CHAPTER 2

MATERIALS AND METHODS

"Go to your task with love in your heart, and you will
Go to it light-hearted and cheerful".

James Allen
2.1 Materials

All solvents used were analytical grade (Merck). Tissue culture plastic-ware and glassware (Nunc) were employed for all cell culture procedures. Cell culture media, Dulbecco’s modified Eagle’s medium (DMEM), foetal calf serum (FCS), antimycotic and antibiotic (Antibiotic Antimycotic Solution (100X)), were purchased from Sigma-Aldrich, India. Microbial culture media, Luria Bertanni Broth and Agar, were obtained from HiMedia, Mumbai, India. Chemicals for various buffer solutions were molecular biology grade (Sigma-Aldrich, India). Protease inhibitor cocktail, phosphatase inhibitor (Sodium ortho-vanadate), lipid raft marker (Cholera toxin B), cholesterol depleting agents like methyl-β-cyclodextrin (MβCD), two-dimensional gel electrophoresis marker were also purchased from Sigma-Aldrich, India. Total RNA extraction kit, Reverse transcription Polymerase chain reaction (RT-PCR) kit, Agarose gel electrophoresis DNA markers and SDS-PAGE protein markers were obtained from Bangalore Genei, India. All reagents and chemicals required for two-dimensional gel electrophoresis were obtained from Bio-Rad Laboratories, USA. Immobiline™ Dry Strip gels (IPG), broad range (pI 3-10, 7cm) and narrow range (pI 3-6, 7cm) as well as the ampholyte mix (Bio-Lyte3/10), were purchased from Amersham Pharmacia, GE Life sciences, USA. Primary antibodies against mouse proteins IRAK and TLR2, were purchased from Imgenex, USA. Antibodies detecting the different Fcγ receptors, CD64 and CD32, and against the co-receptor molecule for LPS, CD14, was a kind gift from Dr. Shilpa Buch, obtained from SantaCruz Biotechnologies, USA. Secondary antibodies namely anti-goat-horse raddish-peroxidase (anti-g-HRP) conjugate and anti-rabbit-horseradish-peroxidase (anti-rb-HRP) conjugate were purchased from Bangalore Genei, Bangalore, India. The
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Apoptosis detection kit (APO-AF) was procured from Sigma-Aldrich, India. High sensitivity silver staining kit for analysis of the second dimension SDS-PAGE gels, was purchased from Invitrogen, USA.

2.2 Cell Culture

Stock cultures of murine macrophage cell line RAW264.7 were purchased from the repository at National Centre for Cell Science (NCCS), Pune, India. The stock cultures were maintained in tissue culture flasks (Nunc), using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with antibiotics and antimycotics and 10% foetal calf serum (FCS). The macrophage cells were incubated in a humidified atmosphere of 5% CO\textsubscript{2} and 95% air at 37°C in a CO\textsubscript{2} incubator (Forma Scientific, Germany). Cells were allowed to grow to confluence, within the culture flasks, and used at a density of $5 \times 10^5$ cells per milliliter for all experiments.

2.3 Bacterial Strains

Bacteria used for the various inductions were obtained from the Departmental repository at M.S. University of Baroda, Gujarat, India. The different strains of gram-negative bacteria obtained were *Escherichia coli* (WT), *Shigella dysenteriae* and gram-positive bacterium was *Staphylococcus aureus* (6532P). All these bacterial cultures were grown to exponential-phase, in Luria-Bertanni Broth (LB) under aeration conditions of 180 rpm at 37°C in a shaker-incubator (Orbitek, India). Culture stocks were maintained both on Luria Agar (LA) Slants and as 20% glycerol stocks. Viability count for all the bacteria were done by counting the colony forming units (CFU) on LA plate assays for cultures grown to optical density (OD) or absorbance of 0.4, measured at 600nm using UV-Visible Spectrophotometer (Thermo, USA). For heat-killed preparation of bacterial strains, all the different bacteria were grown to exponential-phase in LB under shaking conditions and washed twice with 0.85%
saline (6000xg, 10mins (Sigma centrifuge)) re-suspended in autoclaved distilled water (DW) and heat-killed at 15psi for 15 minutes in an autoclave.

