Chapter 5: LPS-neutralization

Study of anti-endotoxin property of the designed short heptadic peptides of different hydrophobic amino acids

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Introduction

Many time, after bacterial infection or treatments, their remains i.e. endotoxins initiates a regulated complex process by cascade of cytokines, growth factors, nitric oxide (NO) and prostaglandin produced by activated host cell like monocyte and macrophages (189, 190). Mainly two pro-inflammatory mediators, NO and tumor necrosis factor alpha (TNF-α) have been recognized as essential component of acute and chronic inflammatory process (191). These mediators recruit other inflammatory cells, which in turn release cytokines and subsequently amplify the immune response. The over expression of cytokines locally can cause serious diseases like rheumatoid arthritis, multiple sclerosis, and psoriasis, while at systemic level, it can cause septic shock (157, 183, 188, 192). Old aged, children and immuno-compromised individuals are more prone to infection and thus to this syndrome, which causes million of death throughout world per year (183, 188, 193). Toll like receptors are key sensory molecule, which recognizes conserved microbial structure, termed pathogen-associated molecular pattern (PAMPs). These PAMPs are nothing but endotoxins like LPS, lipotechoic acid and CpG nucleotides etc. PAMP recognition leads to activation of signaling pathways resulting in the production of pro-inflammatory cytokines (194-196). In addition to the production of cytokines and chemokines, activation of the innate immune system leads to the synthesis and mobilization of antimicrobial peptides (AMPs). These peptides, an evolutionarily antique component of the innate immune system, can obstruct endotoxin-induced pro-inflammatory mediators' production and diminish the mortality (197-199). Current therapeutic approaches to the treatment of inflammatory diseases are centered on the suppression of the NO or TNF-α production (200). Therefore, in order to chuck out, there is urgent need of such antimicrobial molecules which also have anti-endotoxin activity.

LPS cover more than 90% of outer leaflet of outer membrane of Gram negative bacterial strain. It creates the primary permeability barrier which makes membrane relatively impermeable to hydrophobic antibiotic, detergent and host protein (201). Among endotoxins, LPS initiates pro-inflammatory response more profoundly than others like lipotechoic acid and CpG nucleotides (202). In previous studies, it has been considered that the interactions of peptides with LPS controls the pro-inflammatory signaling through TLR and primarily govern

their anti-endotoxin activity (65, 203, 204). Though, antimicrobial peptides are relatively different from each other with respect to sequence, length, secondary structures, and charge to hydrophobicity ratio, but all these peptides are generally cationic, amphipathic and exhibits similar biological functions like antimicrobial activity, immunomodulatory property etc. The anti-inflammatory activities of different antimicrobial peptides on the basis of interaction either with LPS or its targets/receptors have been studied, but blurred & limited outcomes gained (205).

In order to get insight on how the differences in hydrophobicity at heptadic positions influences the cytotoxic and antibacterial properties of the perptides, a set of short peptides were synthesized and characterized as described in chapter-4. In same line, to know the activity of these peptides in LPS-neutralization, inhibition of LPS induced-inflammatory response onto macrophage cell line RAW 264.7 by these peptides were evaluated which has been described in this chapter.

Results

In contrast to VRP and ARP, LRP and FRP inhibited LPS induced NO production more widely

There are several reports that the some antimicrobial peptides inhibit pro-inflamatory response induced by endotoxin LPS, LTA etc (197-199). In order to evaluate the anti-endotoxin property of the designed peptides, LRP, FRP, VRP and ARP, LPS induced NO production was evaluated in macrophage cells in the absence and presence of these peptides by Gries reagent as described in the Material and Methods section. The result of dose dependent response showed that LRP inhibited ~90% NO level at 3μM, FRP ~80% at 4.5μM, VRP ~50% at 6-16μM and ARP did not exhibit any significant activity , even up to more than 18μM concentration (Fig.-1A, B & C). The result of inhibition of LPS induced NO production indicated that the variation of hydrophobicity of the residues at heptadic positions differently affect the anti-inflammatory activity of the peptides, since the positions and the number of cationic residues were the same in all four peptides. The hydrophobicity of leucine, phenylalanine, valine and alanine are in order of leu $\approx$ phe $\approx$ val > ala. Yet heptadic peptide with leucine residues at 'a' and 'd' positions most efficiently inhibited, LPS induced NO production (Fig.-1).

