Study of Modulation of Host Innate and Adaptive Immune Responses by *Vibrio cholerae* Porin OmpU

Sanica C. Sakharwade

Thesis submitted for the partial fulfilment of the degree of

Doctor of Philosophy

Department of Biological Sciences

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Declaration

The work presented in this thesis has been carried out by me under the supervision of Dr. Arunika Mukhopadhaya at the Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali.

This work has not been submitted in part or full for a degree, a diploma, or for fellowship to any other university or institute. This thesis is a bona fide record of the original work done by me. All sources cited within have been listed in the bibliography.

Date:

Place:

Sanica C. Sakharwade

In my capacity as the supervisor of the candidate's thesis work, I certify that above statements by the candidate are true to the best of my knowledge.

Dr. Arunika Mukhopadhaya

Supervisor

Acknowledgment

I would maintain that thanks are the highest form of thought;
And that gratitude is happiness doubled by wonder.
~G.K. Chesterton

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He who dwells in the secret place of the Most High
Shall abide under the shadow of the Almighty.
I will say of the Lord, “He is my refuge and my fortress;
My God, in Him I will trust.”
~Psalm 91:1,2

Table of Contents

List of Tables

List of Illustrations

List of Figures

List of Abbreviations

ACE: Accessory cholera enterotoxin

ACP: Accessory colonization factors

APC: Antigen-presenting cells

APPs: Acute phase-response proteins

ASC: Apoptosis associated speck-like containing a CARD

BMDC: Bone marrow-derived dendritic cell

BPI-2: Bactericidal/permeability-increasing protein

BSA: Bovine serum albumin

CARD: Caspase-recruiting domain

CCL: Cysteine–cysteine ligand

CFA: Complete Freud's adjuvant

CT: Cholera toxin

CXCR: Cysteine-x-cysteine receptor

DAMP: Danger-associated molecular patterns

DC: Dendritic cell

ELISA: Enzyme-linked immunosorbent assay

HA: Hemaglutinins

IEC: Intestinal epithelial cells

IL: Interleukin

IRAK: Interleukin-1 receptor-associated kinase

LAL: Limulus amebocyte lysate

LDAO: N,N-Dimethyldodecylamine-N-oxide

LDH: Lactate dehydrogenase

LPS: Lipopolysaccharide

LRR: Leucine-rich repeat domain

MKP-1: MAPK phosphatase 1

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MyD88: Myeloid differentiation factor 88

NACHT: Nucleoside triphosphatase domain

NFκB: Nuclear factor kappa B

NK: Natural killer

NO: Nitric oxide

PAGE: Polyacrylamide gel electrophoresis

PAMP: Pathogen-associated molecular patterns

PBS: Phosphate buffer saline

PKA: Protein kinase A

PMA: Phorbol mystistate acetate

PmB: Polymyxin B sulfate

PRR: Pattern-recognition receptors

PYD: Pyrin domain

RNI: Reactive nitrogen intermediates

ROS: Reactive oxygen species

SDS: Sodium dodecyl sulphate

SHIP-1: SH2-containing 5'-inositol phosphatase

SOCS: Suppressor of cytokine signaling

TBST: Tris buffer saline with Tween

TCP: Toxin co-regulated pilus

TF: Tissue factor

TLR: Toll-like receptor

TNFα: Tumor necrosis factor alpha

TOLLIP: Toll-interacting protein

VCC: *V. cholerae* cytolysin

ZOT: Zonula occludens toxin

I

Introduction

1.1 Gram-negative enteric bacteria

Enteric bacteria are gram-negative facultative anaerobes that reside in the intestines. Some enteric bacterial species are present as commensals. They constitute the normal microbial flora and are not pathogenic generally at their usual bodily location, however, pathogenic species constitute a dominant proportion of enteric bacteria [1].

Commensals, such as, *Escherichia coli*, *Salmonella enteritidis*, *Klebsiella* spp., *Enterobacter* spp., *Proteus mirabilis*, *Pseudomonas aeruginosa*, etc., provide an important barrier against pathogens. Their presence prevents pathogen colonization as they compete for nutritional and spatial resources [2-4]. Furthermore, the host possesses multiple barriers, which prevent pathogen survival. The gut environment acts as a barrier for invading pathogens as it offers a hostile environment for them. The acidic pH of the gastric juices is not hospitable for microbes. Bile acids metabolize fats and are harmful to the lipid bilayers of microbes. Anti-microbial factors, such as, defensins, histatins, cathelicidins, lysozymes, ribonucleases, etc., secreted by specialized intestinal cells known as Paneth cells, as well as, colicins secreted by commensal *E. coli* provide another level of protection [5,6].

In spite of these multiple barriers, several pathogens establish themselves in the gut. The most well-known pathogenic genera of enteric bacteria include members of *Shigella*, *Salmonella*, *Yersinia*, *Vibrio*, *Serratia*, *Klebsiella*, *Enterobacter*, etc. [7,8]. However, normal gut microbiota may become pathogenic when the host's immune system is compromised. Coliform bacteria, such as, *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia*, and *Citrobacter* are opportunistic pathogens [9]. For example, non-pathogenic strains of *E. coli* are present in the large intestine but pathogenic strains, such as, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enterohemorrhagic *E. coli*, enteropathogenic *E. coli*, and enteroaggregative *E. coli* are responsible for urinary tract infections, meningitidis, and gastroenteritis.

1.1.1 Gram-negative enteric pathogens employ multiple strategies to overcome the host's protective barriers

Enteric pathogens can lead to diarrhea and enteric fever and cause up to more than 3 million deaths every year [10]. The traditional infectious cycle of pathogenic enteric bacteria involves entry into the host, establishment and multiplication in the gut,

avoidance of host defenses during pathogenesis, induction of damage, and finally, release from the host.

Pathogens can establish themselves in the gut by overcoming multiple host-protective barriers. They adhere to the intestinal epithelial cells (IECs) and avoid being swept away by mucus secretions and the peristaltic movement of the alimentary canal [11]. Adhesins are proteins that mediate this attachment process. They can be pili proteins or non-pili proteins that have immunoglobulin domain-like structures [12]. Further, formation of biofilm protects bacteria from anti-microbial factors [6]. Certain bacterial species can degrade and efflux out anti-microbial peptides or modify the charge on their membrane to prevent binding of these factors altogether [11]. Following colonization in the intestine, enteric pathogens may produce secretory exotoxins, cytotoxins, and other virulence factors. Pathogens may employ secretion systems, which are needle-like structures that allow the transport of toxins and pathogenic effectors from the bacteria to the host cells. These effectors alter and exploit immune responses and ensure bacterial survival [12].

Furthermore, enteric pathogens can also evade immune recognition and responses. They may proteolytically cleave antibodies present on host mucosal surfaces that mediate opsonization [11]. Bacteria may modify or shield their exposed proteins that may be recognized by the immune system [11]. They may also delay or interfere with intra-cellular trafficking to prevent their internalization by immune cells. Pathogens can evade phagocytosis by forming capsules, triggering toxin-mediated damage, and by inducing apoptosis in phagocytes [13,14]. *Salmonella* and *Shigella* trigger apoptosis in phagocytes [15,16], whereas, *Yersinia* promotes anti-inflammatory responses to prevent elimination [17].

In contrast to avoidance of immune recognition, some pathogens may trigger pro-inflammatory responses. For example, *Shigella* induces release of cytokines. This in turn, helps in cellular invasion as the vasculature becomes permeabilized [18]. It contains genes on virulence plasmids that facilitate this process. Pathogenicity islands also play a major role in virulence. Pathogenicity islands are usually genes incorporated in the bacterial genome acquired by horizontal gene transfer from other bacteriophages, plasmids, or transposons. One such island in *Helicobacter pylori* is CagA, which initiates pro-inflammatory responses in the stomach epithelium [19]. Thus, pathogens can take advantage of the host-defense mechanisms to establish themselves and cause diseases.

1.2 *Vibrio cholerae is the causal organism of cholera, a human diarrheal disease*

Cholera is characterized by nausea, vomiting, fever, and diarrhea and may be fatal if left untreated. Cholera has relentlessly affected the world and continues to pose a threat to many of the countries even today. There have been seven pandemics of cholera since the 1800s with the seventh still continuing presently [20]. As of December 2015, several Middle East and East African countries, such as, Iraq, Syria, Bahrain, Kuwait, Uganda, Tanzania, Haiti, and Myanmar faced outbreaks. *V. cholerae* is a gram-negative, comma-shaped facultative anaerobe that colonizes in the small intestine of the human host and causes cholera. John Snow, a physician, first proposed that cholera was caused by a micro-organism present in human waste in 1849 [21]. The bacterium was first isolated by Italian anatomist Filippo Pacini. Robert Koch further proved that the causal organism of cholera was bacterial in origin in 1883 [22].

Scientific classification of *V. cholerae*

Domain: Bacteria	Family: Vibrionaceae
Phylum: Proteobacteria	Genus: *Vibrio*
Class: Gammaproteobacteria	Species: *cholerae*
Order: Vibrionales	

Not all of the *V. cholerae* strains are pathogenic. There are about 200 different serogroups of *V. cholerae* based on differences between the O antigens present in the lipopolysaccharide (LPS) [21-25]. Out of these, only two are known to be toxigenic: O1 and O139, which produce the cholera toxin (CT). The other strains induce mild dysentery. The O1 serogroup can further be divided into two biotypes based on phenotypic characteristics: Classical serotype and El Tor biotype. These two biotypes differ in their polymyxin B (PmB)- and phage susceptibility; hemolytic and agglutination abilities; and production of acetylmetylcarbinol, a carbon source [26]. These biotypes can be further divided into three serotypes: Inaba, Ogawa, and Hikojima. However, many believe that the Hikojima serotype is unstable and does not exist independently. The Ogawa and Inaba serotypes have a common O1 antigen, known as the A-antigen, which is a homopolymer of amino sugar D-perosamine substituted with 3-deoxy-L-glycerotetronic acid [27]. But these serotypes differ in the 2-O-methyl group present in the non-reducing terminal sugar

of the Ogawa O antigen (B-antigen) which is absent in the Inaba O antigen (C-antigen) [28]. Seroconversion between the Ogawa and Inaba serotypes is known to occur [29].

1.2.1 Life cycle of V. cholerae

V. cholerae naturally persists in aquatic environments, where it is found attached to crustaceans and feeds on algae and planktons. Ingestion of contaminated seafood or unsterilized water, leads to colonization in the small intestine, after which the disease symptoms begin [21,22]. The bacteria are shed via stool and the spread of the disease occurs via the fecal–oral route. Thus, the prevalence of cholera is much more in developing nations, where sanitation and hygiene practices are poorly followed [20].

Quorum sensing plays an important role in the pathogenesis of *V. cholerae*. Quorum-sensing signals allow communication between bacterial communities and shape group behavior. The levels of quorum sensing molecules known as autoinducers, vary during different stages of colonization. At a high bacterial density, when *V. cholerae* is present in aquatic environments, the levels of autoinducers repress the production of biofilm and virulence factors. During colonization in the human gut, the bacterial density decreases and the levels of autoinducers are reduced. This leads to virulence-gene expression and biofilm formation, and thus, pathogenesis initiation [30,31].

1.2.2 History of cholera

The earliest records of cholera are found in the writings of Hippocrates. He coined the word *cholera* that is derived from the Greek word—*kholē*, meaning bile. He thought that the diarrhea was caused due to an excess of bile. The modern occurrence of cholera was first reported in 1817, in the Indian subcontinent. Since then, there have been a total of seven pandemics that have been summarized below (Table 1).

Pandemic	**Time period**	**Affected Areas**
First	1816–1826	India, China, and Indonesia
Second	1829–1851	Russia, Hungary, Germany, UK, France, Canada, and USA
Third	1852–1860	Russia, Indonesia, Phillipines, Korea, India, Iran, Iraq, Arabia, USA, Spain, Venezuela, Brazil, and Tunisia

Fourth	1863–1875	Zanzibar, Netherlands, Belgium, Italy, Algeria, and UK
Fifth	1881–1896	Russia, Persia, Egypt, Japan, and Germany
Sixth	1899–1923	Russia, India, USA, and Philippines
Seventh	1961–1975	Indonesia, Bangladesh, and Russia
Outbreaks	1991–Present	Peru, India, Republic of Congo, USA, Iraq, Vietnam, South Africa, Zimbabwe, Haiti, Dominican Republic, Cuba, Venezuela, Iraq, Nepal, Pakistan, Iran, Bangladesh, Laos, Cambodia, Vietnam, Afghanistan, India, China, Nigeria, Sierra Leone, Kenya, Zimbabwe, Zambia, Angola, Somalia, Ethiopia, Ivory Coast, Congo, Mozambique, Ghana, Guinea, Mali, and Ukraine

Table 1. Cholera pandemics [21]

1.2.3 Symptoms of cholera

V. cholerae may not produce symptoms in approximately 75% of the people. However, people can still act as carriers and the bacteria may be present in the feces for about 1–2 weeks [32]. The incubation period for the bacteria can last from 1–5 days. Cholera is characterized by grayish rice-water diarrhea and up to 1 liter of fluid may be lost per hour in acute cases [33]. This may lead to sunken eyes, poor skin turgor, excessive thirst, increased heart rate, low blood pressure, no urine output, and sometimes loss of consciousness. The mortality rate is less than 1% when cholera is treated on time [34]. However, if cholera is left untreated the mortality rate can rise to 50–60%. About 3–5 million people are affected by cholera globally, and it can lead to 58,000–130,000 deaths per year as of 2010 according to WHO report [35].

1.2.4 Cholera treatment and vaccines

Currently, oral rehydration therapy or ORS is the first step taken to combat dehydration caused by extreme diarrhea. ORS is an isotonic solution of glucose and electrolytes. WHO data indicate that 80% patients can be treated with ORS, however the other 20% might need intra-venous administration of electrolytes. Antibiotics, such as, tetracycline, doxycycline, furazolidone, or ciprofloxacin may be used to control the disease [35].

Jaume Ferran i Clua, a Spanish physician, was the first to develop a cholera vaccine in 1885. In 1892, a Russian–Jewish bacteriologist Waldemar Haffkine developed another version of the vaccine [36]. Currently, two oral vaccines against cholera are available, Dukoral and Shanchol [35]. Dukoral consists of killed whole cells of *V. cholerae* O1 along with recombinant CTB subunit. Whereas, Sanchol consists of killed whole cells of *V. cholerae* O1 and O139. Both vaccines offer short-term protection of 85–90% till 4–6 months following immunization. The National Institute of Allergy and Infectious Diseases, NIH, USA, is currently developing live vaccine candidates. One candidate, Peru-15, is in the final stages of development. The *V. cholerae* O1 live oral vaccine has a deletion of CT genetic element, insertion CTB subunit gene, and is non-motile [37]. The other candidate, CVD–103HgR, is available in Europe and America. It is a live attenuated strain of *V. cholerae* O1 that has a deleted CTA subunit along with a mercury-resistance gene that serves as a selection marker [38].

1.2.5 V. cholerae pathogenesis factors

V. cholerae genome contains two pathogenicity islands that are crucial for its pathogenesis in the human gut. The first island includes genes for CT (acquired from the CTX phage) and the other island includes genes for accessory colonization factors (ACP) and toxin co-regulated pilus (TCP) (carried by M13-like VPI phage) [20,24,39]. Several virulence genes are also present in the genome apart from these two islands. These factors have been summarized below (Table 2).

Toxins	CT
	Zonula occludens toxin
	Accessory cholera enterotoxin
	Cytolysin
Hemagglutinins	Hemagglutinin/protease
	Mannose-sensitive HA
	Mannose fucose-resistant HA
Toxin co-regulated pilus	Eight factors: TCPA–F, TCPQ–S
Accessory colonization factors	Four factors: ACPA–D

Table 2. Pathogenesis factors of V. cholerae

1.2.5.1 Toxins

The most notorious *V. cholerae* pathogenic factor is the CT. It consists of two types of subunits; A subunit, is single unit that has ribosylase enzymatic activity; and five B subunits, which form a ring around the A subunit and are involved in membrane binding. The intact toxin binds to GM1 gangliosides and is endocytosed. The toxin undergoes cleavage in the endoplasmic reticulum and the free A subunit acts on G protein, leading to an increase in cAMP levels and subsequently protein kinase A (PKA). PKA phosphorylates the Cl^- channel, CFTR (cystic fibrosis transmembrane receptor), leading to a ATP-dependent efflux of Cl^- and thus, a loss of K^+, Na^+, HCO^- and water into the intestinal lumen [20,21,40].

Another toxin, the zonula occludens toxin (ZOT) increases intestinal cell permeability by phospholipase C- and protein kinase C-dependent polymerization of actin present in the intestinal tight junctions [41]. The accessory cholera enterotoxin (ACE) affects ion transport across channels by increasing the potential difference across the intestinal cell membrane [42]. Thus, these three toxins behave in a synergetic manner to initiate loss of electrolytes from intestinal cells. Another toxin, *V. cholerae* cytolysin (VCC) is a pore-forming toxin that lyses intestinal cells [43].

1.2.5.2 Hemagglutinins

Hemagglutinins (HA) are lectins or glycan-binding proteins that cause agglutination of erythrocytes. *V. cholerae* has a few HAs that contribute to its pathogenesis. Hemagglutinin/protease (HA/protease) is zinc-dependent metalloprotease that acts on the host intestinal cell proteins, such as, mucin, fibronectin, and lactoferrin and activates the CTA subunit and other cytolysins [44]. Mannose-sensitive HA encodes for a type IV-pilus subunit in El Tor strains and helps in colonization [45]. Mannose fucose-resistant HA is another HA that is present in all O1 strains and is also involved in bacterial colonization [46].

1.2.5.3 TCP and ACFs

Pili play an important role in adherence to human-gut epithelial cells. The TCP complex consists of different genes that encode for multiple pili proteins that help in evasion of an immune response, which might be generated against pili proteins. Four ACF–ORFs are

present. Their specific function has not been elucidated, but one of the ORFs codes for a lipoprotein [39,47,48].

1.2.5.4 Levels of quorum sensing signals modulate V. cholerae survival and virulence mechanisms in the intestine

During initial stages of *V. cholerae* entry into the intestine, when the bacterial population is dense, the quorum-signaling mediators, autoinducers, maintain high levels of HapR, a transcription factor that represses virulence gene expression and biofilm formation [31]. However, environmental conditions, such as, low pH, presence of bile acids, anti-microbial factors, and osmotic stress are barriers that need to overcome for successful establishment. *V. cholerae* employs multiple strategies to combat these protective mechanisms. Intestinal cells produce hydrogen peroxide, which is an important anti-microbial factor. In response, *V. cholerae* increases levels of RpoS, a stationary phase alternative sigma factor, which confers resistance to the hydrogen peroxide generated [31]. In addition, RpoS also ensures survival in hyperosmolaric and carbon-deficient conditions. CT secreted by *V. cholerae* leads to nitric oxide (NO) production and release of defensins. At this stage, HapR induces production of HA/protease, which mediates detachment from the intestinal epithelium. This phenomenon is termed as the "mucosal evasion response" that occurs due to the combined effect of increased levels of RpoS and quorum signals, which ensure high levels of HapR. Therefore, the mucosal evasion response allows *V. cholerae* to occupy new sites in the intestinal lumen. This response also ensures that the virulence-mediator production does not occur during this transition and also, prevents a high-energy expenditure cost [31].

On implantation at a new site, the low density of bacteria co-relates with decreased levels of autoinducers. Due to a low quorum signal, HapR levels decrease, promoting expression of virulence mediators [30]. Loss of HapR, results in increased production of AphA and AphB transcription factors that regulate the ToxR regulon, which is a master virulence-gene controller. ToxR regulates around 20–30 virulence genes, such as, the CT (ctxAB), TCP (tcpPH), and ACP (acpI–IV) [49-51].

AphA and AphB induce production of two membrane proteins TcpP/H. TcpP/H in conjunction with two other membrane proteins, ToxR/S, mediate virulence-factor production. The TcpP/H genes are found in pathogenic strains, whereas the ToxR/S genes

are found in all strains [52]. Without signals from TcpP/H, ToxR/S does not mediate virulence. Instead, ToxR/S control genes involved in metabolism, such as, maltose- and glycerol metabolism, peptide transport, acetyl phosphate kinases, and genes involved in motility and chemotaxis [52].

The TcpP/H-induced ToxR/S activation triggers expression of another transcription factor, ToxT, which stimulates production of CT, TCP, and ACP factors [49,50,52-58]. The ToxR regulon also controls expression of two channel proteins, OmpU and OmpT porins. The expression of OmpU is positively regulated and that of OmpT, negatively, by the regulon; but in a ToxT-independent manner (Illustration 1) [24,39,59].

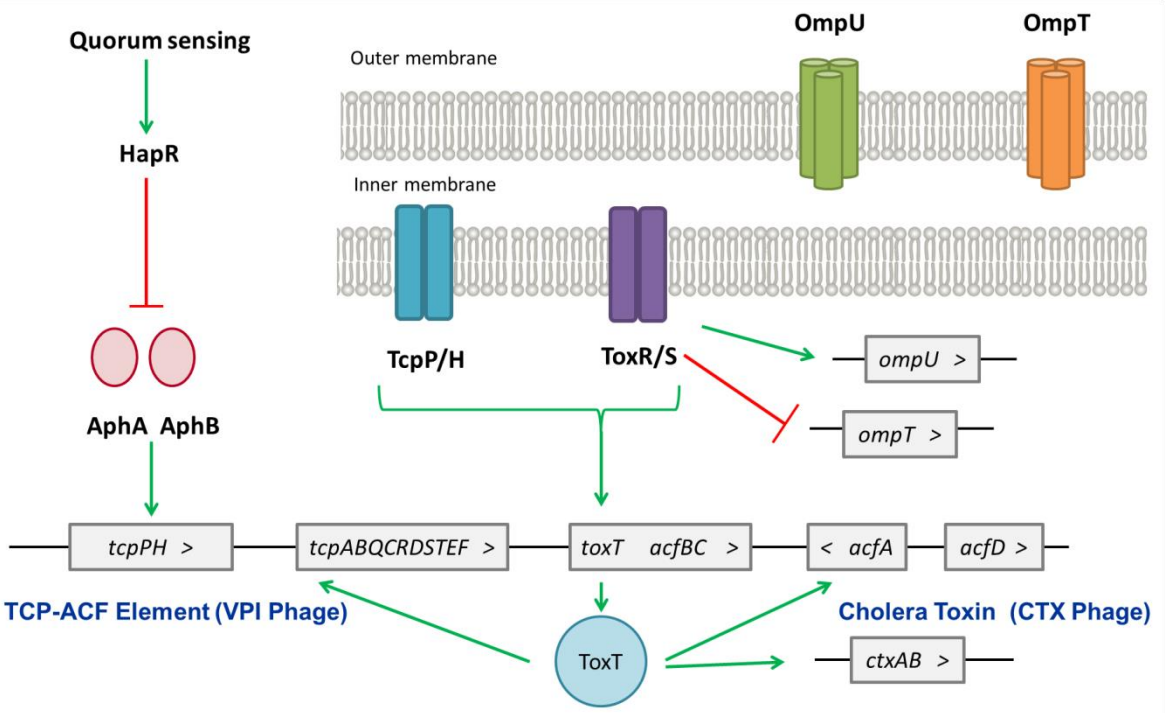


Illustration 1: Regulation of ToxR-mediated virulence factor production by quorum signals in V. cholerae

1.3 Outer membrane of gram-negative bacteria

Gram-negative bacteria contain an additional membrane, the outer membrane apart from the peptidoglycan layer that is present in both, gram-positive- and gram-negative bacteria. The outer membrane forms the interface between the cell and its external environment

and thus, acts as a physical and functional barrier. It controls transport of solutes and prevents entry of antibiotics, detergents, and other toxic chemicals into the bacterial cell.

The outer membrane consists of protein complexes, lipoproteins, and LPS (Illustration 2). These proteins account for ~50% of outer membrane mass and are encoded by approximately one-third of the bacterial genome [60]. They perform multiple roles: they can have enzymatic activity (phospholipase A), have structural roles, act as siderophore transporters, or aid in solute transport [61].

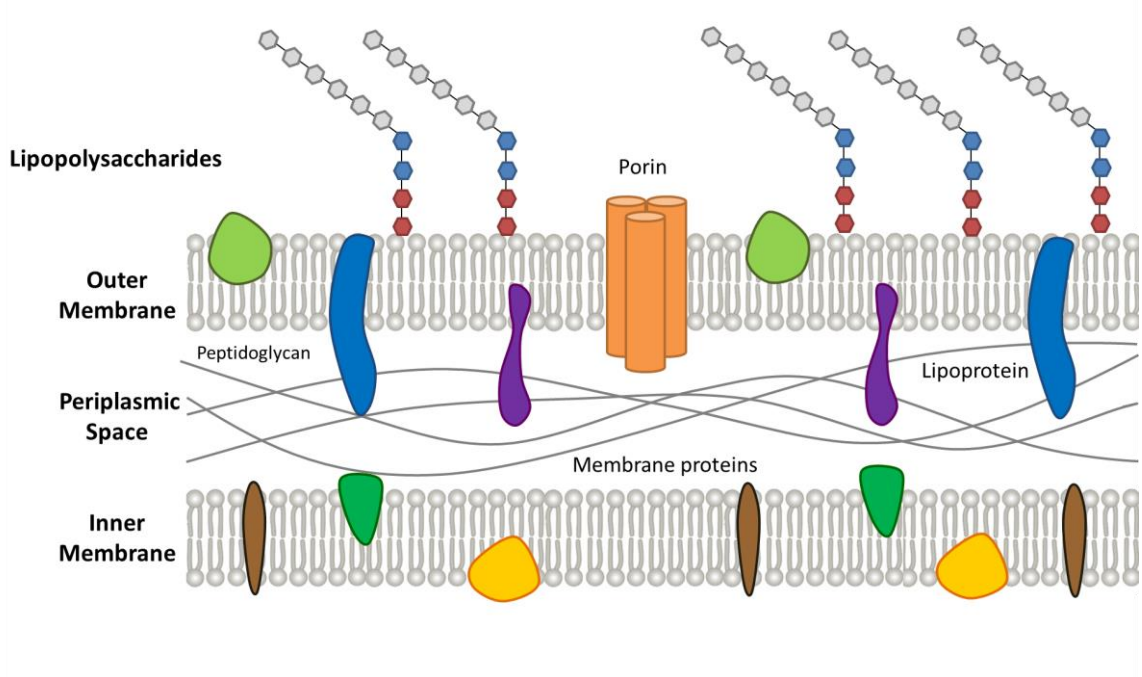


Illustration 2: Outer membrane of gram-negative bacteria

1.3.1 Porins: A class of outer membrane proteins (OMPs)

A class of OMPs involved in solute transport (< 600 Da) is porins, which form pores across the outer membrane. Their expression ranges from 10^4–10^6 copies per cell, depending on the type of bacterium and environmental conditions [62]. Porins can be general diffusive porins or can have specificities for certain solutes. They are β-barrel in structure and usually contain 16–18 β-strands that join together to form a cylindrical structure [60]. Porins can exist as monomers or trimers (Illustration 3). Most sequences among porins are conserved, however, the most variable regions are found in the sequences that form loops between the β-sheets and the loop that invades the interior pore

region or the eyelet region of the porin [63]. The amino acid residues present in the eyelet region create an electrostatic gradient that allows the passage of oppositely charged solute through the porin [63].

