# Chapter 3: Materials and Methods

## Materials:

Rink amide MBHA resin (loading capacity: 0.4-0.8 mmol/g) and all the N-α Fmoc and necessary side-chain protected amino acids were purchased from Novabiochem, Switzerland. Coupling reagents for peptide synthesis like 1-hydroxybenzotriazole (HOBT), di-isopropylcarbodiimide (DIC), 1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and N, N'-diisopropylethylamine (DIPEA) were purchased from Sigma, USA. Dichloromethane, N, N' dimethylformamide (DMF) and piperidine were of standard grades and procured from reputed local companies. Acetonitrile (HPLC grade) was procured from Merck, India. Trifluoroacetic acid (TFA), trifluoroethanol (TFE), N-[2-hydroxymethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), sodium dodecyl sulfate (SDS), calcein, FITC-dextrans, FITC-annexinV, valinomycin, Sephadex G-50, dimethyl sulfoxide (DMSO) and cholesterol (Chol) were purchased from Sigma. Egg phosphatidylcholine (PC) and egg phosphatidylglycerol (PG) were obtained from Northern Lipids Inc., Canada while 3,3'-dipropylthiadicarbocyanine iodide (diS-$C_3$-5), NBD-fluoride (4-fluoro-7-nitrobenz-2-oxa-1, 3-diazole) and tetramethylrhodamine succinimidyl ester were procured from Invitrogen, India.

E.coli 0111:B4 Lipopolysaccharide (L3012), FITC-LPS E.coli 0111:B4 (F3665), Sodium Nitroprusside (228710) from sigma, while P-phenylenediamine (151830) and paraformaldehyde (150146) were procured from MP biomedicals. Components of griess reagents viz sulfanilamide (S92510) and p-naphthylethylenediamine dihydrochloride (N 9125) were purchased from sigma. Rests of the reagents were of analytical grade and procured locally; buffers were prepared in milli Q (USF-ELGA) water.

***Antibodies***- For protein expression study by immunoblotting we have used mouse anti iNOS2 (610328 BD transduction), rabbit anti TNF-α polyclonal (654300 calbiochem USA), IL-1β rabbit polyclonal (sc-7884), COX-2 mouse monoclonal (sc-19999), Mouse anti β-actin monoclonal (CP01) was procured from calbiochem. For immunoblotting and ELISA experiments alkaline phosphatase conjugated anti-mouse (401212), anti rabbit (401352) and peroxidase conjugated anti-mouse (401215), anti-rabbit (401315) were taken from calbiochem USA. Fluorescent labeled antibodies anti mouse Alexa-fluor 488 (A11001) and anti rabbit alexa-fluoro 488 (A11008) were purchased from molecular probes, Invitrogen corporations.

Raw 264.7, murine macrophage like cell line was obtained from National Center for Cell Sciences, Pune, India. For Cell culture Iscove's Modified Dulbecco's Media IMDM (MED 216) and Fetal Bovine Serum (EU-000-F) were purchased from sera laboratories west sussex UK. The GIBCO 100X Antibiotic–antimycotic (15240) was purchased from invitrogen corporations. Sterile polystyrene tissue culture flasks (690175), 96 well plates (655180), 24 well plates (662-160) were procured from Greiner Bio-one, while 6 well plates (3506) were from Corning incorporated costar. Trypsin EDTA (GIBCO-25200) was purchased from invitrogen corporations, Canada. The cell line was maintained in Innova CO2 incubator.