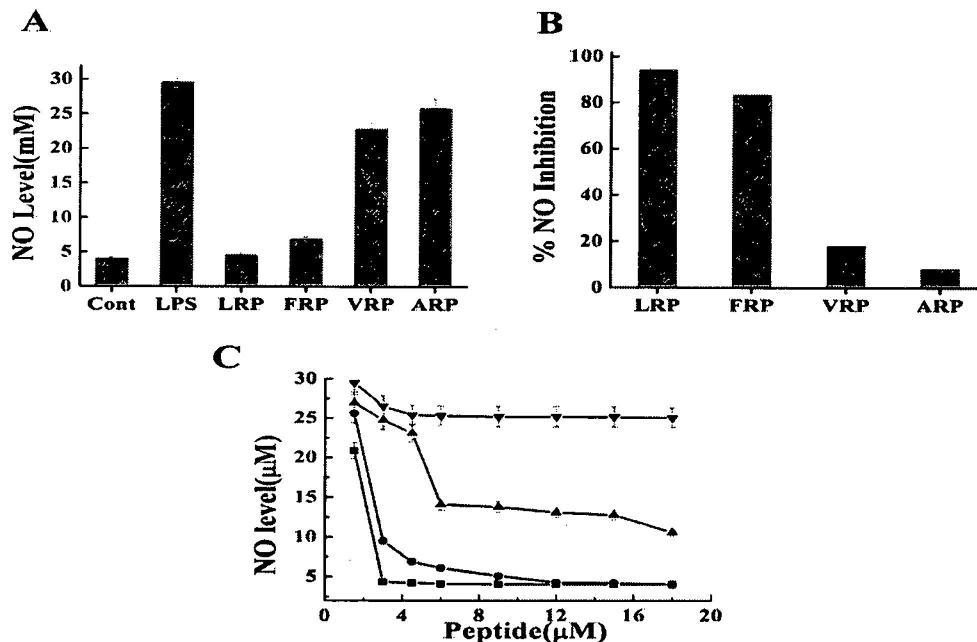

Figure-1: Effect of designed antimicrobial peptides on NO production from LPS induced macrophage RAW 264.7 cell line. *Panel A,* the amount of nitric oxide produced in LPS stimulated RAW 264.7 cells, the ctrl and LPS stands for untreated control and LPS (1μg/ml) treated cells respectively, while LRP, FRP, VRP and ARP represent the amount of NO produced by the LPS treated cells in presence of these peptides (4.5μM). The μM values of nitric oxides were determined by using a standard sodium nitrite curve. *Panel B,* Percentage inhibition of NO production in LPS-stimulated cells in the presence of different peptides, at 4.5μM. *Panel C,* dose dependent response of peptides onto LPS induced NO production. Symbols square; LRP, circle; FRP, up-right triangle; VRP and inverted triangle; ARP.

Inhibition of iNOS-2, TNF-α, IL-1β, and COX-2 induced by endotoxin LPS

The over secretion of nitric oxide is proportional to over expression of iNOS2 (inducible nitric oxide synthase-2). In order to determine the inhibitory effect of peptides on LPS-induced over expression of iNOS-2, Western blotting experiments were performed. The result shows that, LRP in contrast to FRP, VRP and ARP more prominently down regulated the iNOS-2 expression. The FRP although exhibited some effect but not as active as LRP, while VRP and ARP were comparably inactive in experimental conditions (Fig.-2).