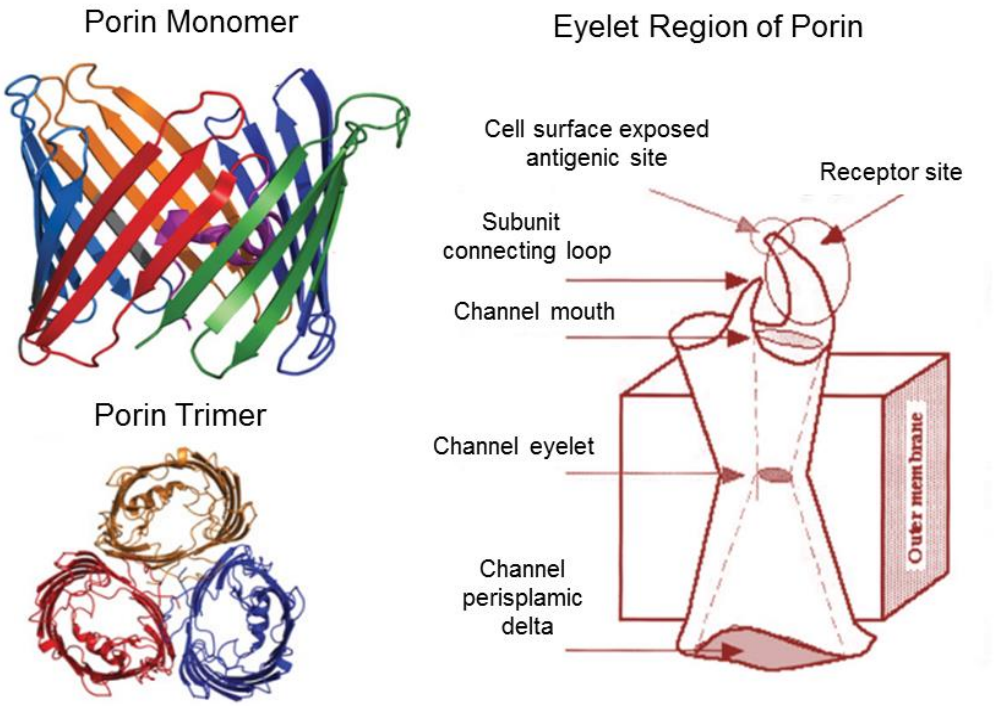


Illustration 3: Structure of Porin
(Modified: Achouck et al, 2001[61]; Zeith and Thein, 2010 [64])

1.3.2 Multiple functions of porins

Porin expression can be regulated by environmental conditions, such as, osmolarity, ionic strength, temperature, heavy metals, antibiotics, bile salts, and aromatic compounds [61]. Bacteria modulate porin expression, size, and amino acid composition to become resistant toward antibiotics [61,65-67]. The loops extending on the outer surface can act as receptors for phages, bacteriocins, epitopes for antibody generation by host immune system, as well as, binding sites for the complement-cascade components [61,68]. Further, porins can aid in pathogenesis. Porins can mediate adhesion and invasion of bacteria into host cells and also induce apoptosis in host cells [69-74].

As the structure of porin is conserved among bacterial species, they can act as pathogen-associated molecular patterns. They can be recognized by the host immune cells because

of their conserved structure and elicit pro-inflammatory responses in host immune cells. Several gram-negative bacterial porins, those of *Salmonella*, *Shigella*, and *Nesisseria* have been extensively studied for their pro-inflammatory responses [69,75-95].

1.3.3 Immune cells possess pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs)

Functionally, the immune system can be characterized by two activities, recognition and response. The innate immune system is the first line of host defense and therefore, plays a crucial role in the recognition of pathogens and subsequent initiation of the inflammatory response. The adaptive system is responsible for elimination of microbes during the later stages of infection and for the generation of memory. The lymphocytes of adaptive system, B cells and T cells together, shape the production of antibodies specific to antigens, whereas, the innate immune response is mediated by phagocytes and antigen-presenting cells (APCs), such as, granulocytes, macrophages, and dendritic cells (DCs). All immune cells are generated from hematopoietic stem cells present in the bone marrow (Illustration 4) [96].

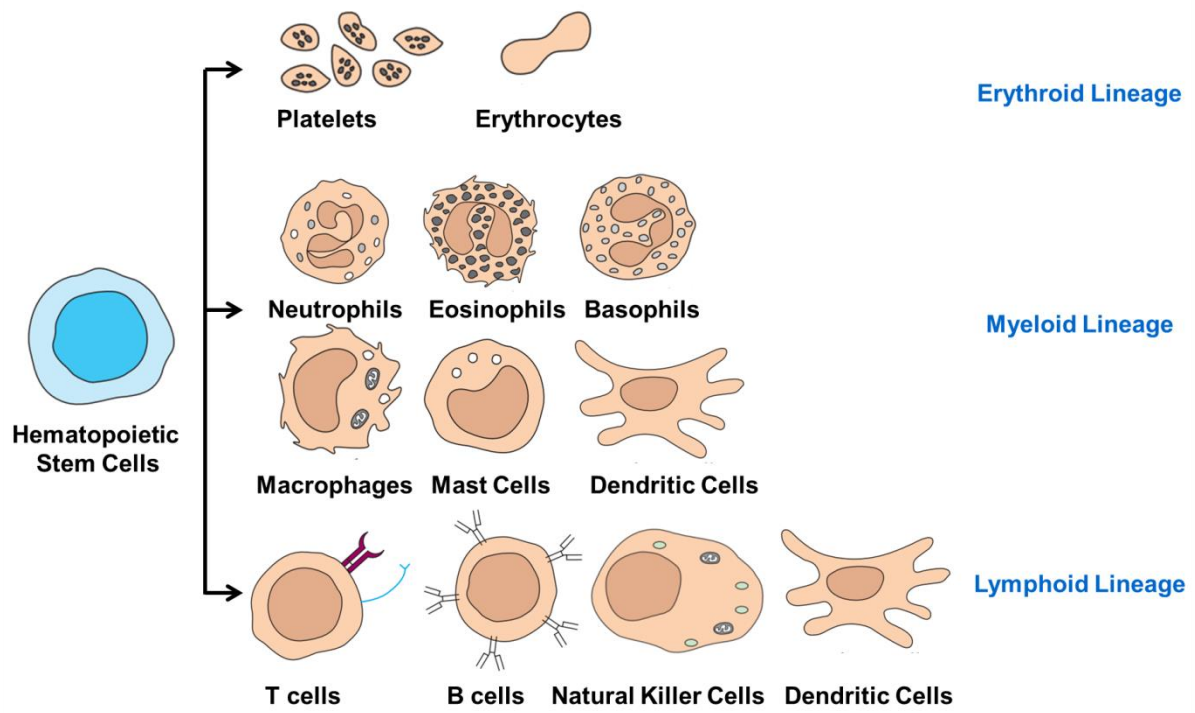


Illustration 4: Cells of the Immune System
(Modified: Immunology, Kuby, 5th Edition [96])

The cellular components of the innate immune system are involved in recognition of microbes and their products, which is necessary for their elimination [97]. Innate immune cells recognize patterns present on pathogens (PAMPs) by PRRs. PAMPs are important for the innate immune system for three main reasons. First, PAMPs are ubiquitous across all microbial species. Every class of microbes is characterized by a certain set of PAMPs. Second, PAMPs are unique to microbes and hence allow discrimination between self and non-self molecules. Third, PAMPs are recognized by the host immune system and thus, make microbial evasion challenging.

The recognition of PAMPs by PRRs, usually leads to inflammatory and anti-microbial responses through signaling cascades, which result in transcription of cytokines, chemokines, cell adhesion molecules, and immune-receptors [98]. This innate immune recognition leads to an early host response to infection, which is subsequently crucial for shaping the adaptive immune response. The nature of PAMP determines the type of innate immune response initiated by monocytes, macrophages, DCs, etc. Furthermore, the epitopes of PAMPs presented by APCs, govern the type of T-cell responses generated. This in turn, defines the B cell-mediated antibody reaction. Therefore, recognition of PAMPs by the host is important for responses initiated by both branches of immunity.

PAMP-recognition by PRRs initiates the innate immune response that may consist of complement activation, phagocytosis, and autophagy. Currently, there are four families of PRRs: Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and RIG-I-like receptors (RLRs), which recognize various PAMPs (Table 3) [97-99]. All classes of PRRs are involved in recognizing multiple PAMPs present on the same pathogen. Thus, no single class of pathogen, such as, virus, bacteria, fungi, protozoa, or parasites is recognized by only a single type of PRR family.

Toll-like Receptors		
Cell surface	TLR1/TLR2	Peptidoglycans, porins
	TLR2/TLR6	Lipoproteins, LPS
	TLR4	LPS
	TLR5	Flagellin
	TLR11	Profilin
	TLR12	Profilin
Endosomal	TLR3	dsRNA
	TLR7	ssRNA
	TLR8	ssRNA
	TLR9	Unmethylated CpG DNA
NOD-like Receptors	NOD1	Muramyl dipeptide (peptidoglycan component)
(Intra-cellular)	NOD2	iE-DAP (peptidoglycan component)
	IPAF	Inflammasome; cytosolic flagellin
	NLRP1	Inflammasome; allergy, autoimmune complexes
	NLRP3	Inflammasome; toxins, urea, asbestos, prion
C-type Lectin Receptors	Galectin-3	β-mannosides
	Minicle	Mannans
(Cell surface)	DC-SIGN	N-linked mannan, viruses, mycobacteria
	Dectin-I	Zymosan, β-glucan
	Dectin-II	Fungal hyphae, N-linked mannan
RIG-I-like receptors	RIG-I	dsRNA
(Intra-cellular)	MDA5	dsRNA, ssRNA
	LGP2	Heterodimerizes with RIG-I and MDA5

Table 3. PRRs families and specificities for PAMPs [100]

1.3.4 OMPs of V. cholerae

Being a gram-negative bacterium, *V. cholerae* harbors OMPs and porins on its surface, which perform a variety of functions that are summarized (Table 4).

Porin	Function
1. OmpW	Hydrophobic channel; porin
2. OmpS	Maltoporin Maltose/maltodextrin high-affinity receptor, phage lambda receptor protein
3. OmpV	Heat shock protein
4. OmpA	Structural role and heat modifiable
5. OmpH	Putative: porin; unfolded protein binding
6. OmpK	nucleoside-specific channel; porin
7. OmpR	Two-component system response regulator OmpR
8. EnvZ	Osmolarity sensory histidine kinase EnvZ
9. OmpX	Putative: virulence related; complement attack resistance; adherence
10. OmpC	Porin; osmolarity related
11. OmpT	Hydrophobic channel; porin
12. OmpU	Hydrophobic channel; porin

Table 4. OMPs of V. cholerae

(The SEED: an Annotation/Analysis Tool Provided by FIG [101])

1.3.5 OmpU, an important candidate in V. cholerae pathogenesis

V. cholerae porin OmpU is a 38 kDa protein with 16 β-strands and exists as trimer in its native state. Its structure is closest to the *E. coli* phosphoporin [102]. OmpU has been implicated in pathogenesis in diverse ways. The surface expression of OmpU on the bacterium increases from 30% to 60% in the gut and is controlled by the ToxR regulon

[103]. These increased levels of OmpU help in bacterial survival amidst the harsh conditions. OmpU confers resistance against bile salts. It mediates acid tolerance by preventing entry of bile salts into the bacterium as the diameter of the channel is about 1.6 nm [104,105]. Further, OmpU confers resistance against cationic anti-microbial peptides, such as, bactericidal/permeability increasing protein (BPI-2) and PmB [106]. Anti-microbial peptides alter the electrochemical gradient across the outer membrane and induce membrane perturbations that cause a conformational change of OmpU. This conformation change of OmpU activates the sigma(E) cascade that restores the electrochemical gradient across the membrane [107].

OmpU from other *Vibrio* species is also involved in several host–pathogen interactions. OmpU of *V. splendidus*, an oyster pathogen, is involved in anti-microbial peptide resistance [74]. *V. splendidus* and *V. vulnificus* OmpU help in adherence to host cells [73,108]. OmpU of *V. alginolyticus*, which infects humans, fish, and crustaceans, mediates antibiotic resistance [66]. OmpU from other species, *V. alginolyticus* and *V. harveyi* have been proposed as vaccine candidates [109,110].

Moreover, the OmpU gene is present in environmental, as well as clinical isolates, but is not present in non-pathogenic strains, that is, non-O1 and non-O139 strains [111,112]. Recently, a study suggested that the sequence of OmpU is conserved among epidemic strains of *V. cholerae*, hence proposing its use as a biomarker [113]. Outer membrane vesicles secreted by *V. cholerae*, predominantly contain OmpU. Immunization of mice with secreted vesicles induces protective immunity [114,115]. Antisera from convalescent patients contain antibodies against *V. cholerae* OmpU and other OMPs [116]. Further, researchers observed that the antisera to OmpU, significantly decreases fluid secretion in rabbit ileal loop model induced by *V. cholerae*, on challenge with homologous strain [116]. Further, *V. cholerae* mutant strains exhibiting impaired OmpU expression, decrease the mRNA expression of several pro-inflammatory factors in the intestinal cell line Int407, by modulating the PI3K/Akt–MAPK–NFκB signaling pathway [117-120]. Therefore, these findings suggest that OmpU may play a far more crucial role in *V. cholerae* pathogenesis.

1.4 Objectives of the study

Although numerous studies have contributed toward the understating the role of *V. cholerae* OmpU in pathogenesis, its immunological role remains poorly characterized. We wanted to explore whether OmpU could modulate the host's immune responses: innate and adaptive.

Toward this study, we had two objectives:

(i) To probe whether OmpU can modulate the inflammatory process induced by monocytes and macrophages.

(ii) To determine whether OmpU can shape adaptive responses by examining its effects on DCs and T cells.

II

Materials and Methods

2. Materials and Methods

2.1 Recombinant OmpU purification

OmpU gene was cloned in a pET14b plasmid (EMD Millipore, Massachusetts, USA) and transformed into *E. coli* BL21 (DE3) vector [102]. The bacterial inoculum (2%) in Luria broth (#M575, HiMedia, Mumbai, India) media was cultured at 37°C till optical density at 600 nm reached 0.5–0.6. The culture was induced by adding IPTG (final concentration 0.1 M, Sigma–Aldrich #I6758, St. Louis, MI, USA) and incubated for 3 h at 37°C. Culture was pelleted down at 2,000 xg, at 4°C for 20 min. Bacterial cells were lysed in lysis buffer (50 mM NaCl, 50 mM Tris, 0.1% Triton-X, pH 7.5) with protease inhibitor cocktail (Sigma–Alrich #P8465) (1 liter culture—10 ml lysis buffer—100 µl protease inhibitor). Cell suspension was sonicated at 25–30 A, with 10 sec on–20 sec off pulse till cells were completely lysed. Suspension was then centrifuged at 18,000 xg, 30 min at 4°C in Oakridge tubes. The pelleted inclusion bodies were washed twice with phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4) supplemented with 100 mM NaCl at 18,000 xg, 15 min at 4°C. Pelleted inclusion bodies were resuspended in 8 M urea (10 ml/ 1 liter culture) and incubated at room temperature for 40 min and subsequently centrifuged at 18,000 xg, 15 min at 4°C.

Solubilized protein was subjected to Nickel–NTA chromatography. Ni–NTA resin (4 ml) (Profinity™ IMAC resin, Biorad, Hercules, California, USA) was packed into column and washed with 50 ml PBS. Column was equilibrated with 4 ml 8 M urea in PBS. Sample was loaded into column and incubated for 1 h. Column was washed with 20 ml of 20 mM imidazole (HiMedia, #RM1864) in 8 M urea in PBS. Protein was eluted using 300 mM imidazole in 8 M urea in PBS and four fractions of 5 ml each were collected. Fractions containing enriched protein content were further used for refolding of the protein. Nickel purified protein 1 ml fraction was refolded in 10 ml of refolding buffer [10% glycerol, 0.5% LDAO (Sigma–Aldrich #40236) in PBS] by rapid dilution method and addition of protein in 50 µl instalments. Protein was allowed to refold overnight at 4°C. Refolded protein was centrifuged at 18,000 xg, 15 min at 4°C to remove aggregates.

Protein was then purified using size exclusion chromatography. Sephacryl S200 prepacked column (GE Life Sciences, Sunnyvale, California, USA) (1.6*60 cm; bed

volume of 120 ml) was equilibrated with 120 ml buffer (10 mM Tris, 10 mM NaCl, 0.5% LDAO, pH 7.5) and protein sample was loaded. Fractions (2 ml) were pooled according to protein content. Protein content was measured using Bradford's assay, aliquoted and stored at –20°C.

2.2 Wild type OmpU purification

Wild type OmpU protein was isolated from outer membrane of *V. cholerae* El Tor O1. *V. cholerae* was cultured in Brain Heart Infusion (Sigma–Aldrich, #53286) liquid broth at 37°C under constant shaking condition until the OD_{600} reached 1.0. Broth was spun at 2,050 xg for 30 min at 4°C, and bacterial cell pellet was suspended in 20 mM Tris–HCl buffer (pH 7.6) (10 ml/liter culture) containing bacterial protease inhibitor cocktail. Cells were lysed by ultrasonic disruption and centrifuged at 18,500 xg for 1 h at 4°C. Supernatant was further ultracentrifuged at 350,000 xg for 20 min at 4°C. Pelleted bacterial membrane was resuspended in PBS and ultracentrifuged under similar conditions. The outer membrane fraction was then treated with 1% Sarkosyl NL-97 in PBS (Sigma–Aldrich, #61747) at 37°C, and the pellet was further washed with 20 mM Tris–HCl (pH 7.6) by ultracentrifugation at 350,000 xg. Pellet was resuspended in 20 mM Tris–HCl (pH 7.6) containing 4% Triton X-100 (Himedia, #MB031), incubated at 37°C for 30 min, and centrifuged at 105,000 xg for 60 min at 4°C. The extracted resuspended membrane proteins (supernatant) were subjected to DEAE–cellulose (Sigma–Aldrich, #D3764) column (10*1.5 cm) equilibrated with 20 mM Tris–HCl (pH 7.6) buffer containing 0.1% Triton X-100. For elution, a continuous gradient of 0–0.2 M NaCl in 60 ml of 20 mM Tris–HCl (pH 7.6) containing 0.1% Triton X-100 was used. Eluted fractions containing OmpU were further purified by Sephacryl S200 size exclusion chromatography column (1.6*60 cm; bed volume of 120 ml) equilibrated with 10 mM Tris–HCl buffer (pH 7.6) containing 10 mM NaCl and 0.5% n-octyl POE (n-octylpolyoxyethylene) (#P1140, Alexis Biochemicals, Farmingdale, NY, USA). Eluted fractions containing the purified OmpU were pooled and protein was estimated by Bradford's assay and stored at –20°C.

2.3 Protein estimation by Bradford's assay

Standards for colorimetric assay were prepared diluting 10 mg/ml bovine serum albumin in Tris buffer (10 mM Tris, 10 mM NaCl, 0.5% LDAO, pH 7.5) as mentioned below (Table 5).

Total Volume 1 ml	Concentration (µg/ml)
0 µl	0.00
25 µl	12.5
50 µl	25.0
75 µl	37.5
100 µl	50.0
125 µl	62.5
150 µl	75.0
175 µl	87.5
200 µl	100.0

Table 5. Standard preparation for Bradford assay

Standard or test sample (5 µl) was added to 95 µl of Bradford reagent in a 96-microwell plate. Absorbance was measured at 595 nm using iMark Microplate Absorbance Reader (BioRad) after 15 min. Values were plotted as a graph and concentration of test sample was calculated accordingly.

2.4 Limulus amebocyte lysate (LAL) assay

LPS contamination in purified protein sample was measured by E-Toxate kit (Sigma–Alrich, #ET0100). Glassware was treated with 1% SDS solution to remove any pyrogen contamination. E-Toxate standard dilutions were prepared by serial dilution such that final concentration of standards ranged from 0.015 to 400 EU/ml and the following working dilutions were prepared (Table 6).

Tube no.	Endotoxin	Pyrogen-free water	Final concentration (EU/ml)
1	0.2 ml of endotoxin stock solution	1.8	400
2	0.2 ml from tube 1	1.8	40
3	0.2 ml from tube 2	1.8	4
4	0.3 ml from tube 3	2.1	0.5
5	1 ml from tube 4	1.0	0.25
6	1 ml from tube 5	1.0	0.125
7	1 ml from tube 6	1.0	0.06
8	1 ml from tube 7	1.0	0.03
9	1 ml from tube 8	1.0	0.015

Table 6. Standard preparation for LAL assay

E-Toxate reagent was reconstituted in pyrogen-free water. Further, different tubes were prepared as follows (Table 7).

	Tube	Sample	Endotoxin Standard Dilution
A	Test sample	0.1 ml	—
B	Negative control	—	—
C	Standard	—	0.1 ml of 0.5 EU/ml
D	Standard	—	0.1 ml of 0.25 EU/ml
E	Standard	—	0.1 ml of 0.125 EU/ml
F	Standard	—	0.1 ml of 0.06 EU/ml
G	Standard	—	0.1 ml of 0.03 EU/ml
H	Standard	—	0.1 ml of 0.015 EU/ml

Table 7. LAL assay procedure

E-Toxate reagent (0.1 ml) was added to all tubes A–H. Samples were mixed gently and incubated at 37°C for 1 h without any disturbance. After incubation, tubes were removed gently and inverted at a 180° angle. A positive test was indicated by the formation of a gel. LPS free samples did not form any gel. LPS content was calculated using the formula: EU/ml = (Dilution factor of standard at which gel was formed)*0.06. 1 EU is equivalent to 0.1–0.2 ng/ml of LPS.

2.5 Cell lines, primary cells, and cell culture conditions

All the cells were cultured at 37°C in 5% CO_2 humidified incubator (#CO170R-230-1000, Eppendorf, Hamburg, Germany). RAW 264.7 (murine macrophage) (NCCS, Pune, India) and THP-1 (human monocytic) (NCCS, Pune, India) cells lines and human peripheral blood mononuclear cells (PBMCs) were cultured in RPMI-1640 media (#11875-119, Life Technologies, Carlsbad, California, USA) with 10% fetal bovine serum (FBS) (Life Technologies, #161400-71) and 100 U/ml penicillin-streptomycin (Life Technologies, #15140-022) **[Complete RPMI media]**. HT29 (human intestinal epithelial) (NCCS, Pune, India) and murine peritoneal macrophages were cultured in DMEM (Life Technologies, #11995-073) with 10% FBS and 1% penicillin–streptomycin **[Complete DMEM media]**. Murine bone marrow-derived dendritic cells (BMDCs), splenic DCs and splenic $CD4^+$ T cells were cultured in complete RPMI-1640 media supplemented with 1% β-mercaptoethanol (Sigma–Aldrich, #M6250), 0.1 mM non-essential amino acids (NEAA) (Life Technologies, #11140-050) and 1 mM sodium pyruvate (Life Technologies, #11360070) **[supplemented complete RPMI media]**.

2.6 Isolation of PBMCs

Venous blood was drawn from a healthy donor and 1.5 mg EDTA (HiMedia, #MB011) per ml of blood was added to prevent coagulation. Diluted blood (1:1 with PBS) was layered over Histopaque-1077 (Sigma–Aldrich, #10771) in a ratio of 1:1 carefully. The sample was centrifuged at 400 xg for 30 min without acceleration or deceleration at ambient temperature. Further, the upper plasma layer was discarded and buffer layer was washed twice with PBS (250 xg for 10 min at room temperature). Cells were resuspended in complete RPMI media. Work with human blood was approved by the Institutional Bioethics Committee.

2.7 Mice

Six- to eight-week-old female BALB/c mice were obtained from Panacea Biotec (Mohali, India) (approved by the Institutional Animal Ethical Committee of Panacea Biotec, Mohali). Mice were sacrificed and bone marrow cells and spleens were isolated.

2.8 Murine peritoneal cells isolation

Ice cold PBS (10 ml) was injected into the peritoneal cavity via a syringe and massaged for flushing out the trapped cells in PBS. Cell suspension was aspirated via the syringe, washed with PBS, resuspended in complete DMEM media, and plated at a density of 1.5 million/ml. After 2 h of incubation, non-adherent cells were removed and adherent macrophages were treated according to the experiment.

