Aggregation of Temporin L and anti-endotoxin property

Introduction of a lysine residue Promotes aggregation of Temporin L in lipopolysaccharides and augmentation of its anti-endotoxin property

Saurabh Srivastava and Jimut Kanti Ghosh*
Molecular and Structural Biology Division
CSIR-Central Drug Research Institute
Chattar Manzil Palace, Post Box No. 173
Lucknow-226001, India

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*To whom correspondence should be addressed
Tel: 091-522-2612411-18 (Ext.-4282)
Fax: 091-522-2623405;
E-mail: jighosh@yahoo.com; jk_ghosh@cdri.res.in

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ABSTRACT:

Temporin L (TempL) is a 13-residue frog antimicrobial peptide that shows moderate bactericidal activity and anti-endotoxin properties in macrophage cells. We envisioned that due to very hydrophobic nature, this peptide may fail to show its desired biological properties. It was predicted by employing the available algorithms that the substitution of a glutamine by lysine at position 3 could appreciably reduce its aggregation propensity in aqueous environment. In order to investigate the structural, functional and biological consequences of substitution of glutamine by lysine at its third position, TempL and the corresponding analog, Q3K,TempL, was synthesized and characterized. Introduction of the lysine residue significantly promoted the self-assembly and oligomeric state of TempL in LPS. Q3K,TempL exhibited augmented binding towards LPS and also dissociated the LPS-aggregates with higher efficacy than TempL. Further, Q3K,TempL inhibited the LPS-induced pro-inflammatory cytokines in rat primary macrophage cells in vitro and in vivo in Balb/C mice to a higher efficacy than TempL. The results showed a simple amino acid substitution in a short hydrophobic antimicrobial peptide, TempL to enhance its anti-endotoxin properties and illustrate a plausible correlation between its aggregation properties in LPS and LPS-detoxification activity.
Conventional antibiotics target important mechanisms or pathways of bacterial survival to inhibit their growth. However, failure of these conventional antibiotics to check the bacterial infections is a major threat to human health in recent years due to emergence of resistance in microbial organisms against these molecules. Inability of the conventional antibiotics to restrict the subsequent effects of dead bacteria and its remains from unwanted stimulation of the host immune system is also a serious disadvantage with the use of conventional antibiotics. The toxins such as bacterial membrane constituents, nuclear materials and cell debris released by bacteria by means of cell divisions or after bacterial death can suddenly provoke the host immune system. Of these, lipopolysaccharides (LPS), also known as endotoxin, which is the main constituent of the outer membrane of gram-negative bacteria is a well-known activator of humoral and cellular immune system in host and can invoke serious pathophysiological consequences (1). Uncontrolled stimulation of host immune system in the presence of LPS could result in excessive release in inflammatory cytokines leading to septic shock which often cause the death of a patient (2, 3). Septicemia is a leading cause of death which can be instigated even by ordinary gram negative bacterial infection. The neutralization of LPS mediated toxic injury has been considered for a long time as a possible therapeutic target in patients.

Antimicrobial peptides (AMPs) are important components of the innate immune system and identified from almost all living organisms including humans (4). These small cationic polypeptides are capable of targeting bacteria, fungi and enveloped viruses and are considered as potential alternatives for conventional antibiotics. These AMPs are mostly membrane active and lyse the microbes by disintegrating their cell membranes and thus not giving them enough opportunities to acquire resistance against these molecules (5). Not only their mode of action makes them better candidates for developing lead bactericidal molecules but also their
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capabilities to bind and neutralize the bacterial remains add to the advantages of antimicrobial peptides in their efficacy against bacterial invasions (6, 7). However not every antimicrobial peptide can neutralize proinflammatory responses stimulated by bacterial debris principally, LPS, yet there are several AMPs that possess both bactericidal and anti-endotoxin properties, and modulation of some parameters may enhance both biological properties in a peptide (8-10). LPS present in the outer leaflet of the bacterial cell membrane makes the outer membrane a highly asymmetric permeability barrier for the resistance of bacteria towards external molecules such as hydrophobic antibiotics, detergents and other membrane permeabilizing agents (11, 12).

Temporin L, a 13-residue cationic antimicrobial peptide which possesses wide spectrum of antimicrobial activities against gram negative and gram positive bacteria as well as appreciable immunomodulatory properties against LPS stimulated proinflammatory responses. Earlier work with Temporin L suggests that it is a self-aggregated antimicrobial peptide and the self aggregation behavior of the peptide is responsible for lesser solubility and relatively lesser bioactivities than expected (13). The N-terminal of Temporin L does not possess any cationic residue; by employing available algorithms, we found that a substitution of glutamine with lysine at third position of TempL could reduce its aggregation in aqueous environment. Antibacterial properties of Temporin L analog [{Arg^3} TL] with an arginine residue in place of glutamine at number three position has been reported recently (14). As compared to the native peptide, this TempL analog exhibited improved bactericidal activity against two Pseudomonas aeruginosa strains among the tested gram negative bacteria (14). However, to our knowledge there is no report on the anti-endotoxin property of [{Arg^3}TL]. In the present study we have incorporated a lysine residue in place of a glutamine residue at position three of TempL which ultimately added an extra charge into the parent molecule and investigated its subsequent effects on structural,
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functional and anti-endotoxin properties of the peptide. Biochemical and biophysical studies revealed the basis of augmentation in anti-endotoxin properties of TempL as a result of introduction of an additional lysine residue.

