6.1 Introduction

Circulating HSP27 has been reported in several inflammatory diseases artherosclerosis (Rayner et al., 2008), multiple sclerosis (Ce et al., 2011). The extracellular HSP27 has been reported to have immunomodulatory properties by stimulating monocyte to predominantly induce IL-10 production (De et al., 2000). IL-10 in turn inhibits monocyte to dendritic cell differentiation and pro-inflammatory cytokine production (Kajino et al., 2007). Unlike large HSP molecules that are considered as danger signals and augment the proinflammatory cytokine by monocytes through Toll like receptors (TLRs) signaling, particularly via TLR4 (Vabulas et al., 2002; Lehnardt et al., 2008). Although, little is known about the receptor for HSP27, a recent study demonstrated that proinflammatory effect of HSP27 is mediated through TLR2 and TLR4 (Jin et al., 2014). Another study reported that HSP27 present in the tumor microenvironment mediates angiogenesis via TLR3 (Thuringer et al., 2013).

TLR4 is a transmembrane receptor which recognizes lipopolysaccharide (LPS), a cell wall component of gram negative bacteria which triggers inflammatory response by activating either myeloid differentiation primary response protein 88 (MyD88) adaptor molecule or TRIF related adapter molecule (TRAM) (also known as TICAM2) (Akira et al., 2004). MyD88 is the central adaptor molecule utilized by all TLRs except TLR3 (Burns et al., 1998, Kawai et al., 1999) for the activation of proinflammatory cytokine through NF-κB whereas, TLR3 induces type I interferons via interferon regulatory factor (IRF) 3. TRAM is selectively involved in TLR4 signaling pathway (Kawai et al., 2006). Though, HSP27 is reported to utilize TLR4 as the receptor but the downstream signaling pathway involved in its immunomodulatory role is not yet clear.

In this study, we investigated the immunomodulatory property of HSP27 on human monocytic THP-1 cell lines. Cells pretreated with HSP27 followed by LPS treatment induced maximal IL-1β expression as compared to stimulation with HSP27 and LPS alone. In
addition, we checked the expression of TLR4 and the two intermediate adaptor molecules; MyD88 and TRAM at mRNA level. Our results show the activation of TRAM pathway by HSP27 for the induction of pro-inflammatory cytokines. This study would help in understanding the pathway involved in the immunomodulatory mechanism induced by HSP27 which could further be employed for the development of therapeutics for inflammatory diseases.

6.2 Materials and methods

6.2.1 Cell culture

Human monocytic cell line THP-1 was procured from National Centre for Cell Science, Pune. Cells were maintained in RPMI-1640 media (Gibco) supplemented with L-Glutamine and antimycotic solution (Himedia) containing 10% fetal bovine serum (Gibco) at 37ºC in humidified chamber and passaged every fourth day.

6.2.2 Stimulation of cells with HSP27 and LPS

Cells growing in log phase were used for the stimulation experiments after washing once with phosphate buffered saline and seeded in 24 well culture plate. For stimulation cell number was maintained upto one million cells per ml. Cells were stimulated either with heat shock protein 27 (HSP27, low endotoxin, Enzo Life Science) at concentration 30ng/ml for 18 hrs or with lipopolysaccharide (LPS, Sigma) at 1µg/ml concentration for 6 hrs. Co-stimulation was done by pretreatment of cells with HSP27 for 18 hrs followed by LPS stimulation for 6 hrs. Cell viability after stimulation was accessed using WST-1 cell proliferation assay kit (Cayman) according to manufacturer’s instruction. After the stimulation, 10ul of WST-1 reagents was added to the each well and incubated for 4 hrs. The absorbance was measured at 450 nm using ELISA plate reader (Bio-Rad laboratories).

6.2.3 RNA isolation from cell lines

Post stimulation cells were harvested and washed with phosphate buffered saline twice
followed by lysing in TRI reagent (Sigma) for RNA isolation as per the manufacturers protocol. Briefly, lysate mixed with the chloroform, centrifuged and the aqueous layer separated was collected. RNA in the aqueous layer is precipitated by isopropanol followed by washing with 70% ethanol. Pellet was then dissolved in nuclease free water. Integrity and purity of RNA was checked on agarose gel. Quantification was done on NanoDrop® ND1000 spectrophotometer.

