CHAPTER 4

HETERO-TOLERANCE INDUCTION
IN MACROPHAGE RESPONSE TO BACTERIA

"Research is to see what everybody else has seen,
and to think what nobody else has thought"

Albert Szent-Gyorgyi
CHAPTER 4
HETERO-TOLERANCE INDUCTION IN MACROPHAGE RESPONSE TO BACTERIA

4.1 INTRODUCTION

Sepsis is characterized by the acute proinflammatory “cytokine storm”, followed by the “silencing” phase signified by suppressed TLR inducible expression of pro-inflammatory cytokines. This “silencing” phase does not globally deactivate monocytes and macrophages since expression of anti-inflammatory mediators and antimicrobial effectors is not inhibited, and has been termed “TLR re-programming” (245). Similar TLR re-programming occurs in endotoxin tolerance that develops after prior exposure to LPS and suppresses expression of pro-inflammatory cytokines in response to subsequent LPS challenge, without inhibiting anti-inflammatory and antimicrobial mediators (297). The development of endotoxin tolerance in monocytes of septic patients curtails excessive inflammatory responses but increases the risk of infections (428).

Detection of bacteria by host cells is mediated by the recognition of conserved and unique microbial structures by pattern-recognition molecules, such as the TLRs and nucleotide-binding oligomerization domain (NOD)3-like receptors (NLRs) (8, 176). TLRs mediate bacterial recognition of several molecules including LPS and microbial nucleic acids at the cell surface or endosomes and induce host immune responses (8). In contrast, NLRs induce innate immune responses through cytosolic recognition of bacterial components (176, 396). Two NLR family members, Nod1 and Nod2, sense molecules produced during the synthesis and/or degradation of bacterial peptidoglycan (192). Nod1 recognizes molecules containing the amino acid meso-diaminopimelic acid that are produced by most Gram-negative and certain Gram-positive bacteria (115). In contrast, Nod2 is activated by muramyl dipeptide (MDP), which is present in both Gram-negative and Gram-positive bacteria (116). Once activated, Nod1 and Nod2 induce transcription of immune response genes through the NF-κB transcription factor and the MAPK signaling pathways (192).
Cytokines represent a family of intercellular mediators that are now recognized to play a central role in virtually all interactions involving cells of the immune system, e.g., communication and differentiation of cellular components of the immune system, induction and amplification of inflammatory responses, and generation of anti-inflammatory reactions (6). Macrophages are considered to be one of the main cellular sources of cytokines released in the body in response to different microbial products, including LPS. In numerous in vitro studies, it has been demonstrated that LPS dependent activation of macrophages results in the release of various secretory products, including pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-12, and anti-inflammatory cytokines, e.g., IL-10. It is of importance that these cytokines can be both produced and utilized by macrophages in processes known as autocrine regulatory pathways. Unlike the early interpretation of endotoxin tolerance as a protection from the lethal effects of bacterial pyrogens (26), the current concept of this phenomenon implies that LPS unresponsiveness is controlled at the cellular level, and that the activity of macrophages may well be instrumental in the development of LPS tolerance (141). Control of macrophage LPS responsiveness may be of primary importance for the host and may function to limit the extent of the pro-inflammatory response to protect the host from excessive destructive processes during inflammation and infection. However, it is becoming increasingly apparent that the acquisition of an endotoxin refractory state is not derived from complete unresponsiveness of exhausted macrophages exposed to chronic LPS stimulus. Rather, this process is mediated by a highly orchestrated compensatory mechanism(s) controlling the balance of pro- and anti-inflammatory cytokines in the host (143). In this respect, it is attractive to hypothesize that some microbial pathogens, and probably malignant cells, may utilize a similar strategy to create an imbalance in the coordinated proinflammatory response of the immune system in an effort to circumvent host immunity. (348).

Studies in LPS-tolerized human monocytes revealed unaltered TLR4 expression, but suppressed TLR4 tyrosine phosphorylation, MyD88-TLR4 and IRAK1-MyD88 interactions, IRAK1 activation and increased expression of negative regulators of TLR4 signaling (297). Endotoxin tolerance is signified by decreased activation of molecules exerting positive regulation of TLR signaling, e.g.
deficient tyrosine phosphorylation of TLR4 and Mal (297), recruitment of MyD88 and TRIF to TLR4 (250), and activation of IRAK1 and TBK1 (220). Enhanced expression of negative regulators, such as Toll-interacting protein, suppressor of cytokine signaling-1, IRAK-M, SH2-containing inositol-5'-phosphatase-1, sterile alpha and HEAT-Armadillo motifs-containing adapter (SARM) and suppressor of IкB-ε (SIKE) also underlies tolerance (359, 407). It is plausible that altered tyrosine phosphorylation of TLR4 and Mal caused by endotoxin tolerization (249), impairs assembly of TLR4-Mal docking platforms and interfere with recruitment of MyD88. MyD88 recruitment to TLR4 enables IRAK4 and IRAKI to interact with the TLR4-Mal-MyD88 complex to assemble the "Myddosome signaling tower" (265, 225). Deficient LPS induced activation of IRAK4 and TAK1, K63-linked polyubiquitination of IRAK1 and TRAF6, and disrupted IRAK1-TRAF6 and IRAK1-IKKγ assembly associated with increased A20 expression and A20-IRAK1 interactions are new determinants of endotoxin tolerance (428). In innate immune cell types, where endotoxin tolerance have been more thoroughly evaluated, the proteins suppressors of cytokine signaling (SOCS) are involved in tolerance phenomena as negative regulators of the pro-inflammatory response, via the TLR4-NFкB pathway (331).