MATERIALS AND METHODS

Rink amide MBHA resin (loading capacity, 0.63 mmol/g) and all N-α-Fmoc- and side chain-protected amino acids from Novabiochem, Coupling reagents for peptide synthesis as 1-hydroxybenzotriazole, N,N’-diisopropylcarbodiimide (DIC), 1,1,3,3-tetramethyluronium tetrafluoroborate, and N,N-diisopropylethylamine from Sigma, while dichloromethane, N,N-dimethylformamide, and piperidine were of standard grades and procured from reputed local companies. Acetonitrile (HPLC grade) was procured from Merck, whereas trifluoroacetic acid (TFA) was purchased from Sigma. TAMRA was purchased from Invitrogen (Molecular Probes, Eugene, OR). E.coli 0111:B4 Lipopolysaccharide (L3012), FITC-LPS E.coli 0111:B4 (F3665) 8-Anilinonapthalene-1-Sulfonic Acid (ANS) (A1028) and, Polymixin B (P0972) were procured by Sigma. Rests of the reagents was of analytical grade and procured locally; buffers were prepared in milli Q ultra pure water (USF-ELGA).

Animals. All animal procedures were performed in accordance with the protocols approved by the Institutional Animal Ethics Committee (79/10/MSB/IAEC) and National Laboratory Animal Center (Lucknow). Animal were properly anaesthetized before experiments and all treatments were performed in accord to minimize sufferings to animals. Our animal protocols are adhered to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Govt. of India.
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Cell culture. Rat bone marrow derived macrophage cells were obtained by inducing differentiation in bone marrow cells isolated from the marrow of femur of healthy wistar rats aging approximately 3-4 weeks. The differentiation was induced by previously known methods using cell supernatant of L929 murine fibroblast cells (15, 16). The culture and processing of these differentiated cells were performed in Dulbecco modified eagle media (Sigma-5648-1L) containing 10% fetal bovine serum (EU-000-F, Sera Laboratories, West Sussex, United Kingdom) supplemented with GIBCO antibiotic antimycotic (Invitrogen 100× antibiotic-antimycotic-15240).

Peptide synthesis, fluorescent labeling and purification. The Stepwise solid phase syntheses of all the peptides was performed on rink amide MBHA resin (0.15 mmol) using standard Fmoc chemistry as reported previously (17). Labeling at the N-terminus of peptides with fluorescent probe, cleavage of the labeled and unlabeled peptides from the resin, and their HPLC purification were achieved by standard procedures (18, 19). Experimental molecular masses of the peptides were detected by ESI-MS analysis.

Structural parameter computation. Hydrophobic parameters such as mean hydrophobicity, hydrophobic moment and mean relative hydrophobic moments of peptides were calculated on Eisenberg Scale of hydrophobicity. In vitro aggregation and secondary structure prediction were performed by use of TANGO software (20). The peptide’s net hydrophobic mean character were calculated by the GRAVY scale (21).

Hemolytic activity of the peptides. Hemolytic activity of the peptides against human red blood cells (hRBCs) in PBS was examined to determine the cytotoxic activity of the peptides as reported earlier (19).
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**Assay for evaluation of LPS neutralizing activity.** Cultured rat bone marrow derived macrophages at 5x 10^5 cells/well in 24-well plate were stimulated with LPS O111:B4 (100 ng/ml) in the presence of 2.5, 5, or 10 μM TempL and its designed analogue Q3K,TempL. Cells stimulated with LPS alone and untreated cells served for maximum and minimum cytokines productions in given set of experiment. The cells were later incubated for 6 h at 37 °C in incubator. Afterwards, samples of the medium from each treatment were collected. Concentrations of TNF-α and IL-6 in the samples were evaluated using mouse enzyme-linked immunosorbent assay kits for TNF α (BD Biosciences cat.no.558534) and IL-6 (BD Biosciences cat.no.555240) according to manufacturers’ protocol and data were presented in terms of percentage inhibitions of LPS induced cytokine production in presence of these peptides.

**Trypan Blue cell exclusion assay.** After LPS and peptide treatments, the viability of macrophage cells was tested by trypan blue cell exclusion assay as previously described (22-24). Following the LPS and peptide treatments, the cells were washed with chilled PBS (pH 7.4) and later trypan blue (4mg/ml) solution was added to cells for 2 minutes. Trypan blue staining of cells from each treatment group was estimated by using hemocytometer under inverted microscope.

**Tryptophan Fluorescence experiments with LPS.** Increasing concentrations of LPS was added to a solution of TempL or its designed analogue (5μM final concentration) in Hepes Buffer (5 mM Hepes, 0.1 mM EDTA, pH 7.0) and fluorescence spectra were measured in a quartz cuvette with a Perkin-Elmer LS55B spectrometer with emission and excitation slits set at 8 and 6 nm. The tryptophan residue of Temporin L was excited at 280 nm, and emission spectra were recorded between 300 to 400 nm, with averaging two scans. Spectra were recorded as a function of the lipid/peptide molar ratio and corrected for the contribution of light scattering in the
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presence of lipid vesicles. Blue shifts were measured as the differences in wavelength of the maxima in emission spectra of lipid-peptide and peptide samples.