## Methods:

***Peptide Synthesis, their Fluorescent labeling and Purification***

All the peptides were synthesized manually utilizing solid phase method on rink amide MBHA resin using Fmoc chemistry (157-159). The functional groups of the amino acid derivatives used in the peptide synthesis are orthogonally protected. Their α-Amino groups are protected with base labile Fmoc whereas their side chains are either free (A, F, G, I, L, M, P and V) or protected with acid labile Trt (N, C, Q, H), OtBu (D and E), Boc (K and W), tBu (S, T and Y) and Pbf/Pmc (R). The carboxyl groups remain unprotected. Initially, 0.15 mM of resin was taken in a sintered funnel having three outlets. For loading the first amino acid from the C-terminal of the peptide sequence to the resin, the Fmoc protecting group of the resin was removed by treating it with 30% piperidine in DMF and deprotection was confirmed by positive Kaiser test(160), which gives blue color on reacting with free α-amino group. Before coupling, the resin was washed several times to remove all the piperidine. Afterwards, the Fmoc protected first amino acid (three fold of the loading capacity of the resin), HOBT and TBTU dissolved in DMF were added into the sintered funnel containing resin and then desired amount of DIPEA was added to it (*in situ* activation). Coupling reaction was checked after four hours by negative Kaiser test, which gives yellow color due to absence of free amino group. Further coupling reaction was carried out by employing DIC/HOBT or TBTU/HOBT/DIPEA reagents to confirm the attachment of the first amino acid to the resin. Subsequently, rests of the amino acids in the peptide sequence from C- to N-terminal were coupled to the existing chain one after another. In case of incomplete deprotection or coupling reaction the process was repeated. Chloranil test was performed for checking the deprotection of imino acids like proline which gives blue color at room

temperature with free imino groups and yellow with bound imino groups(161). After the synthesis was over, each peptide was cleaved from the resin with simultaneous de-protection of side chains by treatment with a mixture of TFA/phenol/thioanisole/1, 2-ethanedithiol/water (82.5:5:5:2.5:5 v/v) for 6-7 hours.

***Labeling of the resin bound peptides with fluorescent molecules***

For labeling of the peptides with fluorescent molecules, 20-30 mg of resin bound peptide was treated with 30% piperidine in DMF to remove the Fmoc group from the N-terminal amino acid. Then the resin was washed with DMF and dried with ether. Then Fmoc de-protected resin-bound peptides were incubated with vigorous shaking to tetramethylrhodamine succinimidyl ester (2-3 equiv.) in dimethylformamide in the presence of 5% diisopropylethylamine for 48-72 hrs, which ultimately resulted in the formation of $N^{\alpha}$-Rho-peptides(162, 163). Similarly, Fmoc de-protected resin-bound peptides were treated with NBD-fluoride (2-3 equiv.) to obtain $N^{\alpha}$-NBD-peptides. After sufficient labeling, the resins were washed with DMF and DCM in order to remove the un-reacted probe. The peptides were cleaved from the resin as above and precipitated with dry ether.

***Purification of the labeled and unlabeled peptides with RP-HPLC***

All the peptides were purified by RP-HPLC on an analytical Waters/Vydac C18 column using a linear gradient of 0-80% acetonitrile in 45 min with a flow rate of 1 ml/min. Both acetonitrile and water contained 0.05% TFA. All the purified peptides were ~95% homogeneous in their HPLC profile. Solvent was removed by freeze drying and each peptide was subjected to ESI-MS or MALDI-TOF analysis for the detection of their molecular masses.

***Assay of hemolytic activity of the peptides***

Hemolytic activity of the peptides against human red blood cells in PBS was performed to check the ability of the peptides to lyse the hRBCs(22, 31). Briefly, fresh human red blood cells (hRBCs) that were collected in the presence of an anti-coagulant from a healthy volunteer were washed three times in PBS. Freshly dissolved peptides in water at desired concentrations were added to the suspension of red blood cells (final density ~$5 \times 10^8$ cells/ml, counted with the help of a LEICA DM 5000 Microscope) in PBS to the final volume of 200 µl and incubated at 37 $^0$C for 35 minutes. The samples

were then centrifuged for 10 min at 2000 r.p.m. and the release of hemoglobin was monitored by measuring the absorbance ($A_{sample}$) of the supernatant at 540 nm. For negative and positive controls hRBC in PBS ($A_{blank}$) and in 0.2% (final concentration v/v) Triton X-100 ($A_{triton}$) were used respectively. The percentage of hemolysis was calculated according to the following equation.