2.4 Macrophage induction

Murine macrophage cell line RAW 264.7 was grown to a cell density of $5 \times 10^5$ cells per ml in T25 tissue culture flasks (Nunc, USA). Viability of the macrophage cells were checked by 0.4% Trypan Blue dye exclusion. Macrophage cells were subsequently infected with different live and heat-killed bacteria at a multiplicity of infection (m.o.i) of 1:100 of macrophages to bacteria, for efficient uptake of bacteria by the macrophages. In the case of heat-killed bacteria, 100 µL of the bacterial preparation (equivalent to $5 \times 10^8$ bacteria per mL) in each case was employed for induction. As for the induction with live bacterium, macrophage cells were grown in DMEM medium lacking antibiotics and antimycotics and subsequently 100 µL of the live bacterial preparation, from the exponential phase grown bacteria after washing the bacterial cells twice with DMEM without antibiotic, was added for an m.o.i of 1:100 and thereafter incubated for 30 mins at 37°C within the CO$_2$ incubator. After this gentamycin (100µg/mL) was added to kill the extra cellular bacteria, and incubated for a further period of 30 mins, within the CO$_2$ incubator. Subsequently the medium from the flasks were discarded and replenished with DMEM containing antibiotic and incubated for the different time periods as per the assays performed.

2.5 Isolation of cytokine mRNA from differentially induced RAW 264.7 macrophages

Murine macrophage cell-line RAW264.7 was grown to confluence in tissue-culture flasks and subsequently the macrophages were treated with live and heat-killed preparation of the different gram-positive and gram-negative bacteria for the following time-intervals of 30mins, 3h and 6h. Following this the spent medium was discarded and the monolayer of the induced macrophage cells was washed twice with
Dubecco’s PBS. Total RNA was extracted from these treated macrophage cells as well as the control untreated macrophage cells, using Total RNA Extraction kit (Banglore Genei). The protocol followed was modified slightly to suit the laboratory conditions. All the glassware and plastic ware, was at first rinsed thoroughly with chloroform to inactivate any RNase present and thereafter sterilised by autoclaving (15psi, 15 mins) before use. All the reagents used were prepared in Diethyl Pyrocarbonate (DEPC) treated and autoclaved water.

The treated and untreated macrophage RAW264.7 cells were layered with 0.5 ml (1 ml in the original protocol for 1 x 10⁶ cells per mL) of lysis buffer/denaturing solution (provided in the kit). Equal volume of water saturated phenol (acidic phenol) and 200 μL of chloroform-isooamyl alcohol mix (freshly prepared in the ratio 49:1) were added and mixed properly by gently inverting the tube several times. Following this, the mixture was kept on ice for 15 min. This was followed by centrifugation at 9300xg, 4°C for 20 min. The upper aqueous phase was then carefully aspirated into a fresh tube and equal volume of 100% isopropanol was added to precipitate RNA. The content was mixed properly and kept overnight (30 min in original protocol) at -20°C, followed by centrifugation at 9300xg, 4°C for 20 min. The pellet was re-suspended in 300 μL of denaturing solution and 300 μL of isopropanol and mixed properly. The mixture was kept at -20°C for half an hour and centrifuged at 9300xg, 4°C for 20 min. Then, 600 μL of 75% ethanol was used to rinse the pellet by incubating at room temperature for 15-20 min, to remove the residual amounts of guanidine. Finally, the tubes were centrifuged at 9300xg, 4°C for 20 min. The pellet thus obtained was dried, followed by re-suspension in 100 μL DEPC treated autoclaved water.