In contrast to the endotoxin of Gram-negative bacteria, no single constituent has so far been identified as the major immunostimulatory element in Gram-positive bacteria. In the last years, increasing evidence has been provided that lipoteichoic acids (LTAs) and peptidoglycans could substitute for whole Gram-positive bacteria in activation of macrophages and induction of multiorgan dysfunction syndrome. Recently, it has been shown that signal transduction and cytokine production in response to several Gram-positive bacteria, peptidoglycan, and lipopeptides from various bacterial species is mediated by the cellular transmembrane receptor TLR2. However, controversial data have been published regarding the involvement of TLR2 or TLR4 in LTA-induced signaling (341, 388). Although previous experiments with bacterial DNA, lipopeptides, or whole bacteria indicated that down-regulation of macrophage responsiveness after stimulation was not restricted to endotoxin from Gram-negative bacteria, it has not been studied whether and how macrophage responsiveness is modulated by LTA (240).
In analogy to LPS tolerance, it has been found that exposure to ligands for TLR2 (349, 260, 70) or TLR9 (69) also induced hyporesponsiveness to subsequent stimulation with the same ligand (self-tolerance) and that TLR ligands can substitute for each other inducing cross-tolerance. Cells treated first with bacterial lipoprotein or MALP-2 (both TLR2 ligands) did not respond to subsequent LPS stimulation (220) and cells pretreated with LPS did not respond to LTA (163) or flagellin (260). However, despite LPS-, LTA-, Pam3Cys-, and CpG DNA-induced self-tolerance, LTA-, Pam3Cys-, or CpG DNA-pretreated cells still (partially) responded to LPS and LPS pretreated cells still responded to MALP-2 or Pam3Cys. In this respect, Dalpke et al. (2005) showed that in vivo pretreatment of mice with LPS, LTA, or CpG DNA induced self-tolerance and that LPS and LTA, but not CpG DNA induced cross-tolerance to the other TLR ligands (69). These findings indicate that tolerance induced by different TLR ligands occurs via distinct mechanisms (10). Murine monocytes/macrophages following prolonged LTA treatment can develop hypo-responsiveness to subsequent LTA-induced IL-1β/TNF-α production (217).

Of note is the identification of a cross-tolerance effect linking TLR4 and TLR2 agonists, which is mediated by a factor that can influence the transduction mechanisms of both receptors in innate immune cells. This suggests a common signaling pathway or protein that can be a target of immune-modulation of these receptors. As these receptors are responsible for recognizing components of gram-negative (TLR4) and gram-positive (TLR2) bacteria, they have become a major target to blunt the pro-inflammatory response in sepsis (331). The disruption of TLR2 signaling leading to decreased IL-1β and TNF-α production in LTA-tolerant cells is not due to inactivation of the TLR2 receptor. LTA-tolerized THP-1 cells are not totally unresponsive to subsequent LTA challenge. Instead, LTA-tolerized cells can still respond to further LTA treatment and selectively express sIL-1RA protein. This indicates that the TLR2 receptor is still functional in LTA-tolerant cells. The decreased expression of inflammatory proteins such as IL-1β and TNF-α is likely due to disruption of intracellular signaling components downstream of the TLR2 receptor and upstream of IRAK. The candidate protein(s) include adaptor protein MyD88, Tollip, TIRAP/MAL, as well as other enzymes such as phosphatidylinositol 3-kinase (PI3K). The LPS/TLR4 signaling can recruit the
specific adaptor protein(s) that leads to IRAK phosphorylation and subsequent degradation whereas LTA/TLR2 signaling recruited adaptor(s) could only lead to IRAK activation, but not phosphorylation/degradation. Indeed, it was observed that LTA cannot induce IRAK phosphorylation. The fact that LTA can induce IRAK kinase activation indicates that IRAK activation and phosphorylation are two independent events and may be controlled by distinct upstream regulatory steps. Cellular innate immunity tolerance toward distinct microbial toxins may involve alteration of distinct TLR signaling components. However, downregulation of the common TLR intracellular signaling factor IRAK will lead to a state of cross-tolerance and decreased IL-1β and TNF-α production upon subsequent challenge with multiple microbial toxins (163).

A previous report shows suppressed cytokine production on stimulation of LPS-pretreated human monocytes with whole S. aureus (179). However, contrasting results were obtained by others showing unaltered or even increased cytokine production upon stimulation with muramyl dipeptide or whole S. aureus after LPS-tolerance induction (317). These discrepancies may be attributable to species differences, as guinea pig and rabbit macrophages were used there, or to overall different experimental settings. Earlier findings by Sato et al. (2000) who demonstrated cross-desensitization of murine macrophages by LPS and the mycoplasmal lipopeptide macrophage activating protein 2 (MALP-2), which has been shown to signal also via TLR2 (329, 389). As a major difference, LPS pretreatment completely inhibited TNF release in response to subsequent LTA stimulation, whereas only a partial reduction of MALP-2 induced TNF production by LPS-desensitized macrophages was found (217).

Expression of CD14 is unaffected or even increased after LPS stimulation, thus it is highly unlikely that tolerance is mediated via expression of this LPS receptor. Refractoriness in response to LPS preexposure has been shown to be associated with altered G protein content (236), phospholipase D and phosphatidylinositol-3 kinase expression (45), and compromised protein kinase C activation (422). Others described suppressed signal transduction via both the mitogen-activated protein kinase cascade and I-kB kinases, resulting in impaired transcription of NF-kB- and AP-1-regulated genes (441). A predominance of transactivation-inactive p50/p50 homodimers of NF-kB also has been found in LPS tolerance (180). Recent data
showing suppression of IL-1R-associated kinase activation and association with myeloid differentiation protein 88 and decreased surface expression of TLR4 on LPS tolerized cells (281) support the notion that very early steps in LPS signaling are altered after LPS exposure. Further evidence for this was provided by Medvedev et al. (2000) who reevaluated in vitro desensitization by IL-1 and TNF, showing induction of cross-tolerance to LPS via the IL-1 receptor but not the TNF receptor (248). Intriguingly, signal transduction of the IL-1R, the LPS receptor TLR4, and TLR2 use similar signaling molecules (217).

In all the studies of induction of cross-tolerance thus far, either the tolerisation is with purified bacterial ligands of different TLRs or upon re-stimulation purified bacterial ligands were employed subsequent to tolerisation with whole bacteria. This is the first study aiming at cross-tolerance induction within macrophages employing heat-killed *S. aureus* for tolerisation of macrophages and subsequently re-stimulation for induction of cross-toleration with live *S. dysenteriae*. The hypothesis behind this set-up for hetero-tolerance is that this would more or less depict the actual environmental conditions that the individuals who are compromised for one type of ligand response during sepsis. Upon tolerisation of the macrophages these individuals are susceptible to infections by opportunistic pathogens and *S. dysenteriae* is one such pathogen which can cause acute rectocolitis. *S. dysenteriae* is a facultative intracellular pathogen, which causes apoptosis of macrophages. Differentiated, macrophage-like THP-1 cells underwent apoptosis when treated with Shiga toxins, but the kinetics of cell death were significantly delayed compared with undifferentiated, monocyte-like cells, with only 11% cell death noted 12 h after toxin treatment (140).