2.9 Murine DC differentiation from bone marrow cells

Epiphyses of isolated femurs were cut and bone marrow cells were flushed in complete RPMI media via a syringe. Cells were washed with supplemented complete RPMI media, treated with 2–3 ml ACK lysis buffer (Life Technologies, #A10492-01), and incubated for 5 min at room temperature for removal of erythrocytes. Supplemented complete RPMI media was added and cells were centrifuged and resuspended in supplemented complete RPMI media and plated in 6-well plates (2.5 million/ml in 2 ml). The media was supplemented with 10 ng/ml of GM-CSF (#315-03, Peprotech, Rocky Hill NJ, USA) and changed every alternate day for 7 days. After 7 days, cells were treated according to the experiment.

2.10 Isolation of DCs from murine spleen

The spleen was excised from mouse and ballooned in 5 ml calcium–magnesium free HBSS [0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 0.1% glucose (Sigma, #G8270)] containing 400 U/ml Collgenase D (#11088866001, Roche, Basel, Switzerland). Cells were incubated at 37°C in humidified 5% CO_2 incubator for 25 min, the reaction was stopped by adding 100 µl of 0.5 M EDTA, and further incubation at 37°C for 5 min. Cell suspension was then passed through a

0.45 μm filter onto supplemented complete RPMI media to obtain a single-cell suspension. Cells were washed with PBS and resuspended in 3 ml of 30% BSA (bovine serum albumin) (Sigma–Aldrich, #A9576) and 1 ml of PBS was layered on the top of BSA. Cells were then centrifuged at 620 xg, at 12°C without acceleration and deceleration for 30 min. Cells at interface of BSA **[CD11c-enriched population]** and PBS were collected, further washed with PBS, and purified using anti-$CD11c^+$ mouse dendritic cell MACS purification kit (Miltenyi Biotec, Bergisch Gladbach, Germany) by positive-selection procedure.

Briefly, upto 10^7 cells were resuspended in 40 μl MACS buffer (0.5% FBS, 2 mM EDTA in PBS) and 5 μl of anti-$CD11c^+$ beads were added and incubated at 4°C for 20 min. Cells were washed with MACS buffer once, resuspended in 500 μl of MACS buffer, and loaded onto preequilibrated LS MACS column placed on magnet. Column was washed thrice with 500 μl of MACS buffer and removed from magnet. MACS buffer (2 ml) was added onto column and eluted using plunger. Cells were washed with MACS buffer, resuspended in supplemented complete RPMI media, and plated according to the experiment.

2.11 $CD4^+$ T-cell isolation from murine spleen

The excised spleen from a mouse was homogenized by squashing it between the frosted ends of sterilized glass slides. Cell suspension was filtered using a 0.45 μm filter to obtain a single-cell suspension and centrifuged at 800 xg, at 4°C for 5 min. Cells were subjected to ACK lysis buffer treatment for erythrocyte removal and washed with PBS. Cells were purified using mouse $CD4^+$ T-cell isolation kit II (Miltenyi Biotec) by negative-selection method.

Upto 10^7 cells were resuspended in 40 μl MACS buffer and 5 μl of biotin-antibody cocktail was added. Cells were incubated at 4°C for 10 min. Further 30 μl MACS buffer and 10 μl of microbead cocktail was added. Cells were incubated at 4°C for 15 min and washed with MACS buffer and resuspended in 500 μl of MACS buffer. Sample was loaded onto preequilibrated MS MACS column and washed thrice with 3 ml MACS buffer. Flow-through cells were collected and washed with MACS buffer. Cells were

resuspended in supplemented complete RPMI media and plated according to the experiment.

2.12 Experimental Designs

2.12.1 Experimental design I: Determination of inflammatory mediator production in response to OmpU in monocytes, macrophages, and human PBMCs

Cytokine analysis: RAW 264.7, THP-1 cells, and human PBMCs were plated at a density of 1 million/ml in 1.5 ml of complete RPMI media in 6-well plate. Cells were treated with OmpU for different doses for several time periods: 4 h, 8 h, 12 h, and 24 h. Thirty minutes prior to OmpU treatment, cells were treated with 5 μg/ml PmB (Sigma–Aldrich, #P4932) for possible LPS contamination in purified protein samples. LPS (Sigma–Aldrich, #L2880, *E. coli* 055:B5) (1 μg/ml) served as a positive control. Elution buffer used in protein purification (10 mM Tris, 10 mM NaCl, 0.5% LDAO, pH 7.5) diluted in 0.5% LDAO in PBS served as a negative control. Culture supernatants were collected after different time periods for time-dependent studies or at the time point at which the production of mediator was maximal for dose-dependent studies and stored at –20°C. Levels of NO, TNFα, and IL-6 levels were further determined in culture supernatants (Illustration 5).

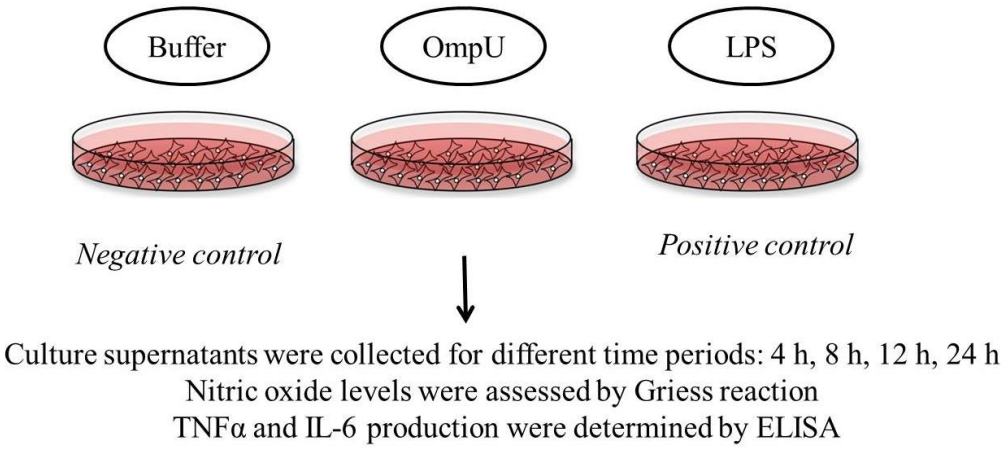


Illustration 5. Experimental design to assess OmpU-mediated effects

Cell viability: THP-1 cells were plated at a density of 1 million/ml in 100 µl complete RPMI media in a 96-microwell plate. Cells were treated with different doses of OmpU for 24 h and buffer treated cells served as a control. Further, cell viability was determined.

2.12.2 Experimental design II: Determination of OmpU-pretreatment on LPS-mediated responses

Cytokine analysis: RAW 264.7, THP-1 cells, and human PBMCs were plated at a density of 1 million/ml in 1.5 ml of complete RPMI media in a 6-well plate. Cells were treated with 5 µg/ml PmB (30 min) and 2 µg/ml OmpU for 24 h **[PmB+OmpU]**. Cells were replated in fresh media without PmB and stimulated with 1 µg/ml LPS for different time periods. Protein buffer-treated cells, stimulated with LPS served as a positive control. OmpU-treated cells further treated with PBS served as a negative control. Culture supernatants were collected after different time periods and stored at –20°C. Levels of NO, TNFα, and IL-6 levels were further determined in culture supernatants (Illustration 6).

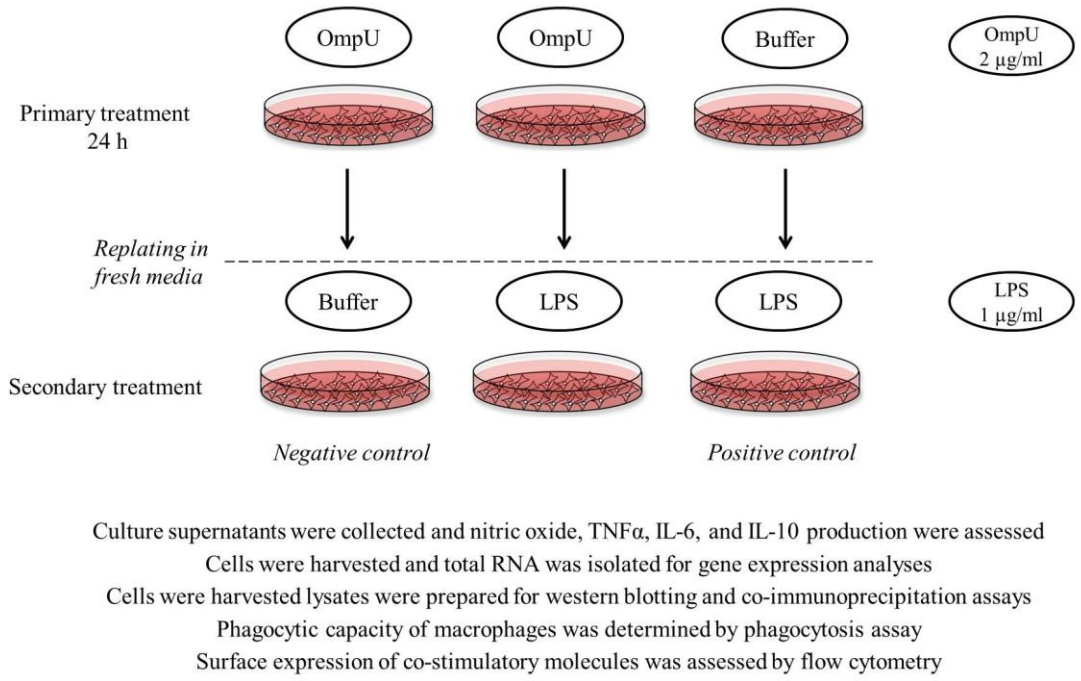


Illustration 6. Experimental design to assess effect of OmpU-pretreatment on LPS-mediated responses

Cell viability: THP-1 cells were plated at a density of 1 million/ml in 100 µl complete RPMI media in a 96-microwell plate. Cells were treated with PmB+OmpU for 24 h, replated in fresh media, and challenged with LPS for 24 h. Cell viability was further determined.

Gene-expression analysis: RAW 264.7 and THP-1 cells were plated at a density of 1.5 million/ml in 2 ml complete RPMI media in a 6-well plate. Cells were treated with PmB+OmpU for 24 h, replated in fresh media, and stimulated with LPS for different time periods. Cells were harvested and total RNA was isolated for subsequent gene-expression analysis by semi-quantitative PCR.

Western blotting and co-immunoprecipitation studies: RAW 264.7 and THP-1 cells were plated at a density of 1 million/ml in 10 ml complete RPMI media in a 100-mm petridish. Cells were treated with PmB+OmpU for 24 h, replated, and challenged with LPS for 1 h for co-immunoprecipitation studies or different time points for immunoblotting. Whole cell or nuclear lysates were prepared after respective incubations.

Phagocytosis assay: RAW 264.7 or murine peritoneal cells were plated at a density of 1 million/ml in 0.5 ml of complete RPMI or complete DMEM media, respectively, in a 24-well plate. Cells were treated with PmB+OmpU for 24 h, replated in fresh media, and challenged with LPS for 24 h. Cells were harvested and assay for phagocytosis was performed.

Flow cytometry for surface-expression analysis: RAW 264.7 and THP-1 cells were plated at density of 1 million/ml in 1 ml in a 6-well plate in complete RPMI media. Peritoneal macrophages were plated in complete DMEM similarly. Cells were treated with PmB+OmpU for 24 h, replated in fresh media, and challenged with LPS for 18 h (TLR2, TLR6; RAW 264.7), 4 h (TLR6; THP-1), 8 h (TLR2; THP-1), and 24 h (CD80, CD86; RAW 264.7, and peritoneal macrophages). Cells were harvested for flow-cytometric analysis.

Neutralization of secreted IL-10 in human PBMCs and its involvement in down-regulation of LPS-mediated TNFα: Human PBMCs were plated as for cytokine analysis. Neutralizing bodies against human IL-10 (#554703, BD Pharmingen, Franklin Lakes, NJ, USA) and TGFβ (#146704, Biolegend, San Diego, CA, USA) were added at a concentration of 0.1 μg/ml and 10 μg/ml along with 24 h PmB+OmpU-pretreatment. Cells were replated in fresh media and stimulated with LPS for 8 h. TNFα levels in culture supernatants were determined by ELISA.

2.12.3 Experimental design III: Determination of OmpU-pretreatment on LPS-mediated IL-12 production in human PBMCs

Human PBMCs were plated at a density of 1 million/ml in 1.5 ml complete RPMI media in a 6-well plate. After 8 h of PmB+OmpU treatment, 100 ng/ml of recombinant human IFNγ (Peprotech #300-02) was added and further incubated for 16 h, such that OmpU treatment incubation was for 24 h. Cells were replated in fresh media and challenged with LPS. As a positive control, IFNγ (16 h)-treated cells were further stimulated with LPS for 24 h. OmpU- and IFNγ-treated cells replated in fresh media and treated with PBS served as a negative control for 24 h. Culture supernatants were collected and IL-12 levels were analyzed by ELISA (Illustration 7).

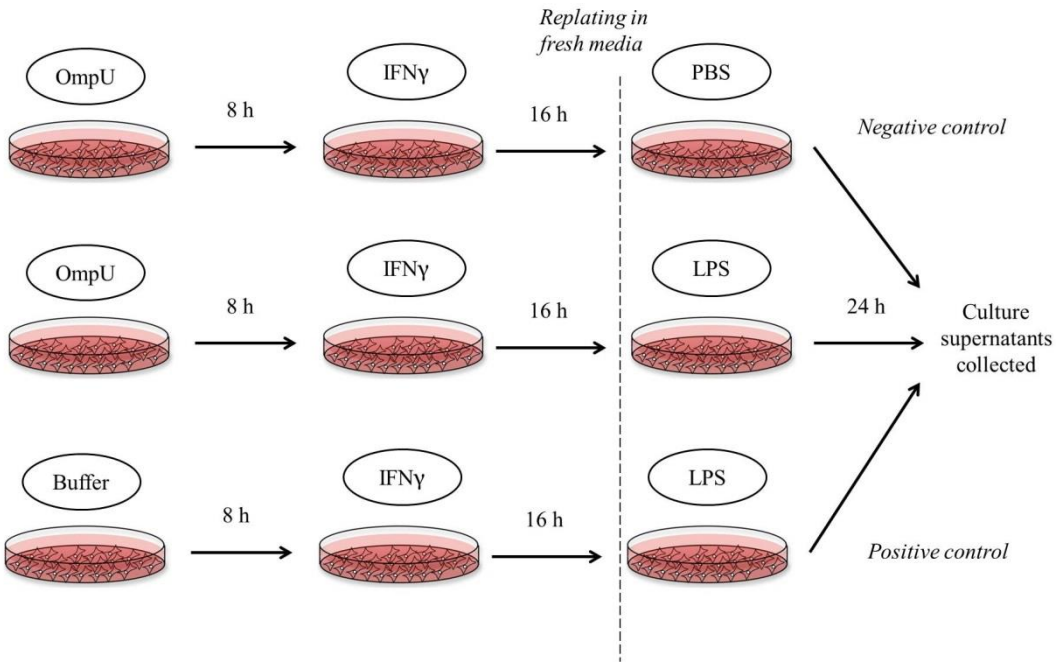


Illustration 7. Experimental design to assess effect of OmpU-pretreatment on LPS-mediated IL-12 production in human PBMCs

2.12.4 Experimental design IV: Differentiation of BMDCs and determination of cellular responses induced by OmpU

Bone marrow cells were plated at a density of 2.5 million/ml in 2 ml of supplemented complete RPMI media. Cells were differentiated with 10 ng/ml of murine recombinant GM-CSF on alternate days (Day 0, Day 2, Day 4, and Day 6). On the 7th day, differentiated BMDCs were treated with PmB+OmpU for 24 h (Illustration 8).

Cytokine analysis: After the incubation, culture supernatants were collected and TNFα, IL-6, IL-1β, IL-12, and IL-10 levels were determined.

Cellular cytotoxicity assay: Culture supernatants were also analyzed for cytotoxicity induced by treatments.

Flow cytometry for surface-expression analysis: Cells were harvested and surface expression of CD80 and CD86 was assessed by flow cytometry.

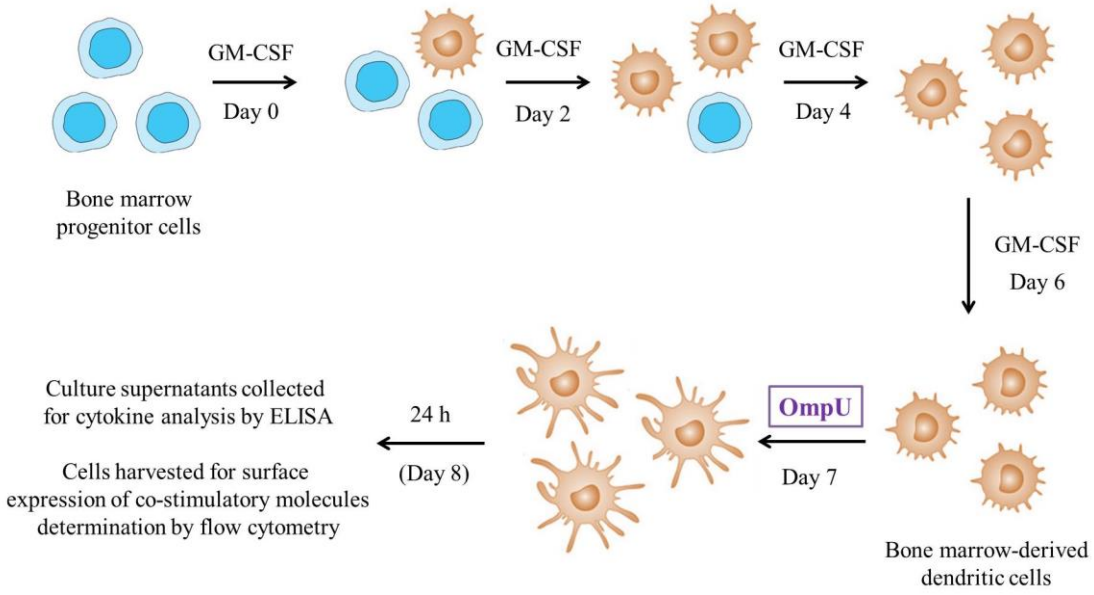


Illustration 8. Experimental design to determine OmpU-mediated responses in mouse

2.12.5 Experimental design V: Determination of murine CD4$^+$ T-cell responses on co-culture with OmpU-primed murine splenic DCs in vitro

Purified splenic DCs were plated at a density of 2x10^4 cells in 200 µl of supplemented complete RPMI media in a 96-microwell plate. Cells were treated with PmB+OmpU for 24 h (Day 0). After the incubation, DCs were serially diluted and purified splenic CD4$^+$ T cells were added such that DC:T cell ratio was 1:10, 1:20, 1:40, and 1:80 in 100 µl of supplemented complete RPMI media (Day 1). After 6 days, BrdU was added to each well and after 18 h, cells were harvested and cell proliferation was assessed (Illustration 9).

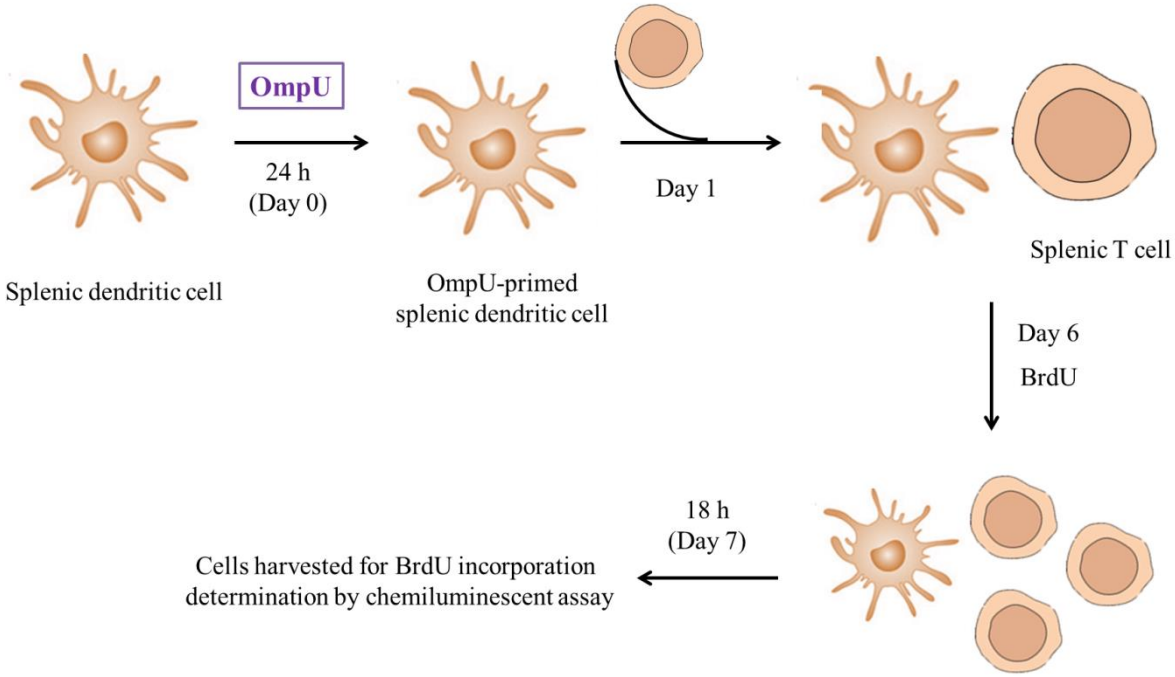


Illustration 9. Experimental design to determine effect of OmpU-primed DCs on CD4$^+$ T cells

2.12.6 Experimental design VI: Determination of TLR, NFκB, AP-1, and inflammasome involvement by neutralization and inhibitor studies

BMDCs and splenic DCs were plated at a density of 1 million/ml in supplemented complete RPMI media, were pretreated with neutralizing antibodies and chemical inhibitors for 1 h, and further treated with OmpU for 24 h. Culture supernatants were collected and levels of cytokines were assessed.

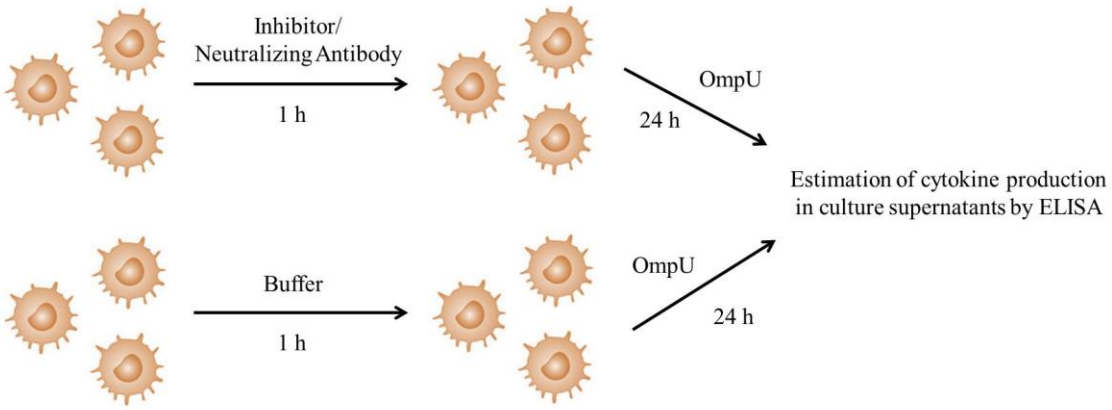


Illustration 10. Experimental design to determine effects of TLR-neutralizing antibodies and inhibitors of signaling mediators on OmpU-mediated response

MLN4924 (500 nM)	R&D systems, #I502 (Minneapolis, USA)	Anti-mouse TLR4	Biolegend, #117608
SP600125 (20 μM)	Sigma–Aldrich, #57067	Anti-mouse TLR2	Biolegend, #121802
VX745 (50 nM)	Santa Cruz Biotechnologies, #sc-361401 (Texas, USA)	Isotype control Rat IgG2aκ	Biolegend, #400516
JNKIN8 (250 nM)	Santa Cruz Biotechnologies, #sc-364745	Isotype control Mouse IgG1κ	Biolegend, #400102
Isoliquiritigenin (80 μM)	InvivoGen, #ihn-ilg (San Diego, CA, USA)		
Parthenolide (18 μM)	InvivoGen, #inh-ptd		

2.13 MTT-cell viability assay

Cells were plated at a density of 1 million/ml in 100 µl in a 96-microwell plate. Cell viability after various treatments was assessed by the EZCount MTT Cell Assay Kit (HiMedia, #CCK003). After incubation periods, 10 µl of MTT reagent (5 mg/ml) was added to 100 µl of cell culture volume and incubated for 37°C in a humidified 5% CO_2 incubator for 3 h. After which, 100 µl of solubilization buffer was added per well and mixed to ensure uniform color development. Absorbance was measured at 575 nm using iMark Microplate Absorbance Reader (BioRad) and viability was calculated as:

[Abs $_{\text{treated cells}}$ − Abs $_{\text{blank}}$]/[Abs $_{\text{untreated}}$ cells – Abs $_{\text{blank}}$]. Blank refers to absorbance of well containing media, MTT, and solubilization buffer but no cells.

2.14 LDH-release assay for measuring cytotoxicity

Cells were plated at a density of 1 million/ml in 100 µl in a 96-microwell plate. Cytotoxicity due to OmpU and chemical inhibitors in BMDCs was measured by the CytoTox 96 non-radioactive cytotoxicity assay kit (#G1780, Promega, Madison, WI, USA). Cells were centrifuged at 600 xg, 5 min at 4°C and 50 µl of supernatant was incubated with 50 µl of assay buffer substrate mix for 30 min in dark at room temperature. The reaction was stopped by adding 50 µl of stop solution and absorbance was measured at 490 nm using iMark Microplate Absorbance Reader (BioRad). Cells treated with Triton-X-100 (0.8%) 15 min prior to assay served as positive control for cytotoxicity.
Cytotoxicity was calculated as:

[Abs $_{\text{treated cells}}$ − Abs $_{\text{blank}}$]/[Abs $_{\text{Triton-X-100}}$ cells – Abs $_{\text{blank}}$]. Blank refers to absorbance of well containing media and assay buffer substrate mix but no cells.