2.6 Reverse transcriptase mediated complementary DNA (cDNA) synthesis

Integrity of the RNA samples was confirmed by visualizing the 28S and 18S rRNA bands following separation by electrophoresis on a 0.8% agarose gel. After confirmation, 5μg of the RNA was taken for subsequent complementary DNA
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(cDNA) synthesis. In a chloroform treated and autoclaved centrifuge tube (1.5mL), 5µg of RNA was taken and the volume made up to 10.5 µL with DEPC treated water, followed by addition of 1µL of solution containing oligo dT (18mer) primer. The contents were mixed properly and incubated at 65°C for 10 min followed by 2 min at room temperature. After this, the following reagents were added sequentially: 1µL containing 10 U/µL RNAsin, 1µL of 100 mM dithiotreitol (DTT), 4µL of 5X reverse transcriptase buffer, 2µL of 30 mM dNTP mix (7.5 mM each dNTP) and 0.5µL of 100 U/µL M-MuLV Reverse transcriptase and further incubated at 37°C for 1 h followed by 95°C for 5 min. At the end of the incubation period, the tubes were immediately cooled on ice for 15 min and stored at -20°C until use.

2.7 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

5µg of Total RNA was used for 1st strand synthesis using M MuLV RT PCR kit (Bangalore Genei). cDNAs coding for pro-inflammatory and anti-inflammatory cytokines, TNF-α, IL-6 and IL-10, as well as an house-keeping protein like β-actin, were amplified from above 1st strand preparations using primer sets specific for the respective cDNAs, as reviewed from available literature. The resultant amplicons were analysed on 1.5% agarose gels and ascertained by the amplicon length, by comparing with the standard Low Range DNA Marker (Bangalore Genei, Bangalore, India).

Nucleotide sequence of primers used for amplification of cDNA coding for TNF-α, IL-6, IL-10 and β-actin is as follows:-

Mouse TNF-α: (239 bp)
Forward- 5’ AGT GAC AAG CCT GTA GC 3’
Reverse- 5’ CTC CTG GTA TGA GAT AGC 3’

Mouse IL-6: (475 bp)
Forward- 5’ CAT GTT CTC TGG GAA ATC GTG G 3’
Reverse- 5’ AAC GCA CTA GGT TTG CCG AGT A 3’
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Mouse IL-10: (183 bp)
Forward- 5' CGG GAA GAC AAT AAC TG 3'
Reverse- 5' CAT TTC CGA TAA GGC TTG G 3'

Mouse β-Actin: (133 bp)
Forward- 5' TGA CAG GAT GCA GAA GGA GA 3'
Reverse- 5' GCT GGA AGG TGG ACA GTG AG 3'

PCR amplifications were then performed with specific primers for cytokines in a 25 µl system with 2 µl of the first strand cDNA, 0.2 mM dNTPs, 1.5 mM MgCl2 and 1.25 pmol each of the forward and reverse primers. This was run for 30 cycles after the addition 0.5 U of Taq polymerase. Amplification conditions were as follows: initial denaturation at 94°C for 6 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 55°C for β-actin, and TNF-α and annealing for 1 min at 55°C for IL-6 whereas at 47°C for IL-10 followed by extension at 72°C for 1 min and a final extension at 72°C for 20 min. PCR products separated on a 1.5% agarose gel were stained with ethidium bromide (0.5 µg/ml) and documented using gel documentation system.

2.8 Cytokine Analysis by Enzyme Linked Immuno Sorbent Assay (ELISA)

Macrophage cells were induced with the different bacterial preparation as explained previously for the following time-interval of 0h, 3h, 6h, 24h, & 48h respectively and the culture supernatants were collected, from the 24-well tissue culture plates within which the inductions were set up, for each of the aforementioned time points. Culture supernatant was also aspirated, for each of the above time points, from control samples (macrophage cells untreated). For the heat-killed induction the 0h sampling was done immediately after addition of the bacteria, whereas in the case of live bacterial inductions, 0h sampling was done after incubation with gentamycin to kill extracellular bacteria (implies that 0h sample was actually 1h after addition of bacteria). Differences in the cytokine profile were analysed performing sandwich
ELISA employing monoclonal primary antibodies specific for each cytokine. Levels of the cytokines were detected, by reading the absorbance of the colored reaction product, at 450nm using ELISA plate reader (BioRad Laboratories, USA). Appropriate controls were also employed for every cytokine ELISA experiment performed.