In the present study the macrophage cells were at first treated with heat-killed *S. aureus* for induction of tolerisation and thereafter treated with live *S. dysenteriae* for determination of induction of cross-tolerance assayed by cytokine response and apoptosis within macrophages. Also the role of the detergent-resistant membranes in the induction of hetero-tolerance was looked at by disruption of raft and estimation of the cytokine profile thereafter.
4.2 RESULTS

4.2.1 Apoptosis detection in macrophages hetero-tolerised with live \textit{S. dysenteriae}

\textit{S. dysenteriae} causes apoptosis in macrophages by activating caspase 1, signaling through the MAPK pathways. IpaB is sufficient to induce apoptosis in macrophages (56). This invasin is secreted by intracellular bacteria and distributed in the cell cytoplasm where it binds to a caspase, and activates apoptosis (392). Mouse Macrophage cell line, RAW 264.7 cells were pre-treated with heat-killed \textit{S. aureus} for induction of tolerisation for 18h at 37°C and thereafter subjected to hetero-tolerance induction with live \textit{S. dysenteriae} for 20h at 37°C. Following hetero-tolerisation the cells were visualized under phase contrast inverted light microscope for visual differences in cell morphology of the macrophages. And as can be seen from Fig 4.2.1.1, there seems to be a visible improvement in the cell survival rates in the microscopic field depicting cells pre-treated with heat-killed \textit{S. aureus} and thereafter induced with live \textit{S. dysenteriae} as compared to macrophages treated with live \textit{S. dysenteriae} without any pre-treatment. However, the macrophage cell-death by apoptosis has not been completely abrogated, as observed by comparing untreated macrophage cell density with the hetero-tolerised macrophages. Also in the experiments of cell viability staining done by 0.4% Trypan blue exclusion, the cell count was marginally lesser taking into account the percentage of hetero-tolerised viable macrophages as compared to the untreated macrophages (data not given). But the percentage viability was much higher of the hetero-tolerised cells in comparison to the live \textit{S. dysenteriae} treated cells, and almost equivalent to the heat-killed \textit{S. aureus} tolerised macrophages (data not given). These preliminary results were further substantiated by analysis of the induction of apoptosis post treatment of macrophages with live \textit{S. dysenteriae}. Fluorescence microscopy images show that there is a definite rescue of macrophages from apoptosis as observed in cells tolerised with heat-killed \textit{S. aureus} and thereafter challenged with live \textit{S. dysenteriae} (fig 4.2.1.2, panel E). Fewer percentage of cells are seen to exhibit green fluorescence related to annexin V binding to phosphatidylserine of the membrane, as compared to the untolerised, \textit{S. dysenteriae} treated cells, where a greater percentage of the macrophages are
**Figure 4.2.1.1 Inverted Microscope Phase contrast images at 10X magnification of hetero-tolerance induction in mouse macrophage cell line RAW 264.7.**

A: Untreated RAW 264.7 cells ($5 \times 10^5$ cells/mL)

B: Mouse macrophage RAW 264.7 treated with heat-killed *S. aureus* (18h)

C: Mouse macrophage RAW 264.7 treated with live *S. dysenteriae* (20h)

D: Mouse macrophage RAW 264.7 treated with heat-killed *S. aureus* (18h) and challenged with live *S. dysenteriae* (20h)
Figure 4.2.1.2 Fluorescence Microscopy images of hetero-tolerance induction in mouse macrophages RAW 264.7 cells

A: Staurosporine treated mouse macrophage RAW 264.7 cells
B: Untreated mouse macrophage RAW 264.7 cells
C: Mouse macrophage RAW 264.7 treated with heat-killed \textit{S.aureus} (18h)
D: Mouse macrophage RAW 264.7 treated with live \textit{S.dysenteriae} (20h)
E: Mouse macrophage RAW 264.7 treated with heat-killed \textit{S.aureus} (18h) and challenged with live \textit{S.dysenteriae} (20h)

# Apoptotic cells were stained with Annexin V (green fluorescence, Ex488nm/Em 530nm). Counter stain employed was Propidium Iodide (red fluorescence, Ex536nm/Em617nm)
stained by Annexin V, depicting pro-apoptotic cells (fig 4.2.1.2, panel D). Staurosporine treated macrophage cells were used as positive control (fig 4.2.1.2, panel A) to detect apoptotic cells and Propidium Iodide was employed as the counter stain for the fluorescence microscopy experiments.

4.2.2 Cytokine response of macrophages hetero-tolerised with live *S. dysenteriae*

Macrophage cell line, RAW 264.7 were treated with heat-killed *S. aureus* for induction of tolerisation at 37°C for 18h and thereafter induced with live *S. dysenteriae* for 20h at 37°C to analyse induction of hetero-tolerance in pre-treated macrophages. Induction of hetero-tolerance was determined by looking at the cytokine profile of the pre-treated macrophages after subjecting them to infection with live gram-negative bacteria. Cytokine ELISAs were performed for both pro- and anti-inflammatory cytokines namely TNF-α, IL-6 and IL-10 (Fig. 4.2.2.1, 4.2.2.2, 4.2.2.3).

The pro-inflammatory cytokine response of macrophages pretreated with *S. aureus* and challenged with live *S. dysenteriae* looks quite diminished in comparison to the macrophages treated only with either heat-killed *S. aureus* and only with live *S. dysenteriae*, suggesting induction of hetero-tolerisation. At the same time the TNF-α and IL-6 response of the hetero-tolerised macrophages is seen marginally higher as compared to untreated macrophages, indicating that the macrophages have not been rendered unresponsive but the inflammatory cytokine response has been down-regulated due to the induction of cross-tolerisation. This indicates that the macrophages have not been anergised but are temporarily rendered hypo-responsive to the insult because of certain re-programming events within the cell signaling machinery.

Anti-inflammatory cytokine response was also looked at by determining amounts of IL-10 released by the differently treated and untreated macrophages and it was observed that the hetero-tolerised macrophages shows diminished response as compared to the different bacteria treated macrophages but the IL-10 cytokine response of the hetero-tolerised macrophages was seen to be similar to that of untreated macrophages. This definitely suggests that the cross-tolerisation is not due to an up-regulation of anti-inflammatory cytokine response but it is pre-
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TNF alpha cytokine levels in RAW 264.7 macrophages subjected to hetero-tolerance

Induction time in hrs

Absorbance values at 450nm

Absolute Control
Staphylococcus aureus (HK) cont
Shigella dysenteriae (L) control
S. aureus (HK)+ S. dysenteriae (L)

TNF-α cytokine levels in RAW264.7 macrophages subjected to hetero-tolerance

Induction time in hrs

Percent Induction

Staphylococcus aureus (HK) cont
Shigella dysenteriae (L) control
S. aureus (HK)+ S. dysenteriae (L)
Absolute Control
**Figure 4.2.2.1** Pro-inflammatory cytokine (TNF-α) response estimated by ELISA of culture supernatants from murine macrophage cell-line RAW 264.7 subjected to induction of tolerance by *S. aureus* (HK)# and challenged with *S. dysenteriae* (L)* for induction of hetero-tolerance. Primary antibody (monoclonal anti-mouse TNF-α) used at 1:1000 dilution whereas secondary antibody (rabbit-anti-mouse HRP conjugate) was used at 1:20,000. Controls for the ELISA were:

Positive control: purified mouse TNF-α @ 1ng/mL.