The over production of secretary cytokines creates condition like inflammation. The interleukin like IL-6 and IL-1β play important role in LPS induced pro-inflammatory responses. Similarly, TNF-α also markedly enhance in presence of LPS, an important cytokine in systemic inflation. In order to detect the effect of these designed peptides on LPS-

induced pro-inflammatory response, Western blotting experiments were performed with specific antibodies. The effect of peptides on the expression of TNF-α and IL-1β showed the same trend as it was observed with iNOS-2 (Figure-2).

The COX-2 not only plays an important role in LPS-induced apoptosis mediated via P53 but also enhances secretions of pro-inflammatory mediators like PGE2 (prostaglandins 2). The Inhibitory effect of these peptides over LPS induced COX-2 production determined similarly. Again, the result of LPS induced COX-2 production also diminished in the presence of these peptides following the same trend as observed in case of iNOS-2 TNF-α and IL-1β.

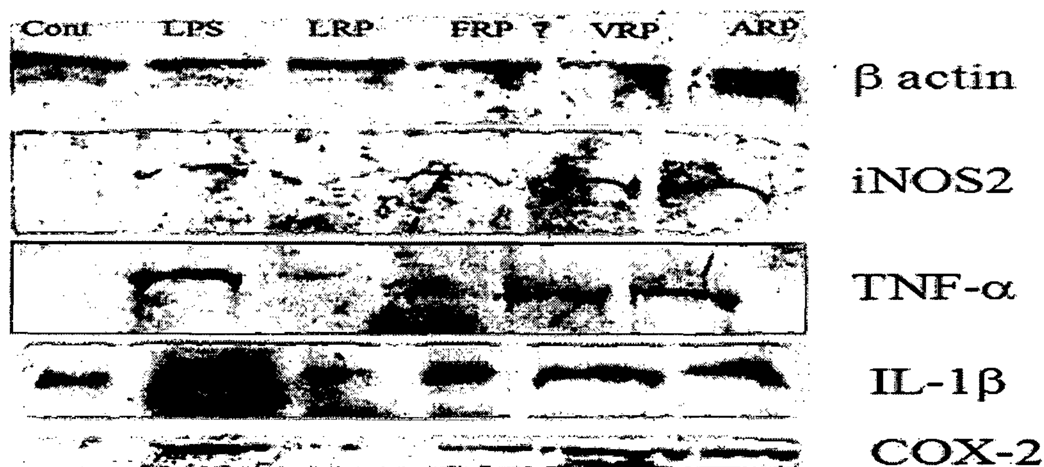

Figure-2: Evaluation of expression level of iNOS-2, TNF-α, IL-1β and COX-2 in LPS-induced macrophage RAW264.7 cells, in the presence of designed peptides LRP, FRP, VRP and ARP (4.5μM). Western blot results represent the effect of treatments of designed peptides on the expression level of iNOS-2, TNF-α, IL-1β and COX-2 in LPS-stimulated cells in 24 hrs. β-actin used as internal control.

Similar to intracellular, secretary pro-inflammatory mediator inhibited selectively by LRP and FRP

Along with intracellular increment, pro-inflammatory mediators are also secreted out in presence of inflammation inducer like LPS, lipotechoic acids etc. Therefore, in order to investigate the effect of these peptides on LPS induced secretion of pro-inflammatory mediators, ELISA experiments with supernatant were performed. We found that LRP and FRP efficiently inhibit the secretion of pro-inflammatory mediators (TNF-α and IL-6), whereas the inhibition induced by VRP and ARP was much lower (Fig.-3A & B).