2.9 Statistical Analysis

Data collected from the cytokine ELISAs of two independent experiments performed in triplicates are statistically represented as mean and standard deviation (SD). Statistical significance of the collected data were determined using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test of all groups versus the control group. P< 0.05 was considered significant.

2.10 Isolation of Detergent Resistant Membranes (DRM)

Macrophage cell-line RAW 264.7, were harvested and subjected to washing with ice cold PBS and homogenized using ice cold TNE buffer (25mM Tris-Cl, pH 7.4, 1%TritonX-100, 50mM NaCl, 1mM EDTA, 100mM sodium orthovanadate, 0.1mM phenylmethylsulfonyl fluoride (PMSF), 10mM di-thio threitol (DTT), protease inhibitor cocktail). Cells were homogenized using a glass-teflon homogenizer on ice and the lysate was solubilised for 30 mins on ice, and centrifuged for 30 min at 4°C at 10,000xg. Post-mitochondrial supernatant thus obtained was subjected to discontinuous sucrose density gradient centrifugation for isolation of membrane rafts or detergent resistant membranes (DRM). Briefly, discontinuous step gradient of sucrose was prepared using 5% to 45% sucrose in TNE buffer at 1.5ml each and the post-mitochondrial supernatant is applied to the 40% sucrose layer after mixing the lysate with equal amount of 80%sucrose in TNE and layer it atop the 45% sucrose placed at the bottom of the centrifuge tube. Following this is a layer of 35% sucrose.
were collected, at the end of the run, from the top of the gradient, and each fraction was analysed using Cholera Toxin-B (Sigma-Aldrich, India) as marker for DRMs or lipid rafts. DRMs specifically isolated from the inter phase between 5%-35%, were termed light DRMs (DRM-L) and 35%-40%, were termed heavy DRMs (DRM-H).

2.11 Extraction of Proteins from DRMs

Proteins were extracted from the DRMs, isolated as detailed above, by using the method of Methanol Chloroform extraction for high lipid containing membranes by Wessel and Flugge (421). Membrane proteins were subjected to solubilisation with 60mM n-octyl β-glucopyranoside (OG) at 4°C for 1 hour. Subsequently they were precipitated employing methanol-chloroform extraction. Flow-chart diagram for extraction:
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To 1 (v/v) of DRM proteins solubilised + 4 (v/v) of Methanol was added (Vortex mix) ↓9000xg, 10 secs
Add 3 (v/v) of Chloroform (Vortex mix) ↓9000xg, 10 secs
Add 3 (v/v) of autoclaved distilled water (Vortex vigorously) ↓9000xg, 1 minute
Remove upper phase and discard leaving the inter-phase with the protein ↓
To this, add 3 vol of Methanol (Vortex mix) ↓9000xg, 2 minutes
Remove the supernatant and dry the pellet under stream of air
The pellet was re-suspended in either IEF Lysis solution or Laemmli Buffer (1X) for sample loading on SDS-PAGE and was analysed for protein profile accordingly.