2.15 NO estimation

NO estimation was determined by Griess reagent (Sigma–Alrich #G4410). Sodium nitrite solution (500 µM) was prepared by stock solution of 0.1 µM (Sigma–Alrich #35273) and following working dilutions were prepared (Table 8).

Total Volume 1 ml water	Concentration (μM)
0 μl	
10 μl (of 500 μM)	2.5
20 μl	5.0
30 μl	7.5
40 μl	10.0
50 μl	12.5
60 μl	15.0
70 μl	17.5

Table 8. Standard preparation for NO estimation

Standard or culture supernatant (50 μl) was incubated with 50 μl of Griess reagent in a 96-microwell plate and incubated in dark at room temperature for 15 min. Absorbance was measured at 540 nm using iMark Microplate Absorbance Reader (BioRad) and values were plotted on a graph. Concentration of test sample was calculated accordingly.

2.16 RNA isolation and purification

RNA was isolated using total RNASure isolation kit (#NP-84107, Nucleo-pore, New Delhi, India). After respective incubations, cells were harvested and washed twice with PBS at 600 xg, 5 min at 4°C. Lysis buffer (350 μl) and 3.5 μl of β-mercaptoethanol were added to the pelleted cells and lysis was carried out by passing through a syringe. The mixture was transferred to shedder columns and centrifuged at 11,000 xg for 1 min. The filtrate was transferred to a new tube and RNA was precipitated by the addition of 350 μl of 70% ethanol (#E193, Amresco, Solon, USA; Molecular-grade water Sigma–Alrich #W4502). Lysate was added to the RNASure column and spun at 11,000 xg for 1 min. Desalting buffer (350 μl) was added to column and centrifuged at 11,000 xg for 1 min. DNase reaction mixture (100 μl) was added to the column and incubated for 15 min. Reaction was stopped using 200 μl of LBA2 buffer and the column was spun at 11,000 xg for 1 min. The column was washed again with 600 μl of LBA2 buffer. WBA3 (200 μl) buffer was added to the column and spun at 11,000 xg for 3 min. Molecular-

grade water (50–60 µl) was added to the column and incubated for 5 min. RNA was isolated by centrifuging column at 11,000 xg for 3 min and absorbance at 230 nm, 260 nm, and 280 nm was measured.

If the absorbance ratio at 260/280 was less than 1.8 or the 260/230 ratio was less than 2, RNA was reprecipitated to obtain better quality RNA devoid of salts and buffer contamination. Volume of RNA sample was made upto 100 µl with molecular-grade water. Autoclaved 1.1 M NaCl (10 µl) was added such that final concentration was 0.1 M. Molecular-grade ethanol (95%; 275 µl, 2.5 volumes) (Amresco, #E193) was added to the sample and incubated at –20°C for 1 h. The sample was centrifuged at 11,000 xg at 4°C for 15 min and washed with 500 µl of 70% ethanol twice. The microcentrifuge tube was allowed to air-dry to ensure complete water removal. Precipitated RNA was resuspended in molecular-grade water and absorbance was measured as mentioned above.

2.17 cDNA synthesis

cDNA was synthesized by the Verso cDNA synthesis kit (#AB-1453/A, Thermo Scientific, Waltham, Massachusetts, USA). cDNA for each RNA sample was prepared as follows (Table 9). The reverse transcription-cycling program included 1 cycle at 42°C for 30 min and 1 cycle at 95°C for 2 min and synthesis was carried out using MyCycler thermocycler (BioRad).

	Volume (µl)	Final concentration
5X cDNA-synthesis buffer	4	1X
dNTP mix	2	500 µM each
RNA primer	1	
RT enhancer	1	
Verso enzyme mix	1	
Template RNA	1–5	1 ng–1 µg
Nuclease-free water	make upto 20	
Total volume	20	

Table 9. cDNA synthesis-reaction mix

2.18 Semi-quantitative PCR

Real-time gene-expression analysis was carried out using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, #KO221). The reaction mix for each cDNA sample was prepared as follows (Table 10).

	Volume (μl)	Final concentration
Maxima SYBR mix (2X)	5	1X
Forward primer	1	0.25 μM
Reverse primer	1	0.25 μM
cDNA	0.5–1	<500 ng
Nuclease-free water	make to 10	
Total volume	10	

Table 10. Semi-qPCR synthesis-reaction mix

Reactions for the gene of interest and house-keeping genes were set. Reactions without cDNA but rest of the components served as negative controls. Two-step cycling protocol was used in Realplex^4 thermocycler (Eppendorf): 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C (15 sec), and 1 cycle at 60°C (60 sec). Fold change was calculated using ΔΔCt method of Livak and Schmittgen [121] from Ct values of each of the reactions obtained.

2.19 Primer sequences used for gene-expression analyses

Primer sequences were sourced from PrimerBank [122].

Primer name	Primer sequence 5'—3'
Mouse CXCR1	F: TGC TGG TTA TCT TAT ACA GGC GA
	R: GCA CGT AGA CAT CCA TGA CGG
Mouse CXCR2	F: ATG CCC TCT ATT CTG CCA GAT
	R: GGT GCT CCG GTT GTA TAA GAT GA
Mouse CCL17	F: TAC CAT GAG GTC ACT TCA GAT GC
	R: GCA CTC TCG GCC TAC ATT GG

Mouse CCL22	F: AGG TCC CTA TGG TGC CAA TGT
	R: CGG CAG GAT TTT GAG GTC CA
Mouse CCL24	F: TCT TGC TGC ACG TCC TTT ATT
	R: CTA ACC ACT CGG TTT TCT GGA AT
Mouse arginase	F: CCT ATG TGT CAT TTG GGT GGA TG
	R: GGT TGT CAG GGG AGT GTT GAT
Mouse SOCS-1	F: CTG CGG CTT CTA TTG GGG AC
	R: AAA AGG CAG TCG AAG GTC TCG
Mouse SOCS-3	F: ATG GTC ACC CAC AGC AAG TTT
	R: TCC AGT AGA ATC CGC TCT CCT
Mouse MKP-1	F: GTT GTT GGA TTG TCG CTC CTT
	R: TTG GGC ACG ATA TGC TCC AG
Mouse SHIP-1	F: GAG ACA CTG TTT CAG CGT CTA C
	R: CGT CTT CAA AAA GTC GGA ATC CA
Mouse TOLLIP	F: CCT CAG CCC CGC TGT AAT G
	R: CAG CAT CTT TGT TCC CTC TCT G
Mouse IRAK-M	F: CTG GCT GGA TGT TCG TCA TAT T
	R: GGA GAA CCT CTA AAA GGT CGC
Mouse GAPDH	F: TGG ATT TGG ACG CAT TGG TC
	R: TTT GCA CTG GTA CGT GTT GAT
Mouse RPL0	F: TGA GAT TCG GGA TAT GCT GTT GG
	R: CGG GTC CTA GAC CAG TGT TCT
Mouse RPL13	F: GGG CAG GTT CTG GTA TTG GAT
	R: GGC TCG GAA ATG GTA GGG G
Mouse HPRT	F: TCA GTC AAC GGG GGA CAT AAA
	R: GGG GCT GTA CTG CTT AAC CAG
Human CCL18	F: TCT ATA CCT CCT GGC AGA TTC
	R: TTT CTG GAC CCA CTT CTT ATT G
Human CCL22	F: ATT ACG TCC GTT ACC GTC TGC
	R: TCC CTG AAG GTT AGC AAC ACC
Human arginase	F: CCC TGG GGA ACA CTA CAT TTT G
	R: GCC AAT TCC TAG TCT GTG CAC TT

Human SOCS-1	F: TTT TCG CCC TTA GCG TGA AGA
	R: GAG GCA GTC GAA GCT CTC G
Human SOCS-3	F: CCT GCG CCT CAA GAC CTT C
	R: GTC ACT GCG CTC CAG TAG AA
Human MKP-1	F: ACC ACC ACC GTG TTC AAC TTC
	R: TGG GAG AGG TCG TAA TGG GG
Human SHIP-1	F: GCG TGC TGT ATC GGA ATT GC
	R: TGG TGA ACC TCA TGG AGA C
Human TOLLIP	F: TGG GCC GAC TGA ACA TCA C
	R: GTG GAT GAC CTT ATT CCA GCG
Human IRAK-M	F: CTG CGG GAT CTC CTT AGA GAA
	R:GCA GAG AAA TTC CGA GGG CA
Human GAPDH	F: AAG GTG AAG GTC GGA GTC AAC
	R: GGG GTC ATT GAT GGC AAC AAT A
Human RPL0	F: AGA AAC TGC TGC CTC ATA TCC G
	R: CCC CTG GAG ATT TTA GTG GTG A
Human RPL13	F: GCC ATC GTG GCT AAA CAG GTA
	R: GTT GGT GTT CAT CCG CTT GC
Human HPRT	F: CCT GGC GTC GTG ATT AGT GAT
	R: AGA CGT TCA GTC CTG TCC ATA A

2.20 Enzyme-linked immunosorbent assay (ELISA)

EIA 96-microwell plates (#13-678-11E, Nunc, Rochester, NY, USA) were coated with 100 µl of diluted capture antibody in coating buffer (0.2 M sodium phosphate buffer, pH 6.5 or 0.1 M sodium carbonate buffer, pH 9.5) and incubated overnight at 4°C. Wells were washed three times with 250 µl wash buffer (PBS with 0.05% Tween-20) per well and blocked with 200 µl 3% BSA (HiMedia, #9048-468) in PBS for 1 h. Standards were diluted according to the instructions as per the kit. Standards and samples (100 µl) were added to the coated wells after three washes. Following 2 h of incubation, wells were washed thrice and 100 µl of diluted detecting antibody (in blocking buffer) was added. After 1 h of incubation with detecting antibody, wells were washed five times. Substrate solution (100 µl) per well was added [1 mg/ml O-phenyldiamine (Sigma–Alrich, #78412) in citric acid buffer, pH 4.5 containing 0.2% hydrogen peroxide

(30% solution, #7722-841, Merck, , Kenilworth, NJ, USA)] and incubated until the color developed. The reaction was stopped by the addition of 100 µl stop solution (2 N sulphuric acid, Merck, #100731) per well. Absorbance was measured at 490 nm using iMark microplate absorbance reader (BioRad). Standard graph was plotted using values and concentrations of test samples were determined.

Mouse TNFα	BD OptEIA, #558534	Human TNFα	BD OptEIA, #555212
Mouse IL-6	BD OptEIA, #555240	Human IL-6	BD OptEIA, #555220
Mouse IL-1β	BD OptEIA, #559603	Human IL-8	BD OptEIA, #555244
Mouse IL-12	BD OptEIA, #555256	Human IL-12	BD OptEIA, #555183
Mouse IL-10	BD OptEIA, #555252	Human IL-10	BD OptEIA, #555157
Mouse TGFβ	R&D DuoSet, #DY1679	Human TGFβ	BD OptEIA, #559119
Mouse IFNγ	BD OptEIA, #555138	(BD, Franklin Lakes, NJ, USA)	
Mouse IL-4	BD OptEIA, #555232		

2.21 Whole cell lysate preparation

For whole cell lysate preparation, harvested cells were washed with PBS twice at 600 xg for 5 min at 4°C. Cells were lysed with 100 µl of lysis buffer (150 mM NaCl, 0.1% Triton X-100, 50 mM Tris HCl pH 8, 0.1% SDS) containing mammalian protease inhibitor (100:1) (Sigma–Aldrich, #P8340) and sonicated at 10 A with two 3–sec pulses using Misonix sonicator (QSonica, Newton, CT, USA). Cells were centrifuged at 24,000 xg for 30 min at 4°C and protein content in the supernatant was estimated by Bradford's assay and stored at –80°C.

2.22 Nuclear lysate preparation

Cells were harvested and washed twice with PBS at 600 xg, for 5 min at 4°C. Nuclear lysis buffer A (100 µl) (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.05% NP-40) containing mammalian protease inhibitor (100:1) (Sigma–Aldrich, #P8340) was added to washed cells. Cells were resuspended by pipetting and vortexing and subsequently centrifuged at 835 xg for 15 min at 4°C. The supernatant, that is, the cytoplasmic fraction was collected. To the pellet containing nuclei, 100 µl nuclear lysis buffer B (5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol

pH 7.9) containing 300 mM NaCl was added and sonicated at 10 A with two 3–sec pulses using Misonix sonicator (QSonica). The lysate was centrifuged at 24,000 xg for 30 min at 4°C. The protein content in supernatant was estimated by Bradford's assay and stored at –80°C.

2.23 SDS-PAGE and Western blotting

Buffers for SDS-PAGE were prepared from HiMedia chemicals as follows.

30% acrylamide solution: 29.2 g acrylamide and 0.8 g bis-acrylamide in 100 ml distilled water
Resolving gel buffer: 1.5 M Tris-Cl, 0.4% SDS, pH 8.8
Stacking gel buffer: 0.5 M Tris-Cl, 0.4% SDS, pH 6.8
Running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3
5x sample loading dye: 250 mM Tris-Cl pH 6.8, 10% SDS, 30% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue, pH 6.8

Buffers were mixed to obtain different percentage gels (Table 11).

	Stacking	**Resolving—10%**	**Resolving—12.5%**
Acrylamide Solution	0.45	3	3.75
Resolving gel buffer	—	2.25	2.25
Stacking gel buffer	0.75	—	—
Distilled water	1.8	3.75	3
10% Ammonium persulfate	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl

Table 11. Buffer composition for SDS-PAGE

Glass plates were cleaned and set on clamps. APS and TEMED were added just before pouring gel between the glass plates. The resolving gel was cast and water was added on top to prevent oxidization. After the resolving gel solidified, the stacking gel was added similarly and comb was set between the two plates. Samples were boiled with sample loading dye for 5 min and cooled.

Lysate samples were loaded onto 10% or 12% SDS-PAGE gels and transferred onto PVDF membrane (Millipore, #162-0177) at 60 V for 60 min (10% gel) or 70 V for 70 min (12.5% gel) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3), using Mini-PROTEAN Tetra Cell (BioRad) and Mini Trans-Blot Module (BioRad). Blocking of blots was performed using 5% BSA (HiMedia, #9048-468) in TBST buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6) for 1 h. All treatments were carried out at ambient temperature under rocking conditions. Subsequently, blots were incubated with diluted primary antibody in TBST buffer for 3 h and washed four times with TBST buffer for 15 min per wash. Blots were further incubated with diluted secondary antibody (Sigma–Aldrich, #A6154) in TBST buffer for 1 h followed by washing. Blots were developed using Immobilon western chemiluminescent HRP substrate (Millipore, #WBKLS0500) and visualized in ImageQuant LAS4000 (GE Healthcare Bio-Sciences).

Anti-TLR2	Santa Cruz, #sc-10739	Anti-p65	Santa Cruz, #sc-372
Anti-TLR1	Santa Cruz, #sc-30000	Anti-c-Rel	Santa Cruz, #sc-71
Anti-TLR6	Santa Cruz, #sc-30001	Anti-IRAK-M	Santa Cruz, #sc-366015
Anti-MyD88	Santa Cruz, #sc-11356	Anti-LaminB1	Santa Cruz, #sc-20682
Anti-IRAK-1	Sigma–Aldrich, #SAB4501559	Anti-GAPDH	Santa Cruz, #sc-25778
Anti-IRAK4	Cell Signaling, #4363	Anti-JunD	Santa Cruz, #sc-74
Anti-caspase1	Sigma–Aldrich, #C4851	Anti-rabbit-IgG	Santa Cruz, #sc-2027

2.24 Co-immunoprecipitation

Whole cells lysates were incubated with different antibodies for 4 h in a rotating shaker at 4°C. Further, 20 µl of protein A/G plus agarose beads (Santa Cruz Biotechnology, #sc-2003) were added to the lysate and incubated overnight under similar conditions. Beads were pelleted at 6,000 xg for 5 min at 4°C and washed thrice with whole cell lysis buffer. Immunoprecipitated proteins were eluted from the beads by adding 60–75 µl of SDS-loading buffer and boiling for 15 min. Samples were loaded onto SDS-PAGE gels and western blotting was performed as above.

2.25 Staining for surface molecules for flow-cytometry assay

Harvested cells were washed with FACS buffer (1% FBS, 0.1% sodium azide in PBS) twice at 600 xg, 5 min at 4°C. Fc Block (50 µl) [anti-mouse CD16/CD32 antibody (Biolegend, #101320); diluted 1:200 in FACS buffer] was added per well and cells were incubated for 15–20 min on ice. For THP-1 cells, appropriate isotype treated (1:200 in FACS buffer) samples served as controls. Cells were washed once with FACS buffer and stained with 50 µl diluted fluorescent-labeled primary antibody (1:200 in FACS buffer) per well and incubated for 1 h at 4°C. Cells were then washed thrice with FACS buffer and acquired by BD FACSCalibur (BD Biosciences) flow cytometer. Data were analyzed by CellQuest Pro (BD Biosciences) and FlowJo (Treestar, Ashland, OR, USA).

APC-anti-mouse TLR6	R&D Systems, #FAB1533A	PE-anti-human TLR6	Biolegend, #334708
FITC-anti-mouse/human TLR2	Biolegend #121806	PE-mouse IgG1κ isotype control	BD Pharmingen, #550617
FITC-anti-mouse CD80	BD Pharmingen, #553768	FITC-mouse IgG1κ isotype control	BD Pharmingen, #550616
PE-anti-mouse CD86	BD Pharmingen, #553692	Anti-mouse CD16/32	BD Pharmingen, #553142

2.26 Phagocytosis assay

After treatments, cells were incubated at 4°C (on ice) for 30 min to render macrophages inactive. Alexa 488-conjugated *E. coli* BioParticles (Invitrogen Life Technologies, #E-12321) were added to cells in a ratio of 10:1 and centrifuged at 300 xg, at 4°C for 5 min. Cells were further incubated at 4°C for 30 min and then at 37°C for 30 min to allow phagocytosis to occur. Fluorescence of non-phagocytosed BioParticles was quenched by adding 50 µl 0.2% trypan blue (Sigma–Alrich, #T8154) in citric acid buffer, pH 4.5. Cells were washed twice with PBS under similar centrifuging conditions and analyzed by BD FACSCalibur flow cytometer. Data were analyzed by CellQuest Pro (BD Biosciences) and FlowJo (Treestar).

2.27 BrdU-incorporation cell proliferation assay

Cell proliferation of $CD4^+$ T cells was determined by the cell-proliferation chemiluminescent assay kit (#5492, Cell Signaling Technologies, MA, USA). As mentioned in section 2.12.5, splenic DCs were treated with OmpU for 24 h and then diluted and co-cultured with splenic $CD4^+$ T cells at different ratios for 7 days. BrdU was added during the last 18 h of incubation periods. Ten microliters of 10X BrdU solution (diluted in culture media) was added to 100 µl of cell culture. After 18 h incubation, cells were centrifuged at 600 xg for 10 min and culture medium was removed. Denaturing solution (100 µl) was added per well and incubated for 30 min at room temperature. Cells were centrifuged and denaturing solution was removed. Detection antibody (100 µl) diluted in detection antibody diluent (1:100) was added per well and incubated for 1 h at room temperature. Cells were then washed thrice with wash buffer and 100 µl of HRP-conjugated antibody diluted in HRP antibody diluent (1:100) was added per well. After 30-min incubation at room temperature, cells were washed thrice. Luminol-enhancer solution (50 µl) and 50 µl of stable peroxide buffer was added per well and chemiluminescence was measured at 425 nm emission wavelength uptil 10 min using POLARstar Omega plate reader (BMG Labtech, Ortenberg, Germany). Values were plotted and proliferation was measured in relative luminescence units.

2.28 V. cholerae growth curve determination

V. cholerae El Tor O1 strain was spread on Luria-agar plate and incubated at 37°C overnight. A single colony was transferred to 3 ml Luria broth and cultured for 12 h at 37°C under constant rotating conditions. From the seed culture, 50 ml of Luria broth was inoculated such that starting absorbance at 600 nm was 0.01. Absorbance was subsequently monitored at 600 nm for 24 h with measurements every 30 min. Values of absorbance were plotted against time and the late log phase was determined to be 11 h.

2.29 Naturally secreted V. cholerae vesicles isolation

One liter of Luria broth was inoculated with 1% seed culture of *V. cholerae* and cultured for 11 h. Bacterial cells were pelleted down at 3,220 xg, at 4°C for 30 min. Supernatant was further centrifuged at 18,500 xg, at 4°C for 30 min for the removal of debris. Further,

the supernatant was centrifuged at 150,000 xg at 4°C for 2 h to pellet down vesicles (Himac CX120GSII micro-ultracentrifuge, Hitachi, Tokyo, Japan). Supernatant at the earlier step can be stored at 4°C on addition of 0.2% sodium azide, 5 μg/ml ampicillin, and 50 mM EDTA. Pelleted vesicles were washed with 10 ml of 20 mM Tris buffer (pH 7.6) and finally resuspended in 500 μl of the same buffer. Protein content in vesicles was measured by Bradford's assay.

2.30 V. cholerae outer membrane isolation

One liter of Luria broth was inoculated with 1% *V. cholerae* seed culture and cultured for 11 h. Bacterial cells were pelleted down at 3,220 xg, at 4°C for 30 min and cells were resuspended in 20 ml 1 M HEPES buffer (pH 7). Cell suspension was sonicated at 25 A for 15 min with 30 sec–on and 10 sec–off pulses and centrifuged at 5,000 xg at 4°C for 20 min. Supernatant was ultracentrifuged at 150,000 xg at 4°C for 2 h and pellet was resuspended in 4 ml of 0.5% N-laurylsarcosine (Sigma, #61739) in HEPES buffer. Sample was ultracentrifuged at 150,000 xg at 4°C for 2 h and resuspended in 1 ml HEPES buffer. Protein content in outer membrane preparation was determined by Bradford's assay.

III

Results

Specific Aim 1: To probe whether OmpU can modulate the inflammatory process induced by monocytes and macrophages

Chapter 1: Modulation of pro-inflammatory mediators by OmpU

Chapter 2: Induction of LPS tolerance by OmpU

Chapter 1: Modulation of pro-inflammatory mediators by OmpU

3.1 Inflammation: The most primitive, primary, and a potent host defense response

The innate immune system is the first line of defense and is present since birth. It involves four kinds of barriers: anatomic-, physiologic-, chemical-, and inflammatory barriers [96]. Inflammation is a complex, regulated, and effective biologic host response against injury, microbial invasion, or damaged host cells. It is defined as a localized physical condition, where tissues of the body become reddened, swollen, heated, and associated with pain [96]. These four cardinal signs of inflammation were first described by Celsus in 10 AD as *rubor* (redness), *tumor* (swelling), *calor* (heat), and *dolor* (pain) followed by *function laesa* or loss of function, which was later suggested by Galen.

Inflammation is characterized by three main stages: acute, subacute, and chronic stages [123]. The acute phase is characterized by redness and swelling due to increase in vascular permeability at the site of infection/injury. Chemical signals induce permeability of blood vessels and expression of cell adhesion molecules on endothelial cells, so that the circulating leukocytes can adhere to blood vessels, by a process called margination [124]. The cells migrate from the blood lumen into the tissue through the spaces between the endothelial cells by diapedesis (extravasation) [125]. Leukocytes are recruited to the site and support the inflammatory process (Illustration 11). The subacute phase initiates the healing and repair of the inflamed area. Angiogenesis and tissue growth occurs during this phase. The chronic stage involves tissue remodeling. Recognition and/or elimination of microbes during the acute phase is mediated by humoral factors, such as, the complement proteins, anti-microbial peptides, etc., and cells, such as, granulocytes, monocytes, macrophages, mast cells, DCs, and natural killer (NK) cells [126].

3.1.1 The acute phase is initiated by phagocytosis: An indispensable immune mechanism for the removal of pathogens and cell debris

The initial responders to pathogens are tissue-resident macrophages. As the inflammation progresses, neutrophils are recruited to the site of infection and support phagocytosis. Macrophages endocytose microbes via a structure called phagosome. The phagosome subsequently binds with lysosomes to form a phagolysosome, which contains cytotoxic

enzymes and radicals that lead to the elimination of the pathogen. Phagocytosis leads to a respiratory burst, which involves production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), which are cytotoxic to the microbe ingested [127]. One of such RNI is NO that mediates vascular permeability, endothelial signaling, and platelet activation for initiation of coagulation [128].