$$\text{Percentage of hemolysis} = [(A_{sample}-A_{blank})/(A_{triton}-A_{blank})] \times 100$$

***Antibacterial activity assay of the peptides***

This bioassay with all the peptides was done in 96-well microtiter plates against different Gram-positive bacteria and Gram-negative bacteria (25, 31). In brief, bacteria were grown at 37°C with shaking at 180 rpm in appropriate growth medium in aerobic condition to the mid-log phase as determined by the optical density at 600 nm, which was 0.4 to 0.5, subsequently diluted in same media, then 50 µl bacterial culture (~$10^6$ cfu /ml) of diluted were added to 50 µl of water containing two fold serially diluted different peptides in each well and incubated for 18-20 h at 37°C. The peptides' antibacterial activities, expressed as their MICs (the peptide concentration which results 100% inhibition of microbial growth), were assessed by measuring the absorbance at 600 nm.

***Antifungal activity assay*:**

The in vitro antifungal activity of these designed peptides was evaluated against C. albicans, C. neoformans, S. schenckii, T. mentagrophytes, A. fumigatus, and C. parapsilosis (ATCC 22019). In this methodology, the minimum inhibitory concentration (MIC) of the peptides was determined according to the standard microbroth dilution technique as per NCCLS guidelines (164). Briefly, testing was performed in flat-bottomed 96-well tissue culture plates in RPMI 1640 medium buffered with MOPS (3-[N morpholino]propanesulfonic acid) for fungal strains. The concentration range of test compounds was 50–0.36 and 32– 0.0018 lg/mL for standard compounds. Initial inocula of fungal were maintained at 1–5 x $10^3$cells/mL. These plates were incubated in a moist chamber at $35^0$C, and an absorbance at 492 nm was recorded on a VersaMax microplate reader after 48 h for C. albicans and C. parapsilosis, 72 h for A. fumigatus, S. schenckii, and C. neoformans, and 96 h for T. Mentagrophytes. The MICs were determined as 90% inhibition of growth with respect to the growth control as observed using SOFTmax Pro 4.3 Software.

***Cell Cultures***

Mammalian cell lines murine 3T3 (mouse fibroblast cells) and macrophage RAW 264.7 were grown in DMEM/IMDM supplemented with 10% fetal calf serum and antibiotics at 37 °C in a humidified atmosphere at 5% $CO_2$ and 95% air. Cells were counted with the help of a LEICA DM 5000 Microscope for the experiments.

***Cell viability assay***

Viability of the cells were determined to check the toxic activity of peptides against murine 3T3 and macrophage cells by a standard procedure as described earlier (165). Cells (10000 per well) were seeded in 96 well plates and overnight incubation was done in $CO_2$ incubator for adherence. Then complete media (DMEM supplemented with 10% fetal calf serum) were discarded from the plate and incomplete media (DMEM minus fetal calf serum) were added followed by addition of different concentration of peptides. Plates were incubated for 2 hrs. in standard condition, now 10 µl of MTT solution (conc. 5mg/ml) were added in each well and further incubated for 3 hrs. Incomplete media were discarded from 96 well plates and 200 µl of DMSO were added in each well to dissolve the crystals. The wells in which no peptide was added served as the control and exhibited 100% viability. Readings of these samples were taken at 550 nm by ELISA reader (165). The viability of the peptide-treated cells was calculated with respect to the control cells of 100% viability.

***Preparation of Small Unilamellar Vesicles (SUVs)***

SUVs were prepared by a standard procedure (166, 167) with required amounts of either of the PC/cholesterol (8:1 w/w) or PE/PG (7:3 w/w) or PC/PG (3:1 w/w) as follows. Dry lipids of required amounts were dissolved in $CHCl_3$/MeOH (2:1 v/v) in small glass vials. Solvents were evaporated under a stream of nitrogen, which resulted in the formation of a thin film on the wall of glass vial. The glass vials containing lipid films were further kept under vacuum for ~ 24 hours in order to remove trace amount of organic solvents. The thin film was then re-suspended in buffer at a concentration of 8.2 mg/ml by vortex mixing. The lipid dispersions were then sonicated in a bath-type sonicator (Laboratory Supplies Company, New York) for 10-20 min until it became transparent. The lipid concentration was determined by phosphorus estimation(168).