Negative controls: a) [-] antigen (mouse TNF-α) [+ ] Primary antibody [+ ] secondary antibody

b) [+ ] antigen (mouse TNF-α) [- ] Primary antibody [+ ] secondary antibody

c) [- ] antigen (mouse TNFα) [- ] Primary antibody [+ ] secondary antibody

# (HK) = heat-killed * (L) = live

**NOTE:** The lower panel shows the TNF-α response in terms of percent induction as compared against values of untreated macrophages equated to zero.
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IL-6 cytokine levels in RAW 264.7 macrophages subjected to hetero-tolerance

Absorbance values at 450nm

0h 3h 6h 24h 48h
Induction in hrs

Cont(Absolute)
S.aureus (HK) cont
S.dysenteriae(L) cont
S.aureus (HK)+ S.dysenteriae (L)

Percent induction

IL-6 cytokine levels in RAW 264.7 macrophages subjected to hetero-tolerance

Percent induction

0 50 100 150 200
Induction time in hrs

S.aureus (HK) cont
S.dysenteriae(L) cont
S.aureus (HK)+ S.dysenteriae (L)
Cont(Absolute)
Figure 4.2.2.2 Pro-inflammatory cytokine (IL-6) response estimated by ELISA of culture supernatants from murine macrophage cell-line RAW 264.7 subjected to induction of tolerance by S.aureus (HK)* and challenged with S.dysenteriae (L)* for induction of hetero-tolerance. Primary antibody (monoclonal anti-mouse IL-6) used at 1:500 dilution whereas secondary antibody (rabbit-anti-mouse HRP conjugate) was used at 1:20,000. Controls for the ELISA were:

Positive control: purified mouse IL-6 @ 1ng/mL.

Negative controls: a) [-] antigen (mouse IL-6)[+]Primary antibody[+]secondary antibody
b) [+] antigen (mouse IL-6)[-]Primary antibody[+]secondary antibody
c) [-] antigen (mouse IL-6)[-]Primary antibody[+]secondary antibody

# (HK) = heat-killed * (L) = live

NOTE: The lower panel shows the IL-6 response in terms of percent induction as compared against values of untreated macrophages equated to zero.
Figure 4.2.2.3 Anti-inflammatory cytokine (IL-10) response estimated by ELISA of culture supernatants from murine macrophage cell-line RAW 264.7 subjected to induction of tolerance by S.aureus (HK)\# and challenged with S.dysenteriae (L)* for induction of hetero-tolerance. Primary antibody (monoclonal anti-mouse IL-10) used at 1:500 dilution whereas secondary antibody (rabbit-anti-mouse HRP conjugate) was used at 1:20,000. Controls for the ELISA were:

Positive control: purified mouse IL-10 @ 1ng/mL.

Negative controls: a) [-] antigen (mouse IL-10)[+]Primary antibody[+]secondary antibody
   b) [+]* antigen (mouse IL-10)[-]Primary antibody[+]secondary antibody
   c) [-]* antigen (mouse IL-10)[-]Primary antibody[+]secondary antibody

# (HK) = heat-killed * (L) = live

NOTE: The lower panel shows the IL-10 response in terms of percent induction as compared against values of untreated macrophages equated to zero.
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dominantly due to regulation of some signaling molecule(s), which might be
common to both the TLR4 and TLR2 mediated pathways. The most important and
novel finding of this study is that the phenomenon of tolerance induction is not
restricted to bacterial determinants alone, this also can be extrapolated to the
induction of tolerance and cross-tolerance with whole bacteria.

4.2.3 Analysis of DRM proteins from hetero-tolerance induced macrophages

Macrophage cells induced for hetero-tolerance were analysed for the role of the
differentially expressed membrane proteins in cross-tolerance induction. Hetero-
tolerised macrophages, tolerised macrophages, live *Salmonella dysenteriae* treated
macrophages and untreated macrophages were all processed for extraction of
DRM proteins as explained in Chapter 2. Samples were collected at 20h post
induction of hetero-tolerance and the membrane proteins were resolved by 2D gel
electrophoresis. Sample was obtained at the above mentioned time point,
subsequent to challenge with live *S. dysenteriae* after induction of tolerance of
macrophages with heat-killed *S. aureus* for 18h, because *S. dysenteriae* is known to
cause apoptosis in macrophages and the induction of macrophage apoptosis
kinetics reported earlier show maximal induction by 20h post infection. As can be
seen from the results (fig. 4.2.3.1, 4.2.3.2), a protein spot corresponding to 30-
35KDa near the 7.8-8.3 pI range is observed to be down regulated in macrophages
treated with live *S. dysenteriae* (fig 4.2.3.1, panel C). Upon LC-MS/MS
identification of the protein spot it was found to be homologous to the
iodotyrosine dehalogenase I precursor (DEHAL I). Also observed was the fact that
the amount of DRM proteins had substantially reduced in the case of the hetero-
tolerised macrophages (fig. 4.2.3.1, panel D).

4.2.4 Identification of DRM recruited proteins during hetero-tolerance
induction in macrophages

In order to analyse the presence of a few known molecules involved in induction
of hetero-tolerance in macrophages, Western blotting using antibodies for known
proteins like TLR2, IRAK 1 and CD 14 was done. As can be seen from the fig.
4.2.4.1, a faint band corresponding to approximately 70KDa can be observed. This
band that is visible corresponds to the protein CD14 which is a co-receptor protein
for LPS and is known to be a raft resident protein. Apart from this band no other
Figure 4.2.3.1 Two dimensional gel electrophoresis analysis of hetero-tolerance induction in mouse macrophage cell line RAW 264.7

(A) Untreated macrophage cells RAW 264.7, DRM*-H fraction (3-10 L)
(B) Heat-killed *S. aureus* tolerised macrophage cells RAW 264.7, DRM-H* fraction (3-10 L)
(C) Live *S. dysenteriae* treated macrophage cells RAW 264.7, DRM-H* fraction (3-10 L)
(D) Heat-killed *S. aureus* tolerised macrophage cells RAW 264.7 challenged with live *S. dysenteriae*, DRM-H* fraction (3-10 L)