6.2.4 cDNA synthesis and Real-time PCR analysis

cDNA was synthesized using high capacity reverse transcription kit (Applied biosystems, Foster City, CA) following manufacturer’s instruction. Briefly, prior to reverse transcription reaction 2µg of RNA was subjected to DNase (NEB) treatment to remove the contaminating genomic DNA. Further, reaction mixture comprising DNase treated RNA, RT Buffer (10X), Random Primer (10X), dNTP mix (25X), Reverse transcriptase enzyme and RNase Inhibitor (10U/µl) were incubated at 25°C for 10 min followed by incubation at 37°C for 120 min. Finally, enzyme is inactivated by incubation at 85°C for 5 min. Whole reaction was carried out in PCR Thermocycler (Applied biosystems, Foster City, CA).

Quantitative real time PCR was performed for proinflammatory cytokines IL-1β; TLR4; Intermediate signaling molecule for MyD88 dependent and MyD88 independent pathway, MyD88 and TRAM respectively. Primers were designed using NCBI/ primer blast and autoprime software (http://www.autoprime.de/ AutoPrimeWeb). Primer sequences and annealing conditions were depicted in the Table 17. All PCR reactions were carried out on Applied Biosystems 7,500 real-time PCR system, using quantifast SYBER green PCR master mix (Qiagen). The thermal profile used for the reactions include denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at temperature depending upon gene specific primer for 1 min as mentioned in Table 17. The comparative Ct method was used to interpret the data as described by Livak and Schmittgen,
2001 (17). Relative expression of each gene among treated and untreated groups was determined using formula, Fold change = $2^{\Delta \Delta Ct}$, where $\Delta Ct = Ct_{(Gene)} - Ct_{(\beta-actin)}$ and $\Delta \Delta Ct = (\Delta Ct_{(treated)}) - (\Delta Ct_{(untreated)})$.

Table 17. Gene specific primers for Real time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
<th>Annealing Temperature</th>
<th>Software Used</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>FP 5’ GGCCCTAAACAGATGAAGTGTAC 3’</td>
<td>60 ºC</td>
<td>NCBI-Primer blast</td>
</tr>
<tr>
<td></td>
<td>RP 5’ AGTGGTGGTGGAGAAATCGGT 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>FP 5’ TGCCTCAAAACTGCTCGTCA 3’</td>
<td>58 ºC</td>
<td>NCBI-Primer blast</td>
</tr>
<tr>
<td></td>
<td>RP 5’ TCCTGGCATCATCCTCATGCT 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MyD88</td>
<td>FP 5’ CTCCTCCAGGTGCCCATC 3’</td>
<td>58 ºC</td>
<td>NCBI-Primer blast</td>
</tr>
<tr>
<td></td>
<td>RP 5’ GGTTGGTGATGAAGCTACAGACG 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAM</td>
<td>FP 5’ ATGCTCTAACAAGCCAGAGGCA 3’</td>
<td>60 ºC</td>
<td>NCBI-Primer blast</td>
</tr>
<tr>
<td></td>
<td>RP 5’ TCTCGTGAGAAGCGATGCTCTCATC 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β- Actin</td>
<td>FP 5’ CTTGCTGGCATGGAGTC 3’</td>
<td>58ºC</td>
<td>Autoprime Software</td>
</tr>
<tr>
<td></td>
<td>RP 5’ TACAGGTCTTTGCAGGTGTC 3’</td>
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6.2.5 Statistical analysis

GraphPad prism software v.5.0 (GraphPad Software, San Diego, CA) was used for the statistical analysis of the data. Statistical differences among treated groups were evaluated by the Student’s $t$ test with the level of significance set at $P < 0.05$. Values are expressed as the mean ± SD from three independent experiments.

6.3 Results

6.3.1 Upregulation of proinflammatory cytokines upon HSP27 prestimulation

IL-1β and TNF-α was significantly upregulated upon LPS stimulation as compared to controls (p value 0.01 for both). The expression of IL-1β and TNF-α in THP-1 cells upon pre-treatment with HSP27 followed by LPS treatment was elevated as compared to untreated
control (p value 0.001 and 0.01, respectively) and beyond the expression level observed with LPS treatment alone (p value 0.002 and 0.01, respectively) (Figure 49A and 49B). HSP27 stimulation alone did not induce the expression of IL-1β as well as TNF-α. Stimulation with HSP27 and LPS or both did not affect the viability of cells as assessed by cell proliferation assay (data not shown).