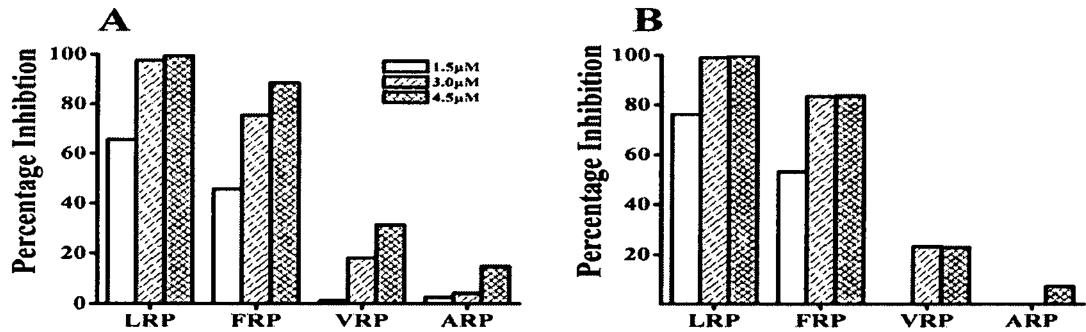

Figure-3: Effect of designed peptides onto the secretion of cytokines in LPS-stimulated RAW 264.7 cells. *Panel A* and *Panel B* show the percentage inhibition of levels of LPS induced secretions of TNF- α and IL-16 respectively in the presence of peptide (LRP, FRP, VRP and ARP) by ELISA experiments.

LRP inhibited LPS induced degradation of Iκβα more prominently among these peptides

The Iκβα degradation is a very important step in nuclear translocation of transcription factor NFkB. LPS responsive cell in presence of LPS, up-regulates certain pro-inflammatory mediator through degradation of Iκβα. The result of inhibition of Iκβα degradation showed that leucine heptadic peptide LRP more efficiently inhibited this intracellular process. As observed in our previous study also (Rghuvendra et al), though leucine, phenylalanine and valine are more or less similar hydrophobic, leucine heptadic peptide, LRP more efficiently inhibited degradation of Iκβα as compared to the other peptides (206) (Fig.-4).

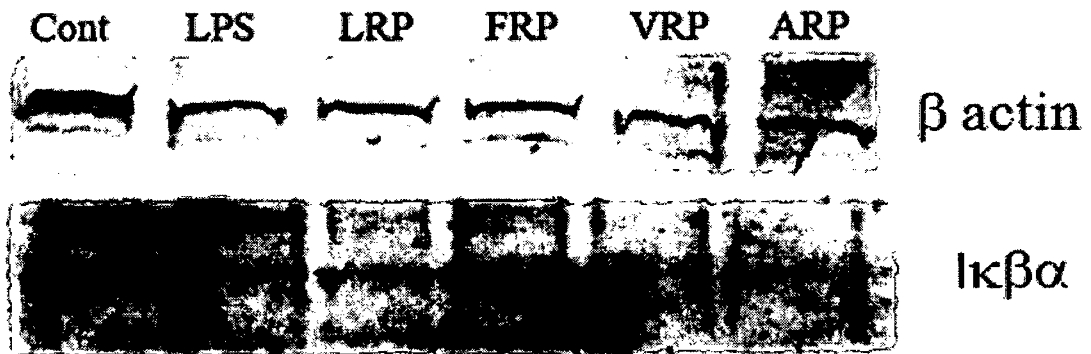

Figure-4: Determination of effect designed peptides on degradation of Iκβα in LPS-stimulated cells. Western blot result shows the effect of (4.5μM) peptide treatment onto level of Iκβα (inhibitor of transcription factor NFκB) in LPS-stimulated macrophage cells Raw264.7.