* DRM-H was collected from a discontinuous sucrose step gradient as the interphase fraction between 35% and 40% sucrose steps.
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Figure 4.2.3.2 Two dimensional gel electrophoresis analysis of hetero-tolerance induction in mouse macrophage cell line RAW 264.7

(A) Untreated macrophage cells RAW 264.7, DRM*-H fraction (3-6 L)
(B) Heat-killed *S. aureus* tolerised macrophage cells RAW 264.7, DRM-H* fraction (3-6 L)
(C) Live *S. dysenteriae* treated macrophage cells RAW 264.7, DRM-H* fraction (3-6 L)
(D) Heat-killed *S. aureus* tolerised macrophage cells RAW 264.7 challenged with live *S. dysenteriae*, DRM-H* fraction (3-6 L)

* DRM-H was collected from a discontinuous sucrose step gradient as the interphase fraction between 35% and 40% sucrose steps.
distinct bands were visualized on the nitrocellulose membrane and so it can be concluded that during hetero-tolerance induction there is no assortment of any of these proteins to the rafts. Probably induction of tolerance and suppression of cytokine release is brought about in macrophages by specifically keeping out these receptor and costimulatory molecules from the rafts thereby disrupting the MyD88 pathway dependent activation of the pro-inflammatory cytokines. As observed from lane 4 (fig. 4.2.4.1), the levels of CD14 is observed to be reduced though not completely absent.

Another possibility is that these molecules are present in the lighter fraction of the DRMs isolated and hence could be specifically enriched within the lighter rafts. This could be explored by looking at the protein profile of the lighter rafts, but within the present study this could not be done as the concentration of proteins extracted was very less within the lighter fraction.

4.2.5 Analysis of the role of the rafts in induction of hetero-tolerance

Induction of hetero-tolerance has been greatly reviewed and it is well established that the immuno-modulation brought about by bacteria in macrophages though is a survival strategy for the organism but may also alter the extracellular milieu of the cells and thereby affect its physiology. The role of the membrane receptors and the down-stream signaling molecules has also been looked at in great details. However it would be interesting to look at this hetero-tolerance induction in the light of role of lipid rafts for the same. For this purpose, the cells were hetero-tolerised and prior to challenge for hetero-tolerance induction subjected to depletion of membrane cholesterol using MβCD. Thereafter the culture supernatants were analysed for cytokine profile by ELISA at time intervals of 0h, 3h, 6h, 24h & 48h. As observed from the cytokine profile, the disruption of rafts by MβCD results in heightened pro-inflammatory cytokine response, indicating that raft integrity is important for the tolerance phenomenon especially with regards to release of cytokines.
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Figure 4.2.4.1 Western Blot identification of DRM-H proteins isolated from macrophages induced for hetero-tolerance with live *S. dysenteriae*.

**Lane 1:** DRM-H from untreated RAW 264.7 cells  
**Lane 2:** DRM-H from RAW 264.7 cells tolerised with heat-killed *S. aureus*  
**Lane 3:** DRM-H from RAW 264.7 cells challenged with live *S. dysenteriae*  
**Lane 4:** DRM-H from RAW 264.7 cells hetero-tolerised.  
**Lane 5:** empty  
**Lane 6:** empty  
**Lane 7:** Protein molecular weight marker (Medium range)

Antibodies (isotype: goat) for detection of different known raft recruited proteins like CD14, TLR2, IRAK1 was used @ 2μg/mL of CD14&TLR2 and @ 1μg/mL of IRAK1. Secondary antibody used was rabbit anti-goat HRP conjugate @1:10,000 dilution.

Molecular weight of CD14= 70 KDa, TLR2= 80KDa, IRAK1= 86KDa. However in RAW264.7 membrane the antibody shows band at 45KDa in addition to the 80KDa band.
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TNF alpha cytokine levels in RAW 264.7 macrophages depleted of cholesterol and subjected to hetero-tolerance

Percent Induction

Absorbance values at 450nm

Induction time in hrs

0h 3h 6h 24h 48h

Absolute Control + MbCD
Staphylococcus aureus (HK) cont+ MbCD
Shigella dysenteriae (L) control + MbCD
S. aureus (HK)+ S. dysenteriae (L) + MbCD

TNF-α cytokine levels in RAW264.7 macrophages depleted of cholesterol and subjected to hetero-tolerance

Percent Induction

Induction time in hrs

0h 3h 6h 24h 48h

Staphylococcus aureus (HK) cont+ MbCD
Shigella dysenteriae (L) control + MbCD
S. aureus (HK)+ S. dysenteriae (L) + MbCD
Absolute Control + MbCD
Figure 4.2.5.1 Pro-inflammatory cytokine (TNF-α ) response estimated by ELISA of culture supernatants from murine macrophage cell-line RAW 264.7 subjected to induction of tolerance by S.aureus (HK)# and challenged with S.dysenteriae (L)* for induction of hetero-tolerance and subjected to cholesterol depletion by MβCD. Primary antibody (monoclonal anti-mouse TNF-α) used at 1: 1000 dilution whereas secondary antibody (rabbit-anti-mouse HRP conjugate) was used at 1: 20,000. Controls for the ELISA were:

Positive control: purified mouse TNF-α @ 1ng/mL.

Negative controls: a) [-] antigen(mouse TNF-α)[+]Primary antibody[+]secondary antibody
   b) [+] antigen(mouse TNF-α)[-]Primary antibody[+]secondary antibody
   c) [-] antigen (mouse TNFα)[-]Primary antibody[+]secondary antibody

# (HK) = heat-killed * (L) = live

NOTE: The lower panel shows the TNF-α response in terms of percent induction as compared against values of untreated macrophages equated to zero.
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IL-6 cytokine levels in RAW 264.7 macrophages subjected to cholesterol depletion and hetero-tolerance

Absorbance values at 450nm

Induction time in hrs

Percent Induction

0h 3h 6h 24h 48h

Induction time in hrs
Pro-inflammatory cytokine (IL-6) response estimated by ELISA of culture supernatants from murine macrophage cell-line RAW 264.7 subjected to induction of tolerance by S.aureus (HK) and challenged with S.dysenteriae (L) for induction of hetero-tolerance and subjected to cholesterol depletion by MβCD. Primary antibody (monoclonal anti-mouse IL-6) used at 1:500 dilution whereas secondary antibody (rabbit-anti-mouse HRP conjugate) was used at 1:20,000. Controls for the ELISA were:

Positive control: purified mouse IL-6 @ 1ng/mL.