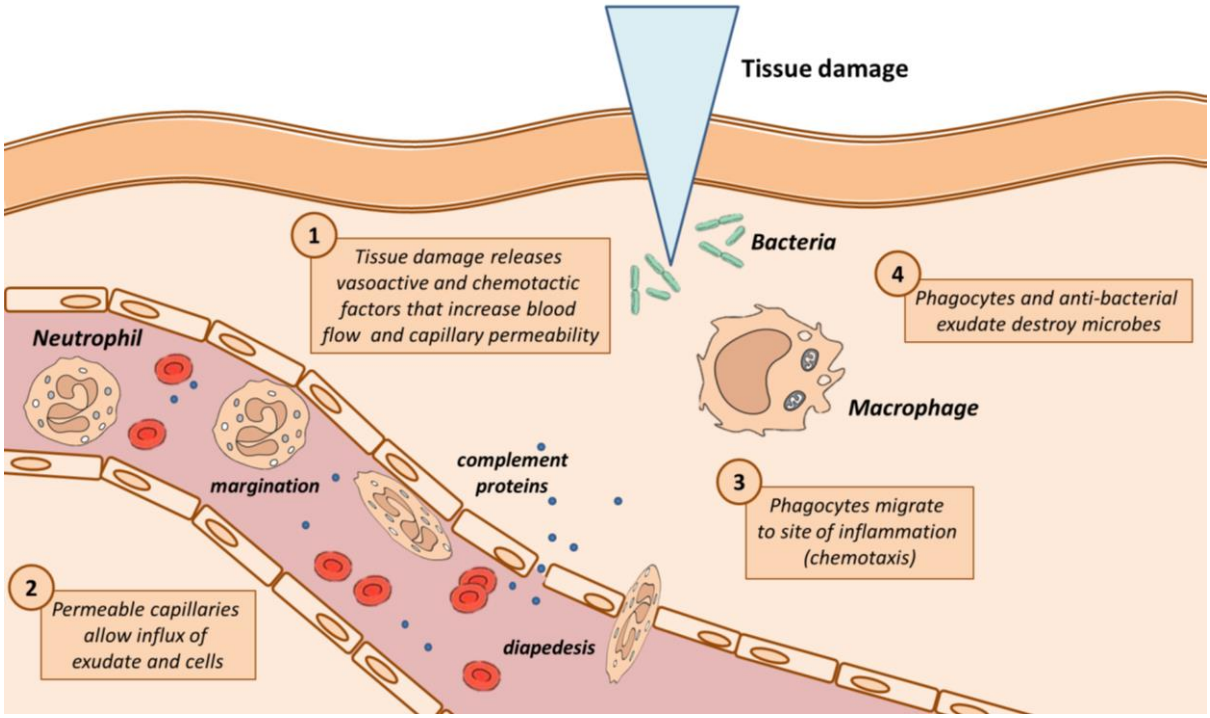


Illustration 11. Multiple steps in the inflammatory response

3.1.2 PAMP and PRR interactions shape the inflammatory response

In response to injury, the inflammatory process is initiated by tissue-resident immune cells, such as, macrophages and DCs. Macrophages and DCs recognize PAMPs by the PRRs present on them. PAMPs may be genetic material of bacteria or viruses, such as, DNA and RNA or molecules present on the surface of microbes, such as, fungal lectins, bacterial LPS, lipoproteins, or flagellins [97]. This recognition activates immune cells and induces the release of chemokines (molecules that attract other immune cells) and cytokines (molecules that signal and activate immune cell function) [98].

Immune cells reach the site of infection by chemotaxis in response to chemoattractants secreted by tissue-resident macrophages. Cytokines, such as, IL-1 and TNFα, secreted by monocytes and macrophages also lead to vascular permeability [129]. TNFα acts as a chemoattractant for neutrophils and increases cell adhesion molecules on endothelial cells. PAMP–PRR binding further alters cytoskeletal structure of phagocytes, which enables phagocytosis to occur, subsequently leading to NO production [130]. TNFα is also an important activator of phagocytosis.

Furthermore, the cytokines IL-1, IL-6, and TNFα act as endogenous pyrogens and mediate fever, which is a crucial anti-pathogen response [129]. Together, they initiate prostaglandin synthesis in the hypothalamus leading to fever, which retards growth of pathogens. Additionally, these cytokines stimulate the release of corticotropin-releasing hormone, which acts on the hypothalamic–pituitary–adrenal axis leading to the release of corticosteroids that are important for the stress response during inflammation.

Activated monocytes and macrophages can release IL-12, which activates NK cells and T cells to produce IFNγ and TNFα, which amplify cell-mediated immunity of the adaptive arm and phagocyte-dependent inflammation. Further, IL-12 increases cytotoxic abilities of NK cells and T cells for effective elimination of pathogens [131-133].

3.1.3 The acute phase response is a systemic response critical for reestablishing homeostasis

The acute phase-response proteins (APPs) are synthesized in the liver and released into circulation on tissue damage by microbes [134,135]. Cytokines IL-1, TNFα, and IL-6 activate the acute phase response [130]. Over 200 APPs mediate the process, whose expression either increases or decreases on stress. APPs can activate the complement-system proteins, which can lead to lysis or phagocytosis of pathogens by the immune cells. The acute phase response involves various processes, such as, pyrexia, leukocytosis, hormone alterations, and muscle-protein depletion, which together reestablish homeostasis after infection [136]. The expression of positive APPs increases during the response. These APPs are important for the entrapment of microbes and their products, neutralizing enzymes, scavenging hemoglobin, and free radicals, as well as, the modulation of the inflammatory process. The response if regulated by a feedback mechanism that limits the time period from 4–7 days [137].

3.1.4 Monocytes and macrophages are crucial mediators of the innate immune response

Tissue-resident macrophages are specialized cells, such as, Kupffer cells (liver), microglia (nervous system), osteoclasts (bone), etc. These macrophages remove apoptotic cells and pathogens. The signals originating from tissue macrophages lead to recruitment of their precursor cells, monocytes, from circulation to the site of inflammation [125] (Illustration 12). Monocytes differentiate into macrophages, acquire phenotypes depending on environmental cues, and augment inflammation. Monocytes, like macrophages, have PRRs; they phagocytose pathogens and secrete cytokines on PAMP recognition [97]. However, their responses are not as pronounced as those of macrophages. Further, monocytes are short-lived whereas macrophages have a longer life span. Together, their partnership ensures a robust inflammatory response.

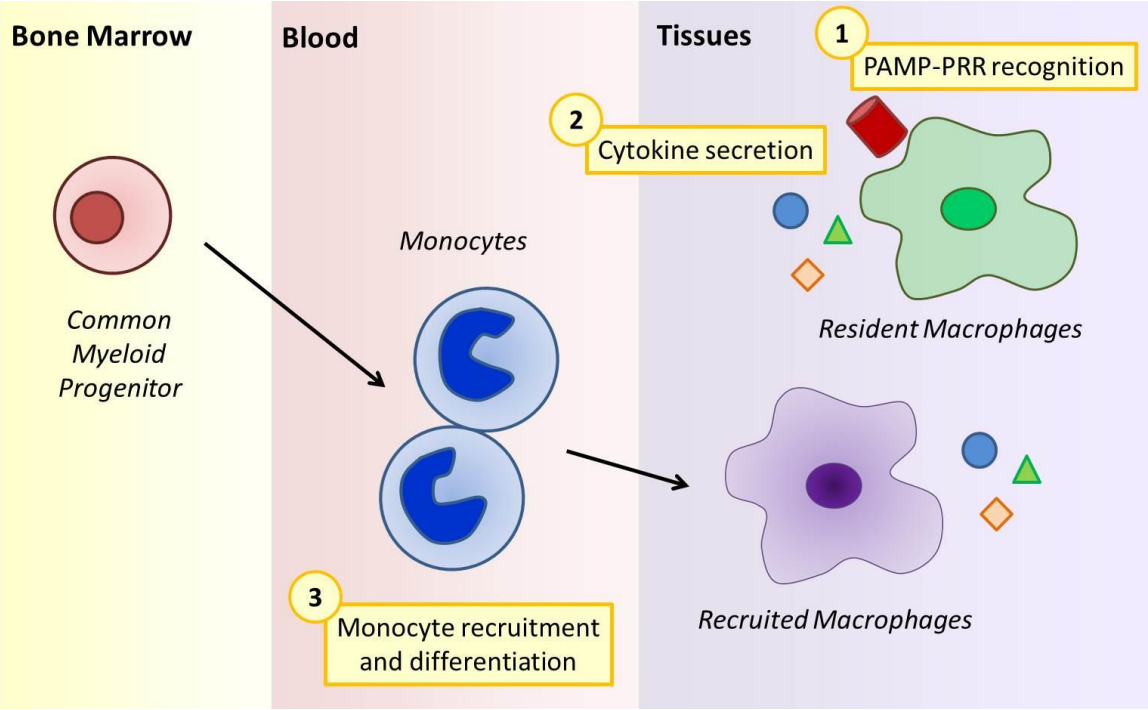


Illustration 12. Monocytes and macrophages mediate inflammation

3.2 Objectives

V. cholerae porin OmpU is involved in pathogenesis as it confers resistance against bile acids and anti-microbial peptides in the gut [104,106,107,138,139]. Further, its expression doubles in the gut environmental conditions [103]. We wanted to probe, whether OmpU can act as a PAMP and activate monocytes and macrophages to induce production of pro-inflammatory mediators.

3.3 Results

3.3.1 OmpU induces NO production in RAW 264.7 macrophages

As mentioned above, NO is one of the major cytotoxic effectors generated during the phagocytic process and mediates vasodilatation, one of the initial steps of inflammation. To study whether OmpU can lead to the production of NO, we treated RAW 264.7 murine macrophage cells with purified recombinant OmpU for various time periods and with different doses. We observed that OmpU induced NO production in a time-dependent manner with significant production at 24 h (Fig. 1A). Further, at this time point, with different doses of OmpU, NO levels increased in a dose-dependent manner (Fig. 1B).

To assess whether any endotoxin contamination was present in the recombinantly purified OmpU samples, LAL assay was performed. Endotoxin levels were non-detectable (<0.06 EU/ml). As a control, we assessed whether the use of PmB, a well-known endotoxin-neutralizing agent would affect NO response by OmpU. Thus, prior to OmpU and LPS treatment, cells were treated with 5 μg/ml PmB and NO levels were measured. The use of PmB led to a decrease in NO levels (Fig. 1C). Therefore, for further experiments, PmB pretreatment was carried out to prevent any response due to possible endotoxin contamination.

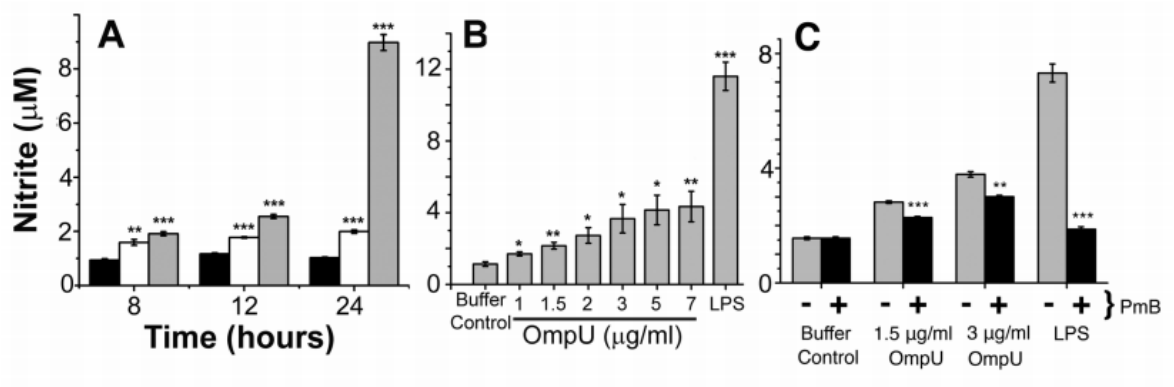


Fig. 1 OmpU induces NO production in RAW 264.7 cells in a time and dose-dependent manner.

(A) RAW 264.7 cells were treated with OmpU for 8 h, 12 h, 24 h and NO levels were measured by Griess reaction. Black solid bar represents negative control, white solid bar represents OmpU, and gray solid bar represents positive control. Buffer-treated cells and 1 μg/ml LPS-treated cells served as controls. (B) RAW 264.7 cells were treated with different doses of OmpU for 24 h and NO levels were estimated. Buffer-treated cells and 1 μg/ml LPS-treated cells served as controls. (C) RAW 264.7 cells were treated with buffer, 1.5 μg/ml, and 3 μg/ml OmpU or 1 μg/ml LPS. Simultaneously, another set of cells were pretreated with PmB and after 30 min, cells were similarly treated. After 24 h, NO levels were assessed. Gray bars represent cells without PmB treatment. Black bars represent cells with PmB treatment. Results are expressed as mean ± SEM and represent the average of three independent experiments. For Fig. 1A and 1B, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus buffer control. For Fig. 1C, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus non-PmB treatments

3.3.2 OmpU induces TNFα production in monocytes and macrophages

TNFα is a cytokine involved in systemic inflammation, fever induction, and in the acute phase reaction [130]. Toward assessing whether OmpU could lead to the production of this cytokine, RAW 264.7, THP-1, and human PBMCs were treated with 1.5 μg/ml OmpU for different time periods: 4 h, 8 h, 12 h, and 24 h. TNFα production was determined by ELISA. In these cell types, TNFα levels were considerable at 24 h in RAW 264.7 cells (Fig. 2A), maximum at 4 h in THP-1 cells (Fig. 2B), and 8 h in human PBMCs (Fig. 2C). Further, with increasing doses of OmpU, TNFα levels also increased in a dose-dependent manner at 24 h in RAW 264.7 cells (Fig. 2D) and at 4 h in THP-1 cells (Fig. 2E).

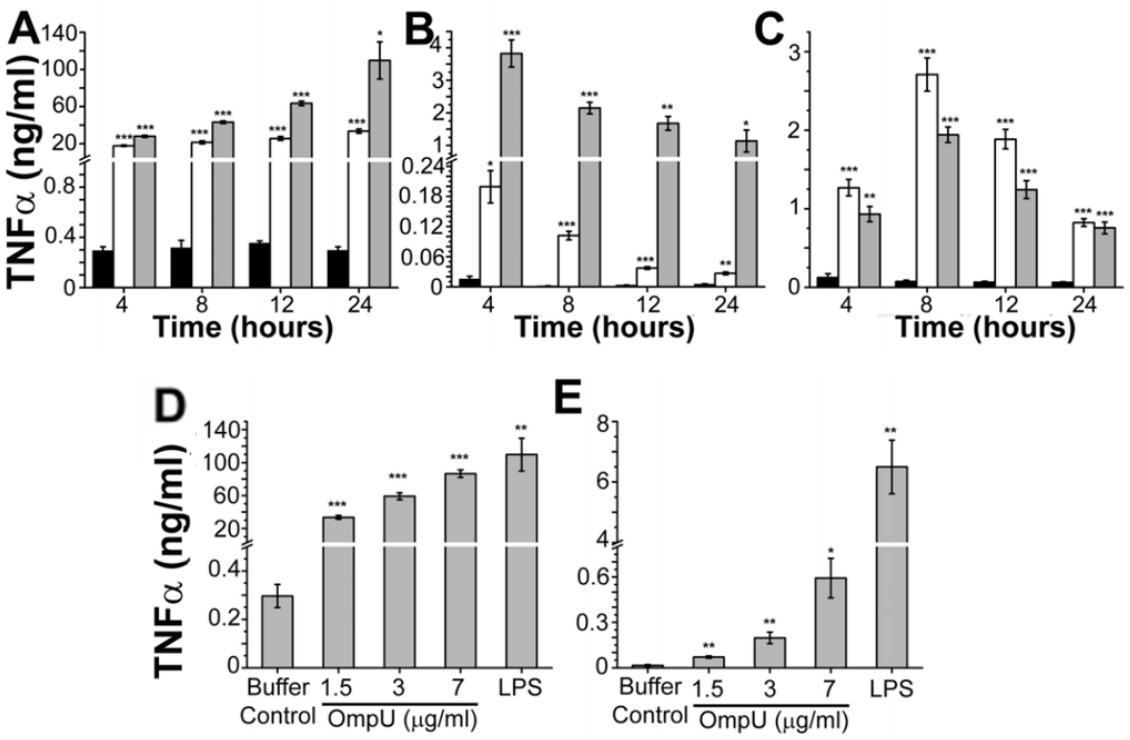


Fig. 2. OmpU induces TNFα in RAW 264.7, THP-1 cells, and human PBMCs.

RAW 264.7 cells (A), THP-1 cells (B), and human PBMCs (C) were treated with 5 μg/ml PmB, 30 min prior to 1.5 μg/ml OmpU treatment for 4 h, 8 h, 12 h, and 24 h. Buffer-treated and 1 μg/ml LPS-treated cells served as controls. Black solid bar represents negative control, white solid bar represents OmpU, and gray solid bar represent positive control. RAW 264.7 cells (D) and THP-1 cells (E) were treated with different doses of OmpU for 24 h and 4 h, respectively, with 30 min PmB pretreatment. Buffer-treated and 1 μg/ml LPS-treated cells served as controls. Culture supernatants were collected and TNFα production was determined by ELISA. Results are expressed as mean ± SEM and represent the average of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus buffer control.

3.3.3 OmpU induces IL-6 production in monocytes and macrophages

IL-6 is most notably known for its role in the acute phase reaction, which is mediated by proteins synthesized in liver [130]. The effect of OmpU on IL-6 was determined in different cell types and levels were assessed by ELISA. IL-6 production in RAW 264.7 (Fig. 3A), THP-1 cells (Fig. 3B), and in human PBMCs (Fig. 3C) increased in a time-dependent manner and was considerable at 24 h. Further, increasing doses of OmpU led to increased IL-6 levels in RAW 264.7 (Fig. 3D), as well as, THP-1 cells (Fig. 3E).

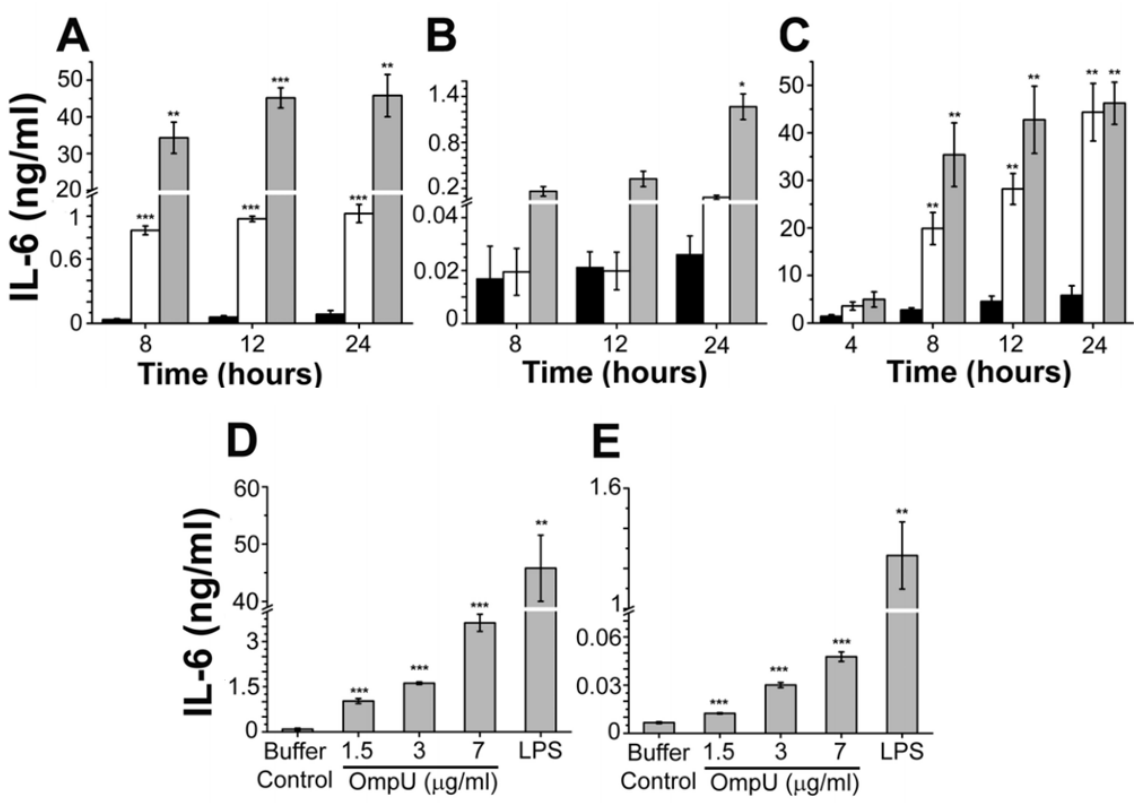


Fig. 3. OmpU induces IL-6 in RAW 264.7, THP-1 cells, and human PBMCs.

RAW 264.7 cells (A), THP-1 cells (B), and human PBMCs (C) were treated with 5 μg/ml PmB, 30 min prior to 1.5 μg/ml OmpU treatment for 4 h, 8 h, 12 h, and 24 h. Buffer-treated and 1 μg/ml LPS-treated cells served as controls. Black solid bar represents negative control, white solid bar represents OmpU, and gray solid bar represent positive control. RAW 264.7 cells (D) and THP-1 cells (E) were treated with different doses of OmpU for 24 h with 30 min PmB pretreatment. Buffer-treated and 1 μg/ml LPS-treated cells served as controls. Culture supernatants were collected and IL-6 production was determined by ELISA. Results are expressed as mean ± SEM and represent the average of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus buffer control.

All these results prove that OmpU acts as a pro-inflammatory agent in cells of mouse- and human origin and leads to production of critical pro-inflammatory mediators, such as, NO, TNFα, and IL-6.

3.3.4 Pro-inflammatory mediator production in response to OmpU purified recombinantly from E. coli and from wild type V. cholerae is similar

We further compared pro-inflammatory responses of recombinantly purified OmpU (rOmpU) and wild type OmpU (wtOmpU) purified from *V. cholerae* El Tor O1 to

determine whether rOmpU behaved similarly as wtOmpU. We assessed NO, TNFα, and IL-6 production in RAW 264.7 in response to both forms of OmpU. Our data indicate that rOmpU and wtOmpU induce a similar pro-inflammatory mediator production (Fig. 4). Therefore for future experiments, rOmpU was used.

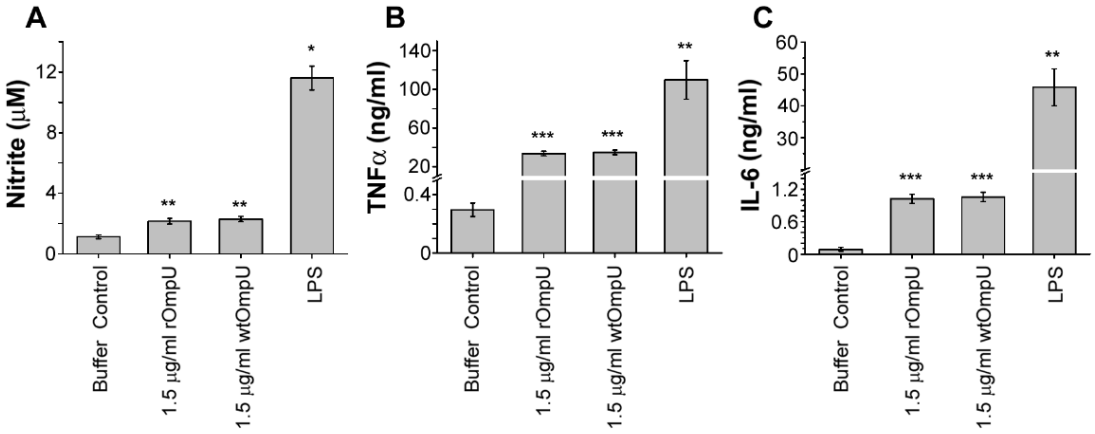


Fig. 4. Recombinant OmpU and wild type OmpU responses in RAW 264.7 cells.

RAW 264.7 cells were treated with with 5 μg/ml PmB, 30 min prior to rOmpU and wtOmpU treatement. Buffer- and LPS-treated cells served as controls. After 24 h, levels of NO (A), TNFα (B), and IL-6 (C) were determined by Griess reaction and ELISA. Results are expressed as mean ± SEM and represent the average of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus buffer control.

3.3.5 OmpU-pretreatment down-regulates LPS-mediated pro-inflammatory mediator production in monocytes and macrophages

Our results indicate that OmpU is pro-inflammatory in nature. We further investigated whether OmpU could differentially regulate pro-inflammatory cytokines. For these experiments, we studied effects of OmpU-pretreatment on cells that were further stimulated with LPS. For down-regulation experiments, we used PmB along with OmpU treatment. After 24 h of incubation, cells were replated in fresh media without PmB and stimulated with LPS. We observed that pretreatment of cells with OmpU suppressed the LPS-induced pro-inflammatory mediators, such as, NO, TNFα, IL-6, and IL-12. The extent and pattern of down-regulation varied across different cell types. Pretreatment

with OmpU substantially decreased LPS-induced nitrite production in RAW 264.7 cell line (Fig. 5A).

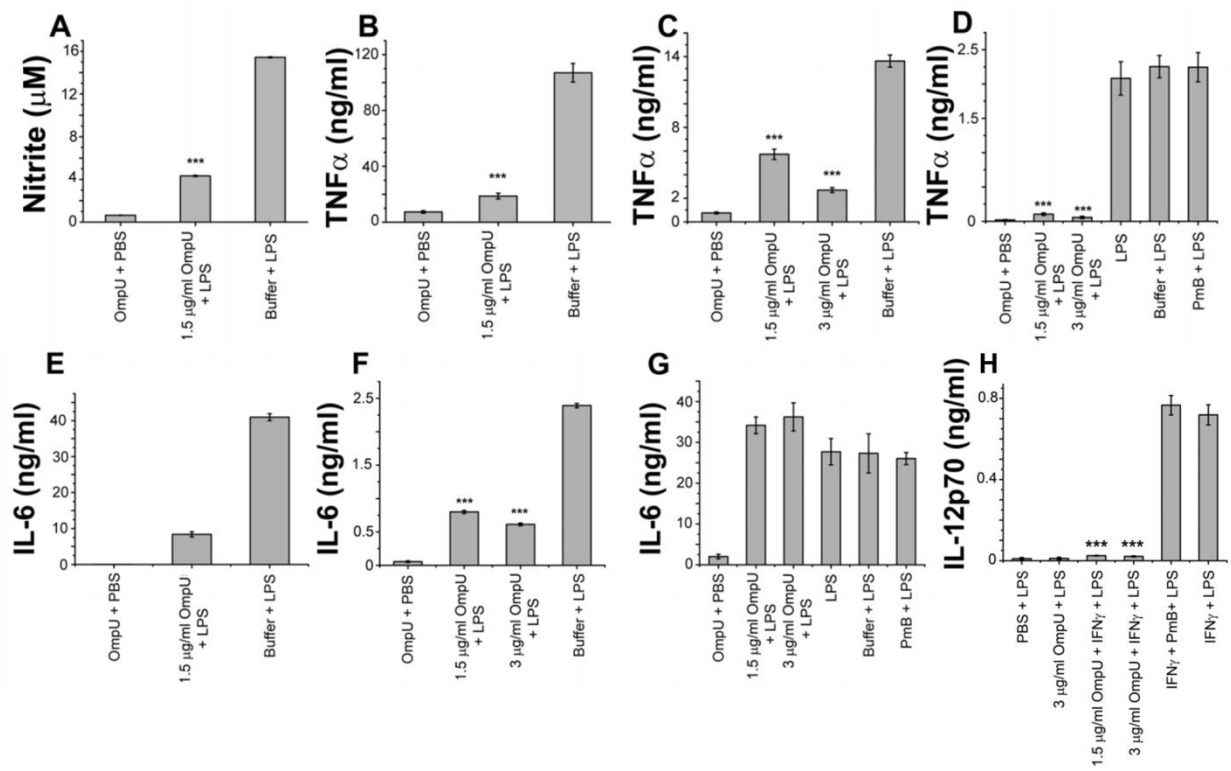


Fig. 5. OmpU-pretreatment decreases LPS-induced pro-inflammatory mediators in monocytes and macrophages.