*Osmotic protection assay*

To determine the diameter of the pores formed by BMAP-28 and its analogue on hRBCs, hemolytic activity of these peptides were examined in the presence of osmotic protectors as described previously (169-172) with 3% hRBCs (final concentration, in v/v). Raffinose, Poly (ethylene glycol) (PEG) 1500, PEG 2000, PEG 3350, PEG 4000 and PEG 6000 of diameters 1.3, 2.2, 2.5, 3.4, 4 and 4.9 nm respectively were used in this experiment as osmotic protectors. Human erythrocytes, prepared in the same way as in hemolytic activity experiment were suspended in PBS and incubated with each of the osmotic protectors at 30 mM concentration at 37°C. After 30 min of incubation of the osmotic protector-treated erythrocytes, ~45 μM peptides were added. Peptide-induced hemolysis was determined after another incubation of 50 min at 37°C by recording the absorbances of the supernatants at 540 nm. When the size of the osmotic protector matched with the size of pores formed by peptide onto the hRBC, inhibition of the hemolytic activity of the peptide was observed. Thus, by observing inhibition of hemolysis in the presence of osmotic protectors, diameter of the pore formed by peptides onto the hRBC was estimated.

*Circular dichroism (CD) studies*

The circular dichroism (CD) spectra of the peptides were recorded on Jasco J-710 spectropolarimeter in phosphate buffered saline (PBS, pH 7.4), zwitterionic PC/Chol (8:1, w/w) and negatively charged PE/PG (7:3 w/w) or PC/PG (3:1 w/w) lipid vesicles. The spectropolarimeter was calibrated routinely with 10-camphor sulphonic acid. The samples were scanned at room temperature (~$30^0$C) with the help of a capped quartz cuvettes of 0.2 cm path length at a wavelength range of 250-190 nm. An average of 4-6 scans was taken for each sample with a scan speed of 20 nm/min and data interval of 0.5 nm. The fractional helicities were calculated by the following formula (173, 174).

$$F_h = ([\theta]_{222} - [\theta]^0_{222}) / ([\theta]^{100}_{222} - [\theta]^0_{222})$$

Where $[\theta]_{222}$ was the experimentally observed mean residue ellipticity at 222 nm. The values for $[\theta]^{100}_{222}$ and $[\theta]^0_{222}$ that correspond to 100 and 0% helix contents were considered to have mean residue ellipticity values of –32,000 and –2,000 respectively at 222 nm (174).

***Detection of peptide-induced membrane damage of hRBCs and bacterial cells***

Peptide-induced phospholipid asymmetry or damage of phospholipid membrane organization of hRBCs was determined by staining the cells (~3.0 × $10^7$ cells /ml) with FITC-annexinV (175, 176) after the treatment with the peptides at room temparature for 5 min. Extent of staining was measured by analyzing peptide treated cells with respect to peptide untreated control using Becton Dickinson FACSCalibur flow cytometer and CellQuest Pro software.

In order to check the peptide induced damage to membrane integrity of *E. coli* ATCC10536 and *S. aureus* ATCC 9144, the cells at mid-log phase were incubated with peptides for 30 min at 37°C with constant shaking. The cells were centrifuged, washed two times with PBS, and incubated further with propidium iodide at 4°C for 30 min, followed by removal of the unbound dye through washing with an excess of PBS and re-suspended in buffer. Peptide-induced damage of bacterial cells was then analyzed by flow cytometer as mentioned above.