Negative controls: a) [-] antigen (mouse IL-6) [+ ]Primary antibody [+ ]secondary antibody

b) [+ ]antigen (mouse IL-6) [- ]Primary antibody [+ ]secondary antibody
c) [- ]antigen (mouse IL-6) [- ]Primary antibody [+ ]secondary antibody

# (HK) = heat-killed * (L) = live

**NOTE:** The lower panel shows the IL-6 response in terms of percent induction as compared against values of untreated macrophages equated to zero.
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IL-10 cytokine levels in RAW 264.7 macrophages depleted of cholesterol and subjected to hetero-tolerance

![Graph](image)

Fold Induction Absorbance values at 450nm

Induction time in hrs

- Absolute Control + MbCD
- Staphylococcus aureus (HK) cont + MbCD
- Shigella dysenteriae (L) Control + MbCD
- S. aureus (HK) + S. dysenteriae (L) + MbCD

![Graph](image)

IL-10 cytokine levels in RAW 264.7 macrophages depleted of cholesterol and subjected to hetero-tolerance

Fold Induction

Induction time in hrs

- Staphylococcus aureus (HK) cont + MbCD
- Shigella dysenteriae (L) Control + MbCD
- S. aureus (HK) + S. dysenteriae (L) + MbCD
- Absolute Control + MbCD
Figure 4.2.23 Anti-inflammatory cytokine (IL-10) response estimated by ELISA of culture supernatants from murine macrophage cell-line RAW 264.7 subjected to induction of tolerance by S.aureus (HK)# and challenged with S.dysenteriae (L)* for induction of hetero-tolerance and subjected to cholesterol depletion by MβCD. Primary antibody (monoclonal anti-mouse IL-10) used at 1: 500 dilution whereas secondary antibody (rabbit-anti-mouse HRP conjugate) was used at 1: 20,000. Controls for the ELISA were:

Positive control: purified mouse IL-10 @ 1ng/mL.
Negative controls: a) [-] antigen (mouse IL-10)[+] Primary antibody[+] secondary antibody
                  b) [+] antigen (mouse IL-10)[-] Primary antibody[+] secondary antibody
                  c) [-] antigen (mouse IL-10)[-] Primary antibody[-] secondary antibody

# (HK) = heat-killed * (L) = live

NOTE: The lower panel shows the IL-10 response in terms of percent induction as compared against values of untreated macrophages equated to zero.
4.3 DISCUSSION

Endotoxin (lipopolysaccharide, LPS) is a molecule found in the cell wall of Gram negative bacteria. Injection of endotoxin initiates a dramatic proinflammatory response that, if untreated, can result in profound hemodynamic derangements and may even progress to shock and death. Interestingly, animals that survive a sublethal exposure to endotoxin are resistant to subsequent challenges with normally lethal doses of LPS during the first few days following the initial exposure (126). Further, exposure to endotoxin may confer resistance to inflammation and injury induced by other challenges including myocardial infarction (257), neural ischemia (214), and gastric insults (199). Although the cytokine profile in LPS tolerant animals is similar to that seen in septic patients, innate immune function in the LPS tolerant state actually seems improved in that bacterial clearance is enhanced and mortality is suppressed after a Gram negative bacterial challenge (216). It was previously shown that LPS pretreatment not only diminishes the inflammatory response but also appears to enhance the effectiveness of the innate immune system to clear both Gram-negative and Gram-positive bacterial challenges. Although Gram-negative bacteria have historically been a major cause of sepsis in clinical patients, the incidence of Gram-positive bacterial sepsis has risen significantly over the last decade. During the same time, investigators have advanced our understanding of the process employed by innate immune cells to detect, and react to, a myriad of microbial organisms (268).

The capacity of circulating leukocytes to respond to endotoxin is impaired in the case of human sepsis, trauma, major surgery, or any systemic inflammatory response syndrome (SIRS) (235). This immune dysregulation is often referred to as tolerance to endotoxin. An alteration in the NF-kB signalling pathway within such tolerized macrophages and within monocytes from patients with sepsis and patients with trauma has been proposed to be responsible for this effect (4). In contrast to the tolerance to endotoxin defined entirely in in vivo or in vitro experimental models, however, analysis of patients has been based on ex vivo experiments. Such studies monitored the consequence of an in vivo insult (e.g., infection, ischemia/reperfusion, blood loss, tissue injury, or endotoxin translocation) in an in vitro culture of isolated leukocytes in the presence of LPS. Although injection of LPS into human volunteers leads to a decreased capacity of
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circulating mononuclear cells to produce cytokines ex vivo (419), the characterization of ex vivo tolerance to endotoxin in animal models has been limited (98).

There is growing evidence for functional plasticity in macrophages as well as for their capacity to adapt reversibly to changing microenvironments. Macrophages can be selectively reprogrammed to a specific phenotype of immune response following relatively short-term exposure to microbial ligands (237). Polarization of human macrophages into proinflammatory type 1 and anti-inflammatory type 2 occurs in response to microbial antigens, and IFN-γ and CD40L-mediated costimulation has been recently reported (408). Depending on the agonist employed, macrophages display different patterns of function relative to cytokines and enzymes produced, and these might not display a strict dichotomy between type 1 and type 2 responses (261). Particularly critical to such macrophage reprogramming are the effects of prior exposure to specific cytokines. It is tempting to speculate that the sensitized macrophage phenotype observed herein is the product of the cytokine milieu generated by treatment with LPS or other TLR agonist. Mechanisms for macrophage reprogramming are as yet poorly understood. One alternative attractive mechanism is chromatin remodeling which has been proposed to mediate innate phenotype changes in several immune and inflammatory disease models (309, 369, 215). Interestingly, TLRs have now been identified as therapeutic targets for a number of diseases (246). Synthetic TLR agonists such as imiquimod, which activates TLR 7/8, are currently being used clinically to treat skin diseases (295).

Previous studies suggested that hypo-responsiveness after pretreatment with LPS was mediated by the action of endogenous mediators such as IL-10, TGFβ, or IL-1β produced on primary or secondary LPS stimulation. Others postulated the existence of soluble yet unidentified suppressor molecules of TNF expression during LPS tolerance (23). But findings that macrophages from mice deficient in TLR2 or carrying a nonfunctional mutant of TLR4 were not rendered refractory by LTA or LPS pretreatment, respectively, when cocultured with wild-type macrophages argue against soluble mediators of desensitization unless factors requiring the presence of TLR2 or TLR4 (i.e., yet unknown soluble ligands of TLR2 or TLR4) are involved (216). However, we cannot rule out that the effects
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of LPS/LTA pretreatment in vivo, i.e., protection against LPS shock and liver injury, are mediated in part by endogenously produced cytokines. Thus, macrophage-derived products such as TNFα and IL-1β, which are released during the LPS/LTA pretreatment, might contribute to the desensitization of target cells (e.g., hepatocytes) against the toxicity of subsequent LPS/LTA challenge in vivo, e.g., by inducing heat-shock and other acute phase proteins (41). In the present study the induction of tolerance in murine macrophages upon treatment with heat-killed *S. aureus*, is observed from the cytokine release profile observed for both pro- and anti-inflammatory cytokine response which shows marginal increase initially and subsiding soon thereafter.