**Circular Dichroism (CD) Spectroscopy.** We performed CD experiments to study the propensity of TempL and its analog to self assemble in LPS and aqueous environments. For this purpose dose-dependent CD spectra of these peptides were recorded in the presence of LPS and in PBS (pH 7.4, 1.5M NaCl) (25) by employing a Jasco J-500A spectropolarimeter which was calibrated with (+)-10-camphorsulfonic acid. Spectra of the samples were recorded at 30°C in a capped, quartz optical cell with a path length of 0.2 cm in the wavelength range of 250-190 nm. Mean residue ellipticity values as expressed in terms of [θ] (degrees square centimeters per decimole) were determined as reported earlier (19, 26).

**LPS destabilization induced by the Peptides.** Collapse of the diffusion potential of LPS membrane due to disintegration of LPS core structure was detected fluorimetrically as described previously (27). The LPS large unilamellar vesicle (LUV) suspension (10mg/mL in 200μL) prepared in K buffer [50mM K₂SO₄ and 25 mM HEPES-sulfate (pH 6.8)] was added to an isotonic K-free buffer [50 mM Na₂SO₄ and 25 mM HEPES-sulfate (pH 6.8)] to a final concentration of 50 μg/mL LPS. Then the membrane sensitive probe di-S-C₃-5 followed by subsequent addition of valinomycin (0.1 μM) created a negative diffusion potential inside the vesicles. Peptide-induced membrane perturbation caused the membrane diffusion potential to dissipate, resulting in an increase in fluorescence. The fluorescence was monitored using excitation and emission wavelengths of 620 and 670 nm, bandwidth kept at 8 and 6nm respectively.
Detection of peptide induced dissociation of LPS aggregates. To study the effect of treatments of TempL or Q3K,TempL on the physical state of LPS, we assayed the peptide-induced dissociation of LPS by employing FITC-labeled LPS as reported earlier (25, 28). FITC-LPS (0.5 μg/ml) was treated with increasing concentrations of either TempL or its analog from 10 μM to 100 μM. The changes in the emission of FITC-LPS as a function of change in aggregation state of LPS in 10 mM sodium phosphate buffer,(pH, 6.9), were monitored at 515 nm using a fluorescence spectrometer (PerkinElmer LS55) with excitation wavelength set at 488 nm and excitation and emission slits of 8 and 6 nm, respectively. The fluorescence data of FITC-LPS at 515 nm were collected with or without peptide additions after background subtractions. Peptide-induced dissociation of the aggregated state of FITC-LPS was measured as an increase in its fluorescence (25, 29).

Binding of peptides to Endotoxin (LAL assay). The abilities of TempL and its designed analogues Q3K,TempL to bind the LPS was assessed using a quantitative chromogenic limulus amoebocyte lysate (LAL) with QCL-1000 (LONZA 50-647U) kit. Experiments were carried out following the protocols recommended by the manufacturer. Stock solutions of peptides were prepared in pyrogen-free water provided with the kit. Peptides at concentrations of 1.875, 3.75, 7.5 and 15μM were incubated with 0.5 endotoxin units (EU) of LPS in non-pyrogenic micro tubes at 37 °C for 30 min to allow the binding of peptides to LPS. A total of 50 μl of this mixture was then added to equal volume of LAL reagent (50 μl), and the mixture was further incubated for 10 min followed by the addition of 100 μl of LAL chromogenic substrate (Ac-Ile-Ala-Arg-p-nitroaniline). The reaction was terminated by the addition of 25% acetic acid, and the yellow color that developed due to cleavage of the substrate was measured spectrophotometrically at
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410nm. The reduction of absorbance at 410 nm as a function of peptide concentrations is directly proportional to the inhibition of LPS by the peptide (30, 31).

Oligomerization of TempL and Q3K, TempL associated with LPS by Tris-tricine SDS Gel electrophorisis. TempL and Q3K, TempL (~30 μg) were incubated with LPS at 37°C for 30 min. Peptide-LPS complexes were treated with freshly prepared solutions of bis-(sulfosuccinimidyl)-suberate (BS3; Pierce) at 37°C for 10 min. The reaction was quenched by addition of 1/3 volume of 10 mM MOPS buffer, pH 7.4, containing 50 mM NaCl and 50 mM lysine (32). Each reaction mixture was treated with polyacrylamide gel electrophoresis (PAGE) loading buffer containing 1% SDS and 5% 2-mercaptoethanol. The peptide-LPS conjugates were subjected to SDS-PAGE in 16.5% per cent Tris-Tricine acrylamide gels as reported earlier (33). An equal amount of the peptides without addition of LPS were also processed for Gel electrophoresis experiments similarly after treatment with BS3 and loading buffer to investigate the relative state of peptide oligomerization in absence of LPS. Images of coomassie blue stained gels were taken and analyzed with respect to apparent molecular weight marker.