RAW 264.7 murine macrophage cells (A), (B), (E); THP-1 human monocytes (C), (F); and human PBMCs (D), (G), (H); were plated and treated with OmpU or protein-buffer and incubated for 24 h. PmB was added to the culture 30 min prior to the treatment. After 24 h of OmpU or buffer treatment, cells were replated in fresh media without PmB and challenged with LPS for defined time period at which LPS-induced response was maximum for the mediator of interest. Control experiments were performed to evaluate the contribution of buffer or PmB for OmpU-mediated down-regulatory phenomenon. Supernatants were collected and analyzed for the presence of pro-inflammatory mediators. Nitrite production was estimated by Griess reaction (A). TNFα (B, C, D); IL-6 (E, F, G); and IL-12 (H) levels were evaluated by sandwich ELISA. Results are expressed as mean ± SEM and represent the average of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus protein buffer + LPS control

Similarly, pretreatment with OmpU suppressed TNFα production in response to LPS in RAW 264.7 cell line, THP-1 cell line, and in human PBMCs (Fig. 5B, 5C, 5D; Table 5).

Moreover, OmpU-pretreatment also suppressed the IL-6 response of LPS-treated RAW 264.7 and THP-1 cells but not that of human PBMCs (Fig. 5E, 5F, 5G; Table 5).

IL-12 is a known regulator of Th1 and Th2 responses and innate immune responses [140]. The possibility of OmpU-mediated suppression of LPS-induced IL-12 production was investigated in human PBMCs as cell lines did not produce IL-12. PBMCs were stimulated with 100 ng/ml IFNγ to induce IL-12 production. A complete suppression of IL-12 production by PBMCs was observed when the cells were preincubated with OmpU (Fig. 5H; Table 5).

3.3.6 OmpU-pretreatment down-regulates LPS-mediated cytokine production in IECs

IECs are vital mediators of intestinal homeostasis and encounter commensal, as well as, pathogenic microbes. As they are at this crucial junction, IECs are specialized epithelial cells that can sense and respond to microbes and their products, similar to immune cells [141]. Thus, they can participate in and co-ordinate immune responses. IECs can produce certain pro-inflammatory cytokines and chemokines, such as, IL-8 and TNFα, which recruit other immune cells at the site of pathogen invasion. As OmpU-pretreatment down-regulated LPS-mediated responses in monocytes and macrophages, we wanted to assess whether OmpU would have a similar effect in IECs, which would be the first cells to encounter *V. cholerae* during pathogenesis. We chose to study this effect in HT29, a human adenocarcinoma cell line.

On OmpU-pretreatment, HT29 cells displayed decreased LPS-mediated IL-8 (Fig. 6A; Table 5), an important chemokine for recruitment of neutrophils and granulocytes. Further, as IECs encounter gram-negative commensal bacteria constantly, IECs may not produce certain pro-inflammatory mediators, such as, TNFα and IL-6 on LPS stimuli. Thus, we stimulated cells with IFNγ and observed that TNFα production was induced by subsequent LPS treatment. In addition to IL-8, OmpU-pretreatment decreased LPS-mediated TNFα production (Fig. 6B; Table 5). Thus, apart from monocytes and macrophages, OmpU down-regulated pro-inflammatory mediators in IECs as well.

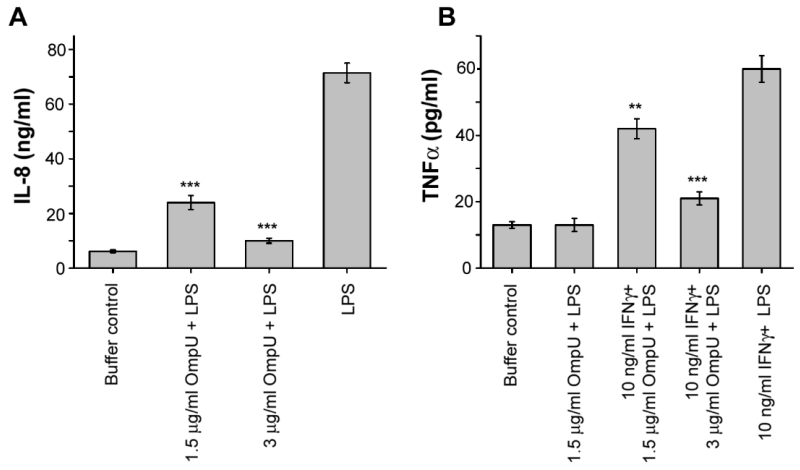


Fig. 6. OmpU-pretreatment decreases LPS-induced pro-inflammatory mediators in HT29 human IEC line.

(A) HT29 cells were plated and treated with OmpU or protein-buffer and incubated for 24 h. PmB was added to the culture 30 min prior to the treatment. After 24 h of OmpU or buffer treatment, cells were replated in fresh media without PmB and challenged with LPS for 24 h. IL-8 levels were assessed by ELISA. (B) HT29 cells were plated and treated PmB+OmpU. After 12 h, 10 ng/ml IFNγ was added. After 24 h of OmpU incubation (and 12 h of IFNγ), cells were replated in fresh media without PmB and challenged with LPS for 4 h. Supernatants were collected and analyzed for TNFα by ELISA. Results are expressed as mean ± SEM and represent the average of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus protein buffer + LPS control.

Cell type	**Extent down-regulation with different OmpU doses**		
		1.5 μg/ml	3 μg/ml
RAW 264.7	NO	73–76%	—
	TNFα	85–91%	—
	IL-6	77–83%	—
THP-1	TNFα	55–67%	80–88%
	IL-6	63–69%	73–75%
Human PBMCs	TNFα	94–96%	96–98%
	IL-6	—	—
	IL-12	94–96%	95–97%
HT29	TNFα	29–32%	60–64%
	IL-8	65–68%	84–86%

Table 12. Extent of down-regulation of LPS-induced pro-inflammatory mediators in monocytes, macrophages, and IECs.

3.3.7 OmpU-pretreated LPS-activated cells do not exhibit decreased cell viability

Cell viability can be affected in response to various stimuli. A common assay employed used to determine cell viability is MTT assay. MTT is a tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The dye is reduced to formazan by NAD(P)H-dependent oxidoreductase enzymes that are present in the cytoplasm. Viable cells are active metabolically and convert MTT into a purple-colored formazan product. However during cell death, cells lose this ability to convert MTT into formazan, thus leading to decreased color development.

To determine whether OmpU treatments affected cell health, THP-1 cells were treated with different doses of OmpU and buffer for 24 h. After incubation, cell viability was measured by MTT assay. Data indicate that OmpU treatments did not affect cell viability significantly (Fig. 7). We performed MTT assay to rule out that the suppressed pro-inflammatory mediator response was due to reduced cell viability. THP-1 cells were treated with OmpU for 24 h and further treated with LPS for 24 h. Cell viability was assessed by MTT assay and we observed that cell health was minimally affected by treatments (Fig. 7).

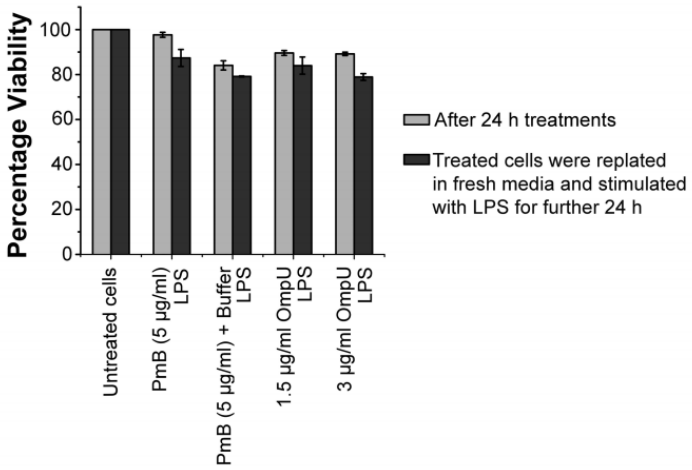


Fig.7. Assessment of cell health by MTT Assay.

THP-1 human monocytes were plated and treated with OmpU, protein buffer, or PmB and incubated for 24 h and cell viability was measured by MTT assay. After 24 h of same treatment, cells were replated in fresh media without PmB and challenged with LPS for 24 h. Cell viability was measured by MTT assay after 24 h of LPS treatment. Results are expressed as mean ± SEM and represent the average of three independent experiments.

3.4 Conclusions

We investigated the role of *V. cholerae* OmpU in monocyte and macrophage activation by determining pro-inflammatory mediator production. The effect of OmpU was studied in RAW 264.7 murine macrophage cell line, THP-1 human monocytic cell line, and human PBMCs. The endotoxin level in purified protein preparation was found to be less than 0.06 EU/ml. Yet PmB was added to the culture prior to OmpU treatment to neutralize possible endotoxin contamination. NO production in response to OmpU in RAW 264.7 cells was considerable at 24 h and increased in a dose-dependent manner (Fig. 1). Further, OmpU induced the substantial amount of TNFα in RAW 264.7 (24 h), THP-1 (4 h) cells, and PBMCs (8 h), whose production increased with increasing doses of OmpU (Fig. 2). Additionally, OmpU induced production of IL-6, in all cell types, considerably at 24 h (Fig. 3). IL-6 production increased in a dose-dependent manner in RAW 264.7 and THP-1 cells (Fig. 3). Moreover, the pro-inflammatory effects of recombinantly purified OmpU are similar to those of wild type OmpU (Fig. 4). Hence, for all future experiments recombinant OmpU was used. Our data indicate that *V. cholerae* porin OmpU classically activates macrophages as it induces secretion of inflammatory mediators such as NO, TNFα, and IL-6 by increasing the mRNA expression of nitric oxide synthase 2 (NOS2, the enzyme that catalyzes NO production), TNFα, and IL-6 [142]. Thus, apart from its role in bacterial survival by conferring resistance against bile acids and anti-microbial peptides, OmpU also activates the innate immunity.

The effect of OmpU-pretreatment was analyzed in LPS-activated cells (OmpU/LPS-treated cells) in terms of suppression or down-regulation of cytokine expression. OmpU/LPS-treated RAW 264.7, THP-1 cells, and human PBMCs exhibited decreased NO, TNFα, IL-6, and IL-12 production (Fig. 5), with the exception of IL-6 in human PBMCs, compared to buffer/LPS-treated cells. Further, OmpU-pretreatment decreased LPS-mediated production of IL-8 and TNFα in HT29 IECs (Fig. 6) as well. Pretreatment of cells with PmB alone did not suppress LPS-mediated effects (Fig. 1C, Fig. 5D, 5G, 5H). Cells can become non-responsive under stress or if the cell health is compromised. We ruled out the possibility that OmpU/LPS treatments were affecting cell viability and thus, leading to decreased responses by MTT assay (Fig. 7). Altogether, these findings suggest that OmpU can differentially modulate pro-inflammatory responses.

Chapter 2: Induction of LPS tolerance by OmpU

4.1 LPS is a potent pro-inflammatory mediator

LPS is a major constituent of the gram-negative bacterial outer membrane. It imparts negative charge to the membrane and helps in membrane stability. Richard Friedrich Johannes Pfeiffer first discovered LPS and termed it as endotoxin as it is part of the bacterial membrane. Contrary to exotoxins that are actively secreted by live bacteria, LPS is released on bacterial damage and destruction and is heat stable [143]. The LPS molecule consists of an O antigen (or O polysaccharide), a core oligosaccharide, and a lipid A moiety. Lipid A is associated with toxicity and polysaccharides with immunogenicity [143].

LPS activates several immune cells, such as, monocytes, macrophages, DCs, B cells, etc., and induces an enormous production of pro-inflammatory mediators and subsequently, fever. This can lead to endotoxemia and septic shock [144]. Septic shock is caused by severe vasculature damage due to extensive inflammation and dilation of blood vessels. This negatively affects cardiac output. Furthermore, increased bacterial proliferation can lead to disseminated intravascular coagulation and loss of function of vital organs, such as, kidneys, liver, lungs, etc., due to decreased blood supply [145]. Therefore, gram-negative bacterial infections, if left unchecked, pose an immense threat to the host.

4.1.1 LPS behaves as a PAMP and mediates its effects via TLR activation

Surface TLRs, such as, TLR1, TLR2, TLR4, TLR5, and TLR6 recognize various PAMPs, which leads to activation of immune cells (Illustration 13). TLR2 heterodimerizes with either TLR1 or TLR6. LPS is classically known to activate immune cells via the TLR4/CD14 complex [146]. However, the recognition of LPS may also involve TLR2 signaling, depending on the source of LPS and immune cell types. LPS from different bacteria can mediate its signaling via TLR1/2 or TLR2/6 heterodimers [147-153]. LPS-mediated TLR activation leads to recruitment of down-stream adapter molecules, such as, MyD88 and TIRAP. This interaction further recruits kinases, such as IRAK1, which recruits IRAK4 or IRAK2 on phosphorylation [150,154,155]. The activated IRAK dimer complex dissociates from the TLR-adaptor molecule complex and interacts with TRAF6 in the cytosol. This may further lead to activation of the transcription factor NFκB.

Furthermore, LPS-mediated TLR signaling can activate the MAPK signaling cascade and result in activation of transcription factors, NFκB and AP-1 that modulate the inflammatory process [146,154-156].

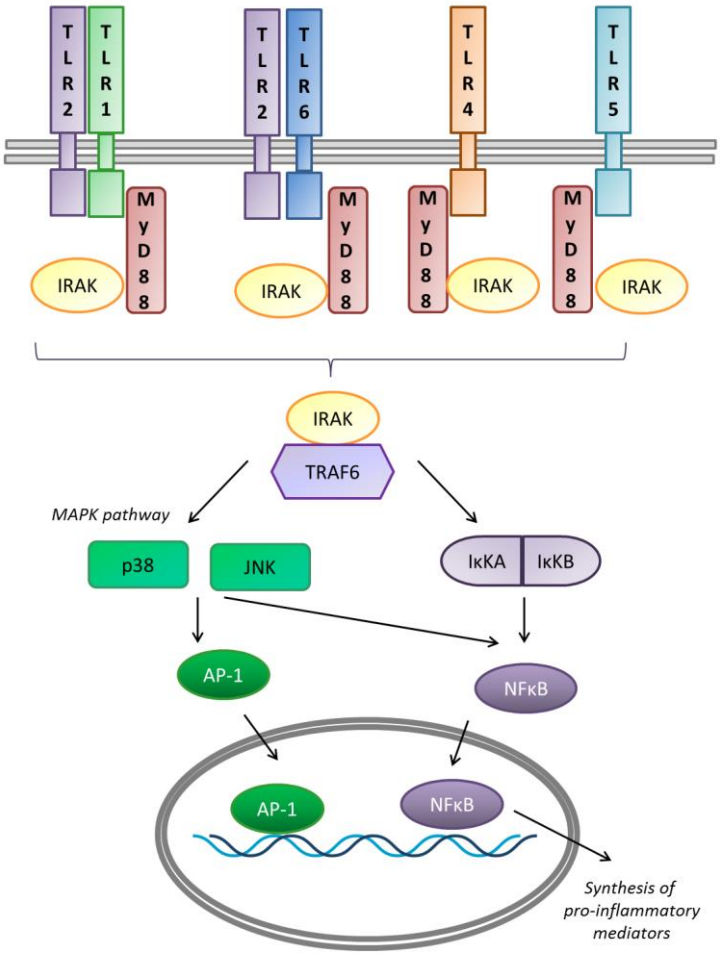


Illustration 13. Cell surface TLR signaling cascade leading to activation of pro-inflammatory transcription factors

Thus, LPS initiates a complex intra-cellular signaling cascade that ultimately leads to production of various inflammatory mediators.

4.1.2 LPS can induce monocyte and macrophage tolerance

Tolerance was first studied about 70 years ago by Beeson [157] in response to LPS. Macrophage tolerance is defined as the reduced capacity of the host (*in vivo*) or of cultured monocytes/macrophages (*in vitro*) to optimally respond to reexposure of an inflammatory stimulus, following exposure to a previous one. Mice administered with

sublethal doses of LPS, when reexposed to LPS, exhibited suppressed immune responses instead of heightened ones, contrary to what was hypothesized [158]. Macrophages of sepsis patients, experiencing heightened whole-body inflammatory responses, exhibit a subsequent compensatory anti-inflammatory response [159].

4.1.3 Monocytes and macrophages can display anti-inflammatory phenotype under certain conditions

4.1.3.1 Monocytes and macrophages can become tolerant

Tolerant monocytes and macrophages display an immune-suppressive phenotype. LPS tolerance is a state of refractoriness to repeated LPS stimulations. Tolerance is characterized by two phases (Illustration 14). In the 1st phase, stimulation by a pro-inflammatory stimulus activates monocytes/macrophages and induces the secretion of inflammatory mediators [157]. In the 2nd phase, when, these activated cells, encounter another pro-inflammatory stimulus, they exhibit attenuated response in terms of secretion of the same mediators, as well as, altered macrophage function in terms of antigen presentation and phagocytic capacity [160-163].

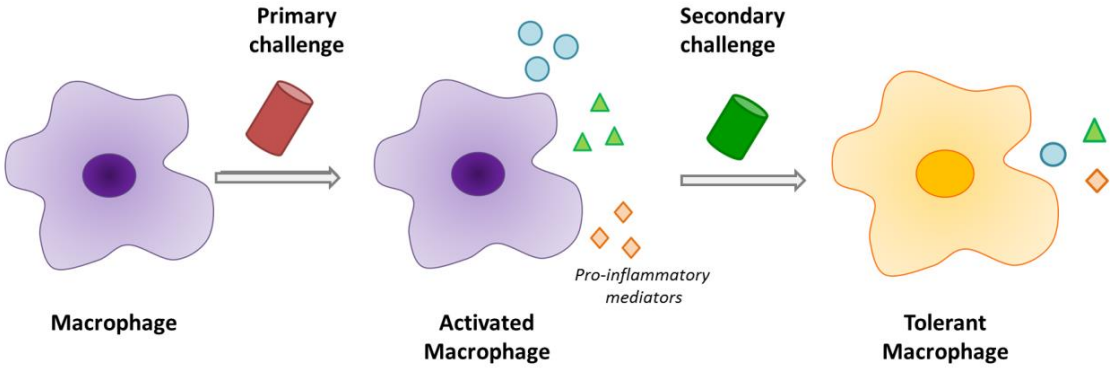


Illustration 14. Tolerant monocytes and macrophages exhibit decreased pro-inflammatory response

Enhanced phagocytic capacity assists removal of pro-inflammatory stimuli and intact bacteria and prevents excess inflammation. Additionally, tolerance involves up-regulation of anti-microbial mediators, which help in resolution of inflammation.

4.1.3.2 Monocytes and macrophages can be polarized toward immune-suppressive phenotype

In the first chapter, the effects of classically activated macrophages were highlighted. However, the very same macrophages that are involved in inflammation, can transform into regulatory macrophages or tissue healing macrophages under different spatio-temporal conditions (Illustration 15). Macrophages are remarkably plastic and can alter their functions in response to various environmental stimuli and display different phenotypes [164]. Macrophages, classically activated by PAMPs, secrete inflammatory mediators and cytotoxic products, which when unchecked, damage host tissues. Initial IFNγ and TNFα signals by NK cells and subsequent IFNγ secretion by T cells enhances the function of classical macrophages [165].

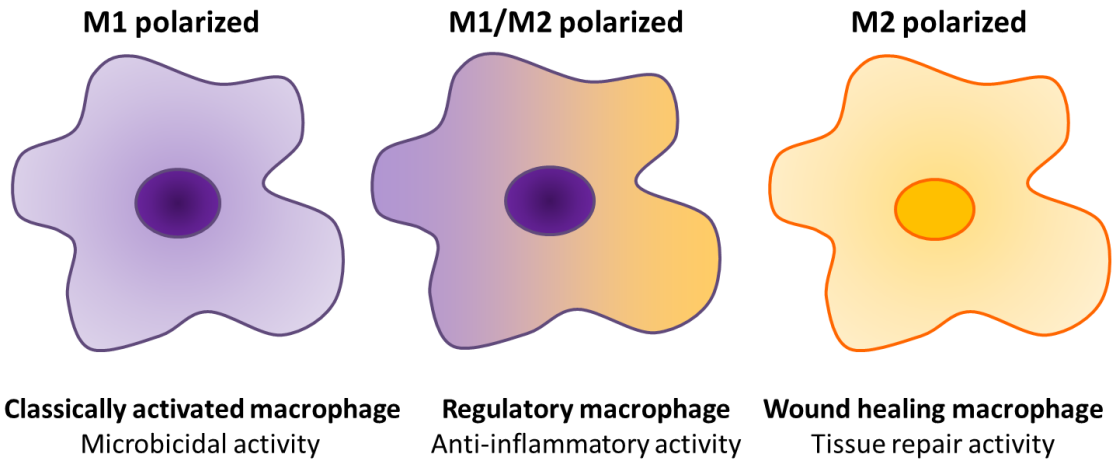


Illustration 15. Different functions of macrophage polarization

When the primary cytokine signal received by macrophages is IL-4, initially secreted by basophils and mast cells, tissue resident macrophages contribute toward wound healing. IL-4 stimulates arginase activity in macrophages, which yields ornithine, a precursor for collagen synthesis, and contributes to extra-cellular matrix production [166]. This activation is further maintained by Th2 cells, which secrete IL-4 and IL-13 and contribute toward anti-inflammatory phenotype. Polyamines secreted by these macrophages inhibits clonal expansion of lymphocytes [166].

Regulatory macrophages exhibit decreased pro-inflammatory activities when glucocorticoids act on them. Glucocorticoids are steroid hormones that are synthesized in

the adrenal cortex and released in response to stress into the blood stream. Glucocorticoid-treated macrophages display defective antigen presentation, thus, biasing T cells toward Th2, the anti-inflammatory phenotype [167].

4.1.3.3 Pathogens can exploit macrophage polarization

Altogether, activation by different environmental stimuli, such as, cytokines, PAMPs, and the network of immune cells at the site of microbe invasion and residence shape macrophage function. Pathogens, however, can exploit this natural process of polarization for their benefit. Pathogens, such as, *Leishmania donovani* and *Mycobacterium tuberculosis* can interfere with classical activation of macrophages by disrupting IFNγ signaling [168]. Pathogens can also polarize macrophages toward wound healing and prevent their clearance by inhibiting antibody-dependent mechanisms, for example, *Leishmania* spp., African trypanosomes, *Coxiella burnetti*, Dengue virus, and Ross River virus [169,170]. Furthermore pathogens, such as, *Crytpcoccus neoformans* and *M. tuberculosis* can persist and multiply when IL-13 and IL-4 levels are high, as these cytokines prevent autophagy-mediated killing [171].

4.1.4 Deciphering the commonalities and differences between the mechanisms and characteristics of macrophage tolerance and alternate activation

An important question that arises is: how do classically activated macrophages maintain their inflammatory status and prevent transition toward tolerance or alternate activation, when they constantly encounter strong pro-inflammatory stimuli? Two explanations can be offered toward this. First, weakly activating stimuli may not trigger feedback inhibition and prevent transition of classically activated macrophages to immune-suppressive phenotype. Second, during on-going inflammation *in vivo*, there is a continuous migration of monocytes that become classically activated. When monocytes enter tissues and inflammation is resolving, these cells can transition toward tolerant or alternatively activated phenotype [172].

As alternatively activated macrophages, as well as, tolerized macrophages exhibit an immune-suppressive phenotype, few studies suggest that these two phenomena maybe co-related [173,174]. Similarities between these two processes include down-regulation of pro-inflammatory-associated cytokines and chemokines along with up-regulation of

anti-inflammatory mediators, as well as, initiation of cellular repair and proliferation, contributing toward wound healing. Both M2-polarized (alternatively activated) and tolerized macrophages display heightened phagocytic capacities; increased expression of scavenging receptors; chemokines CCL2, CCL17, and CCL22; and NFκB p50 homodimers [173,174]. Further, the expression of metallothionein proteins involved in metabolism of heavy metals (such as, zinc, copper, selenium, silver, cadmium, mercury, and arsenic) and protection against oxidative stress is increased in both cases [173,174]. Apart from cellular responses, tolerance and alternate polarization alter intra-cellular signaling initiated by TLRs. Feedback mechanisms are usually present in every signaling cascade, which regulate pathway activation (Illustration 16). The negative regulators of the TLR-signaling pathway, such as, IRAK-M, A20, SOCS-1, and ST2L are up-regulated in tolerant and alternatively activated macrophages [173,174].