Tolerance to endotoxin in vivo has been defined in terms of reduced fever in experimental models after repeated injections of bacteria or endotoxin (LPS) or in infected patients after a single injection of bacteria or LPS (278). This finding was further corroborated by the observation of reduced levels of certain circulating cytokines, particularly tumor necrosis factor (TNF), in both tolerized animals and human volunteers. Macrophages have been shown to contribute in vivo to tolerance to endotoxin, which is consistent with the fact that in vitro cultures of LPS primed monocytes/macrophages produce diminished levels of TNF and other cytokines on subsequent challenge with LPS (130). Although suggestive, these in vitro experiments, however, are unlikely to accurately reflect the in vivo process in which, for example, glucocorticoids have also been implicated in the etiology of tolerance to endotoxin (382).

Although tolerance to endotoxin has long been thought to be specific to LPS, it is now recognized that this is not the case. Recent experimental models of tolerance to endotoxin have demonstrated a cross-tolerance with whole bacteria (201), bacterial toxin, and Toll-like receptor (TLR) ligands. Hyporeactivity of circulating leukocytes has been demonstrated not only with LPS but also with *Streptococcus pyogenes*, *S. aureus*, and superantigens, in patients with sepsis, patients undergoing surgery, or human volunteers receiving an injection of LPS. However, it has been shown that pretreatment of mouse peritoneal macrophages with LPS primed the cells to a further-enhanced production of TNF induced by heat-killed *S. aureus* (42), and it has been shown that circulating leukocytes from resuscitated patients after cardiac arrest and from patients with trauma had an ability to
produce TNF in response to heat-killed *S. aureus* that was similar to that of healthy control subjects (94).

Another layer of complexity was added when it became clear that TLRs differ in their requirements for signal transduction molecules (7, 285). All TLRs seem to use a common signalling pathway which involves the recruitment of the adaptor protein myeloid differentiation factor 88 (MyD88). However, additional adaptor proteins have been identified which mediate TLR-specific signal transduction (432, 96, 154, 290). Thus, in TLR-4 signalling, toll-interleukin 1 receptor domain containing adaptor protein (TIRAP)/ MyD88 adaptor like protein (Mal) can partly substitute for MyD88. Furthermore, TLR-3 and TLR-4 make use of toll-interleukin 1 receptor domain containing adaptor inducing interferon beta (TRIF)/toll-interleukin 1 receptor domain (TIR) containing adaptor molecule (TICAM)-1 and TRIF related adaptor molecule (TRAM)/TICAM-2, thereby inducing interferon (IFN)-b, which in turn activates a set of IFN-inducible genes in a paracrine mode. In the light of this knowledge, Sato et al. observed that MALP-2, while inducing cross-tolerance for MyD88-dependent LPS signalling, was not able to confer tolerance for the MyD88-independent LPS-specific signalling pathway (330). Also, TLR-7 only induced tolerance for MyD88-dependent LPS genes, while TLR-3-mediated cross-tolerance was restricted to MyD88-independent signalling. More generally, it was stated that tolerance affects more genes than cross-tolerance (78). Thus, tolerance as well as cross-tolerance can be differentially developed depending on the specific TLR-activating compound.

Diarrhea-causing pathogens employ a variety of sophisticated strategies to colonize the intestinal epithelium. In essence, ingested pathogens have evolved the abilities to: (1) resist non-specific host defenses, such as stomach acidity, peristalsis, mucosal cell exfoliation, intestinal mucins, and bacteriocins (261); (2) adhere to intestinal epithelia; and (3) ultimately colonize the epithelia. Colonization may, or may not, involve cellular invasion. When cellular invasion occurs, it can be followed either by intracellular multiplication and spread of the bacteria to other tissues, or by bacterial persistence. Although bacterial pathogens employ different strategies, they share common targets in the host cell and often cause the same cellular responses. Virulence factors act either from the extracellular milieu mimicking cell ligands, or are injected into the intracellular...
milieu, where they act in concert to manipulate signaling pathways, for example to subvert the plasticity of the cytoskeleton, interrupt the endocytic traffic, and, in some cases, mediate resistance to phagocytosis. The host cell, in turn, defends itself against infection by initiating an inflammatory response and by altering the intestinal fluid balance in order to extrude the unwanted bacteria (311).

In *Shigella*, the bacterial proteins responsible for adhesion are the same as those that initiate the process of invasion. Following adherence, *Shigella* initiates activation of T3SS and secretion of effector proteins into the host cell. The genes encoding the translocon itself and the effector proteins, including four bacterial chaperons, are located in a 31-kb pathogenicity island within a large virulence plasmid (213 kb) (311). After traversing M cells, *Shigella* exposes itself to an area of the follicle associated epithelium (FAE) intensively populated with macrophages and dendritic cells. There, like *Salmonella*, it triggers macrophage pyroptosis: the translocated IpaB activates caspase-1, which in turn cleaves IL-1β and IL-18 to their mature, active forms. Besides requiring membrane cholesterol (335), activation of caspase-1 by IpaB appears to require Ipaf, a Nod-like receptor localized on plasma and endosomal membranes and implicated in bacterial recognition (377). IL-1β release causes rupture of the epithelial barrier enhancing bacterial dissemination, which in turn increases inflammation and subsequent tissue destruction. Conversely, the parallel release of IL-18, a potent interferon-γ inducer, aids host killing of *Shigella* by activated immune cells, thus demonstrating a protective role in shigellosis (418). In the present investigation, this property of induction of apoptosis in macrophages was looked at and subsequent to hetero-tolerance induction, the hypothesis was that the macrophages challenged with live *S. dysenteriae* should be rescued from apoptotic cell death due to induction of hetero-tolerance. As seen from the results of fluorescence microscopy this indeed seems to be true. There is a decrease in the cell death by apoptosis in macrophages which were tolerised with heat-killed *S. aureus* in comparison to those untolerised macrophages challenged with live *S. dysenteriae*.