ANS fluorescence measurements. To investigate the relative change in extent of oligomerization in TempL and Q3K, TempL with the addition of LPS in peptide solutions, we performed ANS binding assays with peptides pre-incubated with LPS. Aliquots of ANS (10 mM stock) were added to peptide-lipid complex pre-incubated for 30 min at 37°C. Fluorescence intensities were measured in emission ranging 400-600 using an excitation of 365 nm in PBS at room temperature. For control experiments the peptides at same concentrations were treated with ANS to observe relative difference in peak shift in peptide’s maxima when added with lipids. The background fluorescence of ANS in lipids and buffer were subtracted from the obtained spectra. (34).
Fluorescence anisotropy experiment with FITC-LPS. Fluorescence anisotropy measurements of FITC-LPS (1 μg/ml) in presence of TempL and Q3K,TempL were performed with a Perkin Elmer spectrofluorimeter using 10 mM sodium phosphate buffer pH 6.0. Excitation and emission wavelengths were set at 470 and 515 nm and both bandwidths were set at 10 nm. For each sample, fluorescence emission intensity data in parallel and perpendicular orientations with respect to the exciting beam were collected ten times each and then averaged. Anisotropy \( r \) was calculated as; \( r = (I - G I_{\perp})/(I + 2GI_{\perp}) \), where \( I \) and \( I_{\perp} \) are the parallel and perpendicular polarized intensities measured with the vertically polarized excitation light and \( G \) is the monochromator grating correction factor. (35). Final anisotropy values were determined after subtracting control anisotropy readings of increasing concentrations of each peptide from the corresponding FITC-LPS and peptide complex in the same solvent.

Detection of aggregation state of TempL and Q3K,TempL by recording the proteolytic cleavage of their rhodamine-labeled analogs. An increase in rhodamine fluorescence following the treatment of proteinase-k to a rhodamine-labeled peptide is implicated to the aggregated state of the peptide (36, 37). Thus by looking at the relative enhancement in fluorescence of rhodamine-labeled TempL and its analog following the proteinase-k treatment it is possible to have a qualitative understanding on the aggregation state of these peptides in aqueous environment. The changes in fluorescence of rhodamine-labeled TempL or Q3K,TempL (both at 0.25 μM) after addition of Proteinase-K (100 μg/ml) was measured with respect to time in PBS. The excitation and emission wavelengths were set at 550 and 570 nm respectively with excitation and emission slit widths at 8 and 6 nm respectively.

In vivo studies of anti-endotoxin property of the peptides. Female Balb/c were given a standard laboratory diet and water ad libitum and housed under controlled environmental conditions.
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conditions (National laboratory animal centre, central drug research institute, Lucknow). They were approximately 30 gms each at the start of experiments. All the mice were divided into experimental groups of 5 each for LPS or peptide or LPS and peptide administrations. To examine the efficacy of the peptides in neutralizing the LPS-induced inflammatory response in mice, the mice were first injected with 10 mg/kg *E. coli* derived 0111:B4 LPS, followed by (in ~5-7 minutes) the addition of respective peptide, TempL or its analog at different sites of intraperitoneal (i.p.) administrations. Similar to the peptide treatments, polymyxin B (1mg/kg) was administered to LPS treated mice to observe the inhibition of LPS induced pro-inflammatory responses in mice and employed as a positive control. The mice treated with only saline or TempL (1mg/kg) or Q3K,TempL (0.25mg/kg) were considered as experimental controls to monitor the basal pro-inflammatory cytokine-levels and peptide-induced proinflammatory responses in mice without LPS-stimulation. The animals under different kinds of treatments as mentioned above were kept under observation for 72 hrs. Survival of mice was monitored for 72 hrs after LPS or/and peptides administrations. For serum TNF-α and IL-6 measurement, mice were anesthetized with ether, and blood was collected either by tail vein or orbital sinus aseptically. Serum levels of TNF-α and IL-6 were determined by enzyme-linked immunosorbent assay kits as mentioned already. Endotoxin concentrations were measured by the commercially available Limulus amebocyte lysate test (QCL-1000 -LONZA 50-647U) kit. Plasma samples were diluted with sterile endotoxin-free water. The endotoxin content was determined as described by the manufacturer, and the concentrations of endotoxin were calculated by comparison with the standard curve.

**Calculation of therapeutic index.** The therapeutic index of TempL and its analog was calculated to analyze the efficiency of TempL and Q3K,TempL as described earlier (38).
estimated therapeutic potential for TempL and Q3K,TempL by calculating by the ratio of MHC_{50} (minimum concentration of peptide that induce 50% hemolysis in hRBCs) and MIC_{anti-endotoxin} (minimum peptide requirement (mg/Kg) to attenuate lethal cytokine response and maintain survival of LPS treated mice) related to the antibacterial and anti endotoxin efficacies of these peptides respectively.

**Statistical analysis.** Each experiment was repeated three times, and results were expressed as mean ± standard deviations. The data was analyzed statistically using computer software Prism 3.0. Statistical analysis was performed using analysis of variance with Newman–Keuls test. A value of P <0.05 was considered statistically significant, whereas value of P> 0.5 were insignificant.

**RESULTS AND DISCUSSION**

**Design and synthesis of novel analog of Temporin L.** The overall biological activity of TempL is governed greatly by the hydrophobic and minimally by the hydrophilic characters of this peptide. Moreover, the number of cationic residues in TempL is only two which is relatively lesser in comparison to other antimicrobial peptides of similar lengths (39, 40). A closer look into the sequence of TempL revealed that there is scope to increase the balance between cationic and hydrophobic residues. Further, as mentioned in the introduction that by employing related algorithms we found that a substitution of glutamine with lysine at third position of TempL could significantly reduce its aggregation propensity in aqueous environment. Therefore, we chose to replace the glutamine residue at this position TempL by a lysine residue. Further, lysine possesses very similar molecular mass, polar character and helical propensity as glutamine. The amino acid sequences, HPLC retention times, and helical wheels are shown in Fig. 1.
To characterize the physicochemical properties of Q3K,TempL and the parent peptide TempL, several structural parameters were calculated (Table-1) by using available methodologies. The higher values of $\mu$ and $\mu$-rel (calculated on the basis of Eisenberg scale) for Q3K,TempL (Table 1) as compared to TempL (0.44 and 0.54 with respect to 0.41 and 0.51) indicate its more helix forming tendency than the parent peptide which was verified by their relative helicities in 30% TFE (data not shown). The reduced mean hydrophobicity of Q3K,TempL (0.03) as compared to TempL (0.06) implies its enhanced polar characters than the parent peptide. Aliphatic index and GRAVY indicated towards equivalent amphipathy (112.31; Table-1) for both the peptides, and lesser hydrophobic character for Q3K,TempL (0.792) than TempL (0.823). Amylo values calculated by TANGO (20, 21) showed significantly lower value for Q3K,TempL (2796.92) with respect to TempL (4794.7) and indicated towards the lesser in vitro aggregation behavior of the TempL analog than its parent peptide (Table 1).