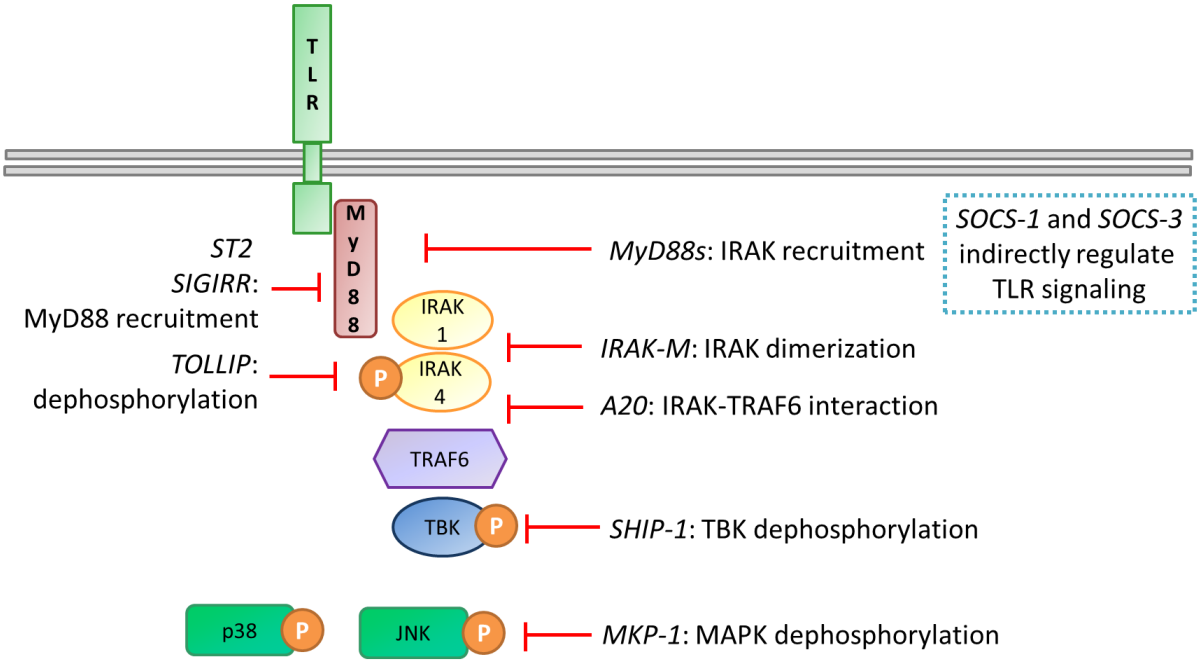


Illustration 16. Negative regulators of TLR signaling

Currently, another critical lacuna present in our understanding is: how does tolerance differ then, from alternatively activated macrophages if both promote resolution of inflammation and healing? Apart from these similarities between the two phenomena, there are certain characteristics that are unique to tolerance. A study has proved that endotoxin tolerance can be established in the absence of IL-4 activation, which is one of the causes of alternate activation [175]. Moreover, *in vitro* macrophages become tolerant

in absence of T-cell cytokines. Therefore, the tolerance mechanism can be independent of alternative polarization status of macrophages.

Functional polarization of macrophages may occur over time *in vivo* that is dependent on different transcription programs. Tolerance may be viewed as a progressive attenuation of pro-inflammatory responses. However, tolerance does not lead to a global attenuation of all genes. A certain group of genes involved in coagulation, acute phase response, antigen presentation, pathogen recognition, anti-inflammatory- and interferon response, chemotaxis, migration, metabolism, growth, and ion transport are up-regulated in tolerant macrophages whereas others are down-regulated [176].

Tolerance may therefore be considered to be a gene-specific program that suppresses pro-inflammatory effects while retaining anti-microbial responses. Although, phenotypically these tolerance macrophages may be characterized as, or maybe even convert into, alternatively activated macrophages; the difference in their transcriptional gene programming may result in varied signaling outcomes.

4.2 Objectives

Work from our laboratory has proved that *V. cholerae* porin OmpU is pro-inflammatory in nature. It induces secretion of classically activated macrophage markers, such as, TNFα, IL-6, NO [142] and chemokines, such as, MIP-1α, MIP-1β (important for granulocyte activation), RANTES (neutrophil recruitment), and CXCL10 (monocyte, macrophage, T cell, NK cell, and DC recruitment) [177]. Furthermore, OmpU-mediated M1-polarization is induced by TLR1/2 activation [177]. We further observed that OmpU-pretreated monocytes and macrophages display suppressed pro-inflammatory mediator production on subsequent LPS challenge.

(i) Considering, these facts, we wanted to explore whether OmpU was inducing a state of macrophage tolerance in LPS-activated cells.
(ii) Further, as classically activated macrophages can polarize toward alternatively activated macrophages, we wanted to determine whether OmpU-pretreated LPS-activated monocytes and macrophages were being polarized toward an anti-inflammatory phenotype.
(iii) Additionally, we were interested in whether both these phenomena were occurring simultaneously in OmpU-pretreated LPS-activated cells.

4.3 Results

4.3.1 OmpU-pretreatment does not enhance M2-polarization markers on LPS-stimulation

Alternatively activated macrophages (M2-type) exhibit increased levels of different chemokines, such as, CCL17, CCL18, CCL22, and CCL24 [164,178,179], which bind to their corresponding chemokine receptors, CCR3 and CCR4, present on eosinophils, basophils, Treg, and Th2 cells, and lead to amplification of Th2-type responses. In addition, M2-polarized macrophages display enhanced expression of chemokine receptors: CXCR1 and CXCR2, which bind to IL-8 [164,180]. Another key feature of M2-polarized macrophages is the up-regulation of the enzyme arginase, which blocks NO production and induces proline production from arginine for collagen synthesis that is crucial for tissue repair [181].

As OmpU-pretreated LPS-activated cells showed a suppression of LPS-mediated pro-inflammatory mediators, mRNA levels of different M2-markers were assessed in response to OmpU/LPS treatments compared to buffer/LPS treatments. In RAW 264.7 cells, we assessed mRNA levels of *Ccl17*, *Ccl22*, *Ccl24*, *Cxcr1*, *Cxcr2*, and *Arginase* (Fig. 8A). Further, in THP-1 cells, we assessed mRNA levels of *CCL18, CCL22,* and *Arginase* (Fig. 8B). In both the cell types, we observed that OmpU-pretreatment did not significantly enhance mRNA levels of these markers as compared to those in cells treated with buffer/LPS (Fig. 8). Hence, our observations indicate that OmpU-pretreatment does not induce M2 polarization in LPS-activated cells.

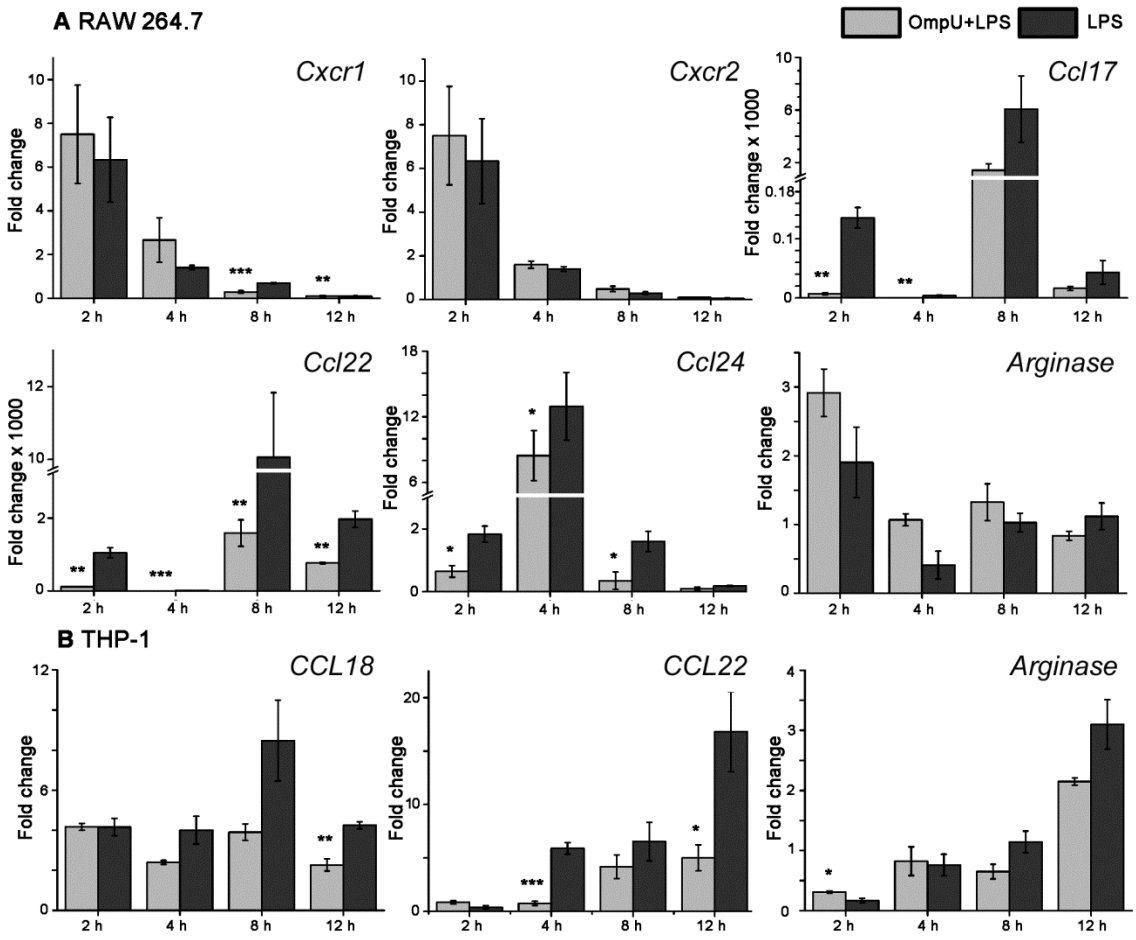


Fig. 8. OmpU-pretreatment does not induce up-regulation of key M2-polarization markers in LPS-activated RAW 264.7 and THP-1 cell lines.

RAW 264.7 (A) and THP-1 cells (B) were treated with OmpU or buffer for 24 h, further replated in fresh media and stimulated with LPS for various time points. RNA was isolated from treated cells and gene-expression analysis for different M2-markers was carried out by semi-quantitative PCR. Results are expressed as mean ± SEM and representative of three independent experiments. **p* <0.05, ***p* <0.01, ****p* <0.001 versus LPS.

4.3.2 IL-10 production is partially responsible for the down-regulation of pro-inflammatory mediators in OmpU-pretreated LPS-activated cells

Cytokines, such as, IL-10 and TGFβ are suppressor cytokines. IL-10 down-regulates production of a Th1 cytokine, IL-12 and impairs APC–T cell interaction by decreasing expression of MHC-II and co-stimulatory molecules [182]. TGFβ inhibits proliferation of monocytes and macrophages and the production of ROS and RNI [183]. Both these cytokines are implicated in M2 polarization and tolerance phenomena. We explored whether induction of immune-suppressive cytokines, such as, IL-10 and/or TGFβ by OmpU might play any role in the down-regulation of LPS-mediated effects. We assessed IL-10 and TGFβ production in response to OmpU in different cell types, such as, RAW 264.7, THP-1, mouse peritoneal macrophages, and human PBMCs. We observed that OmpU did not induce production of these cytokines in any of these cell types, except IL-10 in human PBMCs (Fig. 9A). Following this, we assessed IL-10 levels in human PBMCs in response to various treatments: buffer, OmpU, buffer/LPS, as well as, OmpU/LPS treatment at different time points (Fig. 9A, 9B). We observed that IL-10 levels in response to OmpU/LPS treatments were higher than OmpU alone at earlier time points, prompting us to probe whether these sustained levels could contribute in the down-regulation of LPS-mediated effects. Our data indicated that the use of neutralizing antibodies against TGFβ did not rescue the down-regulation of LPS-mediated TNFα production in human PBMCs (Fig. 9C). However, neutralization of IL-10 secretion could partially rescue TNFα production in OmpU-pretreated LPS-activated cells (Fig. 9C). Rescue in LPS-mediated TNFα production, with the use of 0.1 μg/ml anti-IL10 mAb was around 15% ($p < 0.001$) and with 10 μg/ml anti-IL10 mAb was about 25% ($p < 0.001$) (Fig. 9C). Hence, our observations suggest that IL-10 but not TGFβ is partially responsible for the down-regulation mechanism.

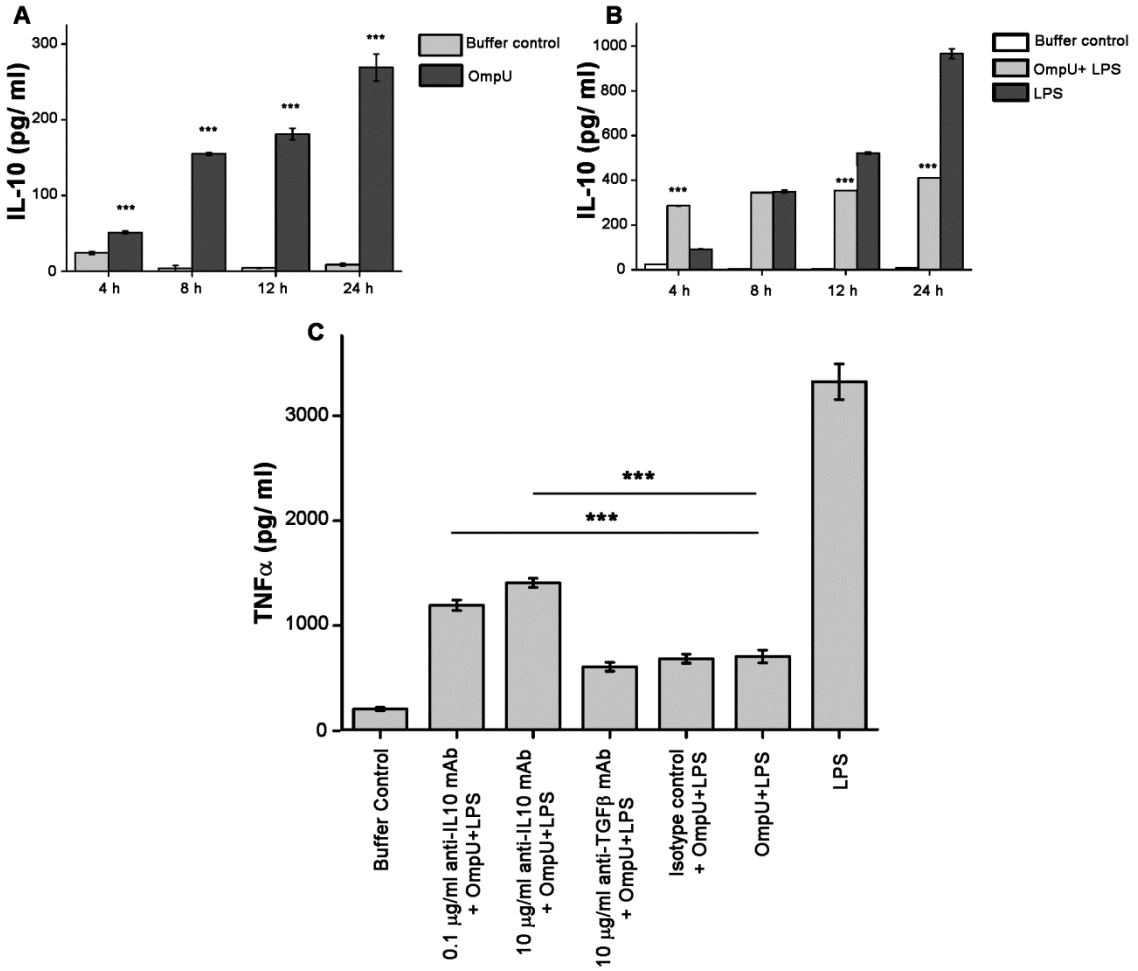


Fig. 9. OmpU-induced IL-10 production is partially responsible for down-regulation of LPS-mediated TNFα production.

(A) Human PBMCs were treated with OmpU or buffer and incubated for different time periods. Culture supernatants were collected after different incubations. Supernatants were assessed for IL-10 production by ELISA. (B) Human PBMCs were treated with OmpU or buffer for 24 h and replated in fresh media and activated with LPS for different time points and culture supernatants were collected. IL-10 levels in supernatants were assessed by ELISA. (C) Human PBMCs were treated with OmpU and incubated with different concentrations of neutralizing anti-IL-10 or anti-TGFβ antibodies for 24 h and further replated in fresh media and stimulated with LPS. Culture supernatants were collected after 24 h of LPS treatment and TNFα levels were assessed by ELISA. Results are expressed as mean ± SEM and representative of three independent experiments. ***$p < 0.001$ versus buffer control (A), versus LPS (B), versus OmpU/LPS (C).

4.3.3 LPS activates TLR signaling by the TLR2/6-MyD8-dependent pathway

As most of the well documented tolerance mechanisms involve alteration in the TLR-signaling pathway, we wanted to explore whether TLR signaling is affected in OmpU-pretreated LPS-activated cells. To determine whether OmpU could modulate LPS-mediated signaling, we first had to establish which TLR(s) get activated by LPS in RAW 264.7 and THP-1 cells. We probed cell surface TLRs, such as, TLR1, TLR2, TLR4, and TLR6. Generally, activated TLR4 homodimerizes and recruits adapter molecules, such as, MyD88 to the receptor complex [184]. In contrast, TLR2 heterodimerizes either with TLR1 or with TLR6, and may further recruit MyD88 to the receptor complex [185-187].

As our above results indicated that OmpU does not induce M2 polarization in LPS-activated cells and partial involvement of IL-10 cannot be distinctly included in M2 polarization alone, we wanted to assess whether OmpU-pretreatment induces tolerance in LPS-activated cells.

We performed co-immunoprecipitation assay with anti-TLR4 or anti-TLR2 antibodies. We observed that LPS-treatment did not cause MyD88 recruitment to TLR4 (Fig. 10A, 10B) in both RAW 264.7 and THP-1 cells. Further, LPS induced heterodimerization of TLR2 with TLR6, but not with TLR1 (Fig. 10A, 10B). Additionally, MyD88 recruitment to TLR2 was enhanced in response to LPS in both the cell types (Fig. 10A, 10B). Further, we assessed surface expression of TLR2 and TLR6 in response to LPS in RAW 264.7 and THP-1 cells. We observed an increase in surface expression of both these TLRs in both the cell lines (Fig. 10C, 10D). Hence, our results indicated that LPS mediates its signaling via TLR2/6-MyD88 pathway in RAW 264.7 and THP-1 cells.

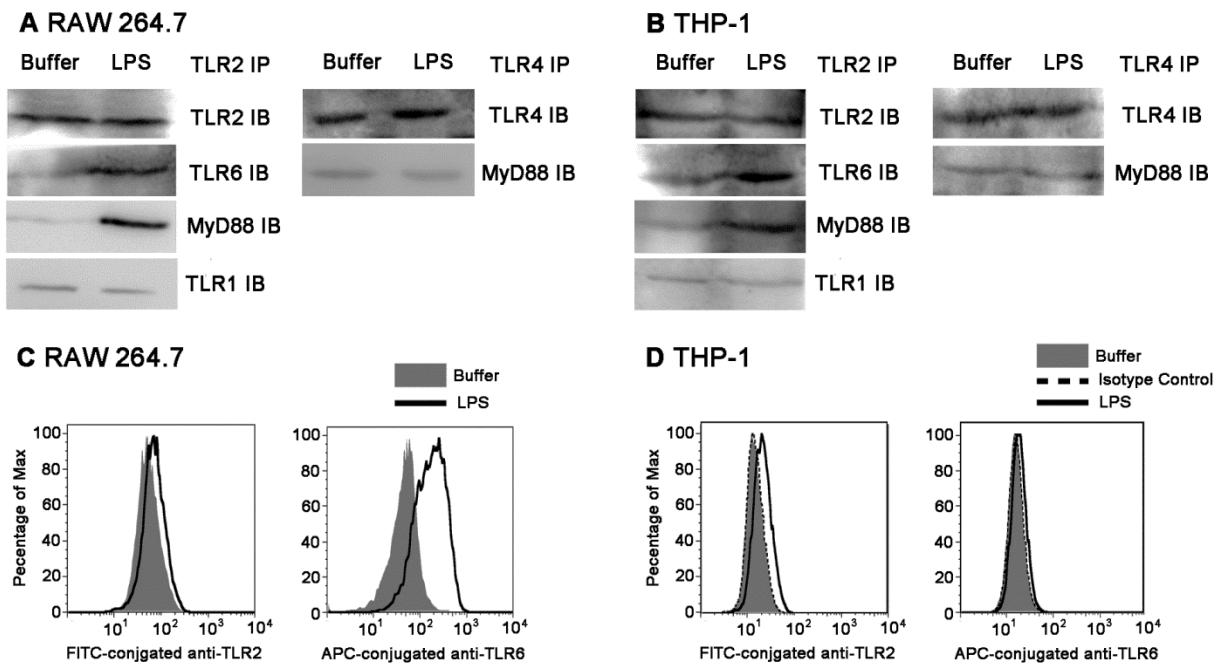


Fig. 10. LPS mediates its signaling in a TLR2/6-MyD88-dependent manner in RAW 264.7 and THP-1 cell lines.

RAW 264.7 (A) and THP-1 cells (B) were treated with LPS or buffer for 1 h and whole cell lysates were prepared. Co-immunoprecipitation (IP) was performed with anti-TLR2 or anti-TLR4 antibody. Immuno-blotting (IB) for various signaling mediators was performed using anti-TLR2, anti-TLR1, anti-TLR6, anti-TLR4, anti-MyD88, anti-IRAK1, or anti-IRAK4 antibody. RAW 264.7 (C) and THP-1 cells (D) were treated with LPS or buffer and surface expression of TLR2 and TLR6 was assessed by flow cytometry. RAW 264.7 cells were treated with Fc block prior to primary antibody staining. For THP-1 cells, isotype-treated cells were used as controls. Blots and histograms are representative of three independent experiments.

4.3.4 OmpU-pretreatment attenuates LPS-mediated TLR signaling by decreasing the association of signaling mediators

Once we established the involvement of TLR2/TLR6 and MyD88 in LPS-mediated signaling in RAW 264.7 and THP-1 cells, we proceeded further to determine whether OmpU could modulate the LPS-mediated signaling by affecting association TLRs and/various signaling mediators. It is known in LPS-mediated signaling that MyD88 further recruits IRAK1 and IRAK4 [146]. IRAK4 phosphorylates IRAK1 and, in turn, activates and recruits other downstream signaling molecules [146].

We observed that OmpU-pretreatment decreased LPS-induced association of TLR6 with TLR2 in both RAW 264.7 and THP-1 cell types, as compared to buffer/LPS treated cells (Fig. 11A, 11B). Further, association of MyD88, IRAK1, and IRAK4 to the receptor complex was also reduced in OmpU/LPS-treated cells (Fig. 11A, 11B). Direct association of IRAK1 and IRAK4 with MyD88 was also decreased in OmpU/LPS-treated cells than in buffer/LPS-treated cells (Fig. 11C, 11D). Hence, our observations suggested that OmpU-pretreatment attenuates LPS-mediated TLR signaling at multiple steps.

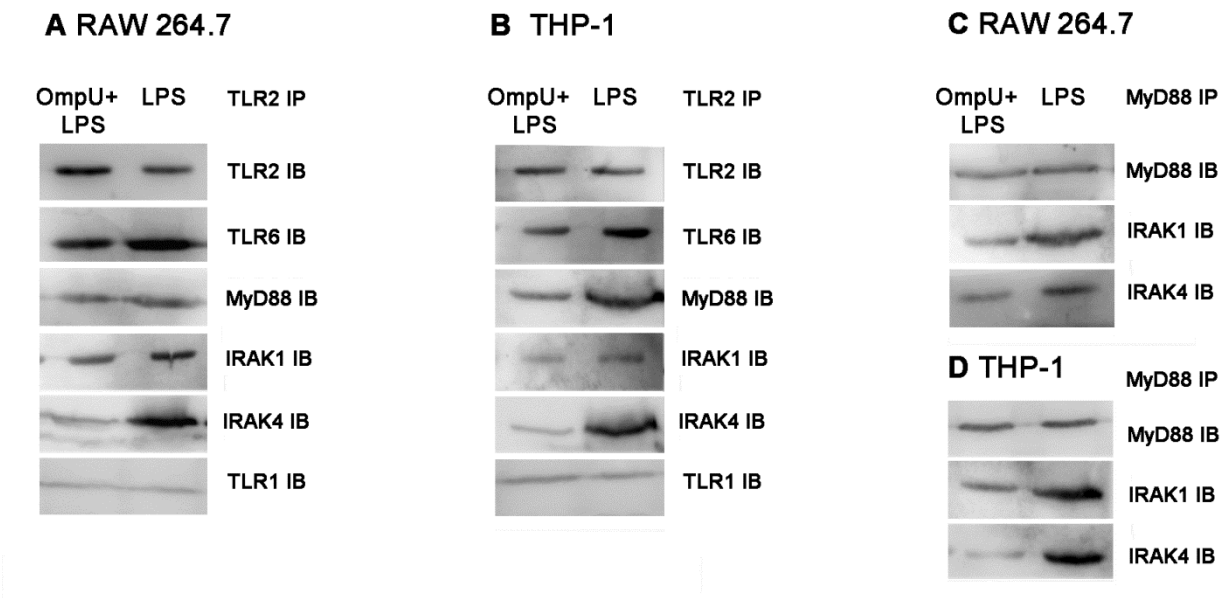


Fig. 11. OmpU-pretreatment affects LPS-mediated TLR2/TLR6 dimerization, MyD88 recruitment to the receptor complex, and IRAK recruitment to MyD88.

RAW 264.7 and THP-1 cells were pretreated with OmpU or buffer for 24 h, further replated in fresh media, and stimulated with LPS for 1 h. Whole cell lysates were prepared from RAW 264.7 (A) and THP-1 cells (B), and co-immunoprecipitation (IP) was performed with anti-TLR2 antibody. Similarly, co-immunoprecipitation (IP) with anti-MyD88 antibody was performed in cell lysates from RAW 264.7 (C) and THP-1 (D). Followed by co-IP, immuno-blotting (IB) for various mediators were carried out using anti-TLR2, anti-TLR1, anti-TLR6, anti-TLR4, anti-MyD88, anti-IRAK1, or anti-IRAK4 antibodies. Each set of blots are representative of three independent experimental sets.