6.3.2 Increased expression of TLR4 on HSP27 prestimulation

The expression of TLR4 was upregulated on treatment with LPS (P value 0.014) or HSP27 (p value 0.014) alone as compared to controls. However, pretreatment of HSP27 followed by LPS showed a cumulative effect on expression of TLR4. TLR4 expression elevated significantly upon HSP27/LPS treatment as compared to stimulation with LPS (p value 0.0359) or HSP27 alone (P value 0.035) (Figure 50).

Figure 49. Expression of proinflammatory cytokines (A) IL-1β and (B) TNF-α in monocyic cells upon LPS and HSP27 stimulation expression. LPS induces the expression of IL-1β and TNF-α (p < 0.05). HPS27 alone did not show any changes in cytokine expression. Prior stimulation with HSP27 followed by LPS treatment augments the expression of IL-1β and TNF-α (p < 0.05) as compared to LPS alone.
6.3.3 HSP27 activates TLR4 pathway via TRAM

The expression of TRAM at mRNA level was upregulated upon HSP27 stimulation alone (p-value 0.018) whereas, no significant difference was observed in the expression of TRAM upon stimulation with LPS alone or HSP27 pre-treatment followed by LPS stimulation (Figure 51A). However, the expression of MyD88 was elevated upon LPS (p-value 0.006) and HSP27/LPS (p-value 0.037) stimulation as compared to untreated control. No significant difference was observed among their expression in LPS and HSP27/LPS treated groups (Figure 51B).

Figure 50. TLR4 expression was upregulated upon stimulation with LPS (P = 0.0144) and HSP27 (P = 0.014). The induction of TLR4 activation after HSP27/LPS treatment together was high as compared to treatment of HSP27 (P = 0.0354) and LPS (P = 0.0359) alone.

Figure 51. Expression of adaptor molecules of TLR4 signaling pathway (A) Stimulation with HSP27 only induces TRAM expression (P = 0.018) whereas LPS alone was not able to induce
TRAM (B) Treatment with LPS (P = 0.006) and HSP27/LPS (P = 0.037) induces the MyD88 expression as compared to untreated controls

6.4 Discussion

We report that HSP27 pretreatment augments the expression of IL-1β in human THP-1 monocytic cell lines. TLR4 expression was also enhanced by treatment with HSP27 prior to LPS stimulation in contrast to HSP27 and LPS treatment alone. This suggests exogenous HSP27 utilizes same receptor for its activity as LPS. This observation is in concordance with the recent study which shows proinflammatory effect of extracellular HSP27 through TLR4 in myocardial ischemia (Jin et al., 2014).

In light of this observation we also evaluated the expression of downstream molecules in TLR4 signaling pathway and observed increased expression of TRAM adaptor molecule by HSP27 treatment alone whereas, no difference was observed in other treated groups. However, HSP27 alone does not induce any change in MyD88 expression. Minimal difference was observed in MyD88 expression upon stimulation with LPS alone or HSP27/LPS stimulation which suggests HSP27 had no contribution in the expression of MyD88. This study indicates that the recruitment of the adaptor molecule, either MyD88 or TRAM following TLR4 activation depends much upon the type of stimulus as previously demonstrated by Zughaier et al., 2005. Thus HSP27 may trigger TRAM pathway following TLR4 activation unlike LPS which triggers MyD88 dependent pathway.

Based on the results obtained we propose a model (Figure 52) which suggests that the augmentation of IL-1β and TNF-α upon HSP27/LPS stimulation could be due to the activation of both MyD88 and TRAM dependent pathways as depicted in Figure 4. Both the MyD88 dependent and independent pathways are involved in TLR4 mediated induction of inflammatory cytokines. TRAM pathways results in the late activation of NF-κB in contrast,
Heat shock protein 27 induces proinflammatory cytokine via TRAM mediated signaling of TLR4

MyD88 dependent pathways leads to early activation of NF-kB (Akira et al., 2004; Kawai et al., 2006). Thus identifying the specific pathway involved in the immunomodulatory mechanism induced by HSP27 would help in better understanding of its implication in inflammatory diseases.

Figure 52. A proposed model to show the pathway followed after activation of TLR4 signaling by HSP27 and TLR4 stimulation. Two adaptor molecules are recruited to TLR4 for the downstream signaling: either TRAM or MyD88 (shown in dashed and bright lines). HSP27 activates TRAM (shown with bright line) only and not the MYD88 (dashed line) after TLR4 activation. LPS activates MyD88 (shown in dashed line only).