2.12 Analysis of membrane proteins by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Membrane proteins were separated according to molecular weight by SDS-PAGE, on a 10% acrylamide gel under reducing conditions (2 mercaptoetanol, 10%), along with standard molecular weight protein markers for molecular weight estimation. Qualitative estimation, of the DRM proteins, was done after silver staining the gels with 20% AgNO₃ solution as per standardized laboratory protocol. Following the SDS-PAGE separation, the proteins were electro-blotted onto nitrocellulose membrane (NCM, 0.2 micron). The membranes were then blocked with 3% gelatin in phosphate buffered saline (PBS) for one hour at room temperature and incubated for further 1 hour with primary antibody (@ 2μg/mL of CD14, CD64 & TLR2 whereas @ 1μg/mL for IRAK) in PBS, after discarding the blocking agent. The membranes were then washed 3 times, 1 min each with PBS containing 0.1% Tween 20 and then...
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incubated with HRP conjugated secondary antibody (anti rabbit, HRP conjugate and anti goat, HRP conjugate) diluted 1:7000 for anti rabbit conjugate and 1: 10,000 for anti goat conjugate in PBS for 1 hour at room temperature, washed 3 times, 1min each with PBS containing 0.1% Tween 20 and developed with TMB/H2O2 reagent (10X).

Reagents for SDS PAGE and Western Blot analysis

**Gel stock solution (30%, 100ml)**

- Acrylamide: 29 g
- Bis acrylamide: 1 g
- Distilled Water (DW): 100 ml

**Resolving gel**

- DW: 1.9 ml
- 30% gel stock: 1.7 ml
- 1.5 M Tris HCl (pH 8.8): 1.3 ml
- 10% SDS: 50 μl
- 10%APS: 50 μl
- TEMED: 7.5 μl

**Stacking gel**

- DW: 1.4 ml
- 30% gel stock: 0.33 ml
- 1 M Tris HCl (pH 6.8): 0.25 ml
- 10 % SDS: 20 μl
- 10 % APS: 20 μl
- TEMED: 5 μl

**Sample buffer (5X, 10 ml)**

250 mM Tris HCl (pH6.8)
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500 mM β-Mercaptoethanol
10 % SDS
50 % Glycerol
0.5 % Bromophenol Blue

Tank buffer (5X, 1 L). Used as 1X

- Tris base: 15.1 g
- Glycine: 94 g
- SDS: 10 g

Transfer buffer (600 ml)

- Tris base: 1.81 g
- Glycine: 8.64 g
- DW: 480 ml
- Methanol: 120 ml

Substrate for Blot

Substrate used for the blot was TMB/H₂O₂ (10X), procured from Bangalore Genei, Bangalore, India as a ready to use solution of substrate for localization. Substrate was employed at a working concentration of 1X, and the membranes were developed in the dark until bands were observed.

2.13 Analysis of DRM proteins by iso-electric focusing (IEF)

Membrane proteins extracted by Methanol-chloroform was re-suspended in IEF lysis solution containing 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS, 4%) and Urea (8M). Protein concentration for each sample was estimated by Bradford’s assay and thereafter 8 µg of protein from each sample was employed per dehydrated strip (pI 3-10 & pI 3-6). Iso-electric focusing was performed for the protein samples as per the standard protocol with minor changes incorporated to accommodate laboratory conditions. Briefly, the IPG strips were firstly re-hydrated at...
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room temperature (RT) over night in 125μL of Rehydration Buffer containing 8 μg of protein, within IEF re-hydration trays (Bio-Rad Laboratories, USA). At first, strips are re-hydrated for 1h at RT and thereafter overlaid with IPG cover fluid (GE Healthcare, USA) and left to hydrate over night. Subsequently the strips are picked up and excess fluid blotted onto a soft blotting sheet, and laid face up into the focusing tray (PROTEAN IEF System, Bio-Rad Laboratories, USA) with the anodic end (+) of the strip resting on the appropriate mark etched on the focusing tray. The strips were subjected to electro-focussing for a total of 40,000–50,000 V-hr with a maximum of 50μA current per strip, subsequent to a conditioning step of 250V for 15 minutes. After focusing, the strips were stored at -80°C until analysed on the second dimension. Before the second dimension, the focused strips were equilibrated in equilibration buffer I for 10mins at RT and thereafter in equilibration buffer II for 10 mins at RT and thereafter the strip is carefully placed on the resolving gel and sealed with agarose. After SDS-PAGE at 100V, the gel was stained by high sensitivity silver staining kit (PAGE SILVER™ Silver Staining Kit, Fermentas Life Sciences, Thermo Fisher Scientific, USA) as per manufacturer’s protocol.