Peptides adopted relatively distinct helical structure in milieu of LPS

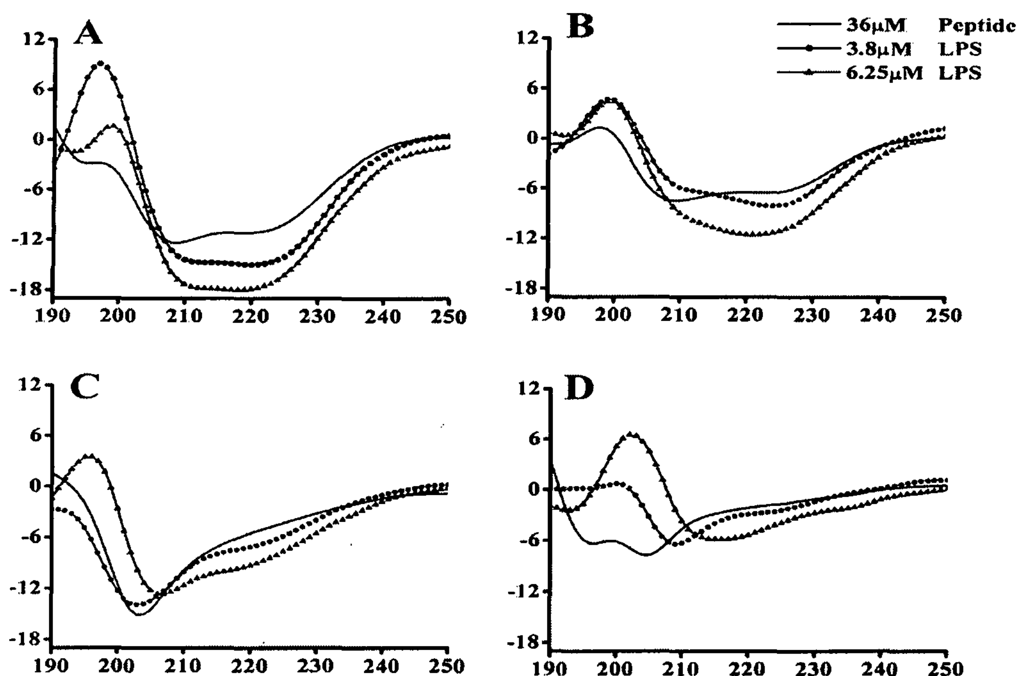

Figure-5: Determination the effect of LPS onto peptides conformation. CD spectra of designed peptides (36μM) recorded at two different concentrations of LPS (3.8μM and 6.25μM). *Panel A*; LRP, *Panel B*; FRP, *Panel C*; VRP and *Panel D*; ARP. Symbol: only line; peptide in aqueous environment, line with square; peptide with 3.8μM LPS and line with upright triangle; peptide with 6.25μM LPS.

LPS-induced pro-inflammatory response is exhibited through cell surface receptor MD-2-TLR complex of monocytes and macrophages. It has been reported that the peptide-LPS interaction plays an important role in anti-endotoxin activity of antimicrobial peptides (65, 203, and 204). Therefore, in order to study the interaction between these designed peptides and LPS CD experiments were performed. The CD spectra of the peptides in presence of LPS shows that the most active peptide, LRP adopted the maximum helical structure among these peptides which was followed FRP (Fig.-5A & B). VRP and ARP adopted lesser secondary structures in comparison to LRP and FRP (Fig.-5C & D). Altogether, the extent of secondary structure adopted by the peptides in presence of LPS follow the trend of their anti-LPS property.

