MODULATION OF TOLL-LIKE RECEPTOR RESPONSES WITH SYNTHETIC LIGANDS AND ITS APPLICATION TO VACCINE DESIGN

by

JIDNYASA GAEKWAD

(Under the Direction of Geert-Jan Boons)

ABSTRACT

Lipopolysaccharides (LPS), which are derived from the outer membrane of gram-negative bacteria, are ligands for Toll Like receptor 4 (TLR4) and have a potential for the development of immune modulators for the treatment of various diseases. However, an important concern of such immune modulators is that over activation of innate immunity may lead to clinical symptoms of sepsis. Hence, a detailed knowledge of structure-activity relationships is important for the design of safe immune modulators.

Various homogenous synthetic lipid A derivatives derived from *E. coli*, *S. typhimurium* and *N. meningitides* LPS and LOS have been employed for a systematic structure-activity relationship study of activation of TLR4 in two mouse macrophage cell lines. The use of the cell lines made it possible to examine a wide range of compounds over a wide range of concentrations on a sufficiently large scale for measuring the production of a panel of cytokines and chemokines corresponding to the two distinct TLR4 activation pathways. Although an additive model could describe the structure activity relationship, some differential activation of

the TLR4 receptor complex leading to differential induction of the pro-inflammatory responses, was observed. However, unlike other studies, no bias towards MyD88- or TRIF-dependent TLR4 induction was detected. Quantitative real-time PCR studies indicated that the modulation of pro-inflammatory responses by the different lipid As occurs upstream of the transcription. In addition, it was shown that modulation occurs in the secretion of IL-1β induced by different lipid A's due to differential activation of the inflammasome complex and differential secretion.

Over-activation of the TLR4 receptor complex can lead to the clinical symptoms of septic shock. Compounds such as synthetic tetra-acylated *P. gingivalis* lipid As that can antagonize cytokine production induced by enteric LPS may have the potential to be developed as therapeutics for the treatment of gram-negative sepsis.

In addition to antimicrobial activities, cytokines also activate and regulate the adaptive immune system. Efficient priming of adaptive immune responses requires presentation of antigen in the context of major histocompatibility complex (MHC) and induction of accessory signals (costimulators and cytokines) on antigen presenting cells (APCs). TLRs expressed on APCs may regulate the accessory signals through their recognition of PAMPs and consequently control activation of antigen-specific adaptive immune responses. To test this hypothesis, a three - component cancer vaccine candidate with Pam_3CysSK_4, a TLR2 ligand as an adjuvant was studied. It has been shown that the TLR2 agonist promotes uptake of the vaccine candidate by macrophage cells. Hence, the immune activation property of a TLR2 ligand can be exploited to increase the antigenicity of otherwise poorly immunogenic tumor associated carbohydrate antigens.

Keywords: Lipopolysaccharide, Toll-like receptors, Sepsis, Cancer Vaccine, Glycolipopeptide

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DEDICATION

To my wonderful parents, loving husband, and all the teachers

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LIST OF ABBREVIATION

AA	any of the 20 natural amino acid
APAF-1	apoptotic peptidase activating factor -1
APC	antigen presenting cells
ASC	apoptosis associated speck like protein containing a CARD
ATP	adenosine triphosphate
BCG	bacille calmette guerin
BMDM	bone marrow derived macrophages
BSA	bovine Serum Albumin
CARD	caspase recruitment domain
CBD	chitin binding domain
CTL	cytotoxic T-lymphocytes
DC	dendritic cell
DC-SIGN	dendritic cell specific ICAM3 grabbing non-integrin
DNA	deoxy ribonucleic acid
DPC	dodecylphosphocholine
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
GAG	glycosylamino glycan
GalNAc	*N*-acetyl galactosamine

Glc	glucose
GlcNAc	*N*-Acetyl glucosamine
Gn.HCl	guanidinium hydrochloride
GPI	glycosylphosphotidylinositol
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
ICE	IL-1β- converting enzyme
IFN-α	interferon α
IFN-β	interferon β
IgG	immunoglobulin G
IgM	immunoglobulin M
IκB	inhibition of NF-κB
IKK	IκB kinase
IL-1	interleukin-1
IL-6	interleukin-6
IL-R	interleukin-1 receptor
iNOS	inductible nitric oxide synthase
IP10	interferon inducible protein-10
IPAF	ICE-protease activating factor
IRAK	IL-1 receptor-associated kinases
IRF	interferon regulatory factor
kdo	2-keto-3-deoxy-D-manno-octulosonate

KLH	keyhole limpet hemocyanine
LBP	lipid binding protein
LOS	lipooligosaccharide
LPS	lipopolysaccharide
LRR	leucine rich repeat
LTA	lipoteichoic acid
MaI	MyD88-adapter like
MAPK	mitogen activated kinase like protein
MCP	monocyte chemoattractant protein
MCF7	human breast adenocarcinoma cell line
MDP	muramyl dipeptide
MHC	major histocompatibilty complex
MM6	mono mac 6
mM	millimolar
mmol	millimole
MPLA	monophosphoryl lipid A
MUC1	mucin 1 glycoprotein
MyD88	myeloid differentiation primary response gene 88
NALP	NACHT-LRR-and pyrin domain containing protein
NCL	native chemical ligation
NF-κB	nuclear factor-kappa B
NK cell	natural killer cell
NOD	nucleotide binding oligomerization domain

OM	outer membrane
Pal	palmitoyl
Pam_3Cys	*N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-propyl]-(*R*)-Cysteine
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PPEtn	phosphoethanolamine
PRR	pattern recognition receptors
PYD	pyrin domain
RANTES	regulated upon activation normal T-cell expressed and presumably secreted
RICK	receptor-interacting serine/threonine kinase
RIG-1	retinoic acid inducible gene 1
mRNA	messenger ribonucleic acid
SARM	sterile α and armadillo motif containing protein
SDS	sodium dodecyl sulfate
SK-MEL-28	human melanoma cells
SPPS	solid phase peptide synthesis
STAT1	signal tranducer and activation of transcription 1
SUV	small unilamellar vesicles
TAA	tumor associated antigen
TACA	tumor-associated carbohydrate antigen
TBK1	TANK binding kinase 1

TCR	T-cell receptor
TEM	transmission electron microscopy
TIR	toll/IL-1R domain
Th1/Th2	T-helper cell 1/ T-helper cell 2
TIRAP	toll/IL-1R (TIR)-domain-containing adaptor protein
TIR	toll-interleukin-1 receptor domain
TLR	toll like receptors
Tn	*N*-α-Fmoc-Thr-(AcO_3-α-D-GalNAc)-OH
TNF-α	tumor necrosis factor-α
TRD	tandem repeat domain
TRAF6	TNF receptor activated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter protein inducing IFNβ
TT	tetanus toxoid
QS-21	*Quillaja sapon*

CHAPTER 1

Introduction

The immune system is one of the important mammalian organ systems. The cells and molecules of the immune system collectively respond to foreign substances that interact with the host. The mammalian immune system is classified into two broad categories: innate (natural/inherent) and adaptive (acquired) immunity. The innate immune system is the first line of defense and consists of physical barriers such as skin and mucous membranes, and physiological barriers such as elevation in temperature and acid in organs of the digestive system. Adaptive immune responses follow innate immune response and are very specific for the invading foreign substance[1,2]. The adaptive immune system is classified into humoral immunity and cell mediated immunity (Figure 1.1).

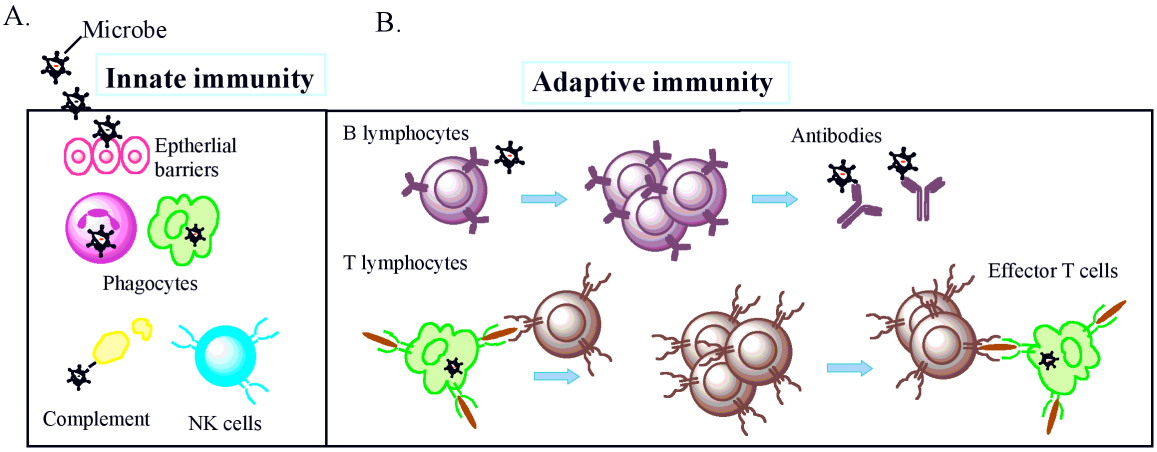

Figure 1.1: Components of innate (A) and adaptive (B) immune system

Charles Janeway coined the term innate immunity and predicted the existence of pathogen-specific pattern-recognition receptors (PRRs) in the late 1980s[3]. PRRs are a set of germline-encoded receptors evolved in host organisms able to recognize microorganism-specific

molecules that are unique to pathogens and called pathogen associated molecular patterns (PAMPs). PRRs can be either secreted proteins present in the body fluids or can be expressed on the cell surface or expressed in the cytoplasm. Functions of PRRs include opsonization of bacteria and viruses for phagocytosis or activation of lectin pathways of complement or uptake of pathogens by phagocyte and dendritic cells or triggering of signaling pathways[4,5].

PAMPs represent molecular structures that are exclusively produced by microbial pathogens but not by host organisms such as peptidoglycan and lipopolysaccharide (LPS). These molecular structures are essential for survival of the microbes and are often shared by large groups of microorganisms. For example all bacteria have peptidoglycan and lipoprotein in the periplasm while, LPS and lipoteichoic acid (LTA) is shared by all gram-negative and gram-positive bacteria respectively and is present in the outer leaflet of the outer membrane. Hence recognition of PAMPs not only indicates the presence of infection, but also provides information regarding the type of invading pathogen. These molecular structures unique to pathogens are recognized by Toll-like receptors (TLRs), Dectin-1, NACHT-,LRR-and pyrin-domain-containing protein (NALP), nucleotide-binding oligomerization domain (NOD), and retinoic-acid-inducible gene I (RIG-I)[6,7].

1.1 Toll-like Receptors:

Toll proteins in the form of receptors were first discovered in *Drosophila* and later in mammals. Both human and *Drosophila* Toll proteins are transmembrane receptors that contain an extracellular leucine-rich repeat (LRR) domain and a cytoplasmic signaling domain, which is homologous to the cytoplasmic domain of the interleukin-1 receptor (IL-1R), and is therefore referred to as the Toll/IL-1R (TIR) domain. The *Drosophila* Toll protein has been found to be required for resistance to fungal infection. *Drosophila* carrying a loss-of-function mutation in the

toll gene, quickly succumb to infections with fungal pathogens, mainly because they are unable to induce the expression of a major antifungal peptide, drosomycin[8]. Flies carrying mutations in another *Drosophila* TLR, showed a complementary phenotype, in that this mutation mainly affected their ability to recognize gram-negative bacterial pathogens and to induce expression of an anti-gram-negative bacterial peptide, attacin. These observations clearly suggest that different members of the Toll family in *Drosophila* may be specialized for recognition of different classes of pathogens. Later it was determined that this recognition is presumably mediated by PAMPs, that is the characteristic of a given pathogen class. An important implication of these studies is that Drosophila, an organism that lacks adaptive immunity and relies entirely on the innate immune system, can not only detect the presence of infection, but can also determine the type of invading pathogen[4]. Similarly mammalian TLRs are also able to discriminate between different classes of pathogens.

TLRs are an important class of mammalian PRRs that recognize structural components unique to a wide range of pathogens such as protozoa, bacteria, fungi, and viruses[9]. Interaction of PAMPs with TLRs results in the activation of downstream signal transduction pathways, which results in triggering of pro-inflammatory responses. Mammalian TLRs are a family of at least 13 paralogues (10 in humans and 12 in mice) membrane proteins. Some TLRs are expressed at the cell surface (*eg*. TLR2, TLR4, TLR1), whereas others are expressed on the membrane of endocytic vesicles (*eg*. TLR9) or other intracellular organelles (*eg.* TLR3)[6]. TLR ligands include lipopeptides (TLR2), lipopolysaccharide (TLR4), flagellin (TLR5), CpG DNA (TLR9), single-stranded RNA (TLR7), and double-stranded RNA (TLR3), which are specific for different classes of pathogens (Figure 1.2). Recognition of these ligands can occur both on the cell surface and inside the cell depending on the sub-cellular localization of each individual TLR[10].

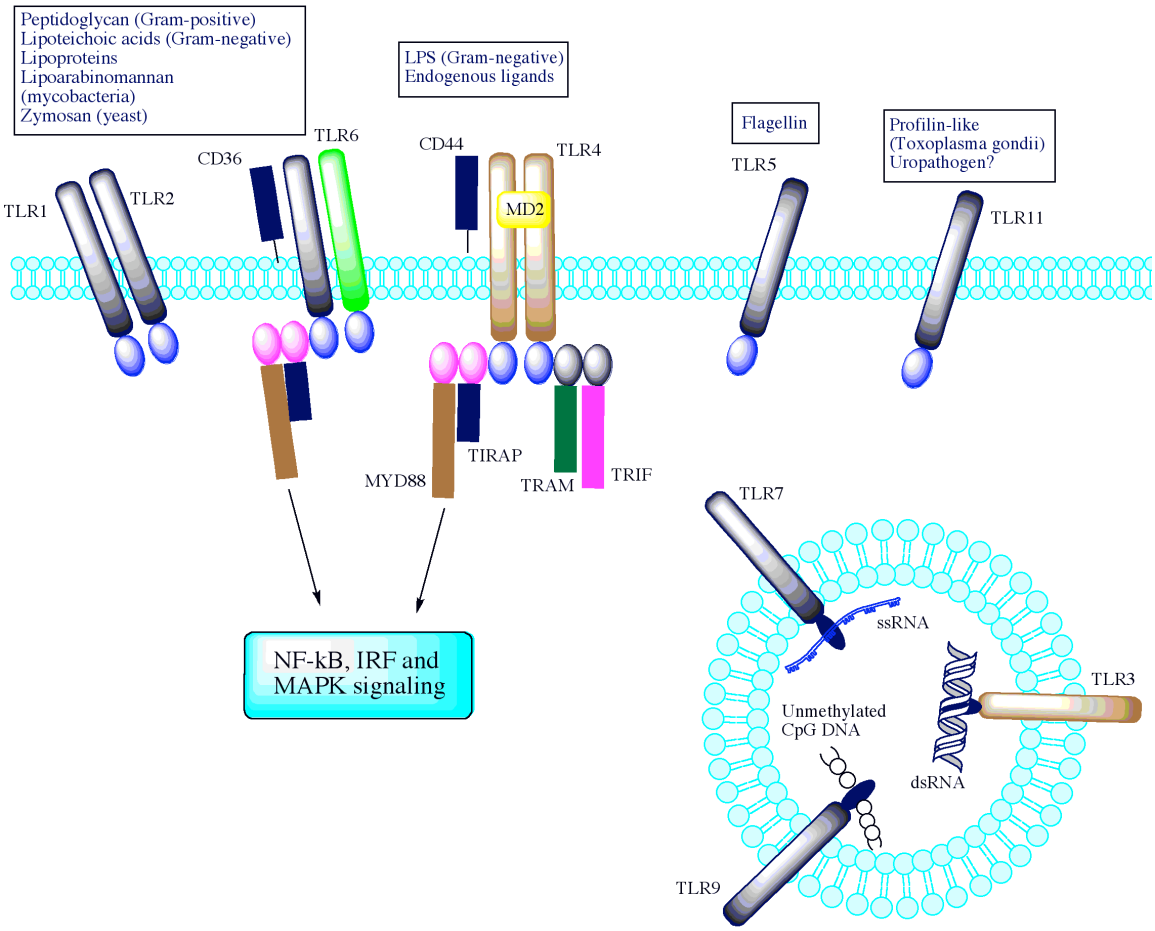

Figure 1.2: Localization of TLRs and their ligands [11].

The induction of defense mechanism by TLRs is mediated through transcriptional activation of immune response genes. Ligand binding to the extracellular domain of TLRs initiates a complex signal-transduction cascade, which ultimately leads to activation of transcription factors nuclear factor-κB (NF-κB) and interferon (IFN)-regulatory factor (IRF), which leads to increased transcription of pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1β, interferon-β (IFN-β), and tumor-necrosis factor (TNF)[5].

The cytosolic domain that is shared by all TLRs has high similarity to the cytosolic domain of the IL-1 cytokine receptor and is essential for downstream signaling. Upon ligand

binding, this domain associates with an adaptor protein, which is necessary for the recruitment of the protein kinases and trigger downstream signaling. There are now five identified cytosolic TIR-containing adaptor proteins Myeloid-differentiation primary-response gene 88 (MyD88), Mal [12,13](MyD88-adaptor like, also called TIRAP), TRIF (TIR domain-containing adaptor that inducing IFN-β, also called TICAM-1), TRAM (TRIF-related adaptor molecule), and SARM (sterile α and armadillo motif containing protein). In addition to the conserved NF-κB activation pathway, there are distinct signaling pathways initiated by individual TLRs that are dependent upon recruitment of specific adaptor proteins. Although nearly all TLRs recruit MyD88, only some recruit Mal, Tram, and TRIF, giving rise to specificity in signaling. TRIF is critical for signaling by LPS *via* TLR4 and for signaling by poly I:C *via* TLR3 leading to the expression of interferon-β (IFN-β), whereas TRAM is required for TLR4 signaling only. No specific role for Mal was described until it was shown that Mal deficient macrophages show total absence of pro-inflammatory cytokine production on either TLR2 or TLR4 stimulation. Later, it was shown that interaction between Mal and TRAF6 results in regulation of serine phosphorylation of the p65 subunit of NF-κB that renders NF-κB active as a transcription factor[14,15]. A specific role for SARM has not yet been described.

Athough, TLRs are an important system for extracellular as well as intracellular microbial sensing, they are not the only PRRs with this function. At the cell-surface, C-type lectin-like molecules, such as the mannose receptor and dectin-1 receptors, also participate in the binding and uptake of microbial components. Components of bacteria and viruses that gain entry into the cytoplasm are recognized by cytosolic receptors such as NALP, NOD, and RIG-I. RIG-I activates IRF-3 resulting in upregulation of type-I interferons, dectin-1 activates NF-κB, NODs

activate NF-κB as well as caspase-1, and NALP activates caspase-1, and all lead to induction of cytokine production and cell activation[6].

1.2 Lipid A-TLR4 Ligand:

Gram-negative bacteria are defined by the presence of outer membrane (OM), which is a unique cellular structure enclosing the peptidoglycan. The OM maintains the shape and integrity of the bacterial body, reacts to changes in environment, inhibits entry of toxic compounds into the cell, and plays an important role in nutrient sensing and transport. The OM is an asymmetric lipid bilayer that is composed of various glycerophospholipids in the inner leaflet and nearly exclusively of the glycolipid LPS in the outer leaflet (Figure 1.3). In *E. coli* and other closely related enteric bacteria, there are ~10^6 LPS molecules per cell covering nearly three quarters of the total outer cell surface area accounting for 30% of the total OM gross weight.[16] LPS is the main OM surface-associated antigen and is involved in a diverse spectrum of physiological and pathophysiological interaction of bacteria with host organism. Hence LPS is referred to as endotoxin. LPS is a complex glycolipid conceptually divided into three regions: the OM-embeded lipid A, an oligosaccharide core, and an O-specific hydrophilic polysaccharide chain that determines the antigenic specificity of the strain[17]. Lipid A is the most conserved LPS domain among gram-negative bacterial genera. Since it is the structural component responsible for most of the biological activities within the host, it represents the endotoxic principle of LPS. The oligosaccharide core connects lipid A to the hypervariable polysaccharide chain, and is further divided into the inner and outer oligosaccharide core regions. The outer core is less well conserved, varying both in saccharide composition and glycosidic linkages while the majority of gram-negative bacteria have an elaborate inner core containing at least one 2-keto 3-deoxy-D-manno-octulosonate (Kdo) molecule which is highly conserved in all LPS structures investigated

so far. The minimal LPS structure required for growth of *E. coli* was considered to be two Kdo residues attached to lipid A[18-20], until a study showed a non-conditional *E. coli* K-12 suppressor strain KPM22 that lacks Kdo and is viable with an OM composed predominantly of lipid IVA; an LPS pathway precursor that lacks any glycosylation[21].

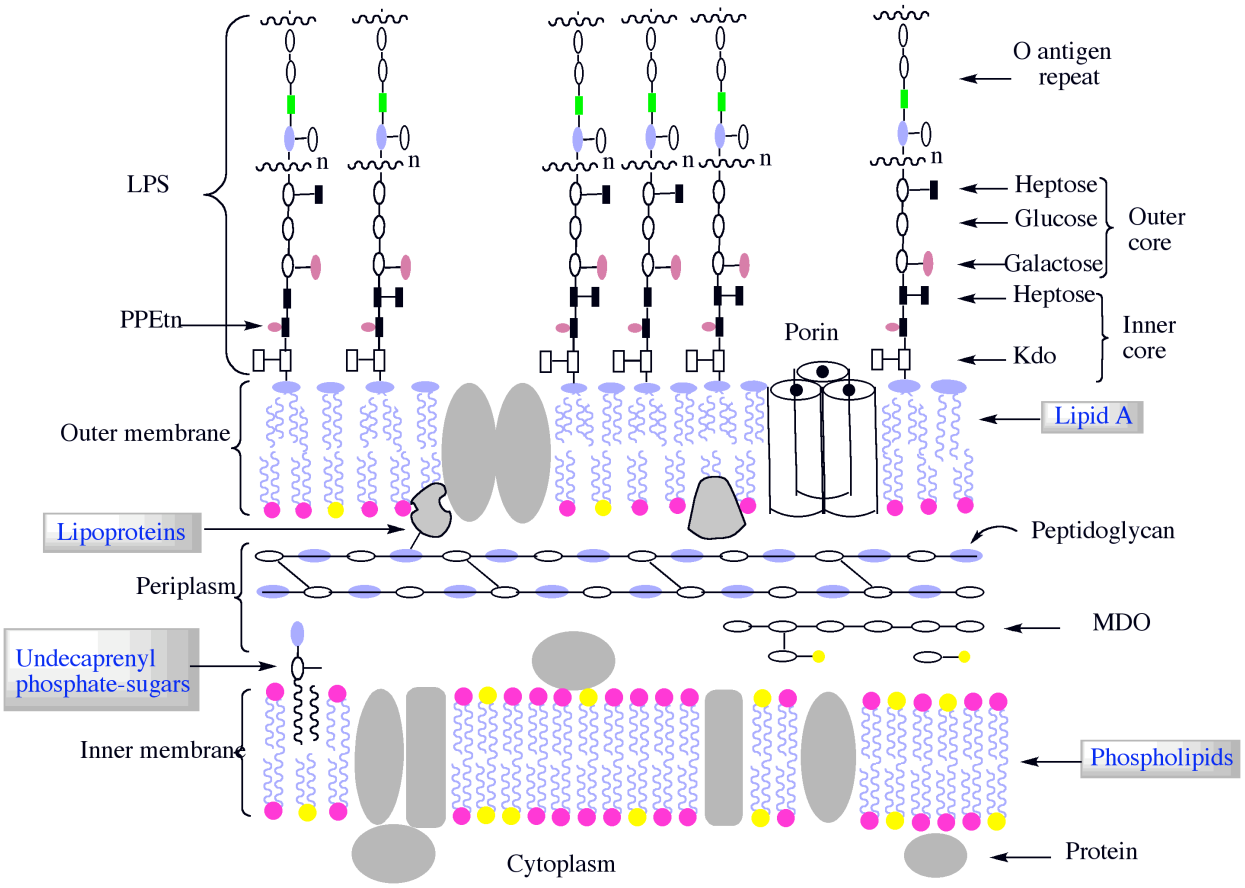

Figure 1.3: Schematic diagram of a gram-negative bacterial cell wall[17].

Lipid A or endotoxin is the major region of LPS to be recognized by the innate immune system[22]. Lipid A is composed of a conserved disaccharide unit of β-D-GlcN-(1-6)-α-D-GlcN with two phosphoryl groups at C-1 and C-4. Four acyl chains are attached to the disaccharide unit *via* ester and amide linkages at C-2, C-3, C-2′, and C-3′, lengths of which vary between different species of gram-negative bacteria. Moreover more fatty acid chains functionalize the acyl chains (Figure 1.4).

Structure-function analysis of lipid A signaling indicates that the presence of both phosphates and length and number of acyl side chains are critical for TLR4 signaling in humans. A variety of studies indicate that hexa-acylated *E.coli* lipid A with side chains of 12 to 14 carbons in length is maximally stimulating in human cells, whereas altering the number or length of the attached fatty acids or altering the charge of lipid A can reduce the magnitude of the signal. Lipid A species consisting of four or five acyl chains, some of which are 16-18 carbons in length are poorly recognized by human TLR4[5]. Lipid A variations between species arise due to nature, number, length, order, and saturation of the fatty acid chains. This variability could have profound implications for disease, particularly in humans, owing to altered recognition by the TLR4-MD2 complex. LPS represents one of the most potent activators of the human innate immune system. A high sensitivity of the host for detecting LPS is mandatory in order to mount an early and rapid response against invading gram-negative bacteria. While a certain set of lipid chains within the lipid A anchor are absolutely required for innate immune recognition, the long O chain consisting of up to 80 repeating oligosaccharide units varies greatly between different bacterial strains and does not play any significant role in pathogen recognition.

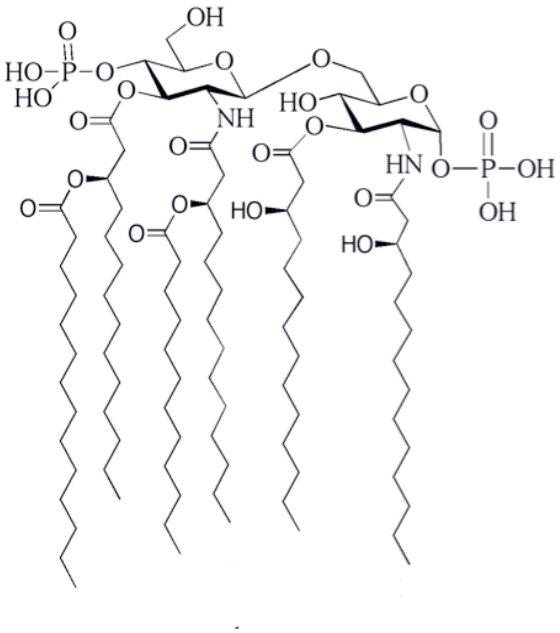

Figure 1.4: Structure of lipid A.

However, numerous reports have shown that the chain length of the carbohydrate unit of LPS that results in either a long (smooth [S]-type) or a short (rough [R]-type) O chain influences the host-pathogen interactions such as colonization of the gut and resistance to complement-mediated killing[23].

1.3 Strategies for the drug development for treatment of sepsis:

Immune detection of lipid A by TLR4 is very sensitive and robust. Overactivation of TLR4 leads to overproduction of downstream mediators such as TNF-α, IFN-β, IL-6, and IP-10 which cause severe inflammation and can ultimately cause a severe complication called endotoxic shock or sepsis. Sepsis is a major clinical problem that leads to about 200,000 deaths in the United States each year.[24] A wide range of approaches have been examined for the treatment of sepsis, which focus at different events in the cascade that lead to severe sepsis. Most of those approaches have demonstrated efficacy in both in vitro and in vivo models, however none of them have proven to be effective in the treatment of sepsis. Hence, efforts are being

made to antagonize the interaction of LPS with its cell-surface receptor, TLR4. One of the strategies is the development of various antibodies against the part structures as well as the whole LPS molecule, which would lead to LPS clearance or neutralize the ability of LPS for cellular activation. However, since LPS is large, complex and the O-antigen as well as the polysaccharide is unique to specific bacterial species, the antibodies raised against the whole molecule would limit its use for neutralization of a variety of bacteria.

To overcome the specificity of the antibodies, another strategy developed for LPS neutralization was the use of lipid A analogs, which interact with the TLR4 receptor complex but fail to trigger the downstream signal transduction pathway. Two naturally occurring lipid A precursors, lipid X and lipid IV_A, are TLR4 antagonist in human cells. Synthetic lipid A analogs derived from *R. sphaeroids*, *R. capsulatus*, (Figure 1.5)[25-29] and *R. sin-1*[30-33] have been shown to possess antagonist activities and can neutralize enteric endotoxin.