Reagents for IEF and SDS-PAGE of 2D electrophoresis

Rehydration Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>8M</td>
</tr>
<tr>
<td>CHAPS</td>
<td>2% (w/v)</td>
</tr>
<tr>
<td>IPG buffer</td>
<td>0.5% (v/v)</td>
</tr>
<tr>
<td>Bromophenol Blue</td>
<td>0.02%</td>
</tr>
<tr>
<td>Milli Q water</td>
<td>to 25mL</td>
</tr>
</tbody>
</table>

# Before use 0.5 M DTT is to be added to the stock solution of rehydration buffer.

Bromophenol Blue Stock Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromophenol Blue</td>
<td>1% (w/v)</td>
</tr>
<tr>
<td>Tris-base</td>
<td>50mM</td>
</tr>
</tbody>
</table>
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Milli Q water to 10mL

Lysis Solution

Urea 8M
CHAPS 4%
Biolyte (3-10) 2%
Milli Q water to 20mL

SDS Equilibration buffer

Tris-HCl, pH 8.8 50mM
Urea 6M
Glycerol (87% v/v) 30% (v/v)
SDS 2% (w/v)
Bromophenol Blue 0.002% (w/v)
Milli Q water to 200mL

# Equilibration buffer I contains 200 mM DTT.
# Equilibration buffer II contains 250 mM Iodoacetamide.

4X Resolving gel buffer

Tris base 1.5M
Milli Q water 750mL
HCl adjust to pH 8.8
Milli Q water to 1L

Acrylamide gel stock

Acrylamide 30%
bis-Acrylamide 0.8%
Milli Q water 200mL
2.14 Liquid Chromatography / Tandem Mass Spectrometry (LC/MS-MS) Analysis

Liquid chromatography-mass spectrometry (LC-MS, or alternatively HPLC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and selectivity. Generally its application is oriented towards the specific detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture). The DRM proteins, resolved two-dimensionally by IEF and SDS-PAGE were stained by high sensitivity silver staining kit (PAGE SILVER™ Silver Staining Kit, Fermentas Life Sciences, Thermo Fisher Scientific, USA) as per the manufacturer's protocol and the differentially expressed spots are cut out of the gel ensuring no keratin contamination. These spots were then sent to The Centre for Genome Application (TCGA), New Delhi, India and identified by LC/MS analysis employing the Mascot Search Engine for homology search based on peptide mass finger printing, Sequence Query and MS/MS Ion Search.
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Figure 2.14.1: Schematic of Electro Spray Ionisation (ESI).

The MS principle consists of ionizing chemical compounds to generate charged molecules or molecule fragments and measurement of their mass-to-charge ratios. In a typical MS procedure: A sample is loaded onto the MS instrument, and undergoes vaporization. The components of the sample are ionized by one of a variety of methods (e.g., by impacting them with an electron beam), which results in the formation of charged particles (ions). The ions are separated according to their mass-to-charge ratio in an analyzer by electromagnetic fields. The ions are detected, usually by a quantitative method. The ion signal is processed into mass spectra. Solvent droplets are separated according to their charge due to the voltage applied between the spray needle tip and the cone. The droplets are expelled from the spray needle tip. During the time of flight between needle and tip, the solvent evaporates until free analyte ions enter the cone.

2.15 Induction of Hetero-tolerance in macrophages

Macrophage cells grown to confluence were subjected to tolerance induction with heat-killed *S. aureus* for 18h at 37°C thereafter these cells were induced with live *S. dydenteriae* for further time-intervals as required by the experiment. For cytokine profiling the incubation periods were 0h, 3h, 6h, 24h, and 48h whereas for membrane protein analysis incubation periods were 15mins, 3h and 12h. As for the analysis of
apoptosis induction, the secondary infection was done for a period of 20h and thereafter apoptotic cells were analysed by fluorescence microscopy and 0.4% Trypan Blue viability staining.