**Fluorescence spectroscopic experiments:**

All fluorescence spectroscopic studies were done utilizing Perkin-Elmer LS55B spectrofluorimeter

***Assay of peptide-induced depolarization of hRBC and bacterial membrane***

Peptide-induced depolarization of the hRBC and bacterial membrane was detected by its efficacy to dissipate the potential across these cell membranes (23-25, 177). Fresh human red blood cells were collected in the presence of an anti-coagulant from a healthy volunteer and washed three times in PBS and re-suspended in the same buffer with a final cell volume of 0.6% in v/v. In case of bacteria, culture were grown at 37°C until it reached to its mid-log phase and centrifuged followed by washing with buffer (20 mM glucose, 5 mM HEPES pH 7.3). Then bacteria were re-suspended (final ~2 × $10^5$ CFU/ml) in the similar buffer containing 0.1M KCl). Both hRBCs and bacteria were incubated with diS-$C_3$-5 probe for 1hr. When the fluorescence level (excitation and emission wavelengths set at 622 and 670 nm respectively) of the hRBCs or bacterial suspension became stable, different amounts of each of the peptides were added to these suspensions in order to record the peptide-induced membrane depolarization of either hRBCs or bacterial membrane. Membrane depolarization as measured by the fluorescence recovery ($F_t$) was defined by the equation (23, 177)

$$F_t = [(I_t - I_0)/(I_f - I_0)] \times 100\ \%.$$

Where $I_f$, the total fluorescence, was the fluorescence levels of cell suspensions just after addition of diS-$C_3$-5; $I_t$ was the observed fluorescence after the addition of a peptide at a particular concentration either to hRBCs or to bacterial suspensions, which were already incubated with diS-$C_3$-5 probe for 1hr and $I_0$, was the steady-state fluorescence level of the cell suspensions after one hr incubation with the probe.

***Assay of peptide induced dissipation of diffusion potential***

Peptides induced disturbance of membrane bilayer was measured by their ability to dissipate the diffusion potential across the membrane in presence of zwitterionic PC/Chol (8:1, w/w) and negatively charged PC/PG (7:3 w/w) lipid vesicles. Small unilamellar vesicles were prepared in $K^+$ buffer (50 mM $K_2SO_4$/25 mM HEPES-sulfate, pH 6.8) by ultrasonication, afterwards appropriate amounts of the lipid vesicles were suspended in isotonic ($K^+$-free) $Na^+$-buffer (50 mM $Na_2SO_4$/25 mM HEPES-sulfate, pH 6.8) followed by the addition of the potential sensitive dye diS-$C_3$-5. Addition of $K^+$ selective ionophore valinomycin to the lipid vesicles caused efflux of these ions thereby developing a potential gradient across the lipid bilayer resulted in a polarized state which in turn quenched the fluorescence of the dye. When the dye exhibited a steady fluorescence level, peptides were added. Membrane-permeability of the peptide was detected by the increase in fluorescence of the dye, which resulted from the dissipation of diffusion potential. The peptide-induced dissipation of diffusion potential was measured in terms of percentage of fluorescence recovery ($F_t$) by the same equation as shown in the previous section of assay of peptide-induced depolarization of hRBC and bacteria. Here $I_t$=the observed fluorescence after the addition of a peptide at time t (~5 min after the addition of the peptide), $I_0$ = the fluorescence after the addition of valinomycin and $I_f$ = the total fluorescence observed before the addition of valinomycin.

***Dye leakage from the calcein-entrapped lipid vesicles***

In order to determine the pore-forming activity of peptides, release of calcein from calcein-entrapped lipid vesicles was measured. Calcein-entrapped lipid vesicles were prepared with a self-quenching concentration (60 mM) of the probe in 10 mM HEPES buffer at pH 7.4 as reported earlier (23, 151, 178). Briefly, thin film of lipid (either PC/Chol or PC/PG) was suspended in calcein solution, vortexed for 1–2 min and then sonicated in a bath-type sonicator. The non-encapsulated calcein was removed from the