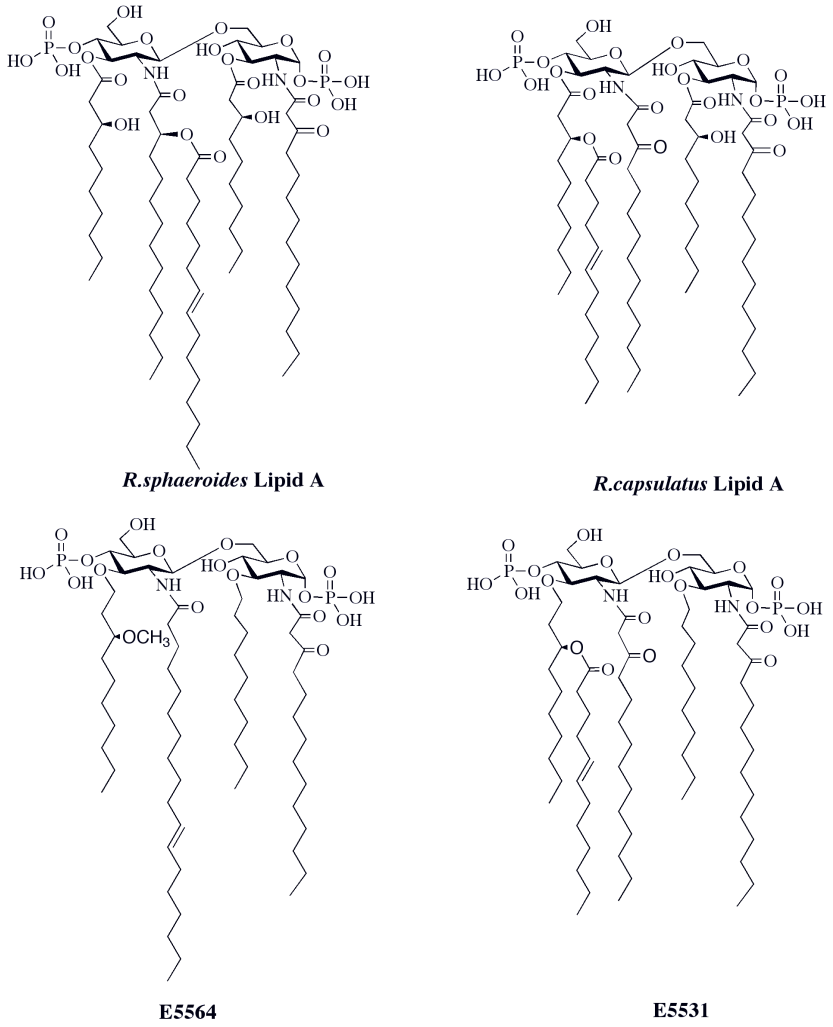

Figure 1.5: Lipid As with antagonist activity[25,34].

However, these synthetic analogs are difficult to synthesize and are unstable for a longer duration of time[35]. Christ and co-workers developed E5531[25] and E5564 (Figure 1.5), which are analogs of *R. sphaeroids* and *R. capsulatus* lipid As, in which the ester linked lipid chains to the glucosamine disaccharide were replaced with alcohol linked lipid chains. Replacement of the linkages increased the stability of the lipid A molecules, and E5531[35-37] and E5564[34] were shown to prevent inflammatory effects of enteric LPS in rabbits, protected against LPS-induced lethality in mice, and blocked the TLR4 mediated activation of NF-κB by enteric LPS. However, the synthetic strategy for E5531 and E5564 is not amenable for large-scale production.

1.4 Signal-transduction by TLR4:

Recognition of the lipid A component of LPS occurs largely by the mammalian LPS receptor-the TLR4-MD2-CD14 complex-which is present on many immune system cell types including macrophages and dendritic cells (DC). Recognition of lipid A also requires an accessory protein – LPS-binding protein (LBP) – which converts oligomeric micelles of lipid A/LPS to a monomer for delivery to CD14[38,39], which is a glycosyl phosphatidylinositol (GPI)-anchored, high affinity membrane protein that can also circulate in soluble form. CD14 concentrates LPS for binding to the TLR4-MD2 complex[40,41].

Although TLR4 is essential for LPS signaling, overexpression of TLR4 does not confer LPS responsiveness on LPS-unresponsive cell lines such as HEK293 cells[42]. Moreover, mutation of the TLR4 gene leads to hyporesponsiveness to LPS in mice. Exogenous expression of TLR4 however does not confer LPS responsiveness on cell lines, suggesting a requirement for an additional molecule. Co-expression of MD-2 imparts LPS responsiveness to TLR4[43]. MD-2 is a small protein that lacks a transmembrane domain and is identified to associate with the extracellular domain of TLR4. Expression of MD-2 confers responsiveness to LPS on cells expressing TLR4 alone. Human MD-2 consists of 160-amino acid residues with a predicted molecular mass of 18 KDa and has 2 potential *N*-linked glycosylation sites. Glycosylation is crucial for TLR4-mediated signal transduction by LPS but not for associating with TLR4. Oligomers of MD-2 associate with TLR4 in endoplasmic reticulum, and this association is assisted by an endoplasmic reticulum chaperone glycoprotein 96[42,44]. TLR4 domain consisting of Glu^{24}-Lys^{631} enables MD-2 binding and LPS recognition but is not sufficient for LPS signaling[45]. The association of TLR4 with the adaptor protein MD2 is a pre-requisite for activation of cells by LPS. TLR4 present on macrophages of different mouse strains was measured, as a TLR4-

MD2 complex, the levels of which correlated with the levels of TLR4 mRNA. However, no significant difference in expression levels of MD-2 mRNA was detected, indicating that MD2 transcription is not influenced by the amount of TLR4 present[46].

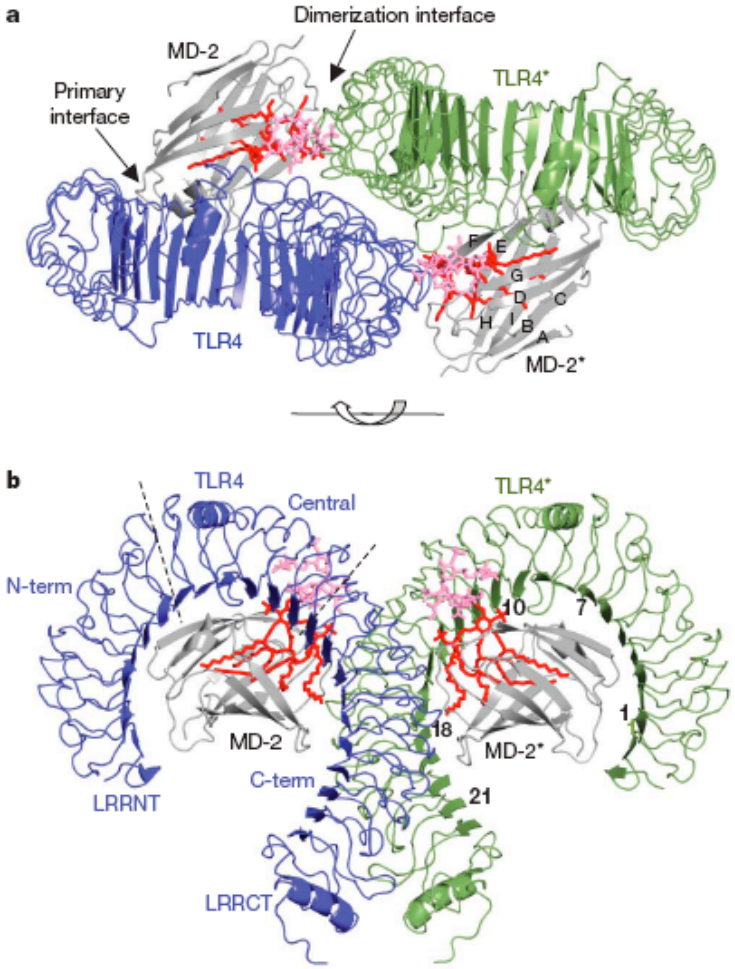

Figure 1.6: X-ray structure of TLR4-MD-2-LPS complex. Top view (a), side view (b) of the symmetrical dimmer complex. Lipid A component of LPS is colored red while inner core carbohydrates are colored in pink. TLR4 is divided into N-terminal, C-terminal and central domains[47].

Recently, three crystal structures of MD-2 with its ligands were resolved, one with human MD-2 bound to lipid IVA[48], another of LPS bound to human TLR4 and MD-2[47], and

third of eritoran[49](TLR4 antagonist) bound to mouse TLR4 and MD-2. The crystal structure with LPS revealed two copies of TLR4, MD-2, and LPS form a symmetrical dimer receptor complex (Figure 1.6). TLR4 adopts a horseshoe like shape characteristic of leucine rich repeat (LRR) superfamily and MD-2 adopts a β-cup fold structure composed of two anti-parallel β sheets forming a hydrophobic pocket for ligand binding. Crystal structure of TLR4-MD-2 with eritoran showed that the primary interface between MD-2 and TLR4 is formed before ligand binding while, binding of lipid A to MD-2 mediates dimerization of the two TLR4-MD-2 complexes. LPS binding and dimerization induces localized changes in the F126 loop of MD-2, and change in radius and bending angle of TLR4. Both, lipid IVA and eritoran are TLR4-MD-2 antagonists and have four acyl chains. Crystal structures with both the antagonists showed that the all the four lipid chains are completely buried in the MD-2 pocket and completely fill the space in the pocket. Hence, it was expected that binding of *E. coli* lipid A (which has six acyl chains) to MD-2 would bring about structural changes in the MD-2 pocket to accommodate the two extra lipid chains of *E. coli* lipid A. However, the crystal structure with *E. coli* LPS bound to MD-2 and TLR4 reveals that the dimensions of MD-2 pocket and shape remain unchanged. MD-2 is able to accommodate the six acyl chains of *E. coli* LPS as the glucosamine backbone of the lipid A is displaced upwards by ~5Å and the lipid A is also rotated by ~180°, giving an additional space to occupy the molecule. Hence all six lipid chains of the *E. coli* lipid A are accommodated in the MD-2 pocket. Phosphate groups in the lipid A structure play an important role in the dimerization of the complex. Both 1-phosphate and 4-phosphate make contacts with MD-2, while 4-phosphate and 1-phosphate interacts with the positively charged lysine and arginine residues in the primary TLR4 and second TLR4 in the dimer complex respectively (Figure 1.6).

This rationalizes the earlier observation that deletion of either of the phosphates reduces the endotoxic activity of LPS by ~100 fold.

CD14, a 55-kDa glycoprotein, is an essential component of the LPS receptor complex.[50] CD14 is present in soluble form in blood or a glycosylphosphatidylinositol-linked form on myeloid lineage cells. CD14-negative cells such as endothelial cells and epithelial cells also respond to LPS because soluble CD14 can substitute for membrane-bound CD14[51,52]. Besides LPS, CD14 is also required for the recognition of other bacterial products including peptidoglycan, lipoteichoic acid (LTA), and lipoarabinomanan. CD14 contains 10 copies of a LRR motif, found in a variety of proteins and is involved in ligand recognition and signal transduction. Mapping studies show that 7 out of 10 LRRs can be deleted without affecting LPS binding. Additional study demonstrated that certain defined residues outside the LRR region of CD14 are necessary for LPS binding. The N-terminal 151 amino acids of human CD14 have been reported to be sufficient for LPS recognition and signal transduction. While for mouse CD14, the C-terminal half of the molecule is found to be required for the signal transduction. The formation of CD14/LPS complex significantly reduces (100-1000 fold) the concentration of LPS required for activation of macrophages as compared with LPS alone. Although CD14 lacks transmembrane and intracellular domains, and thus cannot by itself initiate signal transduction, it seems to possess an ability to discriminate between bacterial products and sort their signals to different TLRs. However, CD14 does not have the fine binding specificity to discriminate lipid A structures[42].

The downstream events of TLR4 activation are known to be mediated by common components, the adaptor protein MyD88, a family of IL-1 receptor-associated kinases (IRAK), and another adaptor protein, TNF receptor-activated factor 6 (TRAF6). Human MyD88 consists

of 296 amino acids and contains 2 protein interaction domains, an N-terminal death domain and a C-terminal TIR domain. The TIR domain of MyD88 associates with the TIR domain of TLR4 and IL-1 receptor, whereas the death domain interacts with the N-terminal death domain of IRAK and recruits IRAK to the receptor complex[53-55]. IRAKs are subsequently phosphorylated and are dissociated from the receptor complex and interact with another adaptor molecule, TRAF6[56]. TRAF6 activates MAPK kinases, which can lead to AP-1 activation or phosphorylation of inhibitors of κB (IκB) kinase (IKK) complex. The IKK complex is composed of 2 catalytic subunits IKKα and IKKβ; and one regulatory subunit IKKγ, which induces phosphorylation of IκB. The phosphorylation of IκBs result in their polyubiquitination, which in turn leads to their 26S proteasome-mediated degradation, allowing NF-κB to translocate to the nucleus and activate the transcription of many κB-dependent genes, including IκBα and inflammatory cytokines TNF-α, IL-6, and IL-1β (Figure 1.7). Newly synthesized IκB binds to NF-κB and thus terminates its transactivating activity[57,58].

It was observed that in MyD88-deficient macrophages, the nuclear translocation of NF-κB and phosphorylation of MAPK remained intact following stimulation with LPS although with delayed kinetics compared to macrophages from wild-type mice[59]. This study led to the identification of another adaptor protein called TRIF, consisting of 712 amino acids and a C-terminal TIR domain while lacking a death domain. Activation of TRIF leads to the recruitment of interferon regulatory factor (IRF)-3 to the activation complex with its subsequent phosphorylation and nuclear translocation leading to the up-regulation of a set of genes including IFN-β[60]. IFN-β activates STAT1, which in turn causes induction of IFN-inducible genes including interferon-inducible protein-10 (IP-10), RANTES, iNOS, and monocyte chemoattractant protein (MCP) 5 (Figure 1.7).

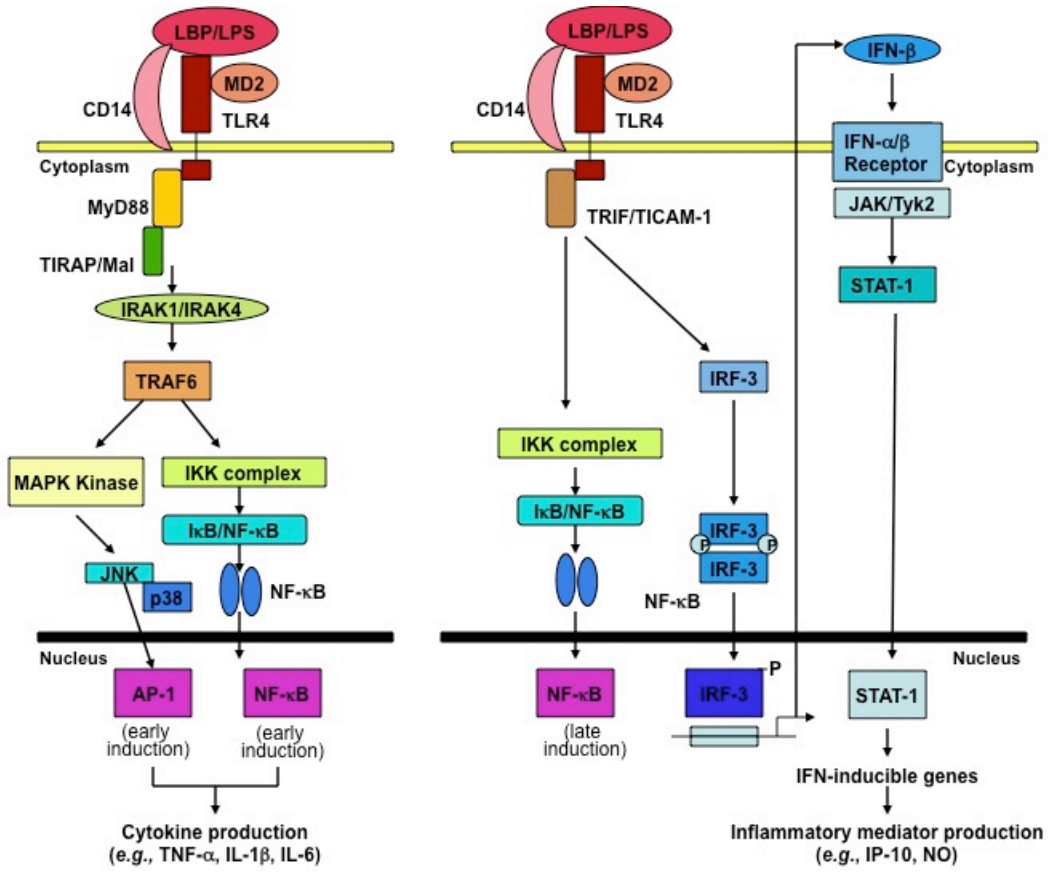

Figure 1.7: Signal transduction pathway of TLR4.

1.5 Caspase-1 Inflammasome:

Unlike IL-1β, most of the cytokines induced by both pathways are synthesized as active proteins. IL-1β is synthesized as an inactive precursor (pro- IL-1β) in response to TLR ligands such as LPS, lipoteichoic acid (LTA), CpG oligonucleotides, anti-viral compound resiquimod, and lipopeptides and has a diffuse distribution in the cytoplasm[61-63]. The cytokine IL-18 shares many of the pro-inflammatory characteristics of IL-1β and it is also synthesized as an inactive precursor. IL-18 induces IFN-γ expression and secretion from IL-12-primed naïve T cells to promote the differentiation of type 1 helper T cells. Unlike pro-IL-1β, however, pro-IL-18 is

expressed constitutively. Biologically active IL-1β and IL-18 are generated from pro-IL-1β and pro-IL-18 respectively by action of aspartate-specific cysteine protease, IL-1β-converting enzyme (ICE)- also known as caspase-1 [64,65]. Caspase-1 itself is synthesized as an inactive 45-KDa zymogen (pro-caspase-1) that undergoes catalytic processing upon receipt of an appropriate stimulus. The active form of the enzyme comprises of subunits p20 and p10, which assemble into a heterotetramer. The enzyme complex responsible for the activation of caspase-1 is named "inflammasome"[66]. Caspase-1 inflammasome is a dynamic complex in which specific adaptor molecules are brought into play depending upon the primary stimulus. Three types of inflammasomes have been proposed NALP1, NALP2, and NALP3 based on biochemical analysis of three Apaf-1-like proteins called NALPs (Nacht, LRR and Pyrin domain-containing proteins), which are also known as NODs and CATERPILLARs and appear to function as intracellular PRRs that detect microbial products within compromised cells. The adapters ASC, Ipaf, and Cryopyrin (Nalp3) have been proposed to bind and regulate caspase-1 through homotypic protein interactions in over-expression studies. ASC (also known as CARD5, Pycard, TMS-1) encodes a 22-KDa protein that contains an amino-terminal PYRIN domain (PYD) and a carboxy-terminal CARD. As an adapter between other PYD- and CARD-containing proteins, ASC is a key component of the inflammasome and has the broadest impact on caspase-1 activation in response to most stimuli. For example, PYD of ASC interacts with PYD of several NALPs, whereas CARD of ASC recruits CARD of pro-caspase-1[67,68]. It was shown that in contrast to wild-type macrophages, which secrete readily detectable levels of IL-1β in response to LPS and ATP, $Nalp3^{-/-}$ macrophages released negligible amounts of IL-1β similar to $ASC^{-/-}$ macrophages.

It is assumed that a specific ligand induces assembly and activation of NALP3-inflammasome (Figure 1.8), but its identity is not very well known. It is now accepted that activation and release of IL-1β requires two distinct signals. The first signal is triggered by TLR activation and leads to the synthesis of pro-IL-1β and other proteins required for inflammasome.

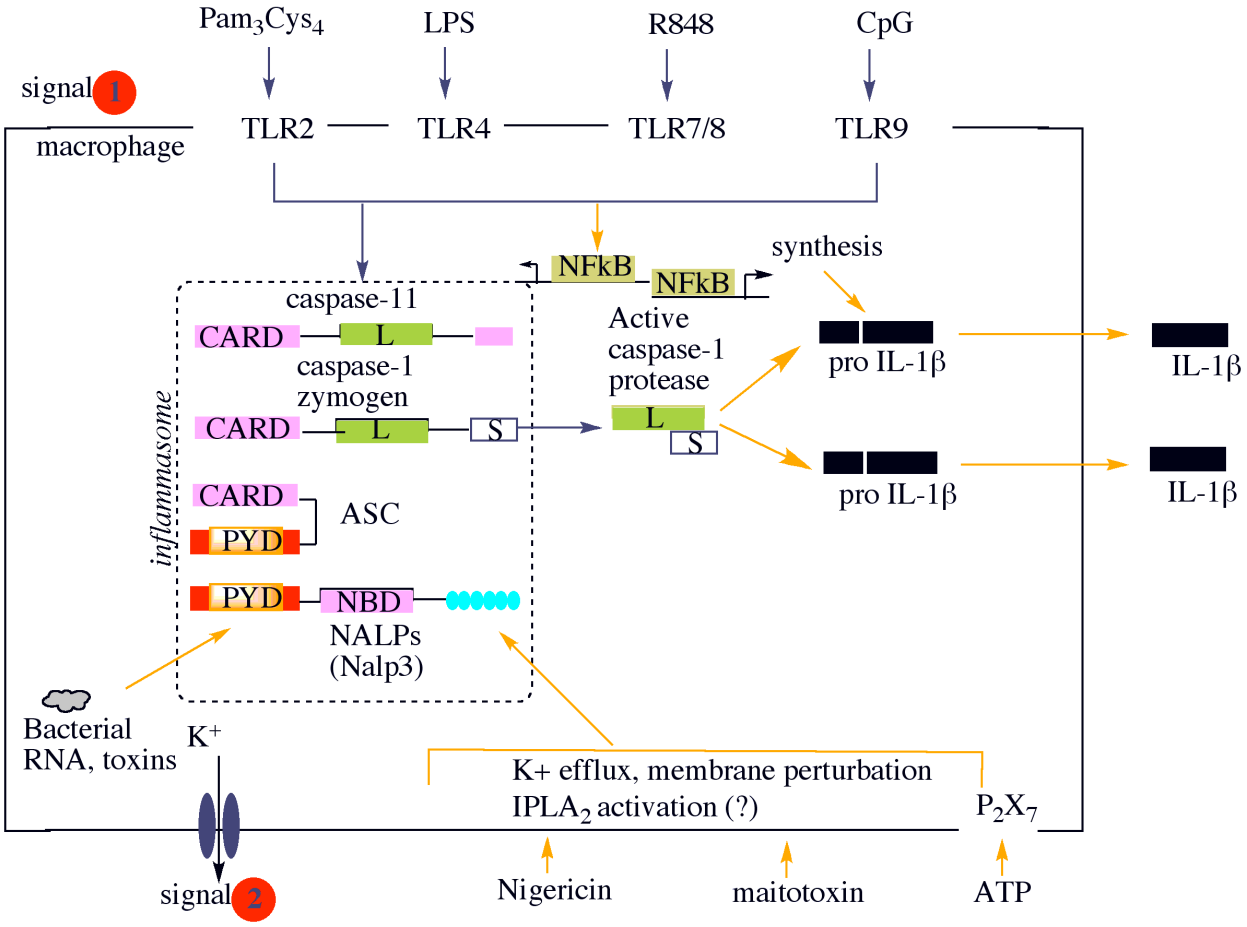

Figure 1.8: Pathways involved in Caspase-1 activation[69].

The second signal that is required for caspase-1 activation and IL-1β secretion appears to derive from the activation of surface expressed purinergic receptors of the $P2X_7$ subtype for which adenosine triphosphate (ATP) is thought to be the major endogenous ligand[70]. $P2X_7$ upon activation causes a rapid K^+ efflux from the cytosol. $P2X_7$ receptor knockout mice have been shown to be completely defective in IL-1β release in response to exogenous ATP.[71] The efflux of

cytosolic K^+ is thought to be a trigger for caspase-1 activation as IL-1β release is also, triggered by potassium ionophores, membrane-permeabilizing agents, pore forming agents, and depletion of K^+ from cell culture media. Other TLR ligands (lipid A, LTA, lipoprotein, CpG oligodeoxyribonucleotides and imidazoquinoline compounds) also prime the cells to activate caspase-1 in response to ATP or other inducers of K^+ efflux such as nigericin and maitotoxin in a NALP3-dependent manner[61]. Thus the NALP3-inflammasome seems to be activated serially first by TLR ligands followed by K^+ efflux. It is likely that TLR stimulation leads to the induction of genes such as NALP3 and caspase-11, that are required for caspase-1 activation, and that K^+ efflux is an upstream trigger of NALP3 activation. The requirement of a second signal for IL-1β production might constitute a fail-safe mechanism that ensures cytokine release only in the presence of a bona fide "danger".

Tschopp and colleagues reported that muramyl dipeptide (MDP), a minimal biologically active moiety of bacterial peptidoglycan could activate caspase-1 in a NALP3-dependent manner in human monocytic cell line[72]. However, unlike human monocytes, murine macrophages did not secrete IL-1β in response to MDP alone, and MDP is dispensable for the activation of NALP3 inflammasome in murine macrophages. In contrast, Nunez and colleagues have reported that NALP3 and ASC are essential for caspase-1 activation by bacterial RNA and imidazoquinoline compounds but not by LPS, lipoteichoic acid or bacterial lipoprotein suggesting an important role for the NALP3 inflammasome in recognition of non-host RNA molecules[73]. To this ongoing debate, Netea and coworkers have shown that synthesis and release of IL-1β differ among monocytes and macrophages. Monocytes constutitively express activated caspase-1, which leads to the activation and secretion of IL-1β after a single stimulation with a TLR ligand[74]. However, macrophages do not express active caspase-1. Hence they need 2 stimuli for IL-1β secretion, one

for stimulation of transcription and translation of pro-IL-1β and a second for caspase-1 activation and release of IL-1β.

1.6 TLRs and immune modulation:

The critical involvement of TLRs in mediating innate immune activation of DCs results in robust activation of adaptive immune responses. In 18th century Deidier observed for the first time, a positive correlation between infection and remission of malignant disease[75]. This led to an inference that microbes could have anticancer properties. Later, at the end of the 19th century, William Coley observed that repeated injections of a mixture of bacterial toxins from gram-positive bacterium *Streptococcus pneumoniae* and the gram- negative bacterium *Serratia marcescens* served as efficient anti-tumor therapeutic agents[11]. This provided evidence that microbial products, rather than live microbes, may mediate an anti-tumor effect. With the advent of new immunological methods, it is shown that DCs are equipped with PRRs such as TLRs that detect and sense components of bacteria, fungi, viruses, and parasites. Once DCs detect the pathogen, a set of signal transduction pathways is triggered resulting in the release of inflammatory mediators. The inflammatory mediators such as cytokines and chemokines augment the antigen presentation by DCs to naïve antigen-specific T cells launching an immune response that results in B-cell response. Thus DCs represent an important nodal point in which pathogen associated signals are integrated and transmitted to the adaptive immune system. Furthermore, in response to signals from pathogens or adjuvants (*eg*. TLR ligands), resident immature DCs at the site of infection or vaccination undergo a maturation program characterized by enhanced expression of costimulatory molecules and inflammatory cytokines and migrate to the draining lymph nodes. The increased cytokine and chemokine levels at the site of infection also result in the recruitment of monocytes and DC precursors from the blood[76]. The influx of

DCs in lymph nodes results in an increased density of antigen-MHC complexes, costimulatory molecules, and proinflammatory cytokines facilitating productive T-cell activation. DCs can also sense pathogens indirectly by detecting inflammatory mediators produced by various cells such as macrophages, NK cells, NK T cells, mast cells, and endothelial cells. TLRs can also play a crucial role in the activation and maturation of the B-cell response during infection. Through T-cell-dependent and T-cell-independent pathways, TLRs regulate the B-cell response by inducing B-cell proliferation, immunoglobulin isotype class switching and somatic hypermutation. TLRs can also regulate the differentiation and maintenance of T and B cells by production of IL-12, IL-23, and IL-27. These cytokines induce T-helper type 1 (TH1) cell development and help to promote the cell-mediated immune response[11]. The nature of microbial stimulus and local microenvironment cues play important roles in tuning T helper responses. Viruses stimulate IFN-α-mediated differentiation of plasmacytoid DCs into Th1-inducing DCs, whereas IL-3 induces such DCs to differentiate into Th2-inducing DCs. Different forms of *Candida albicans* instruct DCs to induce either Th1 or Th2 responses and *E. coli* LPS induces IL-12(p70) in DCs which elicit Th1 responses, while *P. gingivalis* LPS, filarial nematode-secreted products, or cholera toxin fail to induce IL-12(p70) and stimulate Th2-like responses[77]. Most TLRs induce IL-12(p70) leading to potent Th1 responses. TLR3, 7, and 9, which induce type I IFNs, stimulate potent Th1 responses and CTLs, while it is shown that TLR2 ligands can stimulate Th2 responses[78] (Figure 1.9).

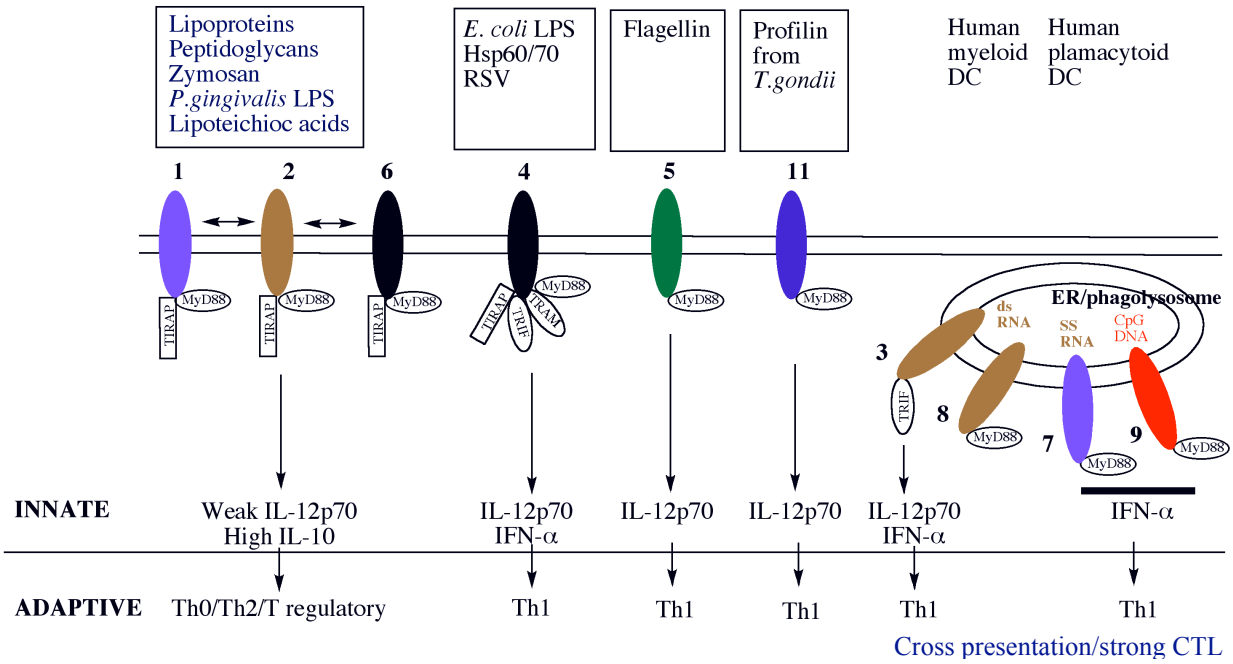

Figure 1.9: Activation of distinct TLRs on DCs induces different cytokines and immune responses[76].