**Designed analog of TempL exhibited significantly higher in vitro LPS neutralization ability than the parent peptide.** TempL is known to inhibit LPS-induced proinflammatory responses in macrophage cells *in vitro* upto 50-60% at nontoxic concentrations (41). We studied TNF-$\alpha$ and IL-6, the two key cytokines associated with LPS-stimulated pro-inflammatory responses in rat macrophage cells and observed that the levels of these cytokines in cell culture supernatant media were 2-3 times lower in presence of Q3K,TempL as compared to that in the presence of TempL at all tested peptide concentrations (P<0.001) (Fig. 2 A & B). The viability of the macrophage cells after the experiment was checked by trypan blue cell exclusion assay (Fig. 2 C) which ruled out any significant cytotoxicity of the peptides onto the macrophage cells (P> 0.05).
Marginal difference between TempL and its analog in their cytotoxic activity. To determine the cytotoxic activity of the peptides, hemolytic activity of Temporin L and Q3K,TempL against human red blood cells (hRBCs) was measured. With gradual increase in peptide concentrations, TempL and its analog showed progressively increasing hemolytic activity against hRBCs. Q3K,TempL exhibited marginally higher hemolytic activity (Fig. 3) than TempL (P<0.001). Thus the results indicate that the substitution of glutamine by lysine to some extent enhances the cytotoxicity of TempL. The data add to the literature on the studies with substitution of amino acid(s) by lysine residue(s) in similar sized peptide, indolicidin (42, 43) and altogether suggest that probably the overall amino acid sequence of the parent peptide, the nature and position of the amino acid(s) that is/are being replaced by lysine residue(s) determine the effect of such amino acid substitution(s).

TempL and Q3K,TempL showed differences in tryptophan fluorescence in LPS vesicles. Both the peptides showed significant blue shifts of tryptophan emission maxima in presence of increasing concentrations of LPS vesicles. However, a significant difference between the tryptophan emission maximum of TempL and Q3K,TempL in the presence of LPS vesicles was observed (Fig. S1A). For example, at 10 and 20 µg/ml LPS concentrations TempL exhibited emission maximum of 356nm and 350nm whereas in the same condition Q3K,TempL showed emission maximum at 347nm and 341.5nm respectively. This higher blue shift of emission maximum of Q3K,TempL in LPS probably indicate that the tryptophan residue of Q3K,TempL was located towards the more hydrophobic environment of LPS as compared to that of its native peptide. The results further suggest that the substitution of the glutamine by lysine residue in TempL probably altered its mode of binding and/or conformation in LPS.
Fluorescence anisotropy experiment with FITC-LPS. The binding of TempL and its analog to LPS in solution was followed by recording the changes in fluorescence anisotropy of FITC-LPS in presence of these peptides (Fig. S2B). The formation of FITC-LPS/peptide complexes led to a significant increase in fluorescence anisotropy, indicating that the binding of TempL or Q3K,TempL to LPS caused mechanical restrictions to the rotational mobility of FITC-LPS. However, the higher increase in anisotropy of FITC-LPS when it was bound to Q3K,TempL could be attributed to its stronger binding to the TempL-analog than TempL resulting in its more restricted movement.

Q3K,TempL exhibited higher self-assembly in LPS environment than TempL. Ellipticity values for both the peptides increased in a dose-dependent manner in LPS environment but Q3K,TempL exhibited appreciably higher ellipticity values than TempL (Fig. 4 A). The data indicate that Q3K,TempL possesses higher tendency to self-assemble in LPS than its parent peptide. In this experiment, we noticed that at higher peptide concentration the ellipticity values for both the peptides reduced which indicate that after certain concentration, both TempL and Q3K,TempL probably undergo a process to form insoluble aggregates, and this tendency was more prominent for TempL than Q3K,TempL.

Q3K,TempL exhibited higher self assembly in presence of LPS than TempL. Fluorescence yield of ANS increases significantly upon its transfer from the aqueous environment to hydrophobic environment as well as its emission maximum shifts towards shorter wavelength (44). Since self-association of a peptide often involves with the rearrangement of its hydrophobic residues that creates well defined hydrophobic patches, ANS is a suitable probe to detect the aggregation property of a peptide. We observed that in presence of LPS, Q3K,TempL induced a marked increase in fluorescence intensity of ANS concomitant with the blue shift of its emission.
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maximum (Fig. 4 B). However, in the same experiment, TempL did not induce any significant enhancement of ANS fluorescence or blue shift of its emission maximum. The results indicate that Q3K, TempL possesses better self-association property in LPS as compared to its parent peptide.