liposome suspension by gel filtration using a sephadex G-50 column. Usually lipid vesicles are diluted to approximately 10 fold after passing through a G-50 column. The eluted calcein-entrapped vesicles were diluted further in the same buffer to a final lipid concentration of ~3.0 μM for the experiment. Peptide-induced release of calcein from the lipid vesicles was monitored by the increase in fluorescence due to the dilution of the dye molecules from its self-quenched state. Fluorescence was monitored at room temperature with excitation and emission wavelengths fixed at 490 and 520 nm respectively. Calcein release as measured by the fluorescence recovery is defined by the equation:

$$F_t = [(I_t - I_0)/(I_f - I_0)] \times 100\ \%.$$

Where $I_t$=the observed fluorescence after the addition of a peptide at time t (after 5 min of the addition of the peptide), $I_0$=the initial fluorescence before addition of the peptide and $I_f$=the total fluorescence, which was determined after the addition of triton X-100 (0.1% final concentration) to the dye-entrapped vesicle suspension.

***Tryptophan blue shift assay***

For the measurement of vesicle-induced changes in the emission spectra of tryptophan, the fluorescence emission spectrum of Trp-containing peptides (designed peptides LRP, FRP, VRP and ARP, and Melittin its analogue MelVal) was monitored in PBS and in the presence of small unilamellar vesicles (SUVs) composed of either PC/Chol (8:1, w/w) or PE/PG (7:3, w/w) or PC/PG (3:1, w/w). The tryptophan was excited at 280 nm and the emission was scanned from 300 to 400 nm. Each of the peptides (~1.1 μM) were added to PBS with subsequent stepwise addition of vesicles upto 400 μM for PC/PG or PE/PG vesicles and 400 μM for PC/Chol lipid vesicles.

***Quenching of Trp Emission by Acrylamide***

To reduce absorbance by acrylamide, excitation of Trp at 295 nm instead of 280 nm was used (22). Aliquots of the 3.0 M solution of this water-soluble quencher were added to the peptide in the absence or presence of liposomes at a peptide/lipid molar ratio of 1:100. The values obtained were corrected for dilution, and the scatter contribution was derived from acrylamide titration of a vesicle blank. The data were analyzed according to the Stern-Volmer equation (23),

$$F0/F = 1 + Ksv\ [Q]$$

Where F0 and *F* represent the fluorescence intensities in the absence and the presence of the quencher (*Q*), respectively, and *Ksv* is the Stern-Volmer quenching constant, which is a measure of the accessibility of Trp to acrylamide. On the premise that acrylamide does not significantly partition into the membrane bilayer (22), the value for *Ksv* can be considered to be a reliable reflection of the bimolecular rate constant for collisional quenching of the Trp residue present in the aqueous phase. Accordingly, *Ksv* is determined by the amount of nonvesicle- associated free peptide as well as the fraction of the peptide residing in the surface of the bilayer.

**Confocal microscopic Experiments:**

All confocal microscopic were performed on Zeiss LSM-510 META confocal microscope using 63x1.4 NA (oil) Plan apochromate lens.

***Localization of peptides onto mammalian and bacterial membranes***

Localization and binding of the peptides onto mammalian and bacterial membranes was studied using the rhodamine-labeled versions of peptides. Fresh hRBCs (3% in PBS) as used in peptides' hemolytic activity assays were incubated with NBD-labeled peptides for 10-30 minutes depending on peptide's toxic activity at 37°C. Cells were washed and fixed with 2% paraformaldehyde (10 min.) after extensive washing with PBS and then confocal microscopic images of cells were taken with argon ion laser set for Rho-excitation at 561 nm. Setting of the photomultiplier was constant during the whole experiments (25).

Localization and binding of the peptides onto the bacterial cells was also examined with the help of NBD-labeled peptides by employing a confocal microscope. Bacteria (~$10^6$ CFU/ml) in LB medium were incubated in the presence of rhodamine-labeled peptides for half an hour and then centrifuged, washed and analysed by the confocal microscope as described above.