The molecular mechanism by which specific TLR2 ligands favor a Th2 bias remains to be established, although recent work suggests that the robust and sustained phosphorylation of ERK1/2 results in stabilization of transcription factor c-Fos in DCs, which in turn suppresses expression of Th1 defining cytokine IL-12(p70) and enhances IL-10, thus favoring a Th2 bias[77,79]. Moreover, signaling via TLR3, 7, and 9 has been shown to enhance cross presentation by DCs, although the mechanism by which this is mediated is not known. In addition to regulating adaptive immune response, TLR agonists have been shown to mediate their anti-tumor effect through many mechanisms. High doses of TLR agonists, such as poly I:C that stimulates TLR3, can lead to apoptosis and have been shown to directly kill both tumor and ancillary cells of the tumor microenvironment, such as the vascular endothelium. TLR activation may also cause tumors to regress by increasing vascular permeability and by directly or

indirectly recruiting leukocytes, resulting in tumor lysis by NK and cytotoxic T-cells[80]. However, one of the most appreciated functions of TLRs in cancer therapy is stimulation of the adaptive immune system. In these studies, tolerance to tumor self antigens is broken, presumably by TLR mediated upregulation of co-stimulatory signals to the adaptive immune response, a property known as adjuvanticity. This has been used in cancer vaccines, as targets of gene therapy and in raising anti-tumor antigen-specific T cells *in vitro* for adoptive transfer[11].

1.7 Vaccines and TLRs:

Vaccines are considered to be the most promising and successful medical interventions to prevent infectious diseases. Vaccines are classified into live-attenuated vaccines and non-replicating vaccines. Live attenuated vaccines, such as smallpox or yellow fever vaccines consists of weakened versions of the pathogen, which are not pathogenic but are highly immunogenic. Non-replicating vaccines include the subunit vaccines, such as hepatitis B vaccine, which consists of antigens derived from the pathogen by chemical approaches or through recombinant DNA techniques. Subunit vaccines are generally poorly immunogenic and need to be administered with adjuvants[81]. Vaccines consist of a preparation of antigen against which adaptive immune response is elicited and an adjuvant, which stimulates the innate immune system. Stimulation of innate immune system leads to the generation of inflammatory cytokines such as TNF-α, IFN-β, and activation of antigen presenting cells like macrophages and DCs. These responses lead to conditioning of the immune system for development of specific adaptive immune responses[82]. Despite the widespread use of adjuvants, such as alum and the oil-based formulation MF59, the immunological mechanisms by which they work are still unclear. However, recent advances in innate immunity are beginning to yield insights into the mechanism of action of adjuvants. In this context, while the role of PAMPs in mediating innate immune

activation via TLRs has been known for 10 years, details on the intracellular networks mediating signaling via TLRs continue to emerge. MF59 adjuvant developed by Chiron vaccines (now Novartis vaccines) consists of an oil-based emulsion (squalene, a natural precursor to synthesis of cholesterol and steroid hormones). MF59 is also a part of an influenza-subunit vaccine, licensed for use in humans in several European countries for more than a decade[83]. It is reported that MF59 is taken up by DCs and stimulate cytokines *in vivo*. However, the role played by TLRs and non-TLRs in mediating these adjuvant effects by MF59 is presently unknown. TLR ligands are known to enhance antibody responses, for example, the hepatitis B vaccine with CpG DNA results in both the proportion of vaccines that seroconvert, as well as an increase in the mean antibody titers. BCG vaccine has been shown to signal via TLR2, 4, and 9 and DC specific intracellular adhesion molecule 3 grabbing non-integrin (DC-SIGN) to elicit proinflammatory cytokines. However, the role played by these receptors in modulating adaptive immune response against BCG remains unknown[84].

Secreted cytokines help in the activation of adaptive immune system by triggering maturation of antigen presenting cells (APCs) and expression of costimulatory molecules on APCs further leading to the formation of stable antigen presentation to the T-cells. This in turn leads to the induction of robust antigen specific T- and B-cell responses. Hence activation of TLRs can be harnessed for immune modulation and antibody production by vaccines. However, over-production of the cytokines and chemokines can lead to septic shock syndrome. [24] Hence it is important to develop a TLR ligand that triggers the immune system without leading to deleterious effects.

1.8 Research Objective:

The goal of the research described in this dissertation is to understand the structure-activity relationship of different lipid A derivatives corresponding to different bacteria for activation of TLR4-MD2 receptor complex, with an aim to develop an adjuvant for vaccine candidates and to develop inhibitors of TLR4-MD2 complex for the treatment of sepsis. The project is divided into two parts. The first part addresses the modulation of activation of TLR4-MD2 receptor complex with different LPS and synthetic lipid A derivatives from different bacteria. In the second part as a model study, the role of TLR2 ligand is studied as it was shown that this ligand could boost immune response when incorporated in vaccine construct.

Lipopolysaccharide (LPS) derived from the outer membrane of gram-negative bacteria is a TLR4 ligand and possesses a potential for the development of an immune modulator. However, an important concern of such immune modulators is that over activation of innate immunity may lead to clinical symptoms of sepsis. Hence, detailed knowledge of the structure-activity relationships is important to design safe immune modulators. Recently, monophosphoryl lipid A from *S. minnesota* was shown to be a TRIF-biased agonist of TLR4[85]. This lipid A has 0.1% of the inflammatory toxicity of its parent LPS molecule, and is likely to be approved as vaccine adjuvant for widespread use [86,87]. Hence attempts are being made to understand the structure-activity relationship of different lipid A derivatives for their development into immune modulators.

Structural studies have demonstrated that the carbohydrate backbone, degree of phosphorylation, and the fatty acid acylation patterns vary considerably among different bacterial species. These structural differences may account for the highly variable *in vitro* and *in vivo* host responses to LPS. There is also some indication that structurally different lipid As may

differentially induce pro-inflammatory responses. In this regard, one of the studies showed that LPS isolated and purified from *E. coli* 055:B5 induced MyD88-dependent pathway more than the TRIF-dependent pathway, *N. meningitides* LPS induced both the pathways equally while *S. typhimurium* LPS induced the TRIF–dependent pathway significantly than the MyD88–dependent pathway in human macrophage cells like THP-1 and MM6[88-90]. However, in these studies compounds were studied over a narrow range of concentration. In another study, various strains of *Neisseria meningitides*[91] and *Neisseria gonorrhoea* have been developed which express altered LPS structures and these LPS molecules have been used for various immune modulation studies. Although in all the above studies, LPS molecules were thoroughly purified from their natural sources, the preparations are suspected to contain a mixture of different structures of LPS and possible contamination with other inflammatory components of the bacterial cell wall. Hence all these studies do not provide insights into the activities of each of the different lipid A structures. In another report it was shown that Salmonella lipid A when purified from Salmonella LPS showed ~10 times lower activity than the parent LPS compound. However, when a chemically synthetic lipid A was used for similar studies, it showed ~100 times lower activity as compared to the parent LPS molecule. These studies were performed in mouse peritoneal macrophages and mouse macrophage like cell-line with TNF-α induction.

To address the issue of heterogeneity and possible contamination in LPS and lipid A preparations, we have employed various homogenous synthetic lipid A derivatives from *E. coli*, *S. typhimurium*, and *N. meningitides* along with LPS and LOS from these bacteria for systematic structure-activity relationship studies for activation of TLR4 in two mouse macrophage cell lines. Unlike others our studies do not reveal bias towards TLR4 induction pathways by different lipid As. Our quantitative real-time PCR studies reveal that there is modulation by different lipid

As at the transcriptional level for all cytokines studied, while by a single lipid A structure, there is no modulation for various cytokines at the transcriptional level. In addition, we show that modulation occurs post-translationally for IL-1β secretion by different lipid A species arising due to modulation of activation of the inflammasome complex.

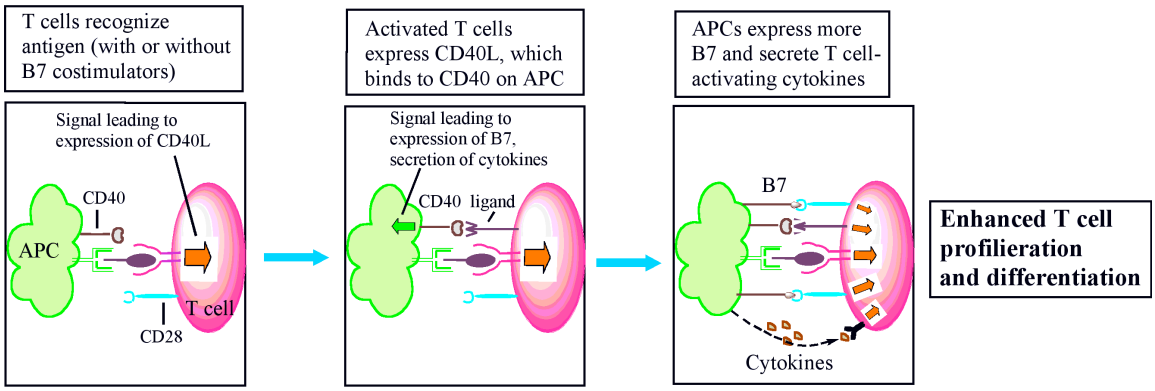

Figure 1.10: Role of co-stimulators in T-cell activation.

In addition to the antimicrobial activities, cytokines generated, also activate and regulate the adaptive immune system. Efficient priming of adaptive immune responses requires presentation of antigen in the context of major histocompatibility complex (MHC) and induction of accessory signals (costimulators and cytokines) on antigen presenting cells (APCs). TLRs expressed on APCs may regulate the accessory signals through their recognition of PAMPs and consequently control activation of antigen-specific adaptive immune responses (Figure 1.10). To test this hypothesis, a three -component cancer vaccine candidate with Pam_3CysSK_4, a TLR2 ligand as an adjuvant was studied. We show that the potency of cytokines induced by this construct is more than 2.5 times less than *E. coli* 055:B5 LPS, which can be appropriate to prime adaptive immune responses without causing sepsis. Moreover, we also show that the TLR2 ligand promotes uptake of the vaccine candidate by macrophage cells.

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CHAPTER 2

Modulation of Innate Immune Responses with Synthetic Lipid A Derivatives

2.1 Introduction:

The innate immune system is an evolutionarily ancient system designed to detect the presence of microbial invaders and activate protective reactions[1]. It responds rapidly to unique molecules that are integral parts of pathogens and are perceived as danger signals by the host. Recognition of these molecular patterns is mediated by sets of highly conserved receptors[2], whose activation results in acute inflammatory responses. These responses include the production of a diverse set of cytokines and chemokines, direct local attack against the invading pathogen, and initiation of responses that activate and regulate the adaptive immune system[3-7].

Evidence is emerging that innate immune responses can be exploited for therapeutic purposes such as the development of adjuvants for vaccines and the treatment of a wide range of diseases including asthma, infections, and cancer. An important concern of such therapies is, however, that over-activation of innate immune responses may lead to the clinical symptoms of septic shock[8,9]. Thus, an important issue for the design of safe immune modulators is detailed knowledge of structure-activity relationships to harness beneficial effects without causing toxicity.

Lipopolysaccharides are structural components of the outer surface membrane of Gram-negative bacteria that trigger innate immune responses through Toll-like receptor 4 (TLR4), a member of the TLR family that participates in pathogen recognition. LPS consists of a hydrophobic domain known as lipid A, a non-repeating core oligosaccharide and a distal

polysaccharid[10,11]. Studies employing chemically synthesized lipid A have unequivocally demonstrated that lipid A is the inflammation inducing moiety of LPS[12,13].

Although LPS-induced cellular activation through TLR4 is complex as many signaling elements are involved, it appears that there are two distinct initiation points in the signaling process, depending upon the recruitment of the adaptor proteins MyD88 or TRIF. The MyD88-dependent pathway leads to early activation of the transcription factor NF-κB, the production of proinflammatory cytokines such as TNF-α, and Th1 responses. The TRIF-dependent pathway induces phosphorylation and dimerization of the transcription factor IRF-3, resulting in interferon-β (IFN-β) production, which in turn activates the STAT-1 pathway leading to the production of mediators such as nitric oxide[7,14]. Other TIR-containing proteins participate in these events, TIRAP in the MyD88 pathway and TRAM in the TRIF pathway, while the role of SARM has yet to be clarified[15-17].

An interplay between the MyD88 and TRIF signaling pathways has been demonstrated by microarray studies that examined LPS-induced changes in gene expression profiles of macrophages isolated from wild-type, MyD88$^{-/-}$, TRIF$^{-/-}$ and MyD88$^{-/-}$ TRIF$^{-/-}$ mice[18,19]. Results from those studies demonstrated that LPS-inducible genes were completely abrogated in the MyD88$^{-/-}$, TRIF$^{-/-}$ mice; these genes can be classified into clusters based on their dependence on MyD88 and/or TRIF for expression. The cluster of genes regulated primarily by MyD88 included genes commonly associated with pro-inflammatory responses (e.g. TNF-α, IL-1ß, and IL-6). The cluster that was dependent on both MyD88 and TRIF included IL-1α and IL-12a, and the cluster that was dependent primarily on TRIF included the IFN-inducible genes, IFN-ß, IP-10, and RANTES.

Of the naturally occurring lipid A structures examined thus far, the vast majority contain a lipid backbone consisting of a β(1-6)-linked D-glucosamine or 2,3-diamino-2,3-di-deoxy-glucosamine in either homo- or hetero-dimeric combinations. However, striking structural differences in lipid As of different bacteria occur in the degree of phosphorylation and patterns of acylation. These structural differences account for the highly variable *in vivo* and *in vitro* host responses to LPS[11,13,20-22]. *E. coli* lipid A is hexa-acylated and the fatty acids are attached to the backbone in an asymmetrical manner (4 fatty acids are attached to one D-glucosamine and 2 fatty acids are attached to the other)[12,13]. *Helicobacter pylori*, *Porphyromonas gingivalis*, and *Chlamydia trachomatis*, three bacteria associated with chronic inflammatory disease[23], contain LPS that are less potent stimulators of inflammatory mediators than LPS from enteric bacteria. This may allow these three organisms to evade the innate host defense system. The lipid A moieties of these bacteria contain either a smaller number of lipids, lipids of longer length, or only one phosphate group.

Gram-negative bacteria such as *S. typhimurium* and *Yersinia pestis* can change the structure of their lipid A in response to environmental factors. Thus, *Y. pestis* grown at 21°C to 27°C (flea temperatures) produces an hexa-acylated lipid A structures which is converted into mainly a tetra-acylated structure at 37°C (host temperature). Hexa-acylated lipid A is a strong activator of human cells, whereas tetra-acylated lipid has low stimulatory activity. Interestingly, *Y. pestis* that can only produce a hexa-acylated lipid is avirulent even at high challenge doses[24,25]. Upon entering cells of the host, *S. typhimurium* modifies its lipid A by conversion of phosphate monoesters into diesters, addition of a palmitate chain to the β-hydroxymyristoyl at C-2, removal of β-hydroxymyristoyl at C-3, and α-hydroxylation of the myristate group at C-3′. These modifications may be incomplete leading to structural heterogeneity including penta-, hexa-, and

hepta-acylated lipid A derivatives having different substitutions at the phosphates[26-28]. These observations provide further support for the notion that the lipid A structure is an important determinant of the host-pathogen interaction.

Based on results obtained prior to the identification of TLRs and their associated intracellular adaptor proteins, lipid A isolated from different bacteria were classified as having either high, moderate, or low endotoxic activity. However, the results of more recent studies[5,7] suggest that relationships between lipid A structure and biological responses are far more complex. However, heterogeneity in the structure of lipid A within a particular bacterial strain and possible contamination with other inflammatory components of the bacterial cell wall have complicated the use of either LPS or lipid A isolated from bacteria to dissect the molecular mechanisms responsible for the biological responses to specific lipid A molecules.

To address this important issue, we have used various synthetic lipid A derivatives corresponding to *E. coli, S. typhimurium,* and *N. meningitidis* with structural modifications in the lengths of the lipid chains, presence of phosphate at the C-1 position, and presence of KDO molecule at the C-6′ position (Figure 2.1), in addition to LPS from *E. coli*, lipopolysaccharide from *N. meningitidis*, and detoxified lipid A from *Salmonella minnesota* R595. These different LPS and lipid As have been studied for the induction of various cytokines and chemokines of the MyD88-dependent as well as the TRIF-dependent pathways in mouse macrophages cells. It has been found that particular modifications had different effects on the potencies (EC_{50}, concentration producing 50% activity) and efficacies (maximum level of production) of induction of various cytokines. However, no bias towards MyD88- or TRIF-dependent response was observed. Thus for the first time, it has been shown that different lipid A derivatives can modulate innate immune responses in a complex manner.

2.2 Materials and Methods:

2.2.1 Reagents:

E. coli 055:B5 LPS and *E. coli* 0111:B4 LPS (ultrapure) were obtained from List Biologicals, *N. meningococcal* lipooligosaccharide (LOS) was a gift from Dr. Carlson (CCRC, UGA), and detoxified lipid A was obtained from Avanti Polar Lipids. Carboxyfluorescein caspase-1 detection kit was from Cell Technology, anti mouse p10 caspase-1 antibody is from Santa Cruz Biotechnology, anti mouse IL-1β antibody was from Chemicon International, and oligo siRNA for ASC/Pycard are from Dharmacon. All synthetic lipid A derivatives were reconstituted in PBS with DMSO (10%) and stored at -80°C. Mouse innate and adaptive immune responses RT^2 Profiler PCR Array is from SABiosciences. The primer pairs for mRNA quantification were designed using the Roche Universal Probe Library and obtained from IDT technology. The primer pairs used were: 18S: Forward- 5'-GCA TGC ACT CTC CCG TTC; Reverse- 5'-ACA GGA CTA GGC GGA ACA GA, TNF: Forward- 5'-TCT TCT CAT TCC TGC TTG TGG; Reverse- 5'-GGT CTG GGC CAT AGA ACT GA, IP-10: Forward- 5'-GCT GCC GTC ATT TTC TGC; Reverse- 5'-TCT CAG TGG CCC GTC ATC, IL-6: Forward- 5'-ACC GCT ATG AAG TTC CTC TC; Reverse- 5'-CTC TGT GAA GTC TCC TCT CC, IFN-β: Forward- 5'-CAC CTA CAG GGC GGA CTT; Reverse- 5'-GAC ATT CTG GAG CAT CTC TTG G, IL-10: Forward- 5'-GGT TGC CAA GCC TTA TCG; Reverse- 5'-TCT TCA CCT GCT CCA CTG, IL-1β: Forward- 5'-ATC TCG CAG CAG CAC ATC; Reverse- 5'-CAG CAG GTT ATC ATC ATC ATC C, RANTES: Forward- 5'-CCT CAC CAT CAT CCT CAC; Reverse- 5'-CTT GGC GGT TCC TTC G, Il1rl2: Forward- 5'- GGG TGT TTT TCC TGC TTC TG; Reverse- 5'- TGC ATA AAA ATG TCC TCA CAC G, Caspase4: Forward- 5'- TCC AGA CAT TCT TCA GTG TGG A; Reverse- 5'- TCT GGT TCC TCC ATT TCC AG, Ccl2: Forward- 5'- CAT CCA

CGT GTT GGC TCA; Reverse- 5'- GAT CAT CTT GCT GGT GAA TGA GT, Cd1d1: Forward- 5'- GTC TGG GGA CAA TCT GAA GC; Reverse- 5'- TCT GTT TGC AAA GGA AGA CAT C, Clec7a: Forward- 5'- ATG GTT CTG GGA GGA TGG AT; Reverse- 5'- GCT TTC CTG GGG AGC TGT AT, Cxcr4: Forward- 5'- TGG AAC CGA TCA GTG TGA GT; Reverse- 5'- GGG CAG GAA GAT CCT ATT GA, Ifngr1: Forward- 5'- TCA AAA GAG TTC CTT ATG TGC CTA; Reverse- 5'- TAC GAG GAC GGA GAG CTG TT, Il12rb2: Forward- 5'- CAG CAA ACA GCA CTT GGG TA; Reverse- 5'- CCA TCA GGA GAT TAT CCG TAG G, Irak2: Forward- 5'- CTA CCG AGC TGC CCA GAT T; Reverse- 5'- GAG GGG AGA TGT GCT CTC AG, Nlrc4: Forward- 5'- TGA TCT CCA AGA GAT GAA GTT GG; Reverse- 5'- GAT CAA ATT GTG AAG ATT CTG TGC, Il1f9: Forward- 5'- GGA CAC CCT ACT TTG CTG CTA; Reverse- 5'- AAC AGG AAT GGC TTC ATT GG, Il1r2: Forward- 5'- CCC ATC CCT GTG ATC ATT TC; Reverse- 5'- GCA CGG GAC TAT CAG TCT TGA, Prg2: Forward- 5'- GGA GCG TCT GCT CTT CAT CT; Reverse- 5'- CCC CTG GAG GAC ACT CTT CT, Trem1: Forward- 5'- TAC AAC CCG ATC CCT ACC C; Reverse- 5'- AAC ACT GGA TGT GGA GAC ACT G, Tnfrsf1a: Forward- 5'- GGA AAG TAT GTC CAT TCT AAG AAC AA; Reverse- 5'- AGT CAC TCA CCA AGT AGG TTC CTT, Nfkb2: Forward- 5'- TGG AAC AGC CCA AAC AGC; Reverse- 5'- CAC CTG GCA AAC CTC CAT, Irf1: Forward- 5'- GAG CTG GGC CAT TCA CAC; Reverse- 5'- TCC ATG TCT TGG GAT CTG G, Cd14: Forward- 5'- AAA GAA ACT GAA GCC TTT CTC G; Reverse- 5'- AGC AAC AAG CCA AGC ACA C, Gusb: Forward- 5'- CTC TGG TGG CCT TAC CTG AT; Reverse- 5'- CAG TTG TTG TCA CCT TCA CCT C, Mapk3: Forward- 5'- TGG AAG CCA TGA GAG ATG TTT; Reverse- 5'- GCT CAG CTG CTG GCT TTT A, Mapk1: Forward- 5'- AAC TCA TTT TTG AAG AGA CTG CTA GA; Reverse- 5'- GTC CTC TGA GCC CTT GTC C, Mapk8: Forward- 5'- AAC

TGT TCC CCG ATG TGC T; Reverse- 5'- TCT CTT GCC TGA CTG GCT TT, Mapk14: Forward- 5'- GAC CTT CTC ATA GAT GAG TGG AAG A; Reverse- 5'- CAG GAC TCC ATT TCT TCT TGG T, Map3k7ip2: Forward- 5'- TTG TTG TAT CCA GGT GCA TGT T; Reverse- 5'- CCA TAA AGA TAC CTT GTA CTC TCC TGA, Mekk1: Forward- 5'- GAT GTG GGG ACT GGG ACT T; Reverse- 5'- TGC TCG GAG GAT GTG TTT CT

2.2.2 Cell maintenance:

Raw 264.7 γNO(-) cells derived from Raw 264.7 mouse macrophage cell line, were obtained from ATCC and maintained in RPMI 1640 medium with L-glutamine (2mM), sodium bicarbonate (1.5 g/l), glucose (4.5 g/l), HEPES (10mM) and sodium pyruvate (1 mM) supplemented with penicillin (100 u/ml)/ streptomycin (100 μg/ml) (Mediatech) and FBS (10%; Hyclone). Bac1.2F5 macrophages were provided by Dr. E. R. Stanley (Albert Einstein College of Medicine, NY) and maintained in Minimum Essential Medium, Alpha 1X supplemented with 25% L-cell medium, penicillin (100 u/ml)/ streptomycin (100 μg/ml), and FBS (10%). All cells were maintained in a humid 5% CO_2 atmosphere at 37°C.

2.2.3 Cytokine induction and ELISAs:

On the day of the exposure assay Raw 264.7 γNO(-) and Bac1.2F5 cells were plated as $2x10^5$ cells/well/180μl and $0.34x10^5$ cells/well/180μl respectively in 96-well tissue culture plates (Nunc). Cells were then incubated with 20 μl, 10X of different stimuli for 5.5 h and 24 h in replicates of six to give final volume 200 μl/well. Culture supernatants were then collected; two replicates of each sample were pooled to give final replicates of three and stored frozen (-80°C) until assayed for cytokine production. After removal of the supernatant, cells were lysed by adding PBS containing Tween 20 (0.01%) and BSA (1%) in the same volume as that of the supernatant and sonicated for 5 min. In a similar way as that of the supernatants, the cell lysates

of two replicates were pooled to give final three replicates and stored frozen (-80°C) until assayed for cytokine production. For estimation of IL-1β secretion in Bac 1.2F5 macrophages, after 24 h incubation with compounds, supernatants were removed and replaced with same volume of medium containing 5 mM ATP (Sigma) for 30 min and harvested supernatants were used for IL-1β measurement.

All cytokine ELISAs were performed in 96-well MaxiSorp plates (Nalgen Nunc International). Cytokine DuoSet ELISA Development Kits (R&D Systems) were used for the cytokine quantification of mouse TNF-α, IL-6, IP-10, RANTES, IL-10, and IL-1β according to the manufacturer's instructions. The absorbance was measured at 450 nm with wavelength correction set to 540 nm using a microplate reader (BMG Labtech). Concentrations of mouse IFN-β in culture supernatants were determined as follows. Plates were coated with rabbit polyclonal antibody against mouse IFN-β (PBL Biomedical Laboratories). IFN-β in standards and samples was allowed to bind to the immobilized antibody. Rat anti-mouse IFN-β antibody (USBiological) was then added. Next, horseradish peroxidase (HRP) conjugated goat anti-rat IgG (H+L) antibody (Pierce) and a chromogenic substrate for HRP 3,3',5,5'-tetramethylbenzidine (TMB; Pierce) were added. After the reaction was stopped, the absorbance was measured at 450 nm with wavelength correction set to 540 nm. All cytokine values are presented as the means of ±SD of triplicate measurements, with each experiment being repeated three times.

2.2.4 mRNA detection by Real-time PCR:

Raw 264.7 γNO(-) and Bac1.2F5 cells were plated as $2.6x10^6$ cells/well/1800 μl in 6 well plates (Nunc) and incubated at 37°C in a humid 5% CO_2 atmosphere for 3-4 hrs. Next, 200 μl, 10X, of different stimuli was added for 90 min. After the incubation, supernatants were removed and

cells were lysed in lysis buffer with β-mercaptoethanol (Strategene) and lysate stored at -80°C. Total RNA was isolated from cell lysates using the Absolutely RNA Miniprep Kit (Strategene) according to the manufacturer's instructions. Isolated RNA was quantitated by measurement of absorbances 260 nm, 280 nm, and 320 nm using a spectrophotometer (Biorad) and 625 ng of RNA was subjected to cDNA synthesis (10 min, 25°C; 30 min, 48°C, 5 min, 95°C) using the TaqMan Reverse transcription Reagents (Applied Biosystems) and thermal cycler (Eppendorf) according to the manufacturer's instructions. The expression of selected genes in each cDNA sample was determined by quantitative real-time PCR using SYBR Green technology (Biorad). Briefly, 5 μl reaction mixture containing iQ™S GYBR Green 2X supermix (2.5 μl, Biorad), forward and reverse primers (400 nM each, IDT), and cDNA (5 ng) was set up in a white shell, clear 96-well PCR plate (Biorad). The reaction mixture was overlayed by 8 μl Vapor-Lock (Corbett) to avoid any evaporation of the reaction mixture and PCR reaction was carried out at 95°C, 3 min; 95°C 15 sec; 60°C, 1 min. Melting curve was performed each time from 55°C to 95°C with increment of 0.5°C for 5 sec to ensure that only one amplification product was formed. The expression of 18S RNA was quantified for each sample and the $\Delta\Delta C_t$ method was used to estimate the differential gene expression between the samples. Each cDNA sample was assayed in replicates of three with each experiment repeated three times. Concentration-response data were analyzed using nonlinear least-squares curve fitting in Prism and EC_{50} concentrations of the stimulus were determined. For the mouse innate and adaptive immune responses PCR array, cells were stimulated and RNA was isolated and quantified in a similar way as above. cDNA was synthesized using the RT^2 First Strand Kit (C-03; SABiosciences) according to manufacturer's instructions. Briefly, 1 μg RNA was subjected to genomic DNA elimination in final volume of 10 μl and incubated at 42°C for 5 min followed by incubation on ice for 1 min.