Q3K, TempL forms higher oligomeric states in presence of LPS than TempL.

Oligomeric states of the peptides in the presence and absence of LPS were investigated by using bis-sulfosuccinimidyl-suberate (BS3) cross-linker with the help of SDS- Polyacrylamide gel electrophoresis experiments. The experiments revealed that Q3K, TempL formed higher oligomeric states ranging from dimer to octamer in LPS (Fig. 4 C), whereas TempL oligomers were mostly limited only unto dimeric forms. In control experiments, TempL and Q3K, TempL were treated with BS3 without prior incubations with LPS to investigate the intrinsic oligomeric states of these peptides in aqueous phase stabilized by the cross linker. We observed lower oligomeric states for both the peptides in comparison to that in the presence of LPS. The data indicate that in presence of LPS, TempL and Q3K, TempL both undergo into self-assembly/oligomerization although Q3K, TempL exhibits higher oligomeric state.

Q3K, TempL induced greater permeabilization in LPS vesicles than TempL. It was examined whether TempL and its analog can disintegrate the core structure of LPS membrane. For this purpose, dissipation of diffusion potential across the LPS bilayer membrane in the presence of TempL and Q3K, TempL was measured (Fig. 5A & B). Q3K, TempL showed significantly higher permeabilization in LPS vesicles as compared to that of TempL. The results may further be implicated towards the stronger ability of Q3K, TempL to destabilize the organization of bilayered structure of LPS vesicles.
Q3K,TempL dissociates LPS aggregates with stronger efficacy than TempL. Physical state of LPS seems to play a key role in determining the LPS-stimulated pro-inflammatory responses, and a direct relation is found between most of the LPS neutralizing peptides with their respective ability to dissociate the aggregated state of LPS (45-48). Q3K,TempL induced the dequenching of FITC-LPS fluorescence more prominently than TempL (Fig. 5C) which suggest that the designed TempL-analog can induce higher disintegration of LPS oligomers than the native peptide. Yet, TempL-induced dissociation up to some extent can be correlated with its relatively lesser LPS neutralizing abilities.

Q3K,TempL possesses higher LPS-neutralization ability than TempL. The effect of introduction of a lysine residue towards the N-terminal of TempL on its binding to LPS was examined by performing the chromogenic Limulus amebocyte lysate (LAL) assay with TempL and Q3K,TempL by employing an endotoxin detection kit (Fig. 6). Binding of the peptides to LPS was determined by measuring their efficacy to inhibit the LPS-induced activation of LAL enzyme. TempL and its designed analog, Q3K,TempL, showed significant binding to LPS as evidenced by the substantial inhibition of activation of LAL enzyme (P<0.001). However, Q3K,TempL showed a considerably higher inhibition of LAL enzyme than TempL at all employed concentrations (P<0.001); for example at 7.5µM, TempL showed only 17% inhibition of LAL enzyme while Q3K,TempL exhibited 92% inhibition of LAL enzyme as a consequence of higher LPS binding efficiency (Fig. 6).

Q3K,TempL inhibited pro-inflammatory mediators in more pronounced manner and promoted the survival of LPS-treated Balb/c-mice at lower concentration than TempL. The inhibitory effects of TempL on LPS stimulated production of TNF-α and IL6 in macrophage cells have been examined earlier (41) and the implication of these two cytokines in endotoxemia
is well documented (49, 50). Therefore, we chose these two cytokines as markers of LPS-induced proinflammatory response either in macrophage cells or in balb/c mice. A significant decline in production of pro-inflammatory cytokines, TNF-α and IL-6, in LPS-stimulated primary rat macrophage cells in presence of TempL and its analog was observed (Fig. 2.

Further, we investigated the changes in serum levels of these pro-inflammatory cytokines over time after LPS administration (10mg/kg i.p.) in Balb/C mice. As shown in Fig. 7 A & B serum TNF-α and IL-6 levels, which were negligible in untreated mice group, rose significantly at higher levels after administration of LPS (P<0.001). The group of LPS (10mg/kg)-treated mice when further treated with TempL (1mg/kg) or Q3K,TempL (0.25mg/kg) demonstrated appreciable inhibition of the cytokine levels in serum (P<0.001), so as obtained with polymixin B treatment to mice, administered with LPS (P<0.001) which served as a positive control for the inhibitory activity against the LPS-induced pro-inflammatory responses (Fig. 7A & B).

However, when the mice group administered with LPS was treated with TempL at a lower dose (0.25mg/kg), only a partial inhibition towards LPS-induced pro-inflammatory response was observed as evidenced by the serum level of IL-6 and TNF-α. Detailed statistical analyses of the same data are provided in Fig S3.