4.3.5 OmpU-pretreatment enhances LPS-mediated IRAK-M expression in RAW 264.7 cells

The most well established mechanism of endotoxin tolerance and some other tolerance mechanisms showed involvement of several negative regulators of TLR signaling [188-206]. As LPS-induced TLR signaling was being affected at the initial stages due to OmpU-pretreatment, we wanted to determine whether any known negative regulator of TLR signaling was involved in the process.

We probed different negative regulators of TLR signaling, such as, SOCS-1, SOCS-3, SHIP-1, TOLLIP, IRAK-M, and MKP-1. SOCS-1 and SOCS-3 have been initially identified as negative regulators of cytokine signaling, however, their role has also been implicated in TLR activation [207,208]. SHIP-1 dephosphorylates TBK1, a down-stream TLR signaling mediator, and hence attenuates TLR signaling [209]. TOLLIP inhibits IRAK activity, after it associates with TLR [210]. IRAK-M prevents dissociation of IRAK dimers from TLRs [211], whereas, MKP-1 is a negative regulator of MAPK signaling, which can be activated by TLR signaling [212].

We did not observe increased mRNA levels of any of these regulators in OmpU/LPS-treated cells as compared to buffer/LPS-treated cells (Fig. 12A, 12B) except that of IRAK-M in RAW 264.7 cells (Fig. 12A). This was further confirmed by western blotting (Fig. 12C). However, IRAK-M overexpression in THP-1, human PBMCs, and mouse peritoneal macrophages did not occur. Therefore, our observation suggests that IRAK-M may contribute toward attenuating TLR signaling in RAW 264.7 cells.

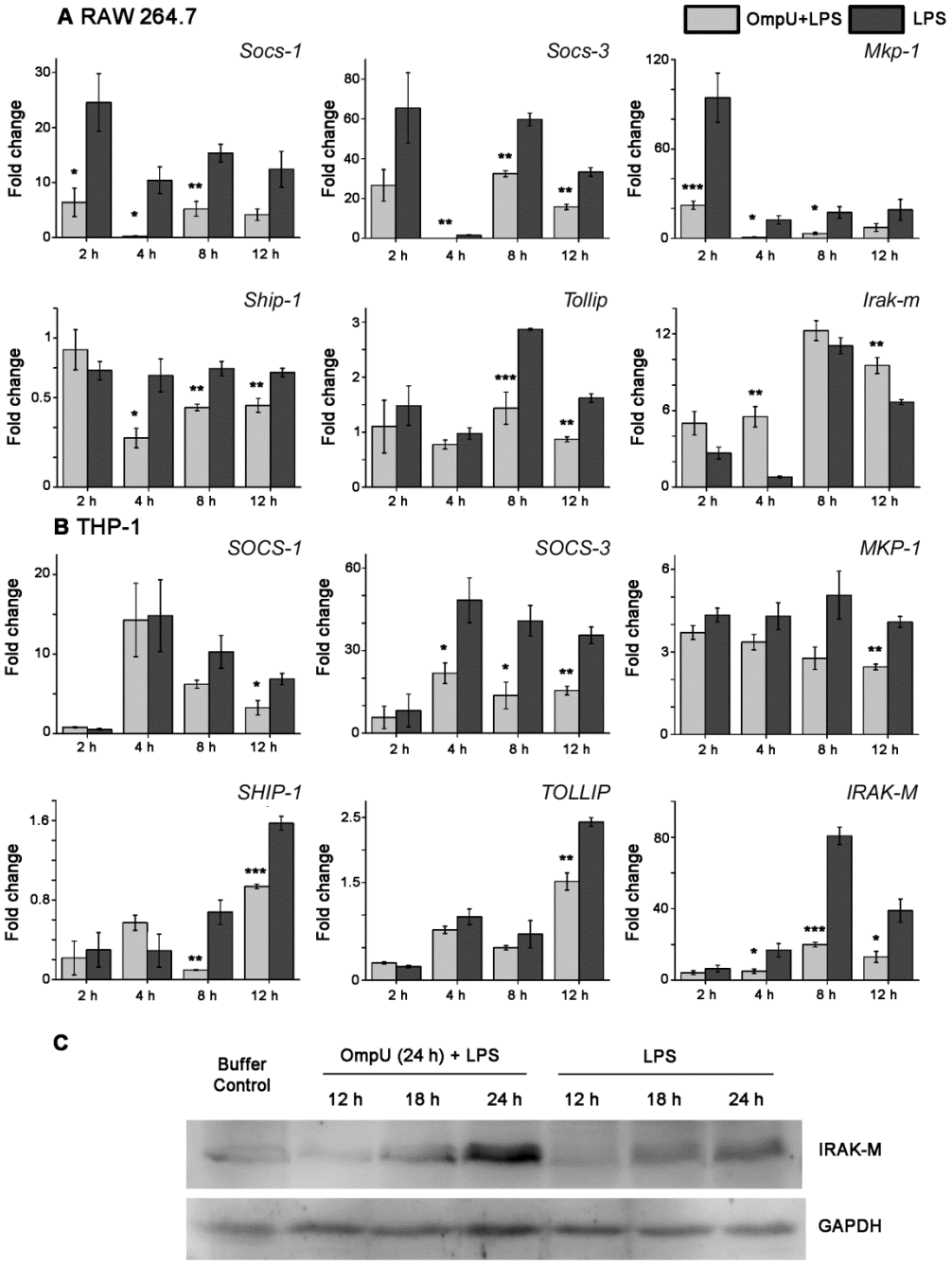


Fig. 12. OmpU-pretreatment does not enhance negative regulators of TLR signaling in LPS-activated RAW 264.7 and THP-1 cells except Irak-m in RAW 264.7 cells.

RAW 264.7 (A) and THP-1 cells (B) were pretreated with OmpU or buffer for 24 h and further activated with LPS. RNA was isolated and gene-expression analysis for *Socs-1*, *Socs-3*, *Mkp-1*, *Ship-1*, *Tollip*, and *Irak-m* was carried out by semi-quantitative PCR. Results are expressed as mean ± SEM and representative of three independent experiments. **p* <0.05, ***p* <0.01, ****p* <0.001 versus LPS. In RAW 264.7 (C), immuno-blotting with anti-IRAK-M antibody was performed under similar treatment conditions. Blot is representative of three independent experiments.

4.3.6 OmpU-pretreatment alters LPS-mediated translocation of transcription factors

TLR signaling usually culminates in activation of the transcription factors, such as, NFκB and AP-1, which are key regulators of pro-inflammatory responses. NFκB and AP-1 promoters are present on cytokine genes, such as, TNFα, IL-6, IL-12, etc. [213]. As OmpU-pretreatment affected the TLR-signaling pathway, we further evaluated whether attenuated TLR signaling resulted in decreased NFκB levels in the nucleus. Toward this, we assessed translocation of two members of NFκB family, by western blotting, that is, RelA (p65) and c-Rel, which are commonly involved in pro-inflammatory responses. We observed that, in both RAW 264.7 and THP-1 cells, RelA and c-Rel translocation to the nucleus was reduced due to OmpU/LPS treatment, as compared to buffer/LPS treatment (Fig. 13). Further, we assessed LPS-mediated translocation of AP-1 family members. Among the seven members present, OmpU increased translocation of suppressive family members: JunD and Fra-1 in RAW 264.7 cells [214], which can contribute toward reduced pro-inflammatory responses. However, this did not occur in THP-1 cells (Fig. 13). Thus, our observations suggested that OmpU modulates LPS-induced TLR-signaling pathway and alters transcription factor levels, ultimately resulting in decreased pro-inflammatory mediator production by LPS [142].

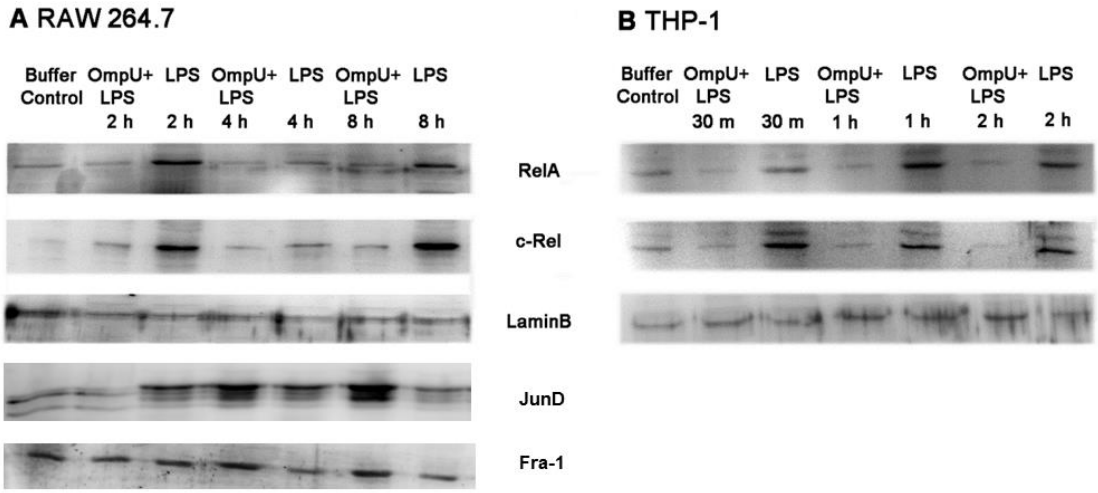


Fig. 13. OmpU-pretreatment alters LPS-mediated translocation of nuclear factors.

RAW 264.7 (A) and THP-1 (B) cells were treated with OmpU or buffer for 24 h and further stimulated with LPS for various time points. Nuclear lysates were prepared and immunoblotting was carried out using anti-RelA and anti-c-Rel antibodies. Blots are representative of three independent experiments.

4.3.7 Assessing effect of OmpU-pretreatment on LPS-driven macrophage function

As all the above results indicated that OmpU-pretreatment probably induces tolerance in LPS-activated cells, we further wanted to confirm it by probing other features of tolerance. Endotoxin-tolerant monocytes and macrophages demonstrate certain features, such as, enhanced phagocytosis and decreased costimulatory molecule expression in macrophages [163,215,216]. Therefore, we further investigated whether OmpU-pretreatment altered phagocytic ability and co-stimulatory molecule expression of macrophages on LPS-stimulation.

4.3.7.1 OmpU-pretreatment enhances phagocytosis of LPS-stimulated cells

Phagocytosis is a central feature of macrophages and can be increased in tolerized macrophages, which helps in clearing pro-inflammatory stimuli and hastening tissue repair. We investigated whether OmpU-pretreatment affected phagocytosis of Alexa Fluor 488-conjugated *Escherichia coli* BioParticles in LPS-stimulated cells. We observed that phagocytic capacity of OmpU/LPS-activated RAW 264.7 cells was enhanced, as compared to buffer/LPS cells (Fig. 14). However, this effect was not observed in mouse peritoneal macrophages.

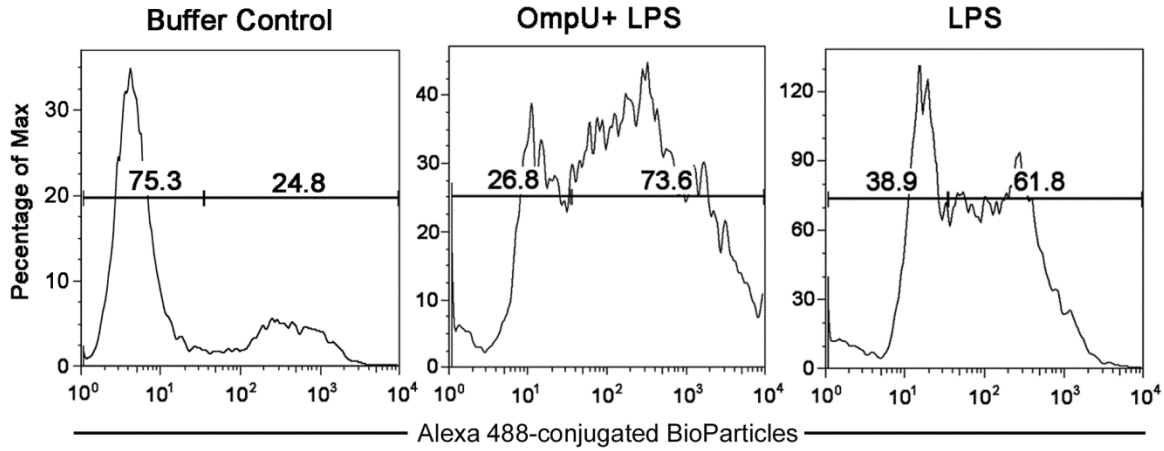


Fig. 14. OmpU-pretreatment enhances phagocytosis of RAW 264.7 cells on stimulation with LPS.

RAW 264.7 cells were treated with OmpU or buffer for 24 h and further stimulated with LPS for 24 h. Phagocytosis of Alexa Fluor 488-conjugated *E. coli* BioParticles was measured by flow cytometry. Gate 1 is set for unstained cells. Gate 2 is set on cells that have phagocytosed BioParticles. Histograms are representative of three independent experiments.

4.3.7.2 OmpU-pretreatment decreases LPS-induced surface expression of co-stimulatory molecules

PAMPs can induce expression of co-stimulatory molecules, such as, CD80 and CD86 on the cell surface of macrophages through PAMP–PRR interactions [154], which is crucial for T-cell activation. Tolerant monocytes and macrophages display decreased co-stimulatory molecule expression and thus, prevent T cells from being optimally activated. This prevents T cells from secreting cytokines that augment pro-inflammatory macrophage functions, thereby, helping in resolution of inflammation.

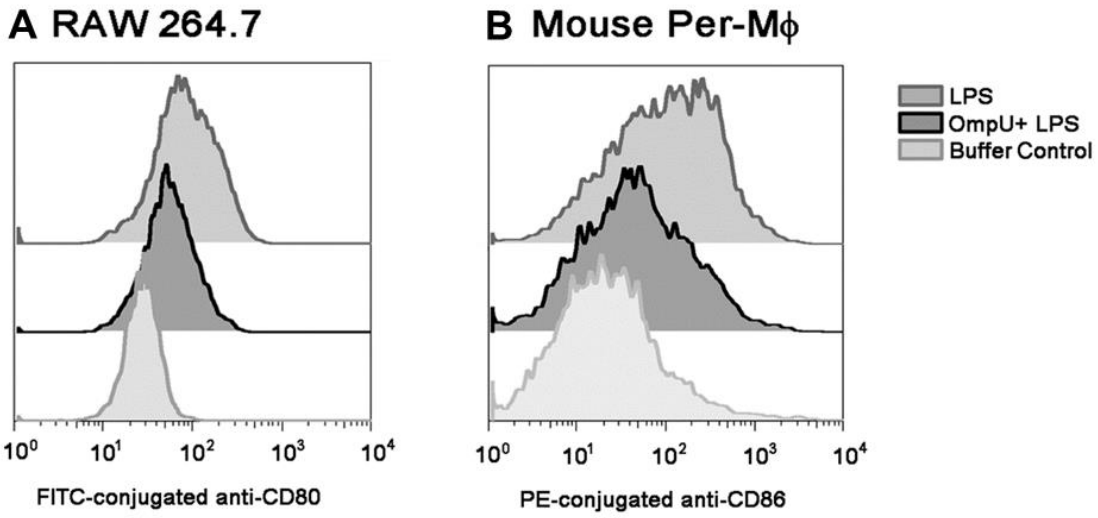


Fig. 15. OmpU-pretreatment decreases surface expression of LPS-mediated co-stimulatory molecules in RAW 264.7 and mouse peritoneal macrophages.

RAW 264.7 cells and mouse peritoneal macrophages were treated with OmpU or buffer for 24 h and further stimulated with LPS for 24 h. (A) Surface expression of CD80 in RAW 264.7 cells following OmpU/LPS or buffer/LPS treatments was determined by flow cytometry. (B) Surface expression of CD86 in mouse peritoneal macrophages following OmpU/LPS or buffer/LPS treatments was determined by flow cytometry. RAW 264.7 cells and mouse peritoneal macrophages were treated with Fc block prior to primary antibody staining. Histograms are representative of three independent experiments.

We observed that CD80 surface expression increased in response to LPS in RAW 264.7 cells, whereas CD86 expression remained unchanged. In mouse peritoneal macrophages, CD86 surface expression increased on LPS treatment but not that of CD80. Further, we wanted to explore whether OmpU-pretreatment could change surface-expression pattern of CD80 or CD86 in LPS-activated RAW 264.7 or mouse peritoneal macrophages. On OmpU-pretreatment, LPS-mediated surface expression of CD80 decreased in

RAW 264.7 cells (Fig. 15A). Similarly, in primary macrophages, CD86-surface expression was reduced in OmpU/LPS-treated cells as compared to buffer/LPS-treated cells (Fig. 15B).

4.4 Conclusions

Classically activated macrophages can transition to immune-suppressive phenotype depending on various environmental stimuli, such as, cytokines and PAMPs [164]. Macrophages can adopt this anti-inflammatory phenotype in two cases: macrophage tolerance and alternative polarization. The initial indicator of tolerant or alternatively activated monocytes and macrophages is the suppression of pro-inflammatory mediator production. As OmpU down-regulated LPS-mediated responses in monocytes and macrophages, we were interested in probing the mechanism behind it. Toward this, we initially had two hypotheses: that OmpU was either polarizing monocytes/macrophages toward M2 type or rendering cells tolerant.

We initially assessed gene expression of M2-polarization markers in RAW 264.7 and THP-1 cells, such as, CXCR1, CXCR2, CCL17, CCL18, CCL22, CCL24, and arginase. We did not observe an increase in gene expression of these M2-associated chemokines in OmpU/LPS-treated cells as compared to buffer/LPS-treated cells (Fig. 8). Therefore, our results pointed out that tolerance may be induced independently of M2-polarization. We then determined whether immune-suppressive cytokines, such as, IL-10 and TGFβ, which are associated with M2 polarization, were involved in the down-regulatory phenomenon. Cell lines did not produce IL-10 and TGFβ in response. But, OmpU-induced IL-10 secretion in human PBMCs played a partial role in the down-regulation of LPS-mediated TNFα production (Fig. 9).

The observation that OmpU-mediated suppression of LPS-dependent responses did not induce heightened expression of some of the key markers of M2-polarization, directed us toward exploring, whether, OmpU could induce tolerance in LPS-activated cells. Macrophage tolerance usually alters TLR-signaling pathways. Our data indicated that LPS signals via TLR2/6-MyD88-dependent pathway (Fig. 10). We therefore, assessed TLR and IRAK levels in whole cell lysates by western blotting, and we did not see any differences in their expression levels in response to OmpU/LPS versus LPS treatments.

This prompted us to study the association of signaling mediators by co-immunoprecipitation. We observed that OmpU-pretreatment attenuated LPS-mediated TLR signaling by decreasing association of TLR6, MyD88, and IRAK1/4 to TLR2, as well as, IRAK1/4 recruitment to MyD88 (Fig. 11).

Numerous negative regulators of TLR signaling are reported to be involved in endotoxin-mediated tolerance [158], as well as, in some other type of tolerance. These include IRAK-M [188-192], SOCS-1 [193,194], SOCS-3 [195,196], MKP-1 [197-200], TOLLIP [201,202], and SHIP-1 [203-206]. In our study, we observed heightened mRNA expression of only IRAK-M in OmpU-pretreated LPS-activated RAW 264.7 cells, which was further confirmed by western blotting (Fig. 12). However, we did not observe enhanced gene expression of other negative regulators assessed.

TLR signaling usually leads to NFκB and AP-1 activation. In our study, we observed that to begin with, there was decreased translocation of NFκB members to the nucleus on OmpU-pretreatment in RAW 264.7 and THP-1 cells (Fig. 13). Further, translocation of two suppressive AP-1 family members: JunD and Fra-1 was augmented in RAW 264.7 cells but not in THP-1 cells (Fig. 13). Thus, OmpU alters transcription factor levels, which contribute toward suppressed LPS-mediated responses.

We further determined whether OmpU could modify LPS-driven macrophage function, such as, phagocytosis and co-stimulatory molecules expression; both of which are important during antigen presentation. Our findings showed that OmpU-pretreatment enhances phagocytosis of RAW 264.7 cells on LPS-stimulation as compared to that of LPS-activated cells (Fig. 14). Further, OmpU/LPS-treated murine macrophages display decreased co-stimulatory molecule expression (Fig. 15).

Altogether, OmpU induces macrophage tolerance in LPS-stimulated cells by attenuating TLR-signaling pathway. Further, this tolerance does not involve M2 polarization [217].

Specific Aim 2: To determine whether OmpU can shape adaptive responses by examining its effects on DCs and T cells

Chapter 3: Dendritic cell activation by OmpU

Chapter 4: Modulation of adaptive responses by OmpU

Chapter 3: Dendritic cell activation by OmpU

5.1 DCs act as sensors, sentinels, and seekers of infectious agents

The mononuclear phagocytic system consists of monocytes, macrophages, and DCs. These cells are important for phagocytosis of microbes and induction of inflammation [96]. The role of monocytes and macrophages has been discussed in detail in the earlier chapters. The third member of this system, DCs derive their name due to the presence of branched membranous extensions or dendrites (Greek, *dendron* meaning 'tree'). DCs can be divided into two types: plasmacytoid DCs (pDCs) and classical DCs (cDCs). pDCs usually are present in blood and lymphoid tissues. They have low expressions of MHC-II, co-stimulatory molecules, and CD11c integrin [218]. They also express limited number of TLRs, namely TLR7 and TLR9, which are important anti-viral responses as they recognize nucleic acid PAMPs. The second type of DCs, cDCs are present in lymphoid and non-lymphoid tissues. cDCs express a larger repertoire of PRRs, including TLRs, NLRs, and RLRs [218]. They also express high levels of CD11c. Due to their presence in non-lymphoid tissues, they are constantly exposed to several antigens and can sense various PAMPs due to diverse PRRs present on them. When immature DCs, which have not encountered an antigen, recognize PAMPs via PRRs, they may become activated and secrete cytokines, such as, TNFα, IL-6, IL-1β, IL-12, etc. [219]. This usually involves NFκB signaling and may involve MAPK signaling, similar to that of monocytes and macrophages [219]. All these aspects make DCs important mediators of innate immunity along with monocytes and macrophages. Moreover, innate activation of DCs subsequently shapes the adaptive responses. The cytokines secreted by DCs and their interaction with adaptive immune cells, determine the type of adaptive response generated. Therefore, DCs are critical mediators between innate and adaptive immunity.

5.1.1 Inflammasomes: Responders to PAMPs and mediators of innate immunity

Along with TLRs, DCs also contain intra-cellular PRRs, such as, NODs and NLRs that are crucial for recognition of PAMPs. Inflammasomes are multi-protein heptameric complexes consisting of caspase-1, PYCARD, and PAMP-binding proteins, such as, NLRs and AIM2 [220]. Inflammasomes convert pro-caspase-1 into active caspase-1. This

in turn, leads to cleavage of pro-IL-1β and pro-IL-18 to yield mature IL-1β and IL-18. However, IL-1β can also be converted into its active form independent of caspase-1, by serine proteases, such as, proteinase 3, elastase, and cathepsin G present in neutrophils and macrophages [221]. IL-1β induces vasodilation, hyperthermia, and increases proliferation and differentiation of lymphocytes. It induces COX-1 and prostaglandins that are involved in inflammatory pain. Additionally, it promotes extravasation of immune cells [130]. IL-18, along with IL-12, contributes toward cell-mediated immunity. It causes T cells and NK cells to release IFNγ (Th1 response) and inhibits Th2 type of response [130].

5.1.2 Different types of inflammasomes recognize several innate immune activators

Inflammasomes can be activated in response to several PAMPs and microbial infections. Currently, there are six types of inflammasomes known, those involving NLR proteins (NLRP1, NLRP3, NLRC4, NLRP6, and NLRP12) and the AIM2 inflammasome (Illustration 17) [222]. The NLRP3 inflammasome also detects danger-associated molecular patterns (DAMPs), such as, uric acid, cholesterol, and silica crystals.

NLR proteins contain a leucine-rich repeat domain (LRR) at the C-terminal that senses PAMPs and DAMPs [223]. NLRs differ in the length and structure of LRR. Further, the nucleoside triphosphatase domain (NACHT) mediates NLR oligomerization and forms the core structure. The N-terminal domain can consist of a pyrin domain (PYD) or caspase-recruiting domain (CARD). Apoptosis associated speck-like containing a CARD (ASC) protein is an adaptor molecule that interacts with NLR via the PYD domain present in AIM2, NLRP1, NLRP3, NLRP6, and NLRP12 and further recruits pro-caspase-1 which also contains a CARD domain [223]. However, NLRP1 and NLRC4 can interact with pro-caspase-1 via CARD domains directly and convert it into active caspase-1 without ASC involvement [224,225]. Moreover, ASC is still required for optimal function.

Inflammasome activation of NLRP1 and NLRP3 involves two steps. Stimulation of NFκB in response to DAMP/PAMP recognition leads to transcription of proteins involved in the inflammasome, such as, NLR, caspase-1, ASC, etc. The second signal initiates oligomerization of these proteins into a complex. These signals may involve

K^+ efflux, ROS generation, mitochondrial changes, release of mitochondrial content and lysosomal cathepsins, and Ca^{2+} influx [221]. Oligomerization of AIM2 and NLRC4 occurs in response to conformational changes on sensing of their respective PAMPs. This oligomerization leads to proximity-induced proteolytic cleavage of pro-caspase-1 into p10 and p20 subunits. These subunits build tetramers and form active caspase-1 (Illustration 18), which further yields active IL-1β and IL-18 [223].