LRP and FRP disintegrate LPS more efficiently than VRP and ARP

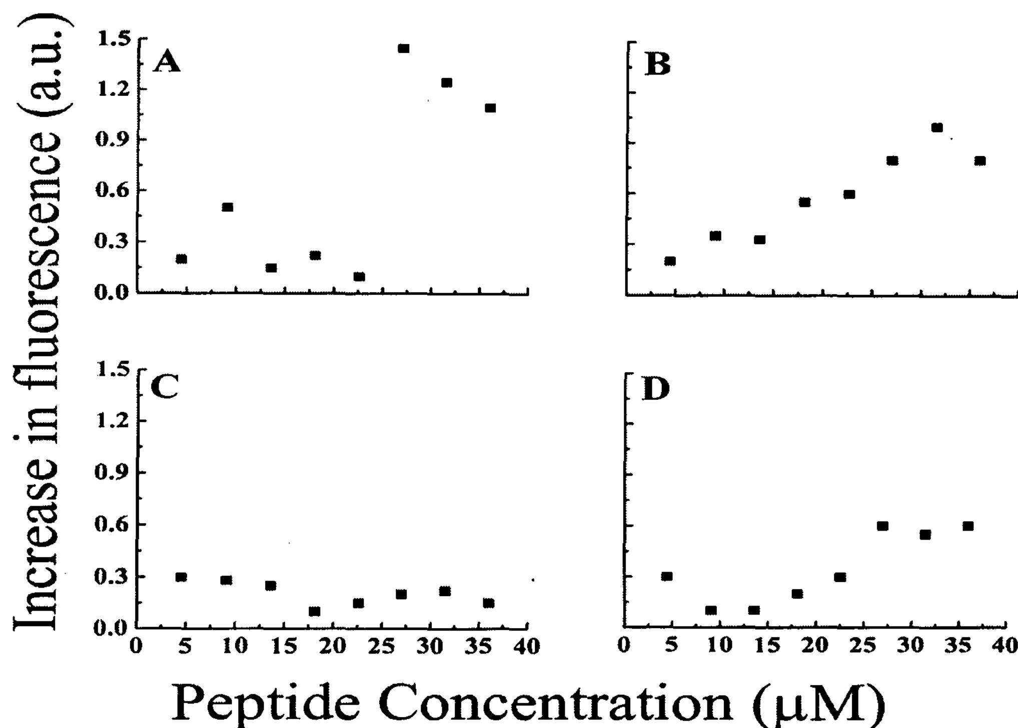

Figure-6: Dissociation of FITC-LPS aggregates in the presence of increasing concentrations of designed peptides. Increase in fluorescence (in arbitrary units) of FITC-LPS has been plotted with respect to peptide concentration in μM. The change in FITC emission after each treatment was monitored until emission reached equilibrium. *Panel A, B, C and D* represent the LRP, FRP, VRP and ARP respectively. FITC-LPS concentration (LPS-FITC 0.5μg/ml) was the same for each experiment and the collected data with a particular peptide has been marked in the X-axis of each plot.

LPS induced pro-inflammatory responses depend on its physical state. In previous studies, it has been well correlated that the dissociation of highly aggregated LPS by peptides directs their extent of neutralization of inflammatory response (65). Therefore in order to investigate the relationship of anti-inflammatory response with its LPS disintegration, peptide induced dequenching of FITC-tagged LPS fluorescence was studied. We observed that the most active anti-inflammatory peptide LRP induced the disintegration of FIT-LPS most profoundly among these peptides (Fig.-6A). FRP also showed significant neutralization towards the LPS-induced proinflammatory response in macrophage cells (Fig.-6B). However,

the VRP and ARP induced significantly lesser disintegration of LPS-aggregates which signifies the lower anti-endotoxin properties of these peptides (Fig.-6C & D).

Discussions:

The results presented in this chapter clearly demonstrate the properties of four heptadic peptides with different hydrophobic amino acids at the respective 'a' and 'd' positions towards the neutralization of proinflammatory response in LPS-stimulated RAW 264.7 cells. Nitric oxide is one of the primary markers of LPS induced pro inflammatory responses in defense cells; we initiated our study with the investigation of inhibitory effects of these designed peptides onto the production of LPS induced nitric oxides in RAW264.6 macrophages. We observed that LRP was the most efficient molecule among these peptides followed by FRP and VRP in neutralization of LPS induced production of nitric oxides. ARP was found to be negligibly active against LPS in inhibition of nitric oxide production in RAW 264.7 macrophage cells (Figure-1). We performed immunoblotting experiments to observe if the changes in production of nitric oxide in presence of these peptides have some correlation with the expression level of the concerned proteins/enzymes such as iNOS2 responsible for LPS-stimulated over production of NO. We found that the expression level of iNOS2 (inducible nitric oxide synthase) altered with the treatments of peptides in presence of LPS Fig (2). In presence of LPS the expression levels were significantly higher than that in untreated cells, while the most active peptide LRP was successfully able to reduce the expression level of iNOS2 to the basal level. FRP also restricted the iNOS levels up to an appreciable extent in LPS treated cells. But, VRP and ARP were proved to be insignificant in inhibiting the expression level of iNOS2 in presence of LPS, at experimental condition. This is an agreeable correlation with their inhibitory effects on LPS induced nitric oxides in RAW 264.7 macrophage cells. Further we investigated the other proteins/cytokines which play important role in establishment of an inflammation like condition in presence of LPS stimulus. We performed immunoblotting experiments for cytokines such as IL-1β and TNF-α, and found the similar responses by these peptides as we found in case of iNOS2 also. We did study the expression of COX-2 which mediates the process of LPS induced cytotoxicity/ cell death and found that in presence of LPS the expression of COX-2 was significantly enhanced