Further, LPS treated mice group showed nearly 10-fold higher endotoxin levels in blood serum which was successfully attenuated by Q3K,TempL (0.25mg/kg) and TempL (1mg/kg) (P<0.001). Though TempL (0.25mg/kg) inhibited such rise in endotoxin levels in mice blood but it is not comparable with the attenuation obtained with Q3K,TempL at similar concentration (Fig. 7C) (P<0.001). This result also verifies that Q3K,TempL is more potent than its parent peptide in neutralizing LPS-induced pro-inflammatory response in vivo in mice. Our control experimental mice groups such as when the mice were administered with LPS and subsequently
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We obtained a significant mortality in LPS treated mice than untreated group of mice (P<0.001). However, the mice groups first treated with LPS and then administered with either TempL (1mg/Kg) or Q3K,TempL (0.25mg/Kg) showed resistance to lethal effects of LPS (P<0.001). The higher LPS neutralization by Q3K,TempL was also evident from the absolute survival of LPS-treated mice when administered with Q3K,TempL (0.25mg/Kg) as compared to the LPS-treated mice, administered with the same dose of TempL (0.25mg/Kg) (Table. S1), which failed to survive against LPS-toxicity. Polymyxin B showed prominent attenuation of LPS toxicity in mice, and showed absolute survival of LPS-treated mice (P<0.001). Control group of mice, treated with TempL or Q3K,TempL only in absence of LPS also showed no sign of inflammatory stress, and did not induce mortality in treated mice (P<0.001 than LPS treated mice) (Table. S1). Probably, the data further indicate that at the levels used the peptides were not per se toxic to the mouse.

Therapeutic potential of the peptides was determined with respect to their in vivo anti-LPS activity (Table S1) in mice. It is evident that introduction of the lysine residue in place of the glutamine residue at the third position appreciably enhances the therapeutic potential of Temporin L with respect to the neutralization of LPS-induced inflammatory response in mice (Table S2).

The introduced lysine residue at the N terminus of TempL probably reduced the propensity and the extents of amyloid like fibril formation by TempL in aqueous solution as also
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predicted on the basis of amylo value in Table-1. In our preliminary experiments, Thioflavin T showed appreciably strong fluorescence with TempL while it showed significantly weak fluorescence in presence of Q3K,TempL (unpublished results). Introduction of the lysine residue significantly reduced the aggregation state of TempL in aqueous environment as evidenced by recording the fluorescence of Rho-labeled peptides after proteolytic cleavage (Fig. S2). To investigate the effect of glutamine to lysine substitution on the bactericidal properties of TempL, the antibacterial assays with the peptides were performed against two gram negative bacteria, namely, *E. coli* (ATCC 10536) and *Pseudomonas auregenosa* (ATCC BAA-427) (Table S3) We observed nearly 2-fold increments in the bactericidal activity of Q3K,TempL against these gram negative bacteria in comparison to TempL indicating that the substitution of glutamine by lysine at position three appreciably enhanced the bactericidal activity of Temporin L (Table S3). The TempL analog with substitution of arginine at the same position also showed improved bactericidal activity against two *Pseudomonas aeruginosa* strains among the tested gram negative bacteria (14).

Taken together, in course of investigating the structure-function relationship of a naturally occurring antimicrobial peptide, TempL, we observed a correlation between the anti-endotoxin properties (Fig. 2 & 7) of TempL with its aggregation in LPS (Fig. 4). The present study demonstrates how the introduction of a lysine residue towards the N-terminus of aggregation prone, TempL, can reduce its inherent propensity to aggregate in aqueous environment and augment its oligomerization in LPS. The enhanced oligomeric state of Q3K,TempL probably led to its better binding to LPS and disintegration of LPS aggregates resulting in augmented neutralization of LPS-induced pro-inflammatory response in rat
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ABBREVIATIONS

PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate; Fmoc, N-(9-fluorenyl) methoxycarbonyl; DIC, differential interference contrast; RP-HPLC, Reversed phase high-performance liquid chromatography; ESI-MS, electro spray ionization- mass spectrometry; LPS, Lipopolysaccharides; LAL, Limulus amebocyte lysate ; ANS, 8-Anilinonaphthalene-1-Sulfonic Acid; DiS-C3-5, 3,3’-dipropylthiadicarbo cyanine iodide; TNF-α, tumor necrosis factor alpha ; Trp, tryptophan.
REFERENCES


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### Table 1: Physicochemical parameters of TempL and designed analogs

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Table 1: Physicochemical parameters of TempL and designed analogs.
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Figures:

**FIG 1**

**FIG 1** Sequences, molecular weights, helical wheel projections and physical properties of Temporin L (TempL) and its analog. Panel A, Amino acid sequences of the peptides, their calculated & ESI-MS obtained molecular weights and RP-HPLC retention times for TempL and Q3K,TempL in water/acetonitrile gradient system (80% to 20% in 40 min at 0.7ml/min flow rate) Panel B, Helical wheel projections of TempL and Q3K,TempL.
FIG 2 Effect of treatments of TempL and Q3K,TempL on production of pro-inflammatory cytokines by rat bone marrow derived macrophages stimulated with LPS (100ng/ml). Panel A represents the percentage inhibition of LPS induced TNF-α production in macrophages by TempL (□) and Q3K,TempL (■) at concentrations of 2.5 μM, 5 μM and 10 μM. Panel B represents the percentage inhibition of LPS induced IL-6 production in macrophages by TempL (□) and Q3K,TempL (■) at concentrations of 2.5 μM, 5 μM and 10 μM. Panel C depicts the data of percentage viability of macrophage cells after the above experiments at
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different peptide concentrations namely 2.5 μM, 5 μM and 10 μM as indicated in the Fig. In order to calculate the percentage of inhibition of cytokine productions in LPS stimulated macrophages by TempL and its analog, the amounts of cytokines produced in LPS untreated and LPS treated cells (without additions of peptides) were taken as control values for minimum and maximum cytokine productions for a given experimental set. Results are presented as mean ± SD, n=3, P values are indicated as *** P < 0.001, ** P < 0.01, * P < 0.05, and # P > 0.05 vs TempL. For Panel C, Results are presented as mean ± SD, n=3, # indicates P >0.05 with respect to TempL whereas (a) P<0.001, (b) <0.01, (c) <0.05 and (d) >0.05 vs untreated cells.
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**FIG 3**