***Determination of peptide-induced pore in Bacillus megaterium***

BMAP-28 and its analogue induced permeabilization of FITC-dextrans in bacteria were performed with slight modification in the protocol of Imura *et. al.(179)*. Calcein and soluble FITC-dextran of 4.4, 20 and 40 kDa were used to determine the diameter of the pores formed in *Bacillus megaterium* (NRRL B-4272) in presence of BMAP-28 and

its designed analogue. Cells were grown upto $10^6$ CFU/ml, washed with PBS thrice to remove media component and $10^5$ cells were incubated in 24 well plate along with calcein, FITC-dextrans- 4.4 kDa, 20 kDa and 40 kDa at 0.16 mg/ml. Peptide concentration was ~4μM (2 time of MIC). In control experiment no peptide was added. After 10 minute incubation cells were transferred poly-lysine coated glass slide for examination.

***Immunomodulatory experiments***

***Assay for NO neutralizing activity***

RAW 264.7 cells were plated at 5x $10^5$cells/well in 24-well plates and then incubated with LPS (1μg/ml) in the presence of 4.5μM peptides (LRP, FRP, VRP and ARP). The cells with and without LPS addition were taken as positive and negative controls for LPS-induced and basal level of nitric oxide production respectively. The nitric oxide production was measured by using Griess reagent. 100μl of Griess reagent was added with 100μl of culture supernatant to observe nitrate accumulation after respective treatments (180). Absorbance was then measured at 548 nm using a 96 well micro titer plate reader from Quant Bio-Tek Instruments, VT. Fresh culture media was used as blank for all experiments. For the determination of NO production by the cells with peptide and LPS treatments, the assay was done in triplicate and the average values were considered for each set. Similarly in another experiment, effect varying dose of peptides on LPS induced NO production determined.

***Immuno-blotting experiments for iNOS2, TNF-α, IL-1β, COX-2 and Iκβα***

RAW 264.7 macrophage cells (approx 2x$10^5$) were stimulated with 1μg/ml LPS in the presence of peptides (4.5μM) in 24 well plates for 24 hrs. LPS treated and untreated cells were taken as positive and negative control respectively representing the stimulated and un-stimulated levels of protein expressions. For immunoblotting cells were harvested, washed with ice cold PBS pH 7.4 and lysed in Laemelli sample buffer (100mM TRIS pH6.8, 4% w/v SDS, 0.2% w/v bromo phenol blue, 20% glycerol and 200mM β mercapto-ethanol). Lysates were resolved by SDS-PAGE on 8% gel for iNOS2 and COX-2 and 12% for TNFα, IL-1β and Iκβα and then transferred to nitrocellulose membrane (Immobilins, Milipore). After blocking with 3% BSA for 3 h at room temperature, membrane was incubated with primary abs for 2 h, washed 3 times with TBST (10 mM Tris (pH 8.0) and 150 mM NaCl solution containing 0.05% tween-20.) and incubated with the appropriate alkaline phsophatase

conjugated secondary Ab. Signals were developed afterwards with substrate NBT/5-bromo-4-chloro-3-indolyl phosphate (calbiochem). β-actin served as loading controls for western experiment.

***Measurement of cytokine expression levels in supernatant***

Enzyme linked immunosorbent assays were performed to estimate the secreted TNF alpha and IL-6 in LPS treated cells in presence of peptide (LRP, FRP, VRP and ARP) after 12 hr incubation. Levels of these cytokines in culture supernatant of untreated and LPS treated cells were taken as minima and maxima to calculate percentage inhibition by peptides as mentioned above (181). The dilution of primary Abs was 1:300 for both TNF alpha and IL-6, while compatible HRPO conjugated secondary abs were diluted 1:1000 with 1% BSA. 3% BSA was used for blocking. *OPD* (o-phenylenediamine dihydrochloride) at 1mg/ml solution along with 0.5µl/ml of 30% Hydrogen peroxide in citrate buffer pH5.0 was used as substrate for calorimetric analysis of HRPO activity. Reaction was stopped by 0.2N H2SO4. The final readings were taken at 492 nm in BIOTEK microtiter plate reader.