Next 10 µl RT cocktail was added to it and incubated at 42°C for 15 min, 95°C for 5 min. Next, cDNA was added in a 2550 µl reaction mixture containing SABiosciences RT^2 qPCR mastermix 2X (1275 µl), and 25 µl reaction mixture was added to each well of the PCR array and overlayed with 8 µl Vapor-Lock (Corbett) to avoid any evaporation of the reaction mixture and PCR reaction was carried out at 95°C, 3 min; 95°C 15 sec; 60°C, 1 min. with melting curve from 55°C to 95°C.

2.2.5 siRNA transfection to downregulate ASC/Pycard:

Prior to the transfection assay (24 h) Bac1.2F5 cells were plated as $3x10^4$ cells/well/100µl in complete medium (MEM supplemented with FBS and penicillin and streptomycin, 25% Ladmac cell medium) in a 96-well tissue culture plate (Nunc). siRNA (100 nM) was incubated with Dharmafect reagent (0.3 µl) at RT for 20 min. before adding to the cells in total volume of 100 µl in MEM supplemented with 25% Ladmac medium. Cells were incubated at 37°C for 24 h. For estimation of knockdown of mRNA by real-time PCR, cell lysates were prepared and isolated RNA was used for reverse transcription, followed by real-time PCR. For estimation of secretion of IL-1β, the transfection medium was replaced with complete medium and stimulated with 10X of increasing concentrations of E. coli 055:B5 LPS for 24 h to give final volume 200 µl/well. Culture supernatants were then collected and stored frozen (-80°C) until assayed for cytokine production. After removal of the supernatant, cells were lysed and total protein was estimated in each sample for correction with total protein.

2.3 Statistical data analysis:

Concentration-response data were analyzed using non-linear least-squares curve fitting in Prism (GraphPad Software, Inc). Concentration-response data were fit with the following four-parameter logistic equation: $Y=E_{max}/(1+(EC_{50}/X)^{Hillslope})$, where Y is the cytokine response, X is

the natural logarithm of the concentration of stimulus, E_{max} is the maximum response and EC_{50} is the concentration of the stimulus producing 50% stimulation. The log EC_{50} and standard errors calculated by Prism were used for further statistical analysis via an additive model: $\log[EC_{50}(i,j)]=mu+a(i)+b(j)+e(i,j)$, where *mu* represents the grand mean, a(i) are deviations about *mu* due to effects of the compounds, b(j) are deviations about *mu* due to variations between the cytokines and e(i,j) are random errors. The data were analyzed by three methods, unweighted ANOVA (OLS), weighted ANOVA, and modified weighted ANOVA. The weighted ANOVA method weights inversely propotionally to the square of the standard error (SE) obtained from Prism. This is theoretically the best weighting if the SEs are correct. However, the Prism SE's are estimates from small sample sizes, and some of the Prism SEs give implausibly small precisions, which in turn would give unduly high weights to observations that are not precise estimates. The modified weighted ANOVA method is the same as the weighted ANOVA, but with a lower bound of 0.100 imposed on SEs.

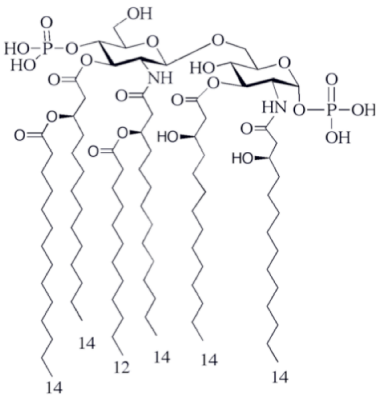
2.4 Results:

2.3.1 LPS and synthetic lipid A molecules from different bacterial species differentially activate TLR4 pathways

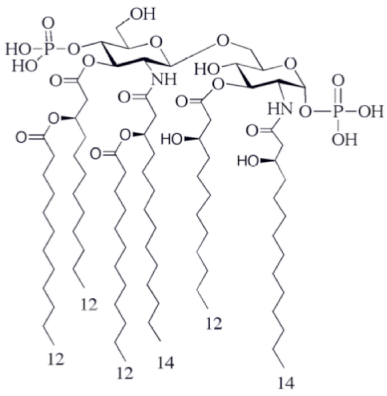
We have employed various synthetic lipid A derivatives corresponding to different bacterial species to study the structure-activity relationship for TLR4 activation. It was anticipated that analysis of potencies and efficacies of the mediators would establish whether structural differences in lipid A show a bias towards either of the pathways. Synthetic lipid As **1**-**7** (Figure 2.1) were used for the TLR4 activation studies. Lipid A derivatives **1** and **2** are *E. coli* lipid As; **3**, **4**, and **5** are *S. typhimurium* lipid As, and **6** and **7** are *N. meningitidis* lipid As (Figure 2.1). The *E. coli* lipid As **1** and **2** are hexa-acylated with asymmetrical pattern of lipid chains containing phosphate residues at C-1 and C-4′ and myristic acids *via* an amide linkage at C-2 and

C-2′ position; and myristic acid *via* an ester linkage at C-3′. Furthermore, both primary (3)-hydroxyacyl chains in the distal glycosamine moiety are esterified with lauric and myristic acids. The myristic acid chains at C-3, C-3′, and at the two primary hydroxyls at the distal glucosamine moieties have been replaced by lauric acids in *E. coli* lipid A derivative **2**. Hence, lipid A **2** has shorter lipid chains than **1**.

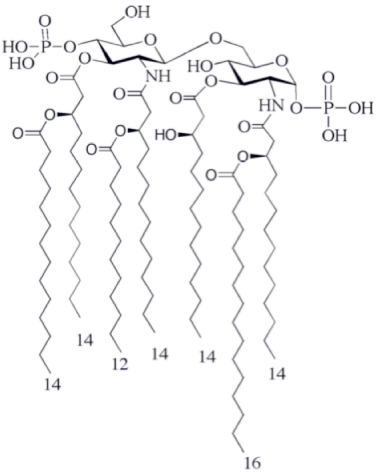
The *S. typhimurium* lipid A is hepta-acylated with asymmetrical pattern of lipid chains and phosphates at C-1 and C-4′ positions. The fatty acid pattern at C-2, C-3, C-2′, and C-3′ is similar to the *E. coli* lipid As. In addition, *S. typhimurium* lipid As **3**, **4**, and **5** have a palmitic acid linked *via* an ester bond at the primary (3)-hydroxyacyl chain of C-2. Lipid A derivatives **4** and **5** have lauric acid chains at C-3, C-3′, and primary hydroxyl of lipid chain at C-3′. Hence they have shorter lipids than lipid A **3**. Moreover, both **3** and **4** have phosphates at C-1 and C-4′ positions while **5** lacks a phosphate at the C-1 position. *N. meningitidis* lipid As **6** and **7** (Figure 2.1) are hexa-acylated with symmetrical pattern of lipid chains of myristic acids linked *via* amide bonds at C-2, and C-2′; and with lauric acids *via* ester linkage at C-3 and C-3′. Furthermore, both the primary (3)-hydroxyl of myristic acids in each of the glucosamine moieties are esterified by lauric acids. Synthetic lipid A **7** is further linked to a 2-keto 3-deoxy-D-manno-octulosonate (KDO) at the C-6′ position. Two KDO molecules connect the lipid A molecule to the oligosaccharide core and are highly conserved in all LPS structures investigated so far.



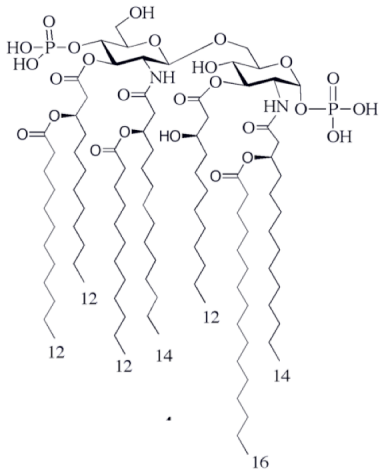
1. *E. coli* lipid A
hexa-acylated
asymmetrical



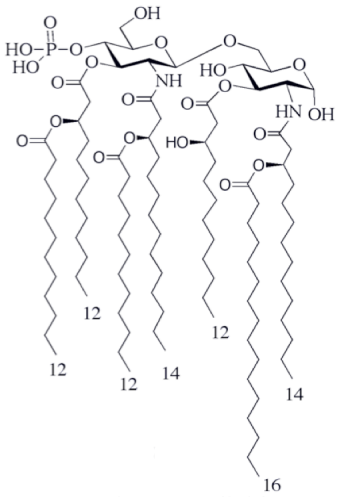
2. *E. coli* lipid A
w/ shorter lipids
asymmetrical



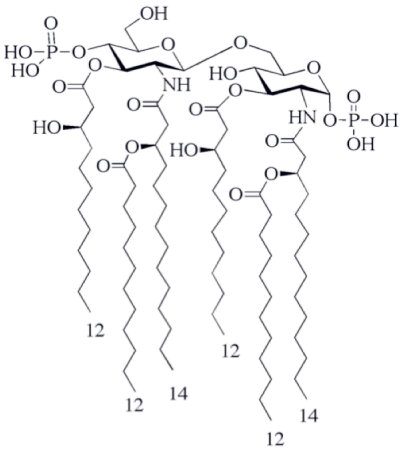
3. *S. typhimurium* lipid A
hepta-acylated
asymmetrical



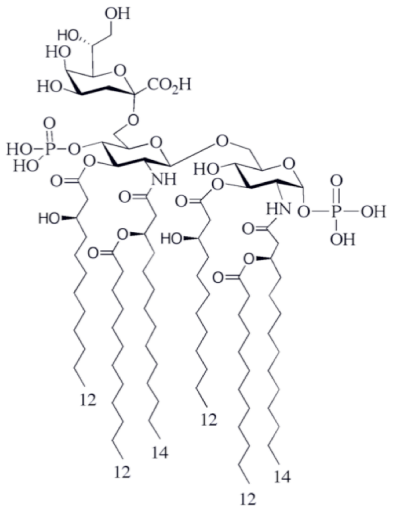
4. *S. typhimurium* lipid A
w/ shorter lipids
asymmetrical



5. *S. typhimurium* lipid A
w/o C-1 phosphate
asymmetrical



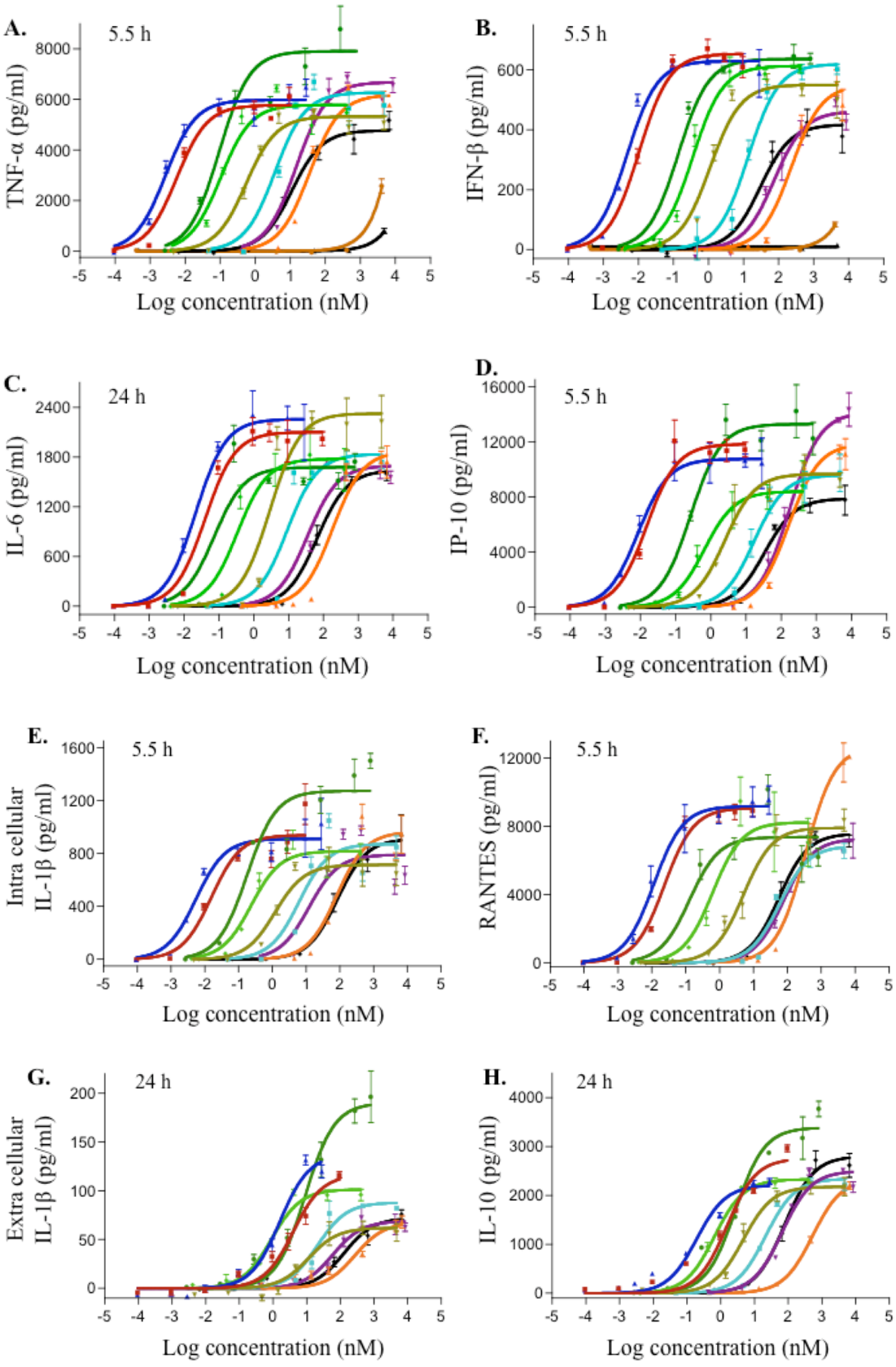
6. *N. mengitidis* lipid A
hexa-acylated
symmetrical

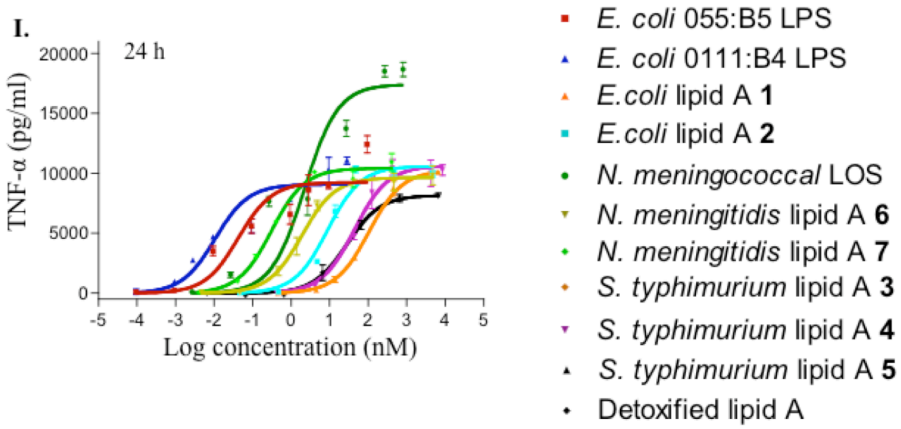


7. *N. mengitidis* lipid A w/KDO symmetrical

Figure 2.1: Chemical structures of synthetic lipid As **1**-**7**.

Synthetic compounds **1**-**7**, *E. coli* 055:B5 LPS, *E. coli* 0111:B4 LPS, *N. meningitidis* LOS, and detoxified lipid A were exposed over a wide range of concentrations to Raw 264.7 γNO(-) cells. Supernatants harvested after 5.5 h and 24 h were examined for the presence of TNF-α and IFN-β. Lipid As **1**, **2**, **4, 6, 7,** *E. coli* 055:B5 and 0111:B4 LPS, *N. meningitidis* LOS, and detoxified lipid A yielded clear dose response curves with large differences in potencies (Figure 2.2). *S. typhimurium* lipid A **3** gave only a partial response at the highest concentration tested, whereas monophosphate lipid A **5** was inactive. Next, the same supernatants obtained from exposure of cells to synthetic compounds **1**, **2**, **4**, **6**, **7**, *E. coli* 055:B5 LPS, *E. coli* 0111:B4 LPS, *N. meningitidis* LOS, and detoxified lipid A were further examined for the presence of RANTES, IP-10, IL-6, IL-1β, and IL-10 using commercial and in-house developed sandwich ELISAs.


A.
5.5 h
TNF-α (pg/ml)
Log concentration (nM)
B.
5.5 h
IFN-β (pg/ml)
Log concentration (nM)
C.
24 h
IL-6 (pg/ml)
Log concentration (nM)
D.
5.5 h
IP-10 (pg/ml)
Log concentration (nM)
E.
5.5 h
Intra cellular IL-1β (pg/ml)
Log concentration (nM)
F.
5.5 h
RANTES (pg/ml)
Log concentration (nM)
G.
24 h
Extra cellular IL-1β (pg/ml)
Log concentration (nM)
H.
24 h
IL-10 (pg/ml)
Log concentration (nM)



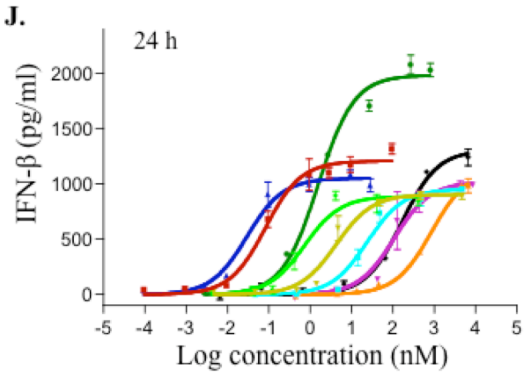
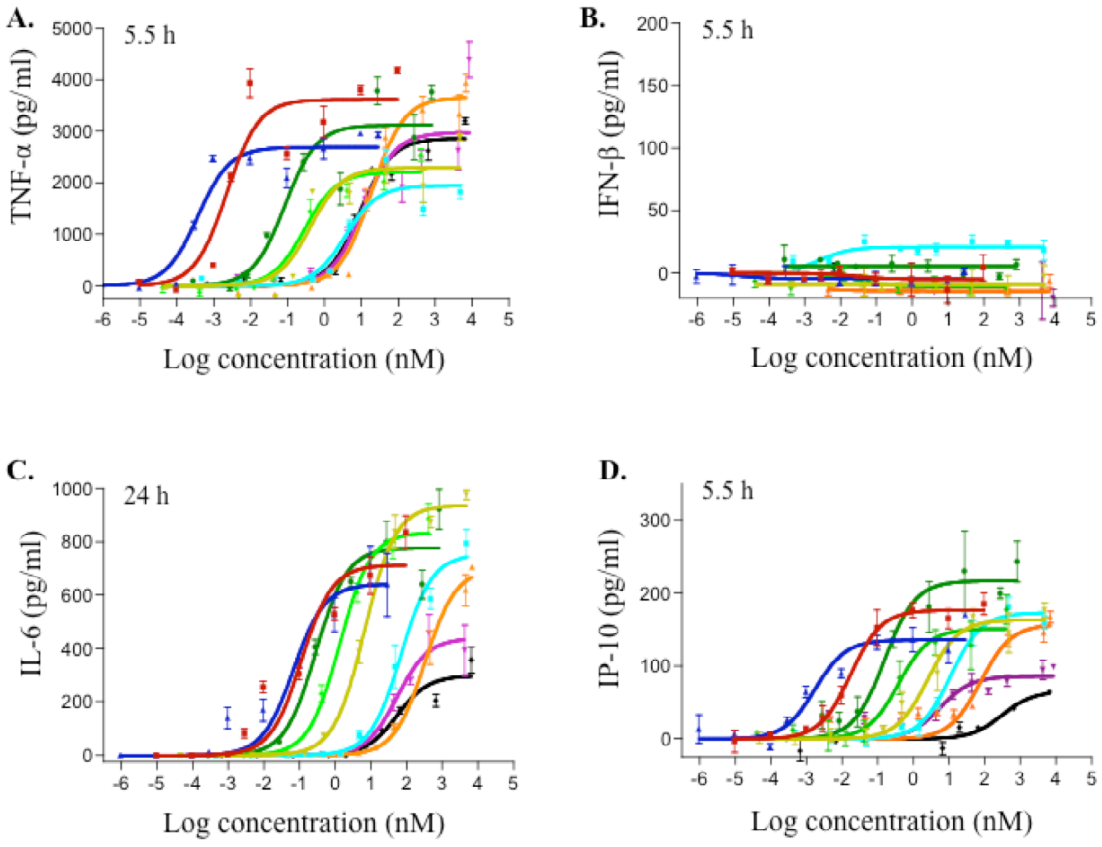
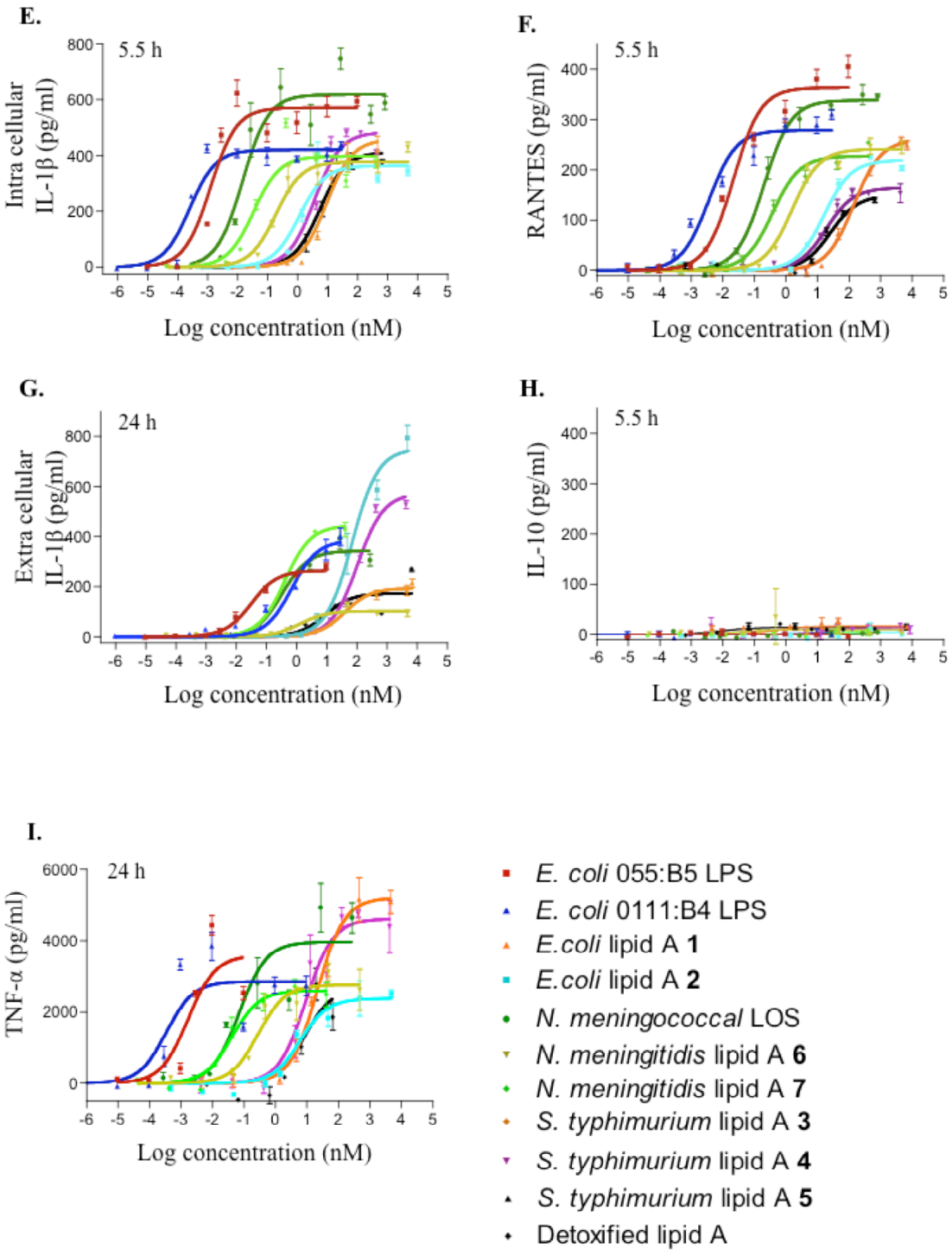


Figure 2.2: Cytokine production by Raw 264.7 γNO(-) macrophage cells after stimulation with LPS and lipid A derivatives. Raw 264.7 γNO(-) cells were incubated with increasing concentrations of different LPS and lipid A derivatives for 5.5 h and 24 h and supernatants were used to measure TNF-α (A), IFN-β (B), IP-10 (D), RANTES (F), and extra-cellular IL-1β (G), IL-10 (H), TNF-α (I), IFN-β (J) respectively using ELISAs. Cell lysates were prepared from

cells induced for 5.5 h and used to measure intra-cellular IL-1β (E). Error bars indicate SD of the mean of three independent experiments.

The range of compounds except, *S. typhimurium* lipid A **3** and **5**, which yielded partial or no response, were also exposed over a wide range of concentrations to Bac 1.2F5 macrophages. It was found that these cells did not secrete IFN-β and IL-10 at 5.5 h and 24 h (Figure 2.3). Moreover, it was observed that for both the cell lines, a short incubation time of 5.5 h led to detection of TNF-α, RANTES and IP-10 (Figure 2.2 and 2.3) however no or low concentrations of IL-6, extra-cellular IL-1β, and IL-10 (data not shown) were detected. The incubation time of 24 h showed clear dose-response curves for IL-6, extra-cellular IL-1β, and IL-10 in both cell lines (Figure 2.2 and 2.3).




E.
5.5 h
Intra cellular IL-1β (pg/ml)
Log concentration (nM)
F.
5.5 h
RANTES (pg/ml)
Log concentration (nM)
G.
24 h
Extra cellular IL-1β (pg/ml)
Log concentration (nM)
H.
5.5 h
IL-10 (pg/ml)
Log concentration (nM)
I.
24 h
TNF-α (pg/ml)
Log concentration (nM)
E. coli 055:B5 LPS
E. coli 0111:B4 LPS
E.coli lipid A 1
E.coli lipid A 2
N. meningococcal LOS
N. meningitidis lipid A 6
N. meningitidis lipid A 7
S. typhimurium lipid A 3
S. typhimurium lipid A 4
S. typhimurium lipid A 5
Detoxified lipid A

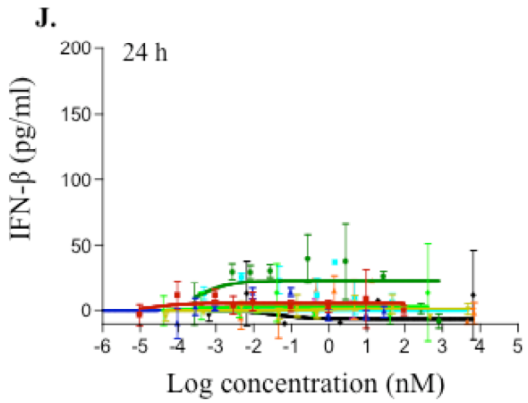


Figure 2.3: Cytokine production by Bac1.2F5 macrophage cells after stimulation with LPS and lipid A derivatives. Bac1.2F5 cells were incubated with increasing concentrations of different LPS and lipid A derivatives for 5.5 h and 24 h and supernatants were used to measure TNF-α (A), IFN-β (B), IP-10 (D), RANTES (F), and IL-6 (C), IL-10 (H) TNF-α (I), IFN-β (J) respectively using ELISAs. For estimation of extra-cellular IL-1β (G) secretion, cells were stimulated with 5 mM ATP for 30 min subsequent to incubation with LPS and lipid A derivatives for 24 h and harvested supernatants were used for ELISA. Cell lysates were prepared from cells induced for 5.5 h and used to measure intra-cellular IL-1β (E). Error bars indicate SD of the mean of three independent experiments.

To detect the possible presence of intracellular cytokines, cell lysates of the cells exposed to the various compounds for 5.5 h were examined for the intracellular intra-cellular IL-1β, TNF-α, IL-6, and IL-10 using ELISA. Cell lysates of the activated cells did not show any TNF-α, IL-6, and IL-10, while a significant quantity of IL-1β was present intracellularly. However, examination of the cell lysates of the cells exposed to the various compounds for 24 h showed minimal presence of IL-1β.

IL-1β is expressed as a pro-protein (pro-IL-1β), which is cleaved by caspase-1 into its active form (IL-1β), which is then secreted. TNF-α is also produced as a pro-protein, which is

proteolytically cleaved by TNF-α converting enzyme (TACE). Interestingly, no detection of TNF-α in the cell lysates after 5.5 h indicates that the proteolytic processing and secretion is not the rate-limiting step for TNF-α, while it is a rate-limiting step for IL-1β. Bac1.2F5 macrophages require the activation by ATP unlike Raw 264.7 γNO(-) cells to secrete IL-1β. It was determined that after stimulation of Bac1.2F5 macrophages with lipid A or LPS for 24 h, addition of 5 mM ATP for 30 min gave a clear dose response curve for the secreted form of IL-1β. Addition of ATP showed a modest decrease in the efficacy of intracellular IL-1β at 24 h. Also, the potency of intracellular IL-1β decreased from 5.5 h to 24 h (Figure 2.4 A). Secreted form of IL-1β in Bac 1.2F5 cells was measured only after treatment of cells with ATP for 30 min after induction of cells with *E. coli* 055:B5 LPS for 5.5 h and 24 h (Figure 2.4 B). However, it was observed that the efficacy increased from 5.5 h to 24 h and potency decreased from 5.5 h to 24 h (Figure 2.4 B). Moreover, clear dose response for secreted form of IL-1β was observed only after stimulation of Bac 1.2F5 cells with other lipid A derivatives for 24 h followed by ATP stimulation for 30 min in Bac 1.2F5 cells (data not shown). Raw 264.7 γNO(-) cells did not secrete any IL-1β after 5.5 h stimulation with *E. coli* 055:B5 LPS and time course showed that efficacy of secreted IL-1β after 24 h was maximum (Figure 2.5). However, when medium was replaced with ATP containing medium for 30 min, it was observed that no IL-1β was measured in the supernatant (Figure 2.4 D), while there was no difference in the efficacy and potency of the intracellular form of IL-1β on ATP stimulation. Hence secreted form of IL-1β was measured in both cell lines after 24 h LPS or lipid A induction in all the earlier experiments. However, since Raw 264.7 γNO(-) cells showed induction of intracellular IL-1β at 5.5 h, which decreased after 24h (Figure 2.5), intracellular IL-1β was measured at 5.5 h in both Raw 264.7 γNO(-) and Bac 1.2F5 macrophages.