Ion channels have been demonstrated to be a central element in the induction and the execution of apoptosis. In particular, mitochondrial ion channels, including not only the permeability transition pore but also a mitochondrial, ATP-sensitive (mKATP) channel as well as a mitochondrial calcium-activated potassium channel

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are involved critically in apoptotic changes in mitochondria. Ion channels in the cell membrane that are altered by induction of apoptosis include potassium, chloride and calcium channels. The Kv1.3 potassium channel belongs to the best-characterized ion channels involved in apoptosis and a genetic model of cells deficient for Kv1.3 has indicated a critical role for Kv1.3, at least in some forms of apoptosis. The mechanisms regulating ion channels during apoptosis are, however, still poorly defined. Earlier studies have suggested a function for distinct membrane domains, termed rafts, in the cell membrane for the regulation of ion channels during apoptosis. Small sphingolipid- and cholesterol-enriched membrane domains are modified by many apoptotic stimuli to form large ceramide-enriched membrane platforms. These platforms serve to cluster receptor molecules, to re-organize intracellular signalling molecules including ion channels, to bring ion channels into close contact with their regulators and/or to separate proteins from a specific ion channel. Finally, the lipid composition of the cell membrane might be involved directly in ion channel regulation (382).

Lipid rafts are subdomains of the plasma membrane that contain high concentrations of cholesterol and sphingolipids. In response to intra or extracellular stimuli, lipid rafts can include or exclude proteins to variable extents. This favors specific protein-protein interactions and modulates the activity of signaling cascades. Recently, a number of proteins involved in apoptotic signals have been located in lipid rafts. Among these proteins is included Bad, a pro-apoptotic molecule belonging to the Bcl-2 family. Bad is attached to lipid rafts in proliferating cells while associated to mitochondria in apoptotic cells, suggesting that the interaction of Bad with rafts is a dynamic process involved in the control of apoptosis (112). Studies involving the role of lipid rafts in the induction of apoptosis in macrophages indicate that they play a very important role in segregating the pro-apoptotic molecules. Therefore, during induction of tolerance and hetero-tolerance it was interesting to look at the role of lipid rafts, if any, in the induction of apoptosis and also rescue of macrophages from apoptosis subsequent to induction of hetero-tolerance. For this the cytokine profile of the macrophages was looked at subsequent to tolerisation and disruption of rafts with MβCD, and it was observed that the cytokine response was of very high
magnitude when rafts were disrupted indicating that induction of tolerance and hetero-tolerance in macrophages is a raft-dependent phenomenon.

Also looked at was the raft proteins subsequent to induction of tolerance and hetero-tolerance and a specific low molecular weight protein (32KDa) was found to show differential recruitment to raft. This protein was shown to be homologous to iodotyrosine dehalogenase I (DEHAL I) precursor, which is an integral membrane protein and employs NADPH as a co-factor. DEHAL I is responsible for the release of iodide from thyroglobulin and thereby aiding in the release of the hormone from the thyroid. This protein was not found in the membrane profile of macrophages treated with only live *S. dysenteriae*, suggesting a role for this protein exclusion from rafts to be responsible for the induction of apoptosis within macrophages either directly or indirectly. The possible role could be that since, *S. dysenteriae* is an intracellular pathogen, it could be secreting certain molecules which might be either forming a complex with the DEHAL I or modifying the protein such that it prevents the translocation of this enzyme within the lipid rafts. This possibly could be the pro-apoptotic pathway within the macrophages. Further studies are required to confirm this hypothesis.

Also looked at was the pattern of distribution of some known proteins of the pathway involved in the induction of tolerance and hetero-tolerance like different receptors and co-stimulatory molecules. In this study the rafts were analysed for differential recruitment of TLR 2, IRAK 1 and CD 14. CD 14 is known to be a raft-resident protein and therefore it can also be employed as a marker for the rafts. Western blot analysis shows absence of TLR2 and IRAK 1 from the DRM-H in all the treated and untreated macrophages. At the same time all the differently treated samples shows presence of a 70 KDa band corresponding to CD14, however the level of this protein was observed to be reduced as per the intensity, and this co-receptor protein could be contributing towards induction of hetero-tolerance because it is known to mediate recognition of the gram-negative bacterial ligand (LPS) through the formation of the CD14/TLR4/MD2 complex. Recent findings have shown that the recruitment of TLR2 within lipid rafts is modulated by soluble TLR2 (sTLR2) by acting as a decoy receptor for bacterial ligands and abrogating the binding of TLR2 with its co-receptor protein CD14. This is suggested to be the mechanism by which the inflammatory response is regulated during conditions of
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tolerance induction within macrophages (307). The present study also supports this finding as the membrane protein analysis shows absence of TLR2 protein within the rafts. Also, the lack of TLR2 expression within rafts in the macrophages treated with live S. dysenteriae indicates that S. dysenteriae does not employ TLR2 recognition system within macrophages. This further supports the findings that gram-positive bacteria are recognized by the TLR2 receptor whereas gram-negative bacteria are not. The absence of any detectable TLR2 within rafts isolated from untreated macrophages, suggests that TLR2 receptors are recruited to rafts only subsequent to infection with bacteria.

IRAK-1 is rapidly phosphorylated, ubiquitinated and then degraded via the proteasome following its activation by TLR ligands or IL-1 (157). Such degradation of IRAK-1 may represent a negative feedback mechanism to prevent prolonged activation of cells in response to TLR ligands or IL-1. IRAK-1 is also subject to negative regulation via its interaction with Tollip and IRAK-M (445). Tollip forms a complex with IRAK-1 in resting cells and prevents its activation in the absence of an appropriate stimulus (e.g. TLR ligand) (445). IRAK-M is a catalytically inactive kinase that suppresses IRAK-1 function by inhibiting phosphorylation of IRAK-1 or preventing its release from the receptor complex (196). The observation that no IRAK 1 is detectable post induction of hetero-tolerance is also in agreement with the finding that IRAK 1 is also involved in the induction of tolerance within macrophages.

Thus it can be concluded that tolerance and hetero-tolerance is induced within macrophages subsequent to treatment with whole bacteria also as has been already documented with the various bacterial determinants. Also the induction of cross-tolerance within macrophages, leads to a reduction of cell death by apoptosis, which is the mechanism employed by gram-negative pathogenic S. dysenteriae, to circumvent the host immune response.

It can also be inferred from the findings presented here that macrophage membrane rafts plays a crucial role in the induction of tolerance and hetero-tolerance within macrophages and DEHAL I might additionally contribute to this, by inhibiting apoptosis induction by S. dysenteriae, by recruiting to the membrane rafts within the tolerised macrophages. Also observed is the absence of TLR2 from
the rafts, which results in the reduced inflammatory cytokine levels observed in conditions of tolerance and hetero-tolerance.