Dose dependent hemolytic activity of TempL and Q3K,TempL against 6% human RBCs. Symbols , ( ) for TempL and ( ) for Q3K,TempL as marked in Fig. also. The plotted data expresses the Results are presented as mean ± SD, n=3, P values are indicated as *** P < 0.001, vs TempL, and (a) P<0.001 (b) P<0.01 vs absolute hemolysis (Triton treated hRBC).
Determination of oligomerization/self aggregation of TempL and Q3K,TempL in LPS environment, Panel A shows the molar ellipticity values for TempL (——) and Q3K,TempL (---) in LPS (12.5µM) environment; peptide concentrations were progressively increased keeping LPS concentration (12.5µM) constant in phosphate buffer saline, Panel B, emission spectra of ANS (50µM) in presence of TempL and Q3K,TempL, —— and —— represent the emission spectra of TempL and Q3K,TempL respectively without ANS treatments, —— and —— represent the
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represent the ANS emission spectra in the presence of TempL and Q3K,TempL respectively in aqueous environment while — and — show the ANS emission spectra in the presence of TempL and Q3K,TempL respectively in LPS environment (50µg/ml). Concentration of either TempL or Q3K,TempL was 10 µM in all these experiments. Panel C denotes the state of oligomerization of TempL and Q3K,TempL in the absence and presence of LPS as observed by Tris-Tricine gel experiment, Lane 1 corresponds to molecular weight marker (from higher to lower 26.6, 17, 14.2, 6.5 and 3.496 Kda values respectively), Lane 2 and Lane 3 stand for self oligomeric states of TempL (30µg) and Q3K,TempL (30µg) respectively, stabilized with BS3 in aqueous environment (PBS) as marked while Lanes 4 & 5 stand for oligomeric states of TempL and Q3K,TempL (30µg) respective in the presence of LPS (10ug), stabilized by BS3 treatment.
FIG 5

Panel A, Dissipation of diffusion potential of LPS vesicles in the presence of TempL and its analog. Panel A shows the plot of percentage fluorescence recovery induced by increasing concentrations of TempL (—) and Q3K,TempL (—). Panel B depicts representative fluorescence profiles for dissipation of diffusion potential induced by TempL and Q3K,TempL in LPS vesicles. Open symbols represent TempL and closed symbols represent Q3K,TempL. Symbols: peptide concentrations of 2.5μM, square; 5μM, circle and 10μM, triangle respectively. Panel C; Increase in fluorescence (arbitrary units) of FITC-LPS as a result of peptide-induced dissociation of its aggregates has been plotted with respect to concentrations of the peptides.
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FITC-LPS (0.5µg/ml) was treated with varying concentrations of peptides to detect the dissociation of LPS aggregates as evident by dequenching of its fluorescence. The collected fluorescence data with a particular peptide (upper half of Panel C, TempL and lower half of Panel C, Q3K,TempL) are shown in the y-axis of each plot.
Binding of TempL and Q3K,TempL to LPS was determined by quantitative chromogenic limulus amoebocyte lysate (LAL) assay. Efficiency of TempL and Q3K,TempL to bind to LPS was analyzed by estimating their ability to inhibit the LPS mediated activation of LAL enzyme. Inhibition in substrate color production as a function of inhibition of LAL enzyme’s activity was estimated and the percentage LPS binding was calculated. Binding of TempL (□) and Q3K,TempL (■) to LPS is denoted by percentage inhibition in color production at various peptide concentrations namely 1.875, 3.75, 7.5 and 15.0 μM as marked. Results are presented as mean ± SD, n=3, P values are indicated as *** P < 0.001, vs TempL.
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FIG 7

Alterations in the level of pro-inflammatory cytokines with time in the serum of LPS-treated (LPS 0111:B4, 10mg/kg) Balb/C mice in the absence and presence of TempL and Q3K,TempL (as described in the Materials and Methods section). Panel A shows the TNF-α levels in blood serum of LPS and peptide treated mice at different time intervals. Panel B shows the IL-6 concentrations in blood serum of LPS and peptide treated mice at different time intervals. Panel C, Endotoxin concentrations in blood serum of LPS and peptide treated mice after 6 hours of peptide or LPS administrations. Results are presented as mean ± SD, n=3, P
values are indicated as *** P < 0.001 and # P > 0.05 vs TempL. (a) P<0.001, (b) <0.01, (c) <0.05 and (d) 0.05 vs untreated mice while (1) P<0.001 vs LPS treated mice. Symbols for Panel A & B; ➥, mice, not-treated with peptide or LPS; ➤, treated with only LPS(10mg/kg); ➥, TempL (1mg/kg) and LPS; ➦, Q3K,TempL (0.25mg/kg) and LPS; ➣, TempL (0.25mg/kg) and LPS; ➜, Polymixin B (1mg/kg) and LPS; ➤, TempL (1mg/kg) with no LPS and •, Q3K,TempL (0.25mg/kg) with no LPS. In Panel C, + or − symbolize the treatment or no treatment of LPS whereas T1 shows TempL (1mg/kg), Q shows Q3K,TempL (0.25mg/kg), T2 shows TempL (0.25mg/kg) and P shows Polymixin B (1mg/kg) peptide treatments.