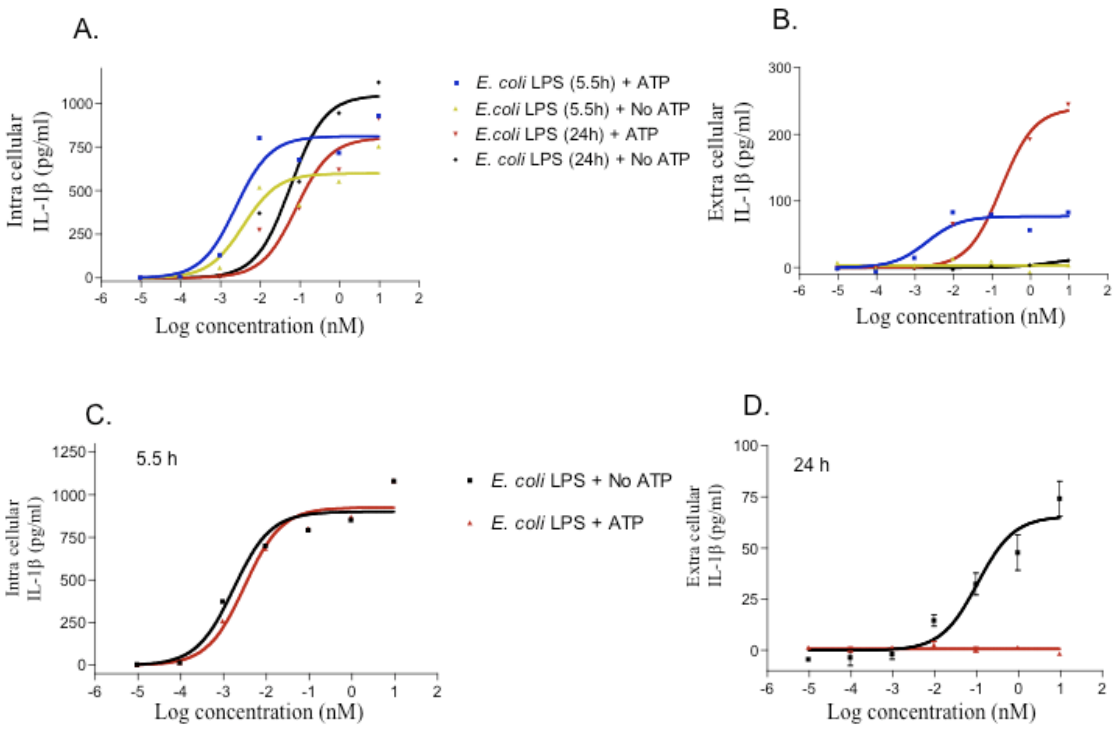


Figure 2.4: Requirement of ATP induction for secretion of IL-1β in Bac1.2F5 macrophages. Bac1.2F5 cells (A, B) and Raw 264.7 γNO(-) (C, D) were incubated with increasing concentrations of *E. coli* 055:B5 LPS for 5.5 h and 24 h and subsequent induction with ATP (5mM, 30 min). Harvested supernatants were used to measure secreted (B, D) form of IL-1β. Cell lysates were prepared from cells induced for 5.5 h and 24 h to measure intracellular IL-1β (A, C). Error bars indicate SD of the mean of three independent experiments.

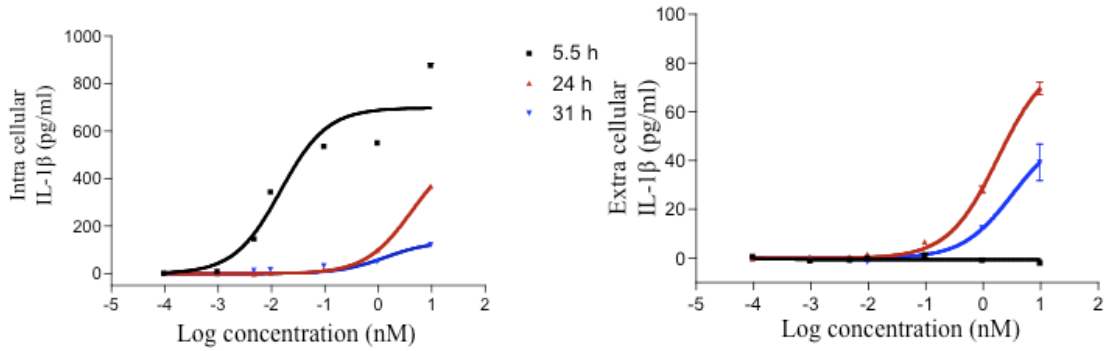


Figure 2.5: Time course of induction of intracellular and secreted form of IL-1β in Raw 264.7 γNO(-) cells. Raw 264.7 γNO(-) were incubated with increasing concentrations of *E. coli* 055:B5 LPS for 5.5, 24, and 31 supernatants were harvested to measure secreted form of IL-1β. Cell

lysates were prepared from cells induced for 5.5, 24, and 31 h to measure intracellular IL-1β. Error bars indicate SD of the mean of three independent experiments.

Potencies (EC_{50}, concentration producing 50% activity) and efficacies (maximum level of production) were determined by fitting the dose-response curves to a logistic equation using Prism software. Examination of the two *E. coli* LPSs from 055:B5 and 0111:B4 strains revealed similar potencies and efficacies in both cell lines, indicating that the variable polysaccharide portion in the LPS from different bacteria do not play significant role in the agonist activity of the lipid A. Moreover, the EC_{50} values for *E. coli* 055:B5 and 0111:B4 LPS were significantly smaller than those of *E. coli* lipid A **1** and **2** (Table 2.1 and Table 2.2). Probably the higher potency of LPS is due to its di-KDO moiety, which is attached to the C-6′ position of lipid A.

Table 2.1: EC_{50} values (nM) of LPS and lipid A derivatives for all cytokines examined in Raw 264.7 γNO(-) cells.

	TNF-α (5.5 h)	IFN-β (5.5 h)	IP-10 (5.5 h)	RANTES (5.5 h)	Intra cellular IL-1β (5.5 h)	IL-6 (24 h)	Extra cellular IL-1β (24 h)	IL-10 (24 h)	TNF-α (24h)	IFN-β (24h)
E. coli 055:B5 LPS	0.005 (0.004-0.007)	0.01 (0.008-0.012)	0.01 (0.01-0.02)	0.02 (0.01-0.03)	0.01 (0.008-0.02)	0.03 (0.02-0.05)	3.94 (3-5)	1.52 (1.05-2.19)	0.04 (0.01-0.09)	0.08 (0.06-0.10)
E. coli 0111:B4 LPS	0.002 (0.002-0.003)	0.004 (0.003-0.005)	0.008 (0.007-0.009)	0.01 (0.009-0.01)	0.005 (0.004-0.007)	0.02 (0.01-0.02)	1.58 (1.12-2.23)	0.19 (0.13-0.27)	0.01 (0.005-0.01)	0.02 (0.02-0.03)
1	35 (25-48)	228 (157-332)	161 (115-226)	336 (287-391)	80 (44-145)	182 (114-290)	268 (152-473)	523 (501-546)	104 (95-114)	845 (742-962)
2	3.64 (2.34-5.65)	13.5 (11-16)	17.0 (13-21)	57 (40-80)	6.19 (3-12)	9.1 (4-17)	20 (14-30)	21 (16-27)	8.94 (6-11)	23 (18-30)
N. men LOS	0.09 (0.05-0.16)	0.13 (0.10-0.17)	0.26 (0.18-0.36)	0.12 (0.05-0.32)	0.17 (0.09-0.34)	0.07 (0.02-0.17)	8.98 (6-12)	2.70 (1.75-4.15)	2.21 (1.09-4.56)	1.54 (1.25-1.89)
6	0.54 (0.42-0.69)	1.11 (0.85-1.45)	2.47 (1.92-3.17)	4.96 (3.90-6.31)	1.20 (0.84-1.72)	2.91 (1.81-4.66)	11.2 (6-19)	4.56 (4.0-5.25)	1.88 (1.73-2.05)	4.18 (3.14-5.59)
7	0.10 (0.07-0.14)	0.34 (0.28-0.43)	0.65 (0.54-0.78)	0.62 (0.35-1.08)	0.23 (0.16-0.34))	0.33 (0.21-0.52)	0.90 (0.62-1.29)	0.65 (0.54-0.78)	0.30 (0.26-0.35)	0.72 (0.51-1.02)
4	14.7 (11-20)	73 (56-95)	170 (147-197)	76 (57-100)	12.2 (4-35)	34 (20-58)	59 (41-85)	65 (47-91)	44 (36-53)	106 (79-142)
Detoxified lipid A	10.9 (8-14)	29 (19-45)	33 (25-42)	55 (45-67)	84 (71-99)	65 (55-77)	127 (86-188)	78 (64-94)	27 (25-30)	162 (135-195)

Further examination of the data revealed that the hexa-acylated *E. coli* lipid A **1** is significantly more potent than the hepta-acylated *S. typhimurium* lipid A **3** in Raw 264.7 γNO(-) cells for TNF-α and IFN-β. Shortening of lipids, such as in compounds **2** and **4**, resulted in higher potencies (smaller EC_{50} values) (Table 2.1 and 2.2). Moreover, the EC_{50} values of the KDO containing lipid A **7** are very similar to the EC_{50} values of *N. meningococcal* LOS for most of the cytokines examined in Bac 1.2F5 and Raw 264.7 γNO(-) cells, which are lower than the EC_{50} values of *N. meningitidis* lipid A **6**. These results demonstrate the importance of the di-KDO unit of LOS for biological activity and presence of at least one KDO moiety can retain the full activity of LOS structure for the induction of a panel of cytokines.

Table 2.2: EC_{50} values (nM) of LPS and lipid A derivatives for all cytokines examined in Bac 1.2F5 cells.

	TNF-α (5.5 h)	IP-10 (5.5 h)	RANTES (5.5 h)	Intra cellular IL-1β (5.5 h)	IL-6 (24 h)	Extra cellular IL-1β (24 h)	TNF-α (24h)
E. coli 055:B5 LPS	0.002 (0.001-0.003)	0.017 (0.014-0.019)	0.02 (0.015-0.029)	0.001 (0.0007-0.0018)	0.11 (0.06-0.19)	0.03 (0.02-0.036)	0.001 (0.0005-0.004)
E. coli 0111:B4 LPS	0.0003 (0.0002-0.0005)	0.001 (0.001-0.002)	0.003 (0.002-0.005)	0.0002 (0.0001-0.0003)	0.06 (0.04-0.10)	0.68 (0.47-0.97)	0.0003 (0.0001-0.0008)
1	18.7 (14.8-23.7)	75 (43-131)	139 (119-162)	9.12 (6.89-12.0)	322 (284-366)	38 (32-45)	20 (18-23)
2	3.62 (2.07-6.30)	10.8 (8.84-13.3)	15.7 (12-20)	1.10 (0.70-1.72)	68 (55-84)	68 (55-84)	5.84 (3.97-8.56)
N. men LOS	0.08 (0.04-0.17)	0.15 (0.10-0.21)	0.20 (0.17-0.22)	0.01 (0.009-0.022)	0.28 (0.18-0.42)	0.33 (0.23-0.45)	0.06 (0.02-0.15)
6	0.40 (0.22-0.73)	2.48 (1.76-3.49)	1.54 (1.21-1.94)	0.18 (0.14-0.23)	6.54 (5.88-7.27)	1.29 (0.78-2.10)	0.28 (0.19-0.38)
7	0.29 (0.20-0.41)	0.38 (0.25-0.55)	0.37 (0.28-0.47)	0.03 (0.01-0.05)	1.31 (1.12-1.53)	0.40 (0.34-0.45)	0.04 (0.02-0.05)
4	11.0 (5.68-21)	5.54 (2.70-11.3)	18.5 (13-25)	3.62 (2.49-5.23)	53 (41-69)	102 (86-121)	9.05 (6.54-12.5)
Detoxified lipid A	8.99 (6.85-11.7)	304 (63-1448)	25 (17-36)	5.33 (4.03-7.04)	52 (35-79)	8.78 (3.67-20)	8.35 (3.33-20)

Subsequently, the calculated log EC_{50} and standard errors calculated were employed for a statistical analysis employing an additive model in which $\log[EC_{50}(i,j)]=mu+a(i)+b(j)+e(i,j)$. where mu is the grand mean, a(i) represents the deviations about the grand mean due to the effects of the compounds, b(j) represents deviations about the grand mean due to the effects of the cytokines, and e(i,j) represents random errors (assumed to be normally distributed with mean 0 and unknown variance. The data were analyzed by an unweighted ANOVA (OLS), weighted ANOVA, and modified weighted ANOVA. Although the order of activity for the compounds and cytokines was consistent for the three models, the modified weighted ANOVA yielded the highest R-squared values for the two cell lines: Raw 264.7 γNO(-) cells (R-squared=0.9652) and Bac 1.2F5 cells (R-squared=0.9216). Hence, this method was employed to calculate the least square means (LSM) estimates for compounds and cytokines.

An additive model assumes that the relative effects of the potency of cytokines (log EC_{50}) are independent of the nature of the compound and *vice-versa*. This assumption can be checked by computing Z-scores in which Z(i,j)=(Obs(i,j)-Exp(i,j))/SE(i,j), for all pairs of compounds (i) and cytokines (j), where, Obs are the values observed in the experiment, Exp are the expected values under the additive model, and SE are the Prism SEs. More than 5% of the Z-scores calculated lied outside the range -2.5<Z<+2.5 (Table 2.3), which indicates that there are some interactions between compounds and cytokines with respect to values of log EC_{50}. However, the additive model yielded a reasonable approximation of the behavior of log EC_{50} for various compound-cytokine combinations indicating only very minor modulation by compound structure.

Table 2.3: Z-scores calculated for each compound and every cytokine examined for Raw 264.7 γNO(-) (A) and Bac 1.2F5 macrophage cells (B). Z-scores are calculated as Z(i,j)=(Obs(i,j)-Exp(i,j))/SE(i,j), where Obs are observed values, Exp are expected values and SE are standard errors for i (compounds) and j (cytokines). Values which are less than -2.5 and higher than 2.5 are in red and green respectively.

A.

	TNF-α (5.5h)	IFN-β (5.5h)	IP-10 (5.5h)	RANTES (5.5h)	Intra cellular IL-1β (5.5h)	IL-6 (24 h)	Extra cellular IL-1β (24 h)	IL-10 (24 h)	TNF-α (24h)	IFN-β (24 h)
E. coli 055:B5 LPS	-0.57	-2.15	-2.21	-3.34	-0.11	0.96	15.7	9.05	0.85	0.35
E. coli 0111:B4 LPS	1.26	-0.45	-0.56	-1.77	0.27	2.58	11.7	5.93	0.88	1.00
1	0.80	2.08	0.63	1.65	0.27	0.87	-1.24	0.94	0.83	2.33
2	1.67	3.24	1.90	3.64	0.50	0.27	-1.30	-0.91	1.74	0.79
N. meningococcal LOS	0.16	-1.80	-1.29	-1.84	-0.26	-1.84	6.53	2.51	2.78	2.08
6	1.46	0.53	1.27	1.86	0.71	1.16	0.79	-1.06	1.98	-0.06
7	1.30	2.10	2.50	0.30	1.11	0.44	-0.87	-1.66	1.94	0.08
4	-0.24	2.78	4.44	-2.00	-1.08	-1.36	-3.02	-3.92	-0.52	-1.74
Detoxified lipid A	0.40	1.62	0.62	0.45	5.77	1.37	0.20	-0.68	1.71	1.78

B.

	TNF-α (5.5 h)	IP-10 (5.5 h)	RANTES (5.5 h)	Intra cellular IL-1β (5.5 h)	IL-6 (24 h)	Extra cellular IL-1β (24 h)	TNF-α (24 h)
E. coli 055:B5 LPS	-0.99	0.82	1.22	-0.31	1.23	-0.10	-0.56
E. coli 0111:B4 LPS	-3.58	-2.33	-1.37	-2.06	4.04	13.06	-1.34
1	-0.17	0.11	1.78	0.90	0.69	-6.52	1.54
2	-0.43	-1.86	-0.42	-0.92	0.23	2.95	1.36
N. meningococcal LOS	0.76	-0.32	0.40	-0.40	-2.12	-0.51	0.79
6	0.04	1.58	0.07	0.98	0.68	-1.87	-0.20
7	3.46	0.25	0.09	-0.03	-0.02	-2.83	-1.80
4	0.78	-2.02	-0.86	1.32	-2.06	2.97	1.89
Detoxified lipid A	0.18	-2.33	-1.37	-2.06	2.58	11.2	-1.34

The cytokines and compounds were separated into groups by the significance calculated using a protected LSD test at α=0.5. As can be seen in Table 2.4 (A), the compounds have more statistically significant differences than the cytokines, with the nine compounds being separated into seven distinct groups for the Raw 264.7 γNO(-) cells and into six distinct groups for the Bac 1.2F5 cells (Table 2.4 (B)). The cytokines were separated into four statistically significant groups for the Raw 264.7 γNO(-) cells and into three significantly distinct groups for the Bac 1.2F5 cells. Importantly, the relative ordering of the potency of the nine compounds was almost identical for the two cell lines.

Table 2.4: Ordering of cytokines and compounds in statistically different groups for Raw 264.7 γNO(-) (A) and Bac 1.2F5 macrophage cells (B). Group 1 has lowest EC_{50} values and Group 4 has highest EC_{50} values. The least square means values normalized to 0 mean in each experiment is also provided.

A

# Group	Cytokine (Least square means)	LPS / Lipid A (Least square means)
1	TNF-α (-0.631)	*E. coli* 0111:B4 LPS (-2.299)
2	Intra cellular IL-1β (-0.280) IFN-β (-0.268) TNF-α (24 h) (-0.150) IL-6 (0.050) IP-10 (-0.033)	*E. coli* 055:B5 LPS (-1.907)
3	RANTES (0.145)	*N. meningitidis* lipid A **7** (-0.720) *N. meningococcal* LOS (-0.685)
4	IFN-β (24 h) (0.403)	*N. meningitidis* lipid A **6** (0.071)
5	IL-10 (0.326) Extracellular IL-1β (0.433)	*E. coli* lipid A **2** (0.755)
6		*S. typhimurium* lipid A **4** (1.501) Detoxified lipid A (1.310)
7		*E. coli* lipid A **1** (1.971)

B

# Group	Cytokine (Least square means)	LPS / Lipid A (Least square means)
1	TNF-α (-0.386) Intra cellular IL-1β (-0.764) TNF-α (24 h) (-0.455)	*E. coli* 0111:B4 LPS (-2.572) *E. coli* 055:B5 LPS (-1.857)
2	IP-10 (0.141) RANTES (0.226)	*N. meningitidis* lipid A **7** (-0.579) *N. meningococcal* LOS (-0.872)
3	IL-6 (0.744) Extra cellular IL-1β (0.498)	*N. meningitidis* lipid A **6** (0.127)
4		*S. typhimurium* lipid A **4** (1.333) *E. coli* lipid A **2** (1.159) Detoxified lipid A (1.407) *E. coli* lipid A **1** (1.857)

Examination of the efficacies for the cytokines in the two cell lines revealed interesting correlations. Considering the different cell number for both cell types in the induction assay, the MyD88-dependent cytokines, TNF-α, IL-6, intra-cellular IL-1β, and extra-cellular IL-1β showed higher efficacies in Bac 1.2F5 cells (Table 2.6) than Raw 264.7 γNO(-) cells (Table 2.5). However, Raw 264.7 γNO(-) cells showed higher efficacies for TRIF-dependent cytokines IP-10 and RANTES. Moreover, the efficacies were maximum for IP-10 and TNF-α in Raw 264.7 γNO(-) and Bac 1.2F5 cells, respectively. Interestingly, the efficacies for different cytokines on induction with each compound varied by about 20-fold and 100-fold in Raw 264.7 γNO(-) and Bac 1.2F5 cells respectively.

Table 2.5: Efficacies (pg/ml) of cytokines for dose-response curves of *E. coli* 055:B5 LPS, *E. coli* 0111:B4 LPS, *N. meningococcal* LOS, detoxified lipid A, synthetic lipid A derivatives **1**, **2**, **4**, **6**, and **7** examined in Raw 264.7 γNO(-) cells.

	TNF-α (5.5 h)	IFN-β (5.5 h)	IP-10 (5.5 h)	RANTES (5.5 h)	Intra cellular IL-1β (5.5 h)	IL-6 (24 h)	Extra cellular IL-1β (24 h)	IL-10 (24 h)	TNF-α (24h)	IFN-β (24h)
E. coli 055:B5 LPS	5782 ±218	654 ±19	11840 ±553	9084 ±326	940 ±75	2103 ±76	116 ±8.39	2761 ±237	9254 ±949	1209 ±35
E. coli 0111:B4 LPS	5987 ±214	630 ±24	10762 ±270	9185 ±246	911 ±42	2259 ±67	137 ±9	2214 ±129	9082 ±825	1052 ±38
1	6197 ±362	550 ±36	11880 ±691	12706 ±375	964 ±101	1872 ±151	67 ±7	2350 ±21	10266 ±164	1132 ±32
2	6281 ±452	620 ±25	9613 ±473	6856 ±523	873 ±117	1838 ±228	88 ±7	2344 ±124	10613 ±604	955 ±54
N. men LOS	7918 ±551	636 ±20	13290 ±600	7374 ±862	1274 ±109	1679 ±182	190 ±9	3391 ±230	17430 ±1935	1985 ±60
6	5337 ±205	550 ±26	9676 ±456	7926 ±353	715 ±45	2328 ±208	62 ±5	2182 ±57	9653 ±154	905 ±48
7	5794 ±381	613 ±24	8413 ±272	8241 ±818	818 ±57	1781 ±149	101 ±6	2333 ±76	10432 ±302	888 ±53
4	6693 ±277	73.6 ±23	14163 ±454	7274 ±397	791 ±112	1700 ±144	69 ±4	2502 ±161	10633 ±374	1019 ±62
Detoxified lipid A	4783 ±184	417 ±30	7898 ±353	7583 ±290	912 ±29	1643 ±50	72 ±5	2806 ±107	8180 ±140	1308 ±49

Table 2.6: Efficacies (pg/ml) of cytokines for dose-response curves of *E. coli* 055:B5 LPS, *E. coli* 0111:B4 LPS, *N. meningococcal* LOS, detoxified lipid A, synthetic lipid A derivatives **1**, **2**, **4**, **6**, and **7** examined in Bac 1.2F5 cells.

	TNF-α (5.5 h)	IP-10 (5.5 h)	RANTES (5.5 h)	Intra cellular IL-1β (5.5 h)	IL-6 (24 h)	Extra cellular IL-1β (24 h)	TNF-α (24h)
E. coli 055:B5 LPS	3618 ±36	177 ±3.6	363 ±16	572 ±36	713 ±65	265 ±8	3594 ±884
E. coli 0111:B4 LPS	2692 ±164	136 ±9	278 ±12	420 ±24	641 ±42	386 ±25	2876 ±435
1	3662 ±220	157 ±8	260 ±8	458 ±16	699 ±16	194 ±7	5215 ±176
2	1945 ±122	173 ±9	219 ±9	364 ±36	752 ±21	752 ±11	2377 ±150
N. men LOS	3120 ±289	217 ±10	338 ±5.3	619 ±40	776 ±43	343 ±18	3972 ±481
6	2295 ±220	163 ±8.3	241 ±8	378 ±16	937 ±16	102 ±7.3	2772 ±176
7	2211 ±122	151 ±9	227 ±9	397 ±36	835 ±21	445 ±11	2602 ±150
4	2978 ±332	86 ±9	164 ±11	482 ±30	439 ±27	573 ±25	4625 ±276
Detoxified lipid A	2864 ±158	65 ±23	149 ±16	412 ±25	298 ±27	173 ±30	2670 ±734

Examination of the efficacies (maximum response) of IL-6 on induction with *E. coli* and *N. meningitidis* LOS and lipid A derivatives in both cell types provided unexpected structure-activity relationships (Table 2.5). *E. coli* 055:B5 and 0111:B4 LPS induced similar efficacies for the production of IL-6, which is higher than *E. coli* lipid A **1** and **2**; while for *N. meningitidis*, the efficacy for the production of IL-6 by *N. meningococcal* LOS and *N. meningitidis* lipid A **7** was similar but lower than lipid A **6**, which lacks the KDO in Raw 264.7 γNO(-) cells. However, similar observations were not found for Bac 1.2F5 macrophages (Table 2.6). Hence these results indicate that the presence of KDO unit is not important for retaining the efficacies of the LPS molecule.

2.3.2 Differential expression of cytokines by lipid As is regulated at transcriptional event:

After establishing the order for potencies of cytokines (as protein) for the different LPSs and synthetic lipid As in two different mice macrophage cells, attention was focused to study the modulation of activation of cytokines at the transcriptional level by examining the potencies for their mRNA. Mouse macrophages (Raw 264.7 γNO(-) cells) were exposed over a wide range of concentrations to two synthetic lipid A derivatives, the *N. meningitidis* lipid As **6** and **7** for 90 min. At the end of incubation, cell lysates were prepared and total RNA was isolated for the various samples. A reverse transcription PCR was performed to obtain the cDNA, which was used for Sybr Green quantitative real-time PCR to determine the levels of mRNA induced for various cytokines. Potencies (EC_{50}, concentration producing 50% activity) were determined by fitting the dose-response curves to logistic equation using PRISM software. A two-tailed T-test with 95% confidence intervals was performed over the log EC_{50} values of each cytokine for the protein and the mRNA. It was observed that for both *N. meningitidis* lipid A derivatives, the potencies of mRNA were not significantly different than the potencies of protein for all the cytokines examined, as shown in Table 2.7. This indicates that for most of the cytokines, the modulation with synthetic lipid A derivatives occurs upstream of transcription of the cytokines.

Table 2.7: T-test analysis (two-tailed, 95% confidence interval) of Log EC_{50} (nM) for various cytokines for mRNA and protein in Raw 264.7 γNO(-) cells induced with *N. meningitidis* lipid A **6** (A) and **7** (B). Log EC_{50} are statistically significant if *p* value <0.05.

A.

	Log EC_{50} (nM) (Std error Log EC_{50})		
	protein	mRNA	
	5.5 h/24 h	90 min	P value
TNF-α	-0.26 (0.10)	-0.38 (0.26)	0.69
IFN-β	0.04 (0.11)	0.61 (0.19)	0.07
Intra cellular IL-1β	0.08 (0.15)	-0.02 (0.18)	0.68
IP-10	0.39 (0.10)	0.63 (0.16)	0.28
RANTES	0.69 (0.10)	0.71 (0.20)	0.92
IL-6	0.46 (0.20)	0.60 (0.29)	0.71
IL-10	0.65 (0.06)	0.63 (0.60)	0.96

B.

	Log EC_{50} (nM) (Std error Log EC_{50})		
	protein	mRNA	
	5.5 h/24 h	90 min	P value
TNF-α	-0.99 (0.15)	-1.54 (0.28)	0.16
IFN-β	-0.45 (0.09)	0.03 (0.15)	0.05
Intra cellular IL-1β	-0.62 (0.16)	-0.66 (0.10)	0.85
IP-10	-0.18 (0.07)	-0.11 (0.17)	0.73
RANTES	-0.20 (0.24)	0.01 (0.16)	0.48
IL-6	-0.47 (0.19)	-0.36 (0.19)	0.71
IL-10	-0.18 (0.07)	0.10 (0.39)	0.50

2.3.3 Genes involved in TLR4 induction pathway are highly upregulated in Bac1.2F5 macrophage cells as compared to Raw 264.7 γNO(-) cells:

To study the differential expression of cytokines in the two cell lines, the expression of genes involved in TLR4 pathway in Raw 264.7 γNO(-) cells and Bac1.2F5 macrophage cells on treatment with 100 ng/ml *N. men* lipid A w/KDO **7**, was examined. Induction of 100 different innate and adaptive immune response genes including transcription factors, adaptor proteins, cytokines, MAPkinases, and proteins involved in inflammasome complex were studied. The CT

values were normalized to the CT values of a housekeeping gene for each sample (18S rRNA, Gusb, ActinB, and Hspb1). Results shown in Figure 2.6 and Table 2.8 are for 18S rRNA and similar results were found for other housekeeping genes (data not shown).

Table 2.8: Different genes (100) studied in both cell types on induction with *N. meningitidis* lipid A **7** (100 ng/ml) using a mouse innate and adaptive immune response array and few separate primer pairs. Delta CT for each gene in both cell types was calculated using the 18 S rRNA as the housekeeping gene. Next, delta delta CT was calculated by subtracting delta CT values of Raw 264.7 γNO(-) from Bac 1.2F5 cells and fold change was calculated as $2^{-\text{delta delta CT}}$.