### 2.16 Fluorescence Microscopy

Macrophage cells were grown in chamber slides (Lab-Tek™-Chamber slide™ System, Nune, Thermo Fisher Scientific, USA) with two chambers, and subjected to induction of cross-tolerance subsequent to tolerance with heat-killed *S. aureus*. *S. dysenteriae* used for induction of cross-tolerance is known to induce apoptosis in macrophages. The analysis of cross-tolerance was therefore based on the induction of *S.dysenteriae* mediated apoptosis in macrophages non-tolerised as compared with tolerised macrophage cells. Induction of apoptosis in macrophage cells was observed and qualitative estimation was done using inverted fluorescence microscope (Nikon TE 2000S, Nikon Corporation, Japan). Working principle of fluorescence microscopy is, when high energy, short wavelength (eg. UV or blue) light is directed onto the preparation and lower energy, longer wavelength (eg. green or red) light is emitted, according to Stokes' Law. The Jablonski Diagram, seen below, shows a number of possible routes by which an excited molecule can return to its ground or room temperature state via unstable triplet states. A rapid return results in fluorescence and a delayed return results in phosphorescence.

![Jablonski Diagram](image_url)

**Figure 2.16.1** Jablonski diagram representing return of exited molecule to ground state through unstable intermediary states.
After fixing the cells on the chamber slides employing 4% formaldehyde solution in Dulbecco’s PBS and incubated for 30 mins at 4°C, staining of the cells was done with the apoptosis detection kit (APO-AF, Sigma Aldrich, St. Louis, MO, USA) as per the manufacturer’s protocol with slight modifications. Briefly, cells were treated with different bacteria and 1μg/mL of Staurosporine for 2h at 37°C (positive control for apoptosis) and thereafter washed with Dulbecco’s PBS. Binding buffer (100 mM HEPES/NaOH, pH 7.5 containing 1.4 M NaCl and 25 mM CaCl2, 10X) was added to each chamber at final concentration of 1X. Add 2.5μL of Annexin V (5μL as per protocol) and 2.5μL of Propidium iodide (PI, 10μL as per suppliers manual) to each chamber and incubate at RT for 10 mins. Documentation was done by fluorescence microscopy at specific excitation/emission (Ex/Em) spectrum for each fluorophore used. Ex/Em for Annexin V is 488nm/530nm (visualized as green fluorescence), whereas that for PI is 536nm/617nm (visualized as red fluorescence).

Figure 2.16.2 Schematic of a fluorescence microscope.

Annexin V-FITC is a fluorescent probe which binds to phosphatidylserine in the presence of calcium. At the onset of apoptosis, phosphatidylserine, which is normally found on the internal part of the plasma membrane, becomes translocated to the external portion of the membrane. The phosphatidylserine becomes available to bind
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to the annexin V-FITC conjugate in the presence of calcium. Propidium iodide binds to DNA and is employed as the counter stain to distinguish the early apoptotic cells.

2.17 Disruption of membrane raft integrity by methyl-β cyclo dextrin (MβCD)

Analysis of the role of lipid rafts or DRMs in the macrophage response to different bacteria was done by reversible disruption of the integrity of these membrane domains by sequestering the cholesterol from these domains. For this purpose, methyl-β-cyclodextrin was employed at a concentration of 5mM. Macrophage RAW 264.7 cells were subjected to treatment with MβCD at 5mM for 30 mins at 37°C, for depletion of membrane cholesterol. After cholesterol depletion, macrophages were subjected to induction with different bacterial preparations, following washes with DMEM medium without FCS. In the case of analysis of role of DRMs in the context of hetero-tolerance, the macrophage cells were subjected to MβCD after induction of tolerance within the macrophage cells. Analysis was done by verifying the cytokine profile as well as membrane protein profile as detailed in earlier sections.