while when in presence of LRP, FRP VRP and ARP the protein levels decreased gradually like that in case of iNOS2 (Fig.-2). We performed ELISA experiments with the supernatant collected from the LPS treated cells both in presence and absence of the peptides and found that the LRP, FRP, VRP and ARP all four molecules exhibited their activities in neutralizing the over expressions of both the cytokines in their regular pattern where LRP showed the maximum inhibition followed by FRP, VRP and ARP respectively (Fig.-3). This proved that the active peptide was able to neutralize cytokines and related proteins at both extra cellular as well as intracellular levels. The maximum inhibition of Iκβα degradation by leucine heptadic peptide LRP in RAW 264.7 cells match with its maximum efficacy to neutralize LPS-induced pro-inflammatory response in RAW 264.7 cells (Fig.-4).

There are certain reports which indicate that LPS detoxification by some peptides or antimicrobial peptides is mediated by the binding/interaction of these peptides with LPS first and then disintegration of the LPS core region takes place which is primarily constituted of Lipid A (65). The gradual decrease in the ability of the peptides to attain a definite secondary structure in LPS from LRP to FRP to VRP to ARP is a consequential phenomenon of their increasing inability to interact with LPS. It is considered that the propensity to get self assembled/ aggregated/oligomerized is one of the key factors governing the immunomodulatory behavior of these peptides (207, 208). It seems that the peptides which tend to get aggregated are more capable of breaking the biochemical organization of Lipid A part of LPS (206-208). We have found a remarkable difference among these peptides in their self-association properties. As already discussed in chapter-4, the LRP is the most helical aggregated peptide, FRP comparatively lesser but VRP and ARP did not exhibit significant helical aggregation behavior in aqueous environment as evidenced by the CD studies (Chapter-4 Fig.-10). In peptide-induced dequenching of FITC-LPS studies, we found that LRP being the most active followed by FRP among these peptides (Fig.-6A & B). This dequenching was the result of its action to disintegrate the LPS core molecule rendering it into a less active/ inactive molecule. VRP and ARP showed to some extent disintegration of LPS core in the similar experiment but definitely not up to the efficiency levels of LRP and FRP (Fig.-6C & D). This was also in correlation with their respective abilities to neutralize LPS-induced pro-inflammatory responses in RAW264.7 macrophages. We as well as others have observed the dominant role of the leucine zipper like sequences in promoting self

oligomerization/aggregation/ self assembly. (22, 23). In this chapter we have focused in studying the ability of peptides with different hydrophobic amino acids at heptadic positions to neutralize LPS-induced pro-inflammatory responses in macrophage cells. The significant and maximum activity of LRP probably indicates that the structural properties of leucine zipper sequence of LRP plays a crucial part in neutralizing LPS-induced pro-inflammatory response.

Recently we have designed several novel analogs of Temporin L (unpublished data) with higher state of aggregation and found it to be more LPS detoxifying than parent molecule. Similarly when we altered the aggregation state of Temporin L parent molecule with substitutions/scrambling we noticed a sharp decline in its immunomodulation against LPS (unpublished data). Hence with the study executed with Temporin L and its designed analogs and the results presented in this chapter, we can conclude that structural motifs like leucine zipper or leucine zipper like heptad repeats which are key factors for regulating oligomeric properties in peptides and proteins can be utilized for designing LPS-neutralizing peptides.