Gene	Fold change	Gene	Fold change	Gene	Fold change	Gene	Fold change
Adora 2a	0.87	IFN-b	0.16	JNK	4.13	Proc	2.29
C8a	2.29	Ifngr2	3.41	Lalba	4.02	Ptafr	3.31
Camp	1.90	Ikbkb	2.58	Lbp	2.00	Pycard	1089
Caspase 1	2.80	IL-10	0.20	Ltf	1.58	Rantes	3.04
Caspase 4	24	IL-18	2.65	Ly96	0.79	Serpina1a	0.24
Ccl2	13.5	Il1a	0.31	Lyz1	1.22	Serpine1	4.82
Ccr3	3.75	IL-1β	51	Mapk14	1.46	Sftpd	2.29
CD14	9.05	Il1f10	2.29	Mapk8	2.15	Stab1	1.76
Cd1d1	12.3	Il1f5	2.29	MEKK1	2.75	TAB1	4.20
Cd55	2.29	Il1f6	2.69	Mif	0.58	TAB2	5.57
Cfp	0.96	Il1f8	16.5	MyD88	1.43	TACE	1.55
Chuk	2.12	Il1f9	109	Nalp-1	190	Tgfb1	2.82
Clec7a	167	Il1r1	0.85	Ncf4	4.56	Ticam 2	11.7
Colec12	2.84	Il1r2	165	NF-κB1	2.26	TLR1	1.47
Crp	2.29	Il1rap	2.09	NF-κB2	11.9	TLR2	2.20
Cxcr4	20.1	Il1rapl2	2.29	Nfkbia	1.79	TLR3	2.07
Cybb	0.93	Il1rl2	3.58	Nlrc4	24	TLR4	1.58
Defb4	0.23	Il1rn	1.74	Nlrp-3	3.36	TLR6	2.28
Dmbt1	2.29	Il2rb2	203	Nos2	2.54	TLR8	0.49
ERK1	9.02	IL-6	12.99	p38	3.84	TLR9	0.80
ERK2	3.75	IP-10	1.66	Pglyrp1	2.00	TNF-α	20
Fn1	2.29	Irak1	2.32	Pglyrp2	2.29	Tnfrsf1α	4.50
Hc	2.29	Irak2	12.6	Pglyrp3	2.29	Tollip	2.01
Hmox1	0.63	IRF-1	29.3	Ppbp	0.76	Traf6	0.88
Ifnar-1	6.63	IRF-3	1.07	Prg2	13.2	Trem1	110

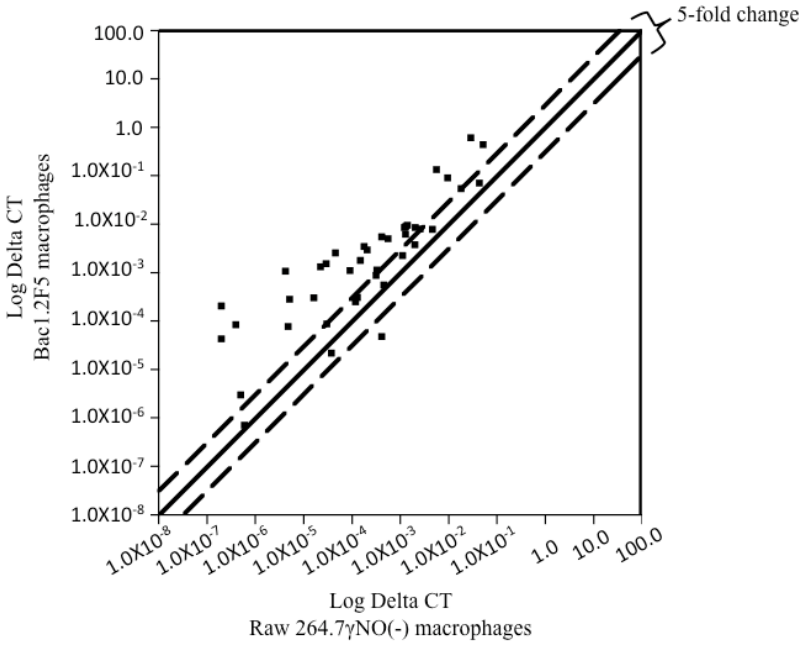


Figure 2.6: Array of genes upregulated 3-fold or higher in Raw 264.7γNO(-) and Bac1.2F5 macrophages on induction with *N. meningitidis* lipid A **7**. Values are plotted as an average of Log Delta CT of three independent experiments. Data were normalized with 18S rRNA. Points lying beyond the dashed lines are expressed more than 5-fold in either of the two cell types.

A two-tailed T-test with 95% confidence interval was performed over the delta CT values of the genes in both cell lines for which a 5-fold change of delta CT in Bac 1.2F5 cells over Raw 264.7 γNO(-) cells was observed (Table 2.9). It was observed that the induction of TRIF-dependent cytokine, IFN-β was more that 5 fold higher in Raw 264.7 γNO(-) cells as compared to the Bac1.2F5 macrophage cells (data not shown) while the expression of MyD88 dependent cytokines, such as TNF-α, IL-6, and extra-cellular IL-1β was higher in Bac 1.2F5 macrophages than Raw 264.7 γNO(-) cells. Moreover, expression of MyD88 was similar in both cell types (data not shown) while that of TRIF/Ticam-2 was more than 10 fold higher in Bac1.2F5 macrophages. Induction of NF-κB, the transcription factor of MyD88-dependent cytokines was

higher in Bac1.2F5 macrophages while IRF-3 the transcription factor of TRIF-dependent cytokines was similar in both cell types.

Table 2.9: T-test analysis for various genes with 5-fold change in Bac1.2F5 cells over Raw 264.7 γNO(-) macrophage cells. Different genes (100) were studied in both cell types on induction with *N. meningitidis* lipid A **7** (100 ng/ml) using a mouse innate and adaptive immune response array and few separate primer pairs. Delta CT for each gene in both cell types was calculated using the 18 S rRNA as the housekeeping gene. Next, delta delta CT was calculated by subtracting delta CT values of Raw 264.7 γNO(-) from Bac 1.2F5 cells and fold change was calculated as $2^{-\text{delta delta CT}}$. T-test further analyzed the genes that were expressed 5-fold or higher in Bac1.2F5 cells. Mean fold change of three independent experiments were used for the analysis. Asterisks indicate statistically significant (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

Gene	Fold Change	Error-	Error+	P-value
Caspase-4	24	6.93	9.72	0.01 (*)
Ccl-2	13.6	3.88	5.44	0.008 (**)
CD-14	9.05	2.61	3.68	0.01 (*)
Cd1d1	12.4	4.34	6.68	0.005 (**)
Clec7a	167	49.2	70	0.005 (**)
Cxcr4	20.1	5.86	8.26	0.0002 (***)
ERK1	9.03	2.63	3.72	0.001 (**)
Ifnar-1	6.63	1.93	2.73	0.01 (*)
Ifngr-1	12.3	3.52	4.93	0.005 (**)
Il12rb2	335	120	188	<0.0001 (***)
IL-1b	52	14.6	20.3	0.01 (*)
Il1f9	110	41.7	67	0.005 (**)
Il1r2	165	62	100	0.0005 (***)
IL-6	130	37.6	53	0.003 (**)
Irak-2	12.7	3.63	5.09	0.001 (**)
Irf-1	29.3	8.42	11.8	0.003 (**)
Nalp-1	190	106	241	0.0001 (***)
NF-kb2	12.0	3.46	4.86	0.001 (**)
Nlrc4	25.0	7.48	10.7	0.0001 (***)
Prg2	13.2	5.28	8.79	0.009 (**)
Pycard	1089	327	467	<0.0001 (***)
Ticam-2	11.8	3.40	4.79	0.007 (**)
TNF	21.9	6.26	8.76	0.002 (**)
Trem1	110	31.2	44	0.001 (**)

Except ERK1, which was more that 5 fold higher induced in Bac1.2F5 macrophages as compared to Raw 264.7 γNO(-) cells, other MAP kinases studied were similarly induced in both cell types. Moreover, proteins involved in the inflammasome complex such as caspase-4, ASC/Pycard, and Nalp-1 (Table 2.9) are highly induced in the Bac1.2F5 macrophages.

2.3.4 Attempts for the measurement of activation of caspase-1 and IL-1β:

Cellular activation studies showed that the potencies for intra-cellular IL-1β and TNF-α were similar while they were as much as 10-1000 fold higher for extra-cellular IL-1β. The potencies obtained from dose-response curves for mRNA indicated that the modulation of activation of extra-cellular IL-1β secretion arises during the processing of intra-cellular IL-1β to secreted form of IL-1β. Moreover, the gene activation profile showed that the Raw 264.7 γNO(-) cells express caspase-1, however, they lack the core component ASC/Pycard, which is essential for the activation of caspase-1 and processing of pro-IL1β to IL-β.

Caspase-1 is known to cleave the leader sequence of pro-IL-1β and convert into IL-1β, which can be secreted. Caspase-1 is constitutively expressed as an inactive 45-KDa zymogen that undergoes catalytic processing by an enzyme complex known as inflammasome upon receipt of an appropriate stimulus. The active form of caspase-1 comprises of subunits p20 and p10, which assemble to form a heterotetramer. ASC/Pycard, Nalp-1, Ipaf, and caspase-4 are the known components, which constitute the inflammasome and are constitutively expressed. Based on our earlier results we propose that different lipid A derivatives differentially activate the inflammasome complex, resulting in differential activation of caspase-1 which ultimately leads to the differential activation of IL-1β. Hence attempts were made to detect the components (p10 and p20) of active caspase-1 by western blots.

Raw 264.7 γNO(-) cells were exposed to *E. coli* 055:B5 LPS for different time intervals (1h to O/N) and RIPA lysis buffer was added directly to the medium containing cells. Cell lysates were harvested, vortexed, and centrifuged to separate the supernatants and pellet. Supernatants were loaded (20 μg protein) on a 4-20% Tris-HCl gel. The proteins were subsequently transferred onto a nitrocellulose membrane and western blot was performed by blocking the membrane in 5% skimmed milk in TBS-T buffer and developed with anti-caspase-1 p10 antibody and anti-rat IgG as primary and secondary antibodies respectively. A 45 KDa band corresponding to the pro-caspase-1 could be observed however, a band corresponding to p10 was not observed (Figure 2.8). Similar assays were also performed using different LPS and lipid A derivatives for induction and different primary antibodies for western blots in Raw 264.7 γNO(-) cells and Bac 1.2F5 macrophage cells. However, none of the results showed the bands for active caspase-1. It could be possible that the caspase-1, being an enzyme is activated in very small amounts, which could not be detected by western blots.

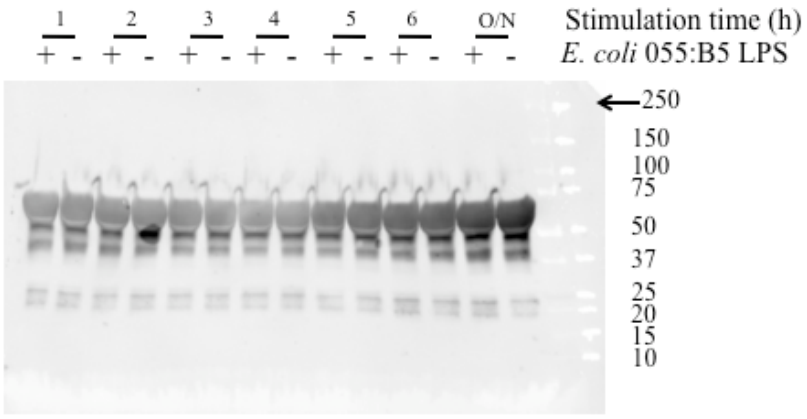


Figure 2.7: Detection of active caspase-1 in Raw 264.7 γNO(-) macrophage cells after stimulation with *E. coli* 055:B5 LPS. Raw 264.7 γNO(-) cells were incubated with 1000 ng/ml of *E. coli* 055:B5 LPS for 1, 2, 3, 4, 5, 6 h and over night. RIPA lysis buffer (3X) was directly added to the cells and the harvested cells and supernatants were maintained on ice for 30 min with vortexing intermitantly. 20 μg protein from each sample was loaded on 4-20% Tris-HCl SDS gel followed by western blot. Blot was developed with primary antibody specific against the p10 subunit.

Attempts were made to detect the secreted forms of IL-1β on stimulation of Raw 264.7 γNO(-) cells with *E. coli* 055:B5 LPS for 24h by western blot experiments. A 33 kDa band corresponding to the pro-IL-1β was observed in the cell lysate treated with *E. coli* 055:B5 LPS, however mature IL-1β (17 kDa) was not observed in either the cell lysates or the supernatant (Figure 2.7). Similar western blot experiments will be performed to detect IL-1β in the cell lysates and supernatants of Bac 1.2F5 macrophages.

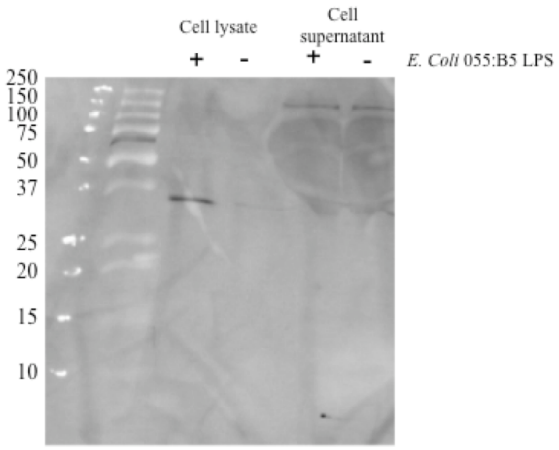


Figure 2.8: Detection of IL-1β in Raw 264.7 γNO(-) cells. Raw 264.7 γNO(-) were incubated with *E. coli* 055:B5 LPS (500 ng/ml) for 24 and supernatants were harvested and concentrated by passing through 10 kDa molecular weight cut-off filter (Nanosep). Cell lysates were prepared by washing the cells with PBS and lysed with RIPA lysis buffer. 12 μg protein was loaded on 15% Tris-HCl gel and western blot was performed using rabbit anti-mouse IL-1β antibody as the primary antibody.

In addition to the detection of active caspase-1, attempts were also made to down-regulate the activation of the inflammasome using sequence specific oligo siRNA for ASC/Pycard and caspase-1 (Dharmacon) in Bac 1.2F5 macrophage cells. Four different designs of siRNAs were screened for both caspase-1 and ASC/Pycard transcripts. siRNAs were transfected into the cells using different Dharmafect transfection reagents, for 24 h and initially knockdown of the mRNA was studied with real time quantitative PCR. It was observed that with each of the four different siRNA sequences used for caspase-1, none of the siRNA showed more than 50% knockdown of the caspase-1 mRNA, while two different siRNAs showed less than 50% knockdown, one showed about 65% knockdown and another sequence showed about 80% knockdown of the ASC mRNA (data not shown). Use of siRNA for ASC/Pycard and the transfection reagents had some effect on IFN-β and TNF-α, which were marginally upregulated.

Next, the transfection with siRNA was followed by *E. coli* 055:B5 LPS induction for 24 h and ATP stimulation for 30 min, after which the supernatants were harvested to measure the release of IL-1β. It was found that the siRNA treated samples showed a clear dose response curve. Moreover, the potency decreased by 5-fold (EC_{50} increased) for siRNA treated cells induced with *E. coli* 055:B5 LPS (Figure 2.9). More siRNA experiments will be performed with different siRNA sequences for ASC/Pycard.

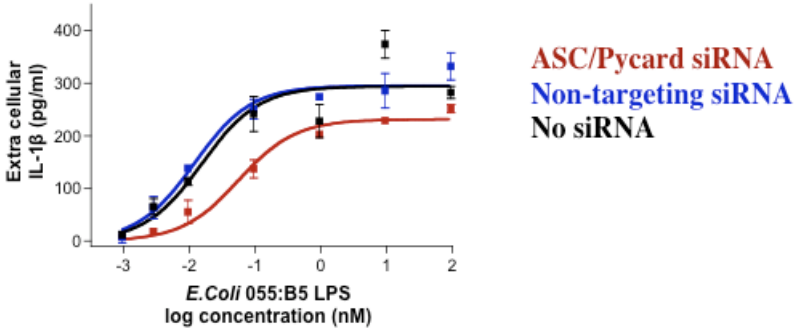


Figure 2.9: Knockdown of ASC/Pycard with siRNA and its effect on release of IL-1β. Bac 1.2F5 cells were transfected with ASC/Pycard siRNA (100 nM, J-051439-11; Thermo Scientific) for 24 h, medium was next replaced and cells were induced with increasing concentrations of *E. coli* 055:B5 LPS for 24 h. Then medium was replaced with ATP (5mM) containing medium and cells were incubated for 30 mins. Next the supernatants were removed and stored at -80°C for measurement of IL-1β by ELISA.

2.5 Discussion:

Structural studies have demonstrated that the carbohydrate backbone, degree of phosphorylation, and fatty acid acylation patterns vary considerably among bacterial species and that these differences account for high variable *in-vivo* and *in-vitro* host responses to LPS[10,11,20,21,29]. There are also indications that structurally different lipid As can differentially induce proinflammatory responses[30-33]. For example, it has been found that the lipooligosaccharide of *N. meningitidis* is a potent agonist of MyD88-dependent and independent cytokines. On the other hand, *E. coli* 55:B5 and *Vibrio cholerae* LPS, at the same picomolar concentration of lipid A, selectively induced the MyD88-dependent pathway, while *S. typhimurium* LPS primarily invoked the MyD88-independent pathway. In another study[34], it was found that bone marrow derived macrophages (BMDMs) exposed over a narrow range of concentrations to monophosphoryl lipid A (MPLA) from *S. minnesota,* which is a promising human adjuvant candidate, induced lower levels of IL-6 and similar levels of IFN-β and IP-10 as compared to *S. minnesota* LPS. These observations led to the conclusion that MPLA is a TLR4 agonist for the TRIF-dependent pathway. The importance of selective activation of the MyD88-dependent and independent pathways has been underscored by the results of *in vivo* and *ex vivo* studies. For example[31], knock-out mice, lacking the ability to synthesize TNF-α, are resistant to lethal amounts of LPS from *E. coli* 055:B5, but remain as susceptible to *S. typhimurium* LPS as wild type mice. In this study, the lethal effects of *S. typhimurium* LPS were due to production of MyD88 independent cytokines such as IFNγ and IL-18.

A clear understanding of the structure-function relationships involved in the activation of TLR dependent inflammatory responses will provide exciting opportunities for the rationale design of adjuvants for immunizations and immune modulators for the treatment of various

diseases that specify both the danger context of the antigens delivered and the Th1 and Th2 pathway to be utilized for the development of protective activities. Also, understanding the structural basis of inflammatory activation will allow a more direct assessment to be made regarding the roles of molecular properties of pathogens in disease pathogenesis.

Heterogeneity in lipid A of particular bacterial strain and possible contaminations with other inflammatory components of the bacterial cell-wall, has made it difficult to dissect specific molecular motifs that may bias an immune response towards MyD88 or TRIF pathway. Also, a cytokine bias at a single or narrow range of concentration of agonist may not necessarily signify modulation by structural features of the agonist.

Therefore, we have examined the ability to induce the production of a wide range of cytokines in two well established mouse macrophage cell lines (Raw 264.7 γNO(-) and Bac 1.2F5) by a range of well defined synthetic homogenous lipid A derivatives derived from *E. coli*, *S. typhimurium*, and *N. meningitidis* and *E. coli* 055:B5 and 0111:B4 LPS, *N. meningitides* LOS over a wide range of concentrations. These mouse macrophage cell lines, which have been obtained from primary monocyte cells by differentiation into macrophages, make it possible to examine a series of compounds over wide range of concentrations on a sufficiently large scale to measure the production of a panel of cytokines and chemokines corresponding to the two distinct TLR4 activation pathways.

It was found that the synthetic compounds and LPS preparation displayed large differences in potency of TNF-α production. Furthermore, for each compound large differences in potencies (EC_{50} values) for the various cytokines was observed, which did not appear to follow a bias towards a MyD88 or TRIF-dependent response. Statistical analysis by an additive model showed the same order of potencies for the various compounds in both cell lines. Thus,

the LPS preparations from *E. coli* were more potent than the LOS from *N. meningitidis* and the KDO containing compound **7**, which in turn were significantly more potent that the prototypic synthetic lipid As derived from *E. coli* (**1**) and *N. meningitidis* (**6**). These data demonstrate that KDO significantly contributes to the inflammatory potency of LPS. It has long been thought that the inflammatory properties of LPS and LOS reside in the lipid A moiety. However, recent studies have shown that lipid A expressed by meningococci with defects in Kdo biosynthesis or transfer has significantly reduced bioactivities compared to Kdo_2 containing meningococcal LOS[35]. Removal of the Kdo moieties of wild type LOS by mild acetic acid treatment also attenuated cellular responses. Interestingly, dendritic cells stimulated with Kdo_2-lipid A from meningococci but not lipid A alone stimulated naïve allogeneic CD4+ cells to secrete enhanced levels of IFN-γ relative to T-cells primed with immature DCs[36]. Several other studies have suggested that the Kdo moiety of LPS or LOS contributes to inflammatory responses. For example, it has been found that *Salmonella* lipid A is inactive whereas the parent LPS is a potent activator of NF-κB in a TLR4-dependent manner in a human monocyte cell line[37]. In addition, a synthetic enteric lipid A containing a di-Kdo moiety was a more potent elicitor of TNF-α and IL-6 compared to its parent lipid A[38]. Furthermore, LPS from a nitrogen-fixing symbiont, *Rhizobium sin*-1 is able to significantly inhibit the *E. coli* LPS-dependent synthesis of TNF-α by human monocytes. A comparison of the biological responses of synthetic and isolated lipid A derivatives and *R. sin-1* LPS indicated that the Kdo moieties are important for optimal antagonistic properties[39-41]. Thus, it is probable that the cell surface receptors that recognize LPS bind to the lipid A as well as to the Kdo moiety of LPS. Indeed, a recent X-ray co-crystal structure of LPS and MD2 and TLR4 showed atleast one H-bond interaction of Kdo with TLR4[42].

The statistical analyses also showed significant differences between the potencies of the various lipid As. Most importantly, the prototypic lipid A from *N. meningitidis* (**6**) was more potent than *E. coli* lipid A. Interestingly, the lipid A of *N. meningitidis* is hexa-acylated in a symmetrical fashion (**6**) whereas enteric bacteria have an asymmetrically hexa-acylated lipid A (**2**) (Figure 1). Also, a number of the fatty acids of *N. meningitidis* are shorter compared to those of *E. coli*. It appears that these structural differences account for differences in potency. It been suggested that the differences in the nature and pattern of fatty acid substitution of enteric and meningococcal lipid A may affect patho-biological properties.

It was also found that *S. typhimurium* lipid A **3** gave only a partial response at the highest concentration tested, whereas the corresponding *S. typhimurium* derived monophosphoryl lipid **5** was inactive. Furthermore, shorter the lipids as in *S. typhimurium* lipid A derivative **4**, greatly increased the potency (**3** *vs*. **4**). Also, *E. coli* lipid A **2**, which has shorter lipids than the parent compound **1**, was more potent albeit the difference was not as large as for *S. typhimurium* lipid As **3** and **4**. Thus, these results indicate that shorting fatty acids of lipid A may increase the potency of the derivative, however, further analogs need to be prepared and studied to obtain a more complete picture. It has been observed that pathogens that cause chronic infections such as periodontitis and peritoneal infections, have fatty acids much longer than what is typically observed in enteric bacteria and hence this type of substitution may diminish detection by the innate immune system[43].

A surprising observation was that for a given compound the potencies of the various cytokines differed substantially. For the Raw 264.7 γNO(-) cells, three statistically different groups were determined whereas for the Bac1.2F5 cell five different groups were found. Importantly, a different order of relative ranking was found in the two cells lines. Furthermore,

the grouping did not follow a pattern of MyD88 and TRIF related cytokines. However, in each case, secretion of TNF-α at 5 hrs was observed at lower concentrations than for any of the other cytokines. Furthermore, the synthesis of intracellular IL-1β was induced at much lower concentrations than the secretion of IL-1β.

An additive model could be employed to describe the potencies of the compounds. However, examination of Z-scores revealed the presence of several interactions between compound and cytokine, suggesting that the expression of some cytokines is modulated by the nature of the compound. A larger number of interactions was found for the Raw 264.7 γNO(-) than for the Bac1.2F5 cell and in particular the secretion of IL-1β seemed subject to immune modulation.

Previously, it was reported that the low toxicity of MPLA's adjuvant function maybe associated with a bias toward TRIF signaling, which was suggested to be caused by active suppression, rather than passive loss of proinflammatory activity of this LPS derivative. This finding might have important implications for the development of future vaccine adjuvants. In this study, murine cells were however, subjected to only one concentration of LPS and lipid A. At this concentration, release of IL-6, IP-10, and IFN-β were measured. It was observed that MPLA induced low levels of IL-6 and similar levels of IFN-β and IP-10 compared to *S. minnesota* LPS and it was concluded that MPLA is a TLR4 agonist for TRIF-dependent pathway.

The results of this study indicate that an observed bias towards the expression of a set cytokine maybe due concentrations of selected agonists rather than due to immune modulation by the structure of the compounds. Firm conclusion about immune modulation can only be drawn when a sufficiently large range of concentrations is examined.

A surprising finding was that for all compounds examined, the potency of intracellularly expressed IL-1β protein was significantly higher than that of secreted IL-1β. Furthermore, exposure of the Bac1.2F5 to compound alone resulted in intracellular expression of IL-1β protein, which was not secreted (Figure 2.4). The addition of ATP resulted, however, in the secretion of IL-1β. In the case of the Raw 264.7 γNO(-) cells, ATP did not affect the intracellular production and secretion of IL-1β.

IL-1β is expressed as a 31-33 kDa proprotein in response to inflammatory stimuli that activate NF-κB gene transcription. Processing of pro-IL-1β by the protease caspase 1 is required for producing and secretion of mature 17-kDa IL-1β[44,45]. Caspase 1, in turn, is constitutively expressed as a pro-protein and is processed to its active form upon assembly of the inflammasome[46]. The inflammasome includes caspase 1 and 11, Nalp proteins and ASC/Pycard. The inflammasome is in turn activated by a secondary stimulus involving engagement of cytotoxic T-cells or activation of purinergic $P2X_7$ receptor by a high concentration of extracellular ATP[44].

Some cell types express low level of ASC and cannot activate caspase-1, which may result in the secretion of immature IL-1β *via* a pathway distinct from caspase-1[47]. The gene expression data show that Raw 264.7 γNO(-) cells express low levels ASC/Pycard, caspase-4, and Nalp-1, which are involved in the formation of the inflammasome. We found that in both cells lines a significantly higher concentration of LPS or lipid A was required for the secretion of IL-1β than for its expression. This finding indicates that LPS and lipid A plays a role the caspase-1 dependent and independent secretion. Furthermore, the statistical analysis showed interactions between several compounds and the secretion of IL-1β suggesting differences in the structure activity relationship for the expression of pro-IL-1β and subsequent secretion.

To examine in more detail the influence of ASC on IL-1β secretion, the expression level of ASC in Bac1.2F5 cells was reduced by siRNA and the potency and efficacies of IL-1β production compared with cells that were exposed to a non-targeting siRNA sequence. Interestingly, a modest reduction of ASC expression (75%) led to statistically significant reduction in the potency of IL-1β secretion. Thus, these results indicate that immune cells can modulate cytokine production by differential expression of proteins involved in the maturation of cytokines. A comparison of gene expression between the two cells demonstrated other differences that may modulate the cytokine pattern in a cell type specific manner. For example, higher expression of the MAP kinase ERK1, the adaptor protein TRIF/Ticam-2, and the transcription factors NF-κB were observed in the Bac1.2F5 cells. Further studies are required to examine the importance of the differential expression of these proteins for the production of various cytokines.

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CHAPTER 3

Synthetic Tetra-Acylated Derivatives of Lipid-A from *Porphyromonas-gingivalis* are Antagonists of Human TLR-4

3.1 Introduction:

Periodontitis is an inflammatory disease that is characterized by loss of an alveolar bone supporting the tooth root and is the leading cause for tooth loss. Removal of dental plaque microbial biofilm remains the most effective treatment for the disease. Studies of dental plaque associated with healthy and periodontitis subjects showed that the levels of *P. gingivalis*, *B. forsythus*, *Treponema denticola*, and *Selenomonas noxia* were significantly elevated in periodontitis subjects[1]. However, little is known about how these different bacterial compositions influence the inflammatory response.

Porphyromonas gingivalis is a gram-negative anaerobic, black, pigmented bacterium that is an important causing agent of human adult-type periodontitis[2]. This bacterium releases large amounts of vesicles containing LPS, which can penetrate periodontal tissue and participate in destructive innate host response associated with the disease. *P. gingivalis* is able to activate human monocytes by s-CD14 (soluble CD14)-dependent mechanism. Several reports have demonstrated that the *P. gingivalis* LPS utilizes TLR2 instead of TLR4 for activation of the murine macrophages. However, it has been suggested that the TLR2 response maybe due to contamination with lipoproteins[3,4]. It has also been found that LPSs of *P. gingivalis* can inhibit IL-6 and IL-β secretion and ICAM expression induced by enteric LPS by U373 and human

peripheral mononuclear cells and human gingival fibroblasts, respectively[5]. Another study found that a purified tetra-acylated monophosphoryl lipid A structure can antagonize E-selectin expression in human cells exposed to enteric or *P. gingivalis* LPS[6]. It appears that MD2 represents the principal molecular component used by these LPS derivatives for inhibition[7].

