR-specific siRNA or using macrophages harvested from TLR2 and TLR4 KO mice. Results obtained in the present study suggest that Mtbhsp60-mediated anti-inflammatory or pro-inflammatory cascade in
macrophages is critically dependent on TLR-mediated endocytosis leading to its differential cellular localization.

The underlying mechanism appears that interaction of Mtbhsp60 with TLR2 cause an increase in endocytosis of Mtbhsp60 leading to the activation of p38 MAPK and IL-10. In contrast, upon interaction with TLR4, Mtbhsp60 remained predominantly localized on the cell surface due to poor endocytosis of the protein that caused a low level of IL-10 production and suppression of p38 MAPK activation. In such a situation an increase in ERK 1/2 phosphorylation and TNF-α was observed. Interestingly, inhibition of clathrin-mediated endocytosis through TLR2 by MDC (a selective cytoskeleton microtubule inhibitor that is known to block clathrin-dependent endocytosis) resulted in surface accumulation of Mtbhsp60 and polarization of immune response towards the pro-inflammatory type characterized with a higher ERK 1/2 phosphorylation and increased production of TNF-α. Thus, Mtbhsp60 activates p38 MAPK signaling to induce an anti-inflammatory response (by up-regulating IL-10 production) via TLR2-mediated clathrin-dependent endocytosis. On the other hand, induction of pro-inflammatory cytokines such as TNF-α by Mtbhsp60 only requires sequestration to the membrane either through TLR2 or TLR4 that results in activation of ERK 1/2 signaling cascades. A role of NF-κB downstream of TLR4 in the regulation of pro-inflammatory cytokines by Mtbhsp60 was demonstrated as higher NF-κB activity was observed when Mtbhsp60 interacted with TLR4 as compared to its interaction with TLR2. Further blocking of NF-κB activity by specific NF-κB inhibitors like BAY 11-7082 as well as PDTC could inhibit TNF-α induction during interaction of Mtbhsp60 with TLR4.
confirming a strong role of these transcription factors downstream of the TLR4 signaling cascade in the regulation of TNF-α/pro-inflammatory cytokines by Mtbhsp60. In fact, many groups have demonstrated earlier that while ERK 1/2 activation is required for TNF-α production (Yadav et al., 2004, Souza et al., 2006), IL-10 activation is predominantly dependent on p38 MAPK signaling (Song et al., 2003, Souza et al., 2006). This study, for the first time, pinpoints that the dichotomous nature of signal transduction through TLRs can be governed primarily by the divergent MAPK-NF-κB signaling transmitted from the endosome against those from membrane which are critical for the regulation of anti-inflammatory and pro-inflammatory signaling in macrophages. Further, these observations indicate for the first time that the TLR2 and TLR4 receptors can regulate two counteracting effector functions by reciprocally modulating p38 and ERK 1/2 MAPK signaling pathways through differential localization of Mtbhsp60 in the endosome or on the cell-surface.

Though the *Escherichia coli* homologue of hsp60 (Ecolihsp60) shares 70% sequence similarity with Mtbhsp60, it is retained mainly on the macrophage surface upon interaction with TLR2 and TLR4. Mtbhsp60 is found to be functionally different from Ecolihsp60 as it activates both anti-inflammatory signaling (during interaction with TLR2 which is followed by endocytosis) and pro-inflammatory signaling (during interaction with TLR4 or TLR2 when it is localized on the cell-surface), while the Ecolihsp60 predominantly induces pro-inflammatory signaling probably because of its retention on the cell surface due to failure of internalization upon interaction with either TLR2 or TLR4. This functional variation between Ecolihsp60 and Mtbhsp60 could be
due to the difference both at the structural level and in their biochemical properties. This relates to the pathogenic property of Mtbhsp60 that can skew the T cell response towards Th2 promoting phenotype through activation of IL-10.

The ability of Mtbhsp60 to induce significant amount of IL-10 when ligated to TLR2 may reflect its ability to specifically down-regulate nuclear c-rel and IL-12p40 induction in PPD-activated macrophages on binding of Mtbhsp60 to TLR2 rather than TLR4 (Khan et al., 2008) and is likely the possible mechanism by which Mtbhsp60 can potentially endow an anti-inflammatory milieu leading to a pronounced Th2 response favoring survival of *M. tuberculosis* inside the host. Though, Mtbhsp60 can also interact with TLR4 to induce a host protective pro-inflammatory response, its ability to increase the surface expression of TLR2 (Khan et al., 2008) can mitigate the TLR4-mediated protective effects. This study provides hints by which *M. tuberculosis* heat shock protein 60 can suppress host protective immune responses and thus can act as a virulence factor. This information is important in devising strategies to regulate macrophage innate responses to engineer host protective immunity not only against the *M. tuberculosis* infection but also against other intracellular pathogens that target the toll receptors.