Several studies have indicated that compounds that can antagonize cytokine production induced by enteric LPS may have the potential to be developed as therapeutics for the treatment of gram-negative septicemia[8]. Success in this area has been limited, and most efforts have been directed towards the synthesis of analogs of lipid A of *Rhodobacter sphaeroides*[9,10]. Analogs of the lipid A moiety of Helicobacter pylori, which have fewer but longer fatty acids and are only phosphorylated at the anomeric center, have also been shown to inhibit IL-6 secretion by human whole blood cells[11]. Recently, it was reported that synthetic lipid As derived from *Rhizobium sin*-1, which lacks phosphates but contains an 2-aminogluconolactone and a very long chain fatty acid 27-hydroxyoctacosanoic acid, can prevent the induction of TNF-α by *E. coli* LPS in human monocytic cells[12-16].

The structure of lipid A, the active moiety of *P. gingivalis* LPS, is different from the classical enterobacterial lipid A. Although *P. gingivalis* LPS contains considerable heterogeneity in its lipid A composition, it shows the presence of two unusual branched fatty acids namely *R*-(3)-hydroxy-15-methylhexadecanic acid and R-(3)-hydroxy-13-methyltetradecanic acid. Moreover, these lipid As exhibit a unique pattern of phosphorylation and acylation. These lipid As lack a phosphate at C-4 position of the non-reducing sugar, and are tetra-acylated rather than the usual hexa-acylated pattern[17,18]. The LPS isolated from *P. gingivalis* as been reported to be a TLR4- and TLR2-agonist in murine macrophages[19]. However, it has been suggested that the TLR2 responses maybe due to contamination with lipoproteins[3,4]. LPS preparation contains a

mixture of many lipid A structures and the structure activity with TLR2 and TLR4 has not been studied. The lipid A structures with antagonist properties may have potential therapeutic properties for the treatment or prevention of septic shock. In this respect, the chemical synthesis of a tri- and penta-acylated lipid A has been reported and the biological studies have shown that these compounds can activate human and murine cells in TLR4-dependent manner. We focus on the activation of human and murine TLR4 and TLR2 receptors by two tetra-acylated lipid As derived from *P. gingivalis* differing in the pattern of acylation.

3.2 Materials and Methods:

Reagents: *E.coli* 055:B5 LPS was obtained from List Biologicals and Pam_3CysSK_4 was obtained from Calbiochem. Synthetic tetra-acylated *P. gingivalis* lipid As were reconstituted in PBS with dry THF(10%) and stored at -80°C.

3.2.1 Cell maintenance:

RAW 264.7 γNO(-) cells were obtained and maintained as mentioned in chapter 2. Mono Mac 6 (MM6) cells, provided by Dr H.W.L. Ziegler-Heitbrock (Institute for Inhalation Biology, Munich, Germany), were cultured in RPMI 1640 medium with L-glutamine (BioWhittaker) supplemented with penicillin (100 u/ml), streptomycin (100 μg/ml; Mediatech), OPI supplement (1%; Sigma containing oxaloacetate, pyruvate and bovine insulin) and fetal bovine serum (FBS; 10%; HyClone). New batches of frozen cell stocks were grown up every 2 months and growth morphology evaluated. Before every experiment, MM6 cells were incubated with calcitriol (10 ng/ml; Sigma) for 2 days to differentiate into macrophage like cells. Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's medium (ATCC) with glutamine (4 mM), glucose (4.5 g/l), and sodium bicarbonate (1.5 g/l), supplemented with penicillin (100 u/ml), and FBS (10%). Stably transfected HEK 293T cells with murine or human

TLR4/MD2/CD14 (InvivoGen), and mouse or human TLR2 were grown in the same growth medium as for HEK 293T cells supplemented with selective agents HygroGold (50 μg/ml; InvivoGen) and blasticidin (10 μg/ml; InvivoGen); and blasticidin (10 μg/ml) respectively. All cells were maintained in a humid 5% CO_2 atmosphere at 37°C.

3.2.2 Cytokine induction and ELISAs:

Cytokine induction assays in Raw 264.7 γNO(-) cells were performed as mentioned in chapter 2. On the day of the exposure assay differentiated MM6 cells were harvested by centrifugation and suspended (10^6 cells/ml) in tissue culture tubes Cells were then incubated with different combinations of stimuli for 5.5 h. Culture supernatants were then collected and stored frozen (-80°C) until assayed for cytokine production. All cytokine ELISAs were performed in 96-well MaxiSorp plates (Nalgen Nunc International). Concentrations of human TNF-α protein in culture supernatants were determined by sandwich ELISA. Plates were coated with purified mouse anti-human TNF-α antibody (Pharmingen). TNF-α in standards and samples was allowed to bind to immobilized antibody. Biotinylated mouse anti-human TNF-α antibody (Pharmingen) was then added. Next, avidin-horseradish peroxidase conjugate (Pharmingen) and ABTS peroxidase substrate (Kirkegaard & Perry Laboratories) were added. After the reaction was stopped, by adding peroxidase stop solution (Kirkegaard & Perry Laboratories), the absorbance was measured at 405 nm using a microplate reader (BMG Labtech). Cytokine ELISAs for murine TNF-α, IFN-β, IL-6, IP-10, and IL-1β were performed as mentioned in chapter 2.

3.2.3 Transfection and NF-κB activation assay:

The day before transfection, HEK 293T wild type cells and HEK 293T cells stably transfected with human and murine TLR4/MD2/CD14 and human and murine TLR2 were plated in 96-well tissue culture plates (16,000 cells/well). Next day, cells were transiently transfected with

expression plasmids pELAM-Luc (NF-κB-dependent firefly luciferase reporter plasmid, 50 ng/well) and pRL-TK (*Renilla* luciferase control reporter vector, 1ng/well; Promega) as an internal control to normalize experimental variations using PolyFect Transfection Reagent (Qiagen). The empty vector pcDNA3 (Invitrogen) was used as a control and to normalize the DNA concentration for all of the transfection reactions (total DNA 70 ng/well). Fourty-four h post-transfection, cells were exposed to the stimuli in the presence of FCS to provide soluble CD14 for 4 h, after which extracts were prepared. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions and a combination luminometer/fluorometer microplate reader (BMG Labtech). Expression of the firefly luciferase reporter gene was normalized for transfection efficiency with expression of *Renilla* luciferase. The data reported as the means ±SD of triplicate treatments. The transfection experiments were repeated at least twice.

3.3 Data Analysis:

Concentration-response data were analyzed using Prism software as mentioned in chapter 2. Inhibition data were fit with the following logistic equation: Y=bottom+(top-bottom)/(1+$10^{(X-\log IC50)}$), where Y is the cytokine response, X is the logarithm of the concentration of the inhibitor and IC_{50} is the concentration of the inhibitor that reduces the response by half.

3.4 Results and Discussion:

It is clear that the enteric LPS induces cellular activation through TLR4 via two distinct pathways differing at the initiation point where either of the two adaptor proteins is recruited at the cytoplasmic domain of TLR4, one being a specific intracellular adaptor protein called MyD88 and the other adaptor protein called TRIF, which operates independently of MyD88[20,21]. TNF-α, IL-6, and IL-1β is the prototypical measurement of MyD88-dependent pathway,

whereas secretion of IFN-β and IP-10 are used as an indicator of TRIF-dependent pathway. The variation of lipid As in the carbohydrate backbone, degree of phosphorylation, and acylation pattern can account for significant differences in inflammatory responses[22].

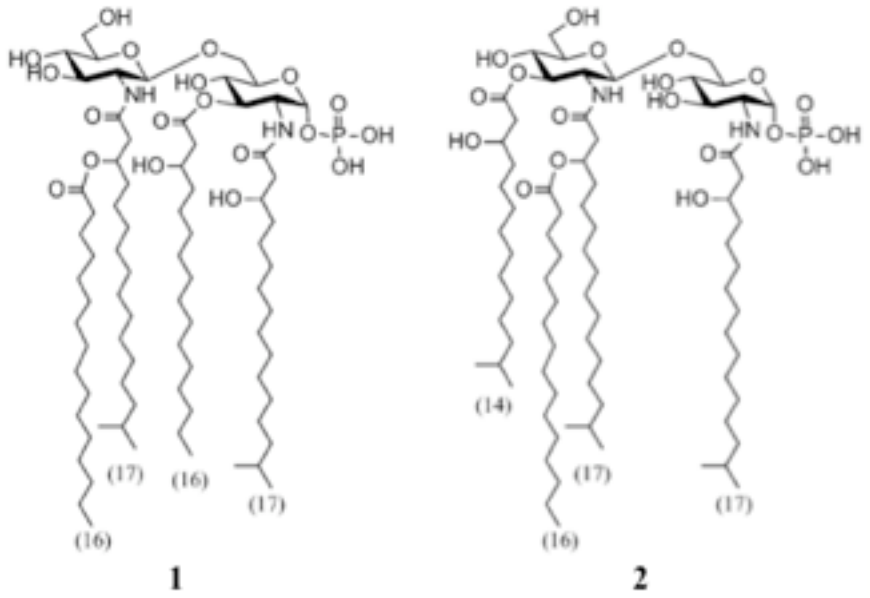

Figure 3.1: Structures of synthetic *P. gingivalis* lipid As.

Several studies indicate that LPS from various bacterial species such as *P. gingivalis*, *L. interrogans*, *L. pneumophila*, *B. fragilis* NCTC-9343, and *P. aeruginosa* PAC-611 can induce cellular activation in a TLR2-dependent manner[23-26]. However, it may be possible that the cellular responses are derived from contamination by lipoproteins. We have used chemically synthesized tetra-acylated lipid As **1** and **2** (Figure 3.1) to study whether lipid As derived from *P. gingivalis* can induce cellular activation in a TLR2- or TLR4-dependent manner. Furthermore, there are indications that LPS of *P. gingivalis* can antagonize cytokine production induced by enteric LPS and therefore these properties have also been studied. Thus, a human monocytic cell line (Mono Mac 6 cells) was exposed over a wide range of concentrations to compounds **1** and **2** and *E.coli* 055:B5 LPS. After 5.5 h, the supernatants were harvested and examined for human

TNF-α using a commercial capture ELISA. Potencies (EC_{50}, concentration producing 50% activity) and efficacies (maximal level of production) were determined by fitting the dose-response curves to a logistic equation using PRISM software. As can be seen in Figure 3.2, *E. coli* 055:B5 LPS is a potent inducer of TNF-α whereas the synthetic compounds **1** and **2** did not exhibit any activity. A similar experiment using mouse macrophages (RAW 264.7 γNO(-) cells) did not lead to secretion of cytokines (eg. TNF-α, IL-6, IP-10, IFN-β, and IL-1β) when exposed to compound **1** and **2** (Figure 3.3).

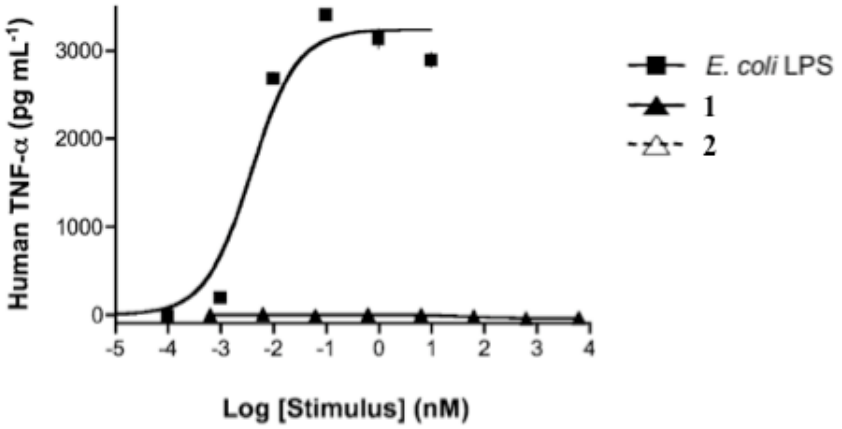

Figure 3.2: Concentration-response curves of *E.coli* 055:B5 LPS and synthetic *P. gingivalis* lipid As **1** and **2** in human monocytic cells. MM6 cells were incubated for 5.5 h at 37°C with increasing concentrations of *E.coli* 055:B5 LPS and synthetic compounds **1** and **2** as indicated. TNF-α protein in cell supernatants was measured using ELISA.

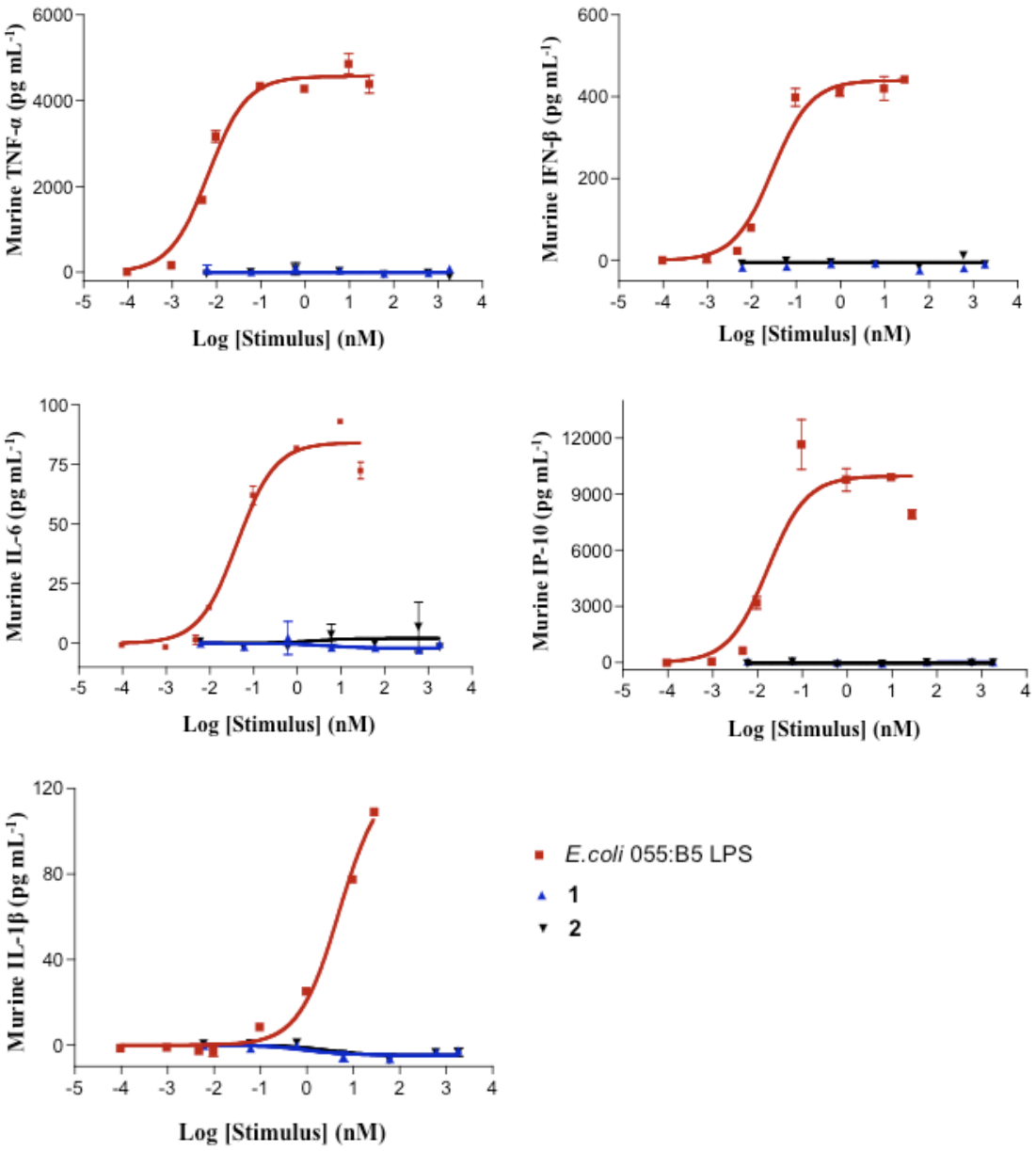

Figure 3.3: Concentration-response curves of *E.coli* 055:B5 LPS and synthetic *P. gingivalis* lipid As **1** and **2** in mouse macrophage cells. RAW 264.7 γNO(-) cells were incubated for 5.5 h and 24 h at 37°C with increasing concentrations of *E.coli* 055:B5 LPS and synthetic compounds **1** and **2** as indicated. TNF-α, IFN-β, IP-10, and Rantes protein was measured after 5.5 h and IL-6, and IL-1β after 24 h in cell supernatants using ELISA.

Synthesis and secretion of TNF-α protein depends on a complex process involving activation of transcription factors, upregulation of the genes responsible for production of the

cytokine, transcription of the message, and then translation of the mRNA and processing of the protein[27-29]. As this process is tightly controlled, it may be possible that a compound can activate NF-κB or induce expression of TNF-α mRNA without causing production or secretion of the TNF-α protein[30]. To examine the ability of the synthetic compounds to induce activation of NF-κB, HEK 293T cells were employed that were stably transfected with various immune receptors and transiently transfected with a plasmid containing the reporter gene pELAM-Luc (NF-κB-dependent firefly luciferase reporter vector) (Figure 3.4). No activation of NF-κB was observed when cells transfected with human TLR4/MD2/CD14 and human or mouse TLR2 were exposed to compounds **1** and **2**. As expected, LPS, which is a potent activator of TLR4, could activate cells transfected with TLR4/MD2/CD14 and Pam_3CysSK_4, which is a known agonist of TLR2, was able to activate the TLR2-containing cells. However, at high concentrations, compound **2** could induce NF-κB activation in cells transfected with mouse TLR4/MD2/CD14. These results clearly demonstrate that compounds **1** and **2** do not induce cellular activation in a TLR2-dependent manner. Although compound **2** is a weak activator of mouse TLR4, it could not induce the secretion of cytokines.

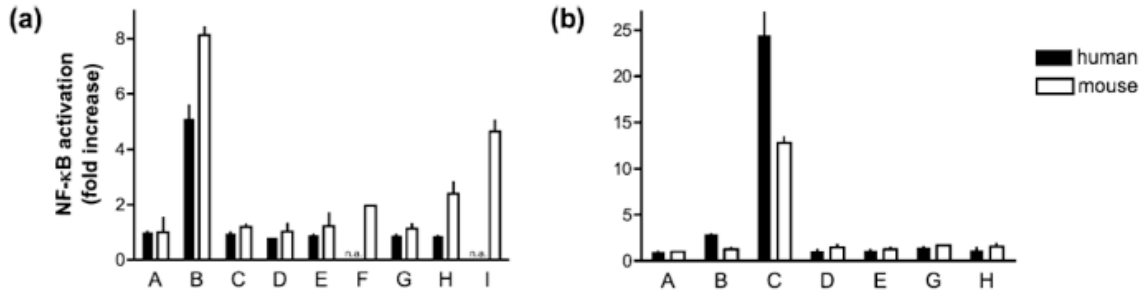

Figure 3.4: Response of HEK 293T cells expressing human or murine TLRs to **1** and **2**. Induction of NF-κB activation was determined in triplicate cultures of HEK 293T cells stably transfected with human or mouse (a) TLR4/MD2/CD14 and (b) TLR2 and transiently transfected with pELAM-Luc and pRL-TK plasmids. Fourty-four h post-transfection, cells were treated with (B) *E. coli* 055:B5 LPS (10 ng/ml), (C) Pam_3CysSK_4 (1 μg/ml), (D, E and F) 1 (0.1, 1 and 10 μg/ml respectively), (G, H and I) 2 (0.1, 1 and 10 μg/ml, resepectively) or (A) were left untreated (control). Fourty-eight h post-transfection, NF-κB activation was determined by firefly luciferase activity relative to *Renilla* luciferase activity. n.a. indicates not analyzed.

Compounds that lack proinflammatory properties may still interact with relevant receptors (TLR4/MD2/CD14) thereby inhibit TNF-α production induced by *E.coli* 055:B5 LPS. Thus the human monocytic cells (MM6) and mouse macrophages (RAW 264.7 γNO(-) cells) were exposed to a combination of *E.coli* 055:B5 LPS (10 ng/ml) and a wide range of concentrations of lipid As **1** and **2** and, after an incubation time of 5.5 h, the supernatant was examined for human or mouse TNF-α. Only marginal inhibition was observed in the mouse cells. However, both compounds were able to antagonize TNF-α production by the human cells (Figure 3.5) and it was found that compound **1** was significantly more potent antagonist than **2** (IC_{50} concentration producing 50% inhibition for **1** and **2** were 160 nM and 3.2 μM respectively).

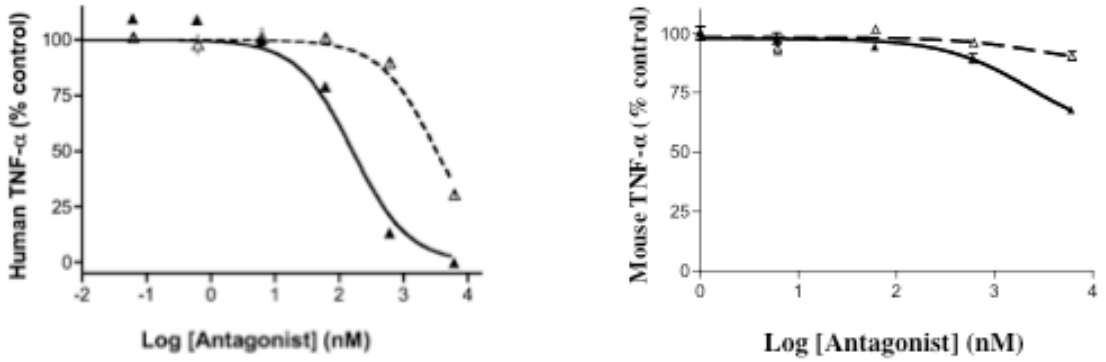

Figure 3.5: Antagonism of *E. coli* 055:B5 LPS by synthetic compounds **1** and **2** in human monocytic cells and mouse macrophage cells. TNF-α concentrations were measured after preincubation of cells with increasing concentrations of **1** and **2** as indicated for 1 h at 37°C, followed by 5.5 h of incubation with *E. coli* 055:B5 LPS (1 ng/ml). Results are expressed as percentage of cytokine concentration of control cells, which are incubated only with *E. coli* 055:B5 LPS.

It has been reported that *P. gingivalis* LPS can initiate innate immune responses in a TLR2- and/or TLR4-dependent manner[19]. The heterogeneity of LPS and lipid A preparations has limited the identification of specific compounds that are responsible for this unsual mode of activation. It has been reported that penta-acylated and tri-acylated lipid As can only activate human and mouse cells in a TLR4-dependent manner[31]. Furthermore, we have found no evidence that the tetra-acylated compounds **1** and **2** can activate human or mouse TLR2. It may be possible that a yet-to-be-identified *P. gingivalis* lipid A may exhibit TLR2-dependent activity; however, it is more likely that lipoprotein contaminants are responsible for the observed activity. An exciting observation reported here is that the tetra-acylated lipid A **1** is a potent antagonist of TNF-α production induced by enteric LPS. The acylation pattern of **1** is critical for optimal

activity because compound **2** exhibits a significantly reduced activity. Antagonists of cell surface receptors that recognize enteric LPS have a potential for being used as therapeutic interventions for patients with gram-negative sepsis. Success in this area has been limited and most efforts have been directed towards the synthesis of analogs of lipid A of *R. sphaeroides*[9,10]. An attractive feature of compounds **1** and **2** is that they are mono-phosphorylated and can be prepared by a highly convergent synthetic approach. Furthermore, it is expected that the analog synthesis will provide more potent compounds that have simpler structures.

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CHAPTER 4

Robust Immune Responses Elicited by a Fully Synthetic Three-Component Vaccine

4.1 Introduction:

Vaccines are considered to be the most promising and successful medical intervention to prevent infectious diseases. A vaccine is composed of an antigen, against which adaptive immune response is elicited, and an adjuvant responsible to stimulate the innate immune system[1]. For the successful development of suboptimal vaccines the selection of an adjuvant is as crucial as the selection of antigen to trigger early innate immune responses, which later aid in the generation of robust and long-lasting adaptive immune response[2]. The stimulation of the innate immune system leads to the generation of inflammatory cytokines like tumor necrosis factor-α and interferon-β, which activate antigen presenting cells (APCs) such as macrophages and dendritic cells. These responses lead to a conditioning of the immune system for development of specific adaptive immune responses[3,4].

The innate immune system uses a wide variety of receptors that detect evolutionarily conserved signatures from pathogens (pathogen-associated molecular patterns, PAMPs). The addition of such molecular patterns to the vaccine formulation leads to the development of robust and durable adaptive immune response[5]. PAMPs are recognized by a set of germline-encoded receptors called pattern-recognition receptors (PRRs), which are expressed on immune cells such as monocytes, macrophages, dendritic cells, B-cells, T-cells, and natural killer cells[6]. Toll-like receptors (TLRs) are one of the families of pathogen recognition receptors (PRRs). Activation of

TLRs leads to the secretion of cytokines and chemokines as well as maturation of antigen-presenting cells (APCs). It has been shown that different TLR ligands can lead to modulation of immune responses when incorporated in vaccine design. Moreover, the distinct TLRs differentially affect the Th1/Th2 balance[3,7-9].

It is very clear that the structures of glycans and the pattern of glycosylation change with the onset of cancer[10]. These changes arise from the changes in expression levels of glycosyltransferases in the golgi compartments of cancerous cells[11]. Furthermore these changes in glycosyltransferases can lead to modification in the core structures of *O*- and *N*-linked glycans, which can also lead to increase in size and branching of the glycans. In addition, the sialyltransferases and fucosyltransferases, which are involved in linking the terminating residues on glycans are also shown to be over-expressed in tumor cells. This leads to the over-expression of terminal glycan epitopes such as sialyl Lewis x (sLe^x), sialyl Tn (sTn), Globo H, Lewis y (Le^y) and polysialic acid (PSA)[12-15].

Mucin glycoproteins are often over-expressed in epithelial tumors and are used as diagnostic markers of cancer[16]. Since cancer cell glycans differ from those on healthy cells, it might be possible to recruit the immune system to specifically target the cancer cells. However, since the glycans are self-antigens and are also expressed on healthy cells, they are well tolerated by the immune system. Various approaches have been used to increase the immunogenicity of these tumor associated carbohydrate antigens for example, by conjugation to carrier proteins such as keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) as a source of helper T-lymphocyte epitopes. However, the carrier proteins can elicit strong B-cell responses, which may lead to suppression of antibody responses against the carbohydrate antigen[17,18]. To circumvent immune suppression caused by carrier proteins, recently a three-component vaccine

was designed, which is composed of a tumor-associated carbohydrate B epitope, a promiscuous peptide T-helper (Th) epitope and a TLR2 ligand. The vaccine design contains as a B epitope a tumor-associated glycopeptide derived from MUC1[19,20] and the well-documented mouse major histocompatibility complex (MHC) class II restricted Th epitope derived from amino acid 103 to 115 region of capsid polypeptide of poliovirus[21]. Pam_2CysSK_4 and Pam_3CysSK_4, which are TLR2 ligands were incorporated as a built-in adjuvant. Three-component vaccine candidates **1** and **2** (Figure 4.1) were then incorporated into phospholipids-based liposomes for immunization into mice. Group of five female BALB/c mice were immunized subcutaneously four times at weekly intervals[32,38]. Sera was collected to determine anti-MUC-1 antibody titers. A group of mice immunized with glycolipopeptide, **2** having covalently linked TLR2 ligand elicited exceptionally high titers of anti-MUC1 IgG antibodies, subtyping of the IgG antibodies (IgG1, IgG2a, IgG2b, and IgG3) indicated bias towards a Th2 immune response.

Table 4.1: ELISA anti-MUC1 and anti-T-epitope antibody titers after four immunizations with various preparations[32].

Immunization	IgG total[(a)]	IgG1	IgG2a	IgG2b	IgG3	IgM
1	20,900	66,900	700	900	7,300	1,400
1 and QS-21	30,200	113,100	23,000	6,600	17,800	1,100
2	169,600	389,300	56,500	42,700	116,800	7,200
2 and QS-21	322,800	371,300	378,900	56,800	263,500	5,000

Anti-MUC1 antibody titers are presented as median values for groups of five mice. ELISA plates were coated with BSA/BrAc/MUC1 conjugate and titers were determined by linear regression analysis, with plotting of dilution versus absorbance. Titers were defined as the highest dilution yielding an optical density of 0.1 or greater relative to normal control mouse sera. (a) Statistically significant difference ($p<0.05$) was observed between **1** and **2**.

In addition, the observed high IgG3 titer is typical of an anti-carbohydrate response. While the group of mice immunized with glycolipopeptide **6**, having an immuno silent lipid

moiety instead of a TLR2 ligand, resulted in significantly lower titers of IgG antibodies. The analysis of these IgG titers demonstrates that TLR engagement is critical for optimum antigenic responses. However, liposomal preparation of glycolipopeptide **6** with the TLR2 ligand **3b** or TLR4 ligand monophosphoryl lipid A elicited IgG titers similar to those of seen with compound **2**. In the case of immunization with mixture of compound **6** and **3b**, the immune response was biased towards a Th2 response. This was evident from the high titers of IgG1 and low IgG2b. On the other hand, immunizations with the combination of compound **6** and monophosphoryl lipid A led to significant IgG1 and Ig2a,b antibody titers, so this preparation elicited a mixed Th1/Th2 response.

TLR2 is one of the prominent pattern recognition receptors of the innate immune system and mediates responses to the most diverse set of molecular structures, including peptidoglycan, lipoteichoic acid, lipoarabinomanan, and bacterial lipopetides/lipoproteins (LPs)[22]. Unlike other TLRs that function through the formation of homodimers, TLR2 is believed to form heterodimers with either TLR1 or TLR6[23-26]. The diacylated and triacylated LPs Pam_2CysSK_4 and Pam_3CysSK_4 induce activation of innate immune cells through TLR2/6 and TLR2/1 respectively[27]. The molecular mechanisms of the recognition of lipopeptides (LP) by TLR2 are not yet very clear. However, there is evidence suggesting that Pam_3CysSK_4 binds to LPS-binding protein (LBP) and CD14 (soluble or membrane bound), which then results in spatial proximity of lipopeptide, CD14, TLR2 heterodimer and subsequent TLR signaling. Signaling through TLR2 heterodimers leads to the formation of an active signaling complex that includes MyD88 as the adaptor protein and the IL-1R-associated kinase (IRAK) proteins, finally resulting in the translocation of NF-κB a transcription factor and subsequent cytokine production[28-30]. Both Pam_2CysSK_4 and Pam_3CysSK_4 have shown to have good adjuvant characteristics such as they

can be synthesized in large quantities, are chemically stable, water soluble, and effective additives for eliciting a cellular immune response in mice[31]. The first objective of this project was to study the agonist characteristics of two isoforms of Pam2CysSK4 and Pam3CysSK4 and determine if they retain the TLR2 agonist characteristics when attached to a vaccine candidate. In the second part of the study, we determined that the TLR2 ligand enhances the entry of the vaccine conjugate inside the macrophages.

4.2 Materials and Methods:

4.2.1 Cell maintenance:

Raw 264.7 γNO(-) cells were obtained and maintained as mentioned in chapter 2. The human embryonic kidney cell line (HEK 293T), HEK 293T cells stably transfected with mouse TLR2, TLR2/6, and TLR4/MD-2/CD14 were obtained and maintained as mentioned in chapter 3.

4.2.2 Cytokine Assay:

Cytokine induction assays in Raw 264.7 γNO(-) cells were performed as mentioned in chapter 2. Cytokine ELISAs for murine TNF-α and IFN-β were performed as mentioned in chapter 2. Concentration-response data were analyzed using Prism software as mentioned in chapter 2. To ensure that LPS did not contaminate the synthetic compounds, leading to induction of cytokine production, all compounds were evaluated by pre-incubation with polymyxin B for 30 min before incubation with the cells. Polymyxin B is an antibiotic that binds to lipid A region of LPS, thereby preventing LPS induced cytokine production.

4.2.3 Transfection and NF-κB Activation Assay:

Transfection experiments in HEK 293T cells and HEK 293T cells stably transfected with murine TLR2, TLR2/6 or TLR4/MD2/CD14 were performed as mentioned in chapter 3.

4.2.4 Binding and Uptake Assay:

Raw 264.7 γNO(-) cells, HEK293T cells and HEK293T cells stably transfected with mouse TLR2, TLR2/6 and TLR4/MD-2/CD-14 ($2.6x10^6$ cells/ml) were exposed to Alexa fluor-488 labelled vaccine candidate (1 mg/ml) for 30 min at 37°C. Cells were harvested and washed in HNE buffer (HEPES, 20mM; NaCl, 150 mM; EDTA, 1 mM). Next, cells were lysed in passive lysis buffer, and fluorescence of cell lysates was measured at excitation 485 nm and emission 538 nm using POLARstar OPTIMA combination luminometer/fluorometer (BMG Labtech). Fluorescence values were normalized for maximum fluorescence (100%) using untreated cell lysates spiked with the fluorescent compound at the time of fluorescence measurement. Samples that were assessed for only internalization were treated with trypsin (500 μg/ml) for 1 min with subsequent wash with the HNE buffer followed by lysis with passive lysis buffer. The data is presented as mean ± s.d. of triplicate treatments, each experiment being repeated 3 times.

4.2.5 Confocal Microscopy Studies:

Raw 264.7 γNO(-) cells ($1x10^5$ cells/ml) were grown on poly-lysine (0.01%) coated glass coverslips for 24 h such that it reached 80% confluency. Next, cells were exposed to either lysotracker (company name and concentration) or golgi tracker (company name and concentration) for 30 min at RT followed by 3 washes with PBS buffer. Next, cells were exposed to Alexa 488-labelled vaccine candidate (concentration) and immediately observed under confocal scanning microscope (SP2M1 Laser Scanning Confocal Microscope at the University's Center for Ultrastructural Research).

4.3 Results and Discussion:

4.3.1 Screening of synthetic TLR2 ligands based on their ability to induce TNF-α in Raw 264.7 γNO(-) cells.

For this study, synthetic Pam_3CysSK_4 and Pam_2CysSK_4 both *as (R)* and *(S)* stereoisomers were used. The rationale behind studying these compounds was that different TLR2 ligands with a stereocenter may induce different profiles of cytokines.

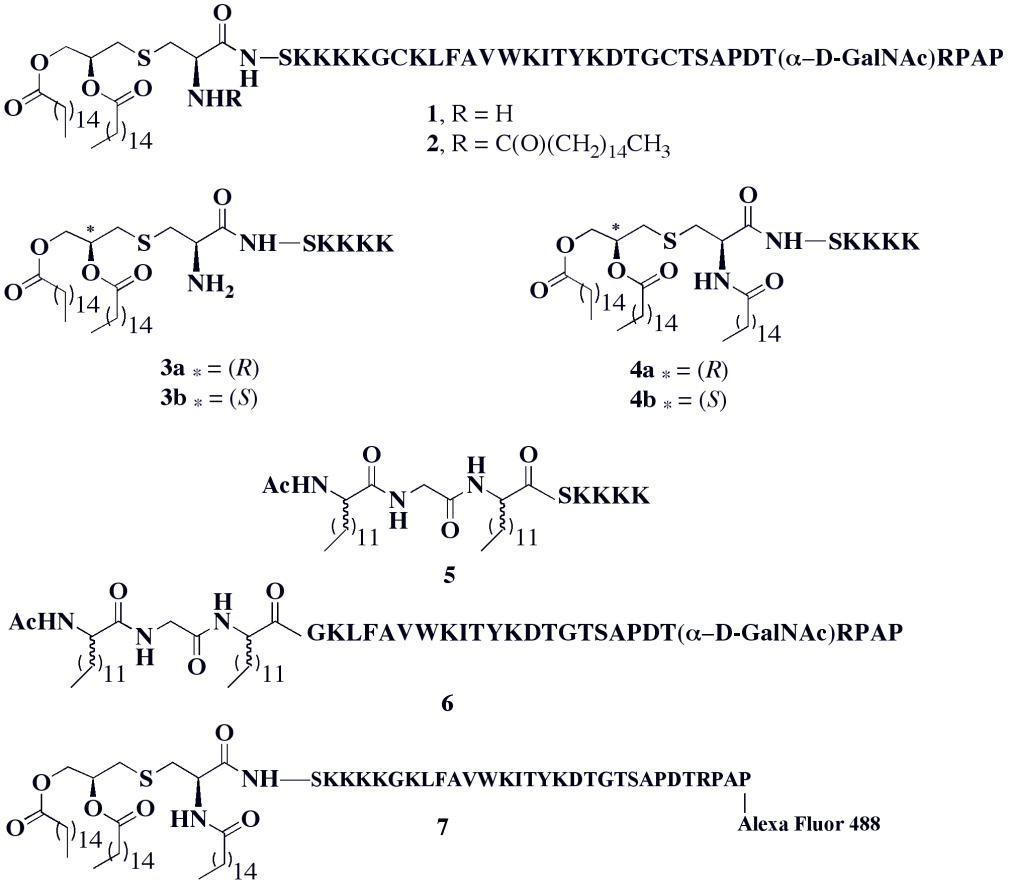

Figure 4.1: Structures of synthetic compounds.

To obtain the two stereoisomers, synthesis of **3a** and **4a** was carried out using *(R)(-)*-glycidol while compounds **3b** and **4b** were prepared by using *(S)(-)*-glycidol[33-35]. Next, compound **5** was studied, which contains lipidated amino acids[36,37]interspersed with glycine

residues. This compound is immuno-silent and does not induce cytokine production, and therefore will allow us to study in more detail the importance of covalent attachment of a TLR ligand to the vaccine candidate. Furthermore, the lipidated amino acid moiety had enabled the incorporation of the compound into liposomes (Figure 4.1).

The cells were induced with compounds **3a**, **3b**, **4a**, and **4b** in triplicates over a wide range of concentrations for 5.5 hours at 37°C. The supernatants were harvested and stored at -80°C and assayed for TNF-α and IFN-β. As the synthetic compounds are agonists for TLR2, it was observed that the compounds yielded clear dose response curves for TNF-α (Figure 4.2) while no IFN-β was detected (data not shown).

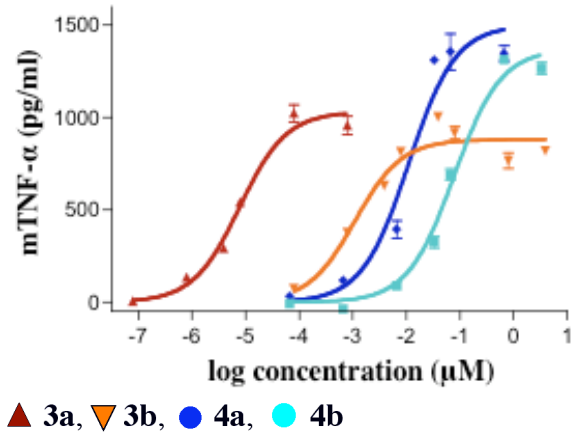

Figure 4.2: Cytokine production by Raw264.7 rNO(-) macrophage cells on stimulation with different lipopeptides. Raw264.7 rNO(-) cells were incubated with increasing concentrations of different lipopeptides for 5.5h and supernatanats were used to measure TNF-a, using ELISAs.. Error bars indicate SD of the mean of three independent experiments.

Dose-response curves were analyzed using PRISM (GraphPad Software, Inc.) and the EC_{50} values and the maximum response of the compounds for TNF-α were compared with that of *E.coli* 055:B5 LPS, as LPS is known to bring about sepsis due to excessive induction of TNF-

α and IFN-β through TLR4. Pam_3CysSK_4 and Pam_2CysSK_4 both in their (*R*)-stereomeric forms have lower EC_{50} values than the (*S*)-isomers. Moreover, both Pam_3CysSK_4 isomers have a higher efficacy than the Pam_2CysSK_4 isomers (Figure 4.2).

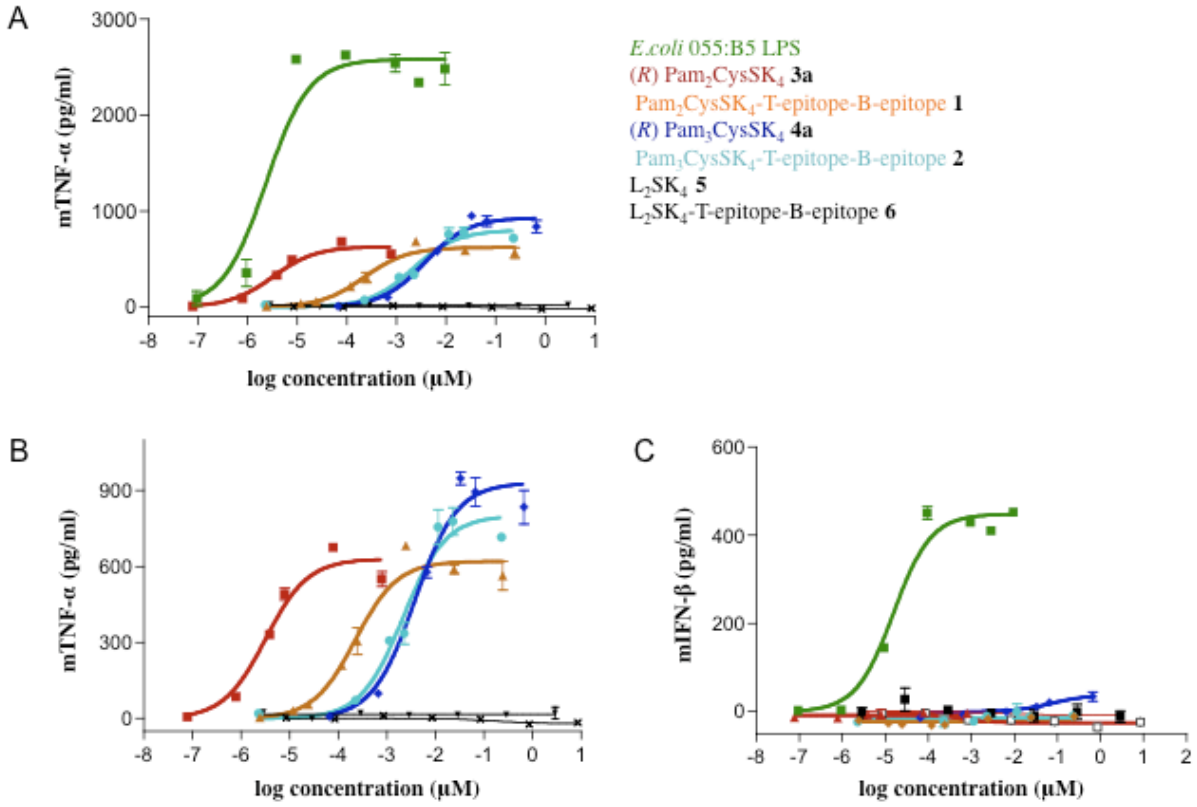

Figure 4.3: Cytokine production by Raw 264.7 γNO(-) macrophage cells on stimulation with *E.coli* 055:B5 LPS different lipopeptides. Raw 264.7 γNO(-) cells were incubated with increasing concentrations of different lipopeptides for 5.5 h and supernatants were used to measure TNF-α (A & B), and IFN-β (C) using ELISAs. Error bars indicate SD of the mean of three independent experiments.

Next, the Pam_3CysSK_4 (*R*) and Pam_2CysSK_4 (*R*) were compared to the vaccine constructs 1 and 2, in which these TLR2 ligands were attached to the T-epitope-B-epitope. It was observed that Pam_2CysSK_4 (*R*) lost 100-fold activity when it was attached to the vaccine construct while

the EC_{50} values for Pam_3CysSK_4 (*R*) did not change significantly on attachment to the vaccine construct (Figure 4.3) for the induction of TNF-α, while no IFN-β was detected. This showed that the vaccine constructs are highly specific for TLR2 and do not contain any endotoxin contamination. To further confirm the specificity of the vaccine constructs, we also studied activation of transcription factor NF-κB in HEK 293T wild type cells and HEK 293T cells stably transfected with murine TLR2, TLR2/6 or TLR4/MD-2/CD-14. Cells were transiently transfected with pELAM-Luc and pRL-TK (internal control to normalize experimental variations). The empty vector pcDNA3 was used as a control and to normalize the DNA concentration for all of the transfection reactions. 20 hour post-transfection, cells were exposed to the vaccine constructs for 4 h, after which cell extracts were prepared for measuring the luciferase activity. Expression of the firefly luciferase reporter gene was normalized for transfection efficiency with expression of *Renilla* luciferase. Activation of NF-κB by the vaccine constructs was TLR2 dependent (Figure 4.4). It was observed that the vaccine candidates **1** and **2**, and the (*R*) Pam_3CysSK_4 resulted in induction of NF-κB activation in HEK 293T cells stably transfected with TLR2 or TLR2/6, while no NF-κB activation was observed in wild type HEK 293T cells and HEK 293T stably transfected with TLR4/MD-2/CD-14. These results confirmed the specificity of the vaccine candidates **1** and **2** towards TLR2.

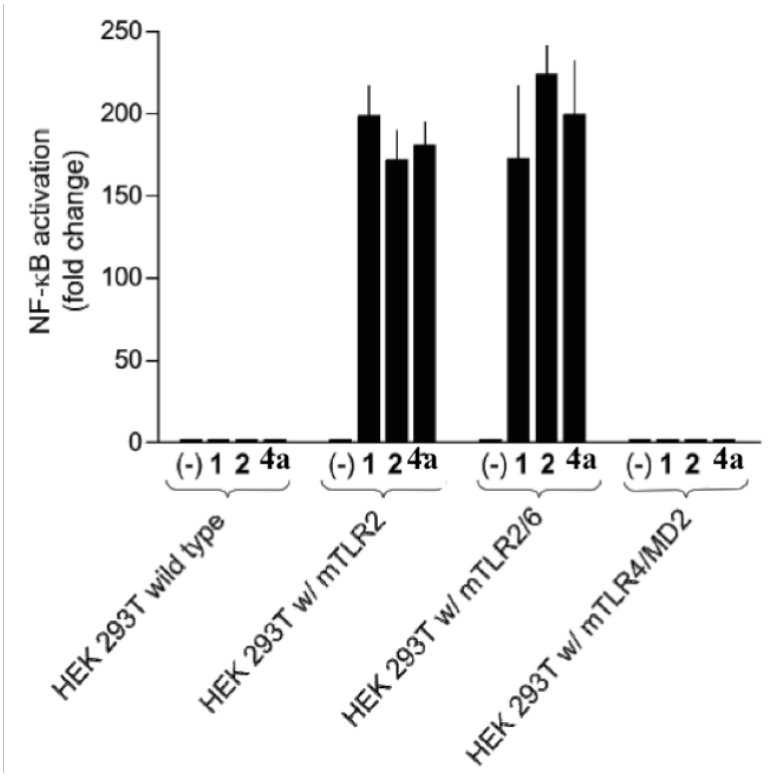

Figure 4.4: Involvement of TLR2 in NF-κB activation by compounds **1**, **2** and **4a**. Induction of NF-κB activation was determined in triplicate cultures of HEK293T cells (wild type and stably transfected with murine TLR2, TLR2/TLR6 or TLR4/MD2) transiently transfected with expression vectors pELAM-Luc and pRL-TK. Fourty-four hour post-transfection, cells were treated with the synthetic compounds **1**, **2** and **4a** (6.1 μM each) or were left untreated (control). Fourty-eight hour post-transfection, NF-κB activation was determined by firefly luciferase activity relative to *Renilla* luciferase activity. Data represent mean values ± s.d. (n=3). In the transfection experiment shown, human TNF-α (10 ng ml^{-1}) 145 ± 11, 82 ± 25, 141 ± 10 and 42 ± 2 fold activation of NF-κB in wild type and stable transfected murine TLR2, TLR2/TLR6 and TLR4/MD2 cells respectively.

4.3.2 TLR2 ligands promote internalization of the vaccine construct.

As antibody production needs the presentation of antigen fragments on the MHC complex of antigen presenting cells to the B-cells and T-cells, the internalization of antigens is very crucial. It was anticipated that in addition to initiating the production of cytokines the lipopeptide Pam_3CysSK_4 might facilitate selective uptake by antigen presenting cells such as macrophages in a TLR2-dependent manner. To test this hypothesis, a vaccine construct with an Alexa Fluor 488 label was synthesized[32] and administered to Raw 264.7 γNO(-) mouse macrophages.

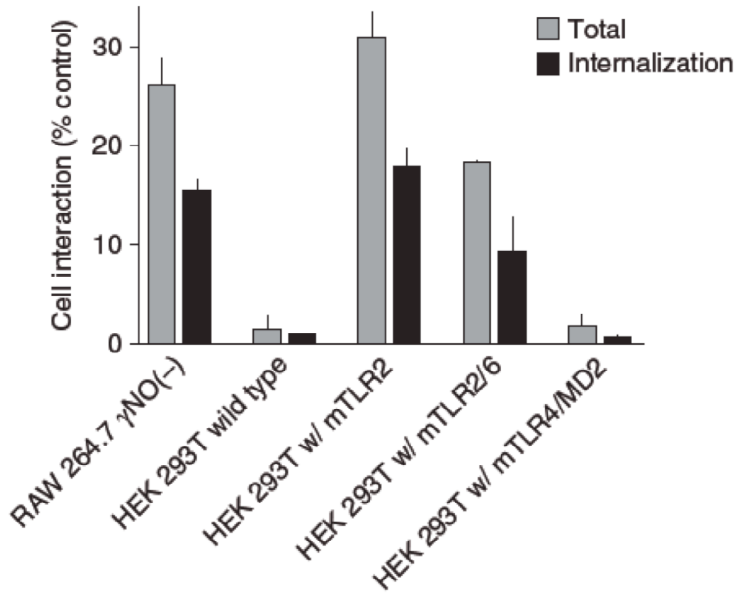

Figure 4.5: Cellular uptake of compound 11. Cells (Raw 264.7 γNO(-), HEK 293T wild type and HEK 293T stably transfected with mouse TLR2, TLR2 plus TLR6 or TLR4 plus CD14 and MD2) were exposed to Alexa fluor 488-labelled compound 11 (1 μg ml^{-1}) for 30 min. After cells were washed and lysed (total cell interaction; gray) or washed, treated with trypsin and then lysed (internalization only; black) fluorescence (absorbance at 485 nm, emission 538 nm) was measured. Fluorescence values were normalized for maximum possible fluorescence (100%). Data represent mean values ± s.d. (n=3).

After 30 min, the cells were harvested and lysed and the fluorescence was measured. To account for all possible cell surface binding without internalization, the cells were also treated with trypsin before lyses and then examined for fluorescence. More than 50% of the labeled compound that interacted with the cells was internalized. Similar assay was also performed in HEK 293T wild type cells and HEK 293T cells stably transfected with mouse TLR2, TLR2/6 or TLR4/MD-2/CD14 to determine if the uptake was mediated by TLR2. Substantial uptake was only observed when cells were stably transfected with TLR2, which indicated that the uptake is receptor mediated (Figure 4.5). The receptor-mediated uptake was further confirmed by confocal microscope images, which show that the Alexa Fluor-488 labeled vaccine construct localized in the lysosomal compartments of the macrophage cells, further indicating a Th2 response (Figure 4.6).

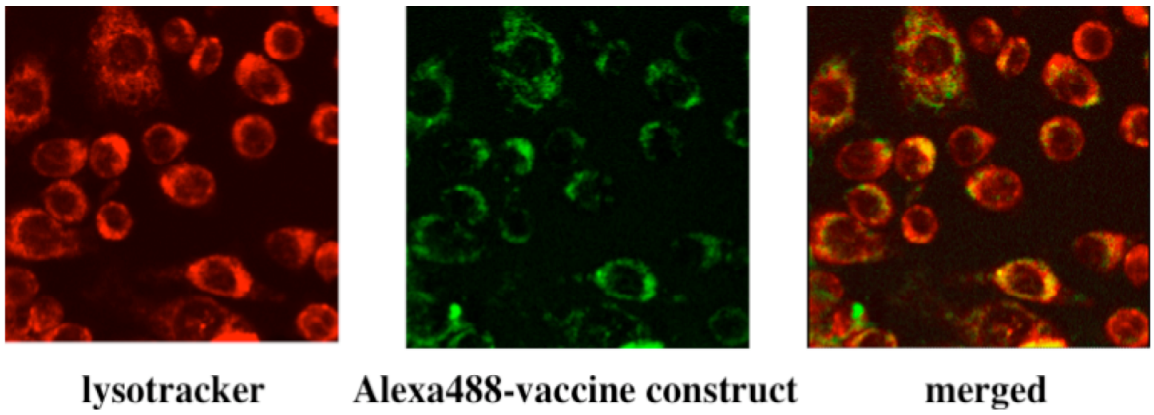

Figure 4.6: Confocal microscope images for compound **7**. Raw 264.7 γNO(-) were exposed to lysotracker for 30 min. followed by 3 washes with PBS. Next, cells were exposed to compound **7** (concentration) and immediately observed under confocal scanning microscope.

The excellent antigenicity of the three-component cancer vaccine is attributed to several features. First, it does not have any unnecessary components that are antigenic and may cause

immune suppression. The chemical attachment of TLR2 ligand to the T- and B-epitopes ensures that the cytokines are produced at the site where the vaccine interacts with the immune cells, this leads to local upregulation of cytokines, thereby facilitating maturation of immune cells. The TLR2 ligand also facilitates uptake by TLR2 expressing immune cells such as antigen presenting cells, which assists antigen processing, antigen presentation and activation of adaptive immune responses. We found that when Pam_2CysSK_4 or Pam_3CysSK_4 are incorporated in the vaccine construct, it leads to elicitation of high titers of IgG antibodies[32] due to local production of cytokines, upregulation of co-stimulatory proteins, and enhanced uptake by macrophage cells.

4.4 References:

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CHAPTER 5

Conclusions

The innate immune system responds rapidly to foreign substances that interact with the host and activate protective reactions. Pattern recognition receptors such as Toll-like receptors, which are evolutionarily ancient receptors of the innate immune system, respond to unique molecules present on the pathogens. Mammalian TLRs are a family of atleast 13 paralogues, which can recognize bacteria, viruses, fungi, and protozoa. Activation of different TLRs results in induction of inflammatory responses by production of a diverse set of cytokines and chemokines. In addition to the inflammatory responses, the cytokines and chemokines augment the activation of antigen presenting cells by expression of co-stimulators such as CD40 and B7, which lead to enhanced antigen presentation to the T-cells. Hence, TLR activation leads to the activation of innate as well as adaptive immune system. This property of TLRs is exploited for the development of immune modulators for the development of immune adjuvants for vaccines. However, over-activation of innate immune responses may lead to the clinical symptoms of septic shock. Hence, the detailed knowledge of structure-activity relationships of TLR ligands is essential for the design of safe immune modulators.

Lipopolysaccharides are structural components of the outer surface membrane of gram-negative bacteria and trigger innate immune responses through TLR4. Lipid A is the hydrophobic domain of LPS and is known to be the inflammation causing moiety of LPS. The TLR4 activation by lipid A is highly complex and results in two sets of inflammatory responses, which depend on the two distinct initiation points that involve the recruitment of adaptor proteins MyD88 or TRIF.

Structural studies have demonstrated that the carbohydrate backbone, degree of phosphorylation, and the fatty acid acylation patterns of the lipid A structure vary considerably among different bacterial species. Various studies have shown that structurally different lipid As from different bacteria may differentially induce pro-inflammatory responses. Most of these studies were performed in primary macrophage cells, which could be a mixture of cell population at different maturation phase arising from monocytes. Moreover, the use of primary cells limits the range of studies that can be performed. This resulted in studies of a small number of compounds over a narrow range of concentrations for a particular time of induction, and most of the compounds were isolated from their natural sources, and could contain heterogeneous mixture.

Hence, to address all these issues we have studied a synthetic homogeneous lipid A derivatives corresponding to *E. coli*, *N. meningitidis*, and *S. typhimurium* over a wide range of concentrations in Raw 264.7 γNO(-) and Bac 1.2F5 murine macrophage cell lines, which are obtained from primary monocyte cells differentiating into macrophages. Unlike primary cells, the use of cell lines allowed us to examine a wide range of concentrations on a large scale for the ability to measure the production of a panel of cytokines and chemokines corresponding to the

two distinct TLR4 activation pathways. However, since a particular cell line does not represent the physiological conditions in the animal, these studies need to be performed in animal model.

Unlike others, our studies do not reveal bias towards either MyD88 dependent or TRIF dependent TLR4 induction pathway by different lipid A derivatives. It was found that most of the lipid A derivatives, *E. coli* LPSs and *N. meningitidis* LOS examined yielded clear dose response curves, except the *S. typhimurium* lipid **3**, which yielded partial response and *S. typhimurium* lipid A **5** which did not show any agonist activity. Statistical analysis of the potencies by an additive model showed the same order of potencies for the various compounds in both cell lines. *E. coli* 055:B5 and 0111:B4 LPS were the most potent, followed by *N. meningitidis* LOS and KDO containing *N. meningitidis* **7**, which were more potent than the *E. coli* lipid A derivative **1** and *N. meningitidis* lipid A derivative **6**. These results indicate that the presence of a single KDO unit can retain the full activity of the LOS structure. *N. meningitidis* lipid A **6** with symmetrical arrangement of the lipid chains showed higher potency than the *E. coli* lipid A **1**, which contains asymmetrical arrangement of lipid chains. Moreover, 3 lipid chains of *N. meningitidis* lipid A **6** are 2-carbons shorter than E. coli lipid A **1**, further indicating that the pattern and length of the fatty acid chains of lipid A can differentially modulate the activation of TLR4 receptor complex. Similar effect was also found by shortening of the lipid chains in *E. coli* lipid A **2** and *S. typhimurium* lipid A **4**, which led to increase in the potency of the lipid As derivatives than their parent compounds **1** and **3**.

Cytokines were grouped into three statistically significant groups for Raw 264.7 γNO(-) cells and into five different groups for Bac 1.2F5 macrophage cells. Moreover, the rankings of the cytokines were different in both the cell lines, which did not follow a pattern of MyD88- and TRIF-related cytokines. However, secretion of TNF-α and expression of intracellular IL-1β was

observed at lower concentrations of the compounds while secretion of IL-1β required the most concentration of the compounds for both the cell lines. This indicated that the activation and secretion of IL-1β is differentially influenced by the different lipid A derivatives.

Also, several studies have shown that the lipid A compounds which can antagonize cytokine production induced by enteric LPS may have the potential to be developed as therapeutics for the treatment of gram-negative sepsis. We studied two tetra-acylated *P. gingivalis* lipid A derivatives, which have unusual branched fatty acids and exhibit unique pattern of phosphorylation. We showed that these lipid A derivatives are potent antagonist of TLR4 and inhibit the induction of TNF-α production induced by enteric LPS.

In addition to the antimicrobial activities, cytokines generated also activate and regulate the adaptive immune system. Efficient priming of adaptive immune response requires presentation of antigen in the context of MHC and induction of accessory signals such as co-stimulators and cytokines on antigen presenting cells (APC). TLRs expressed on APCs may regulate the accessory signals through their recognition of PAMPs and consequently control activation of antigen specific adaptive immune responses. We showed that lipopeptide ligands Pam_2CysSK_4 and Pam_3CysSK_4 induce TNF-α *via* induction of TLR2, which also promotes uptake of the vaccine candidate by macrophages cells when incorporated in the vaccine design as an in-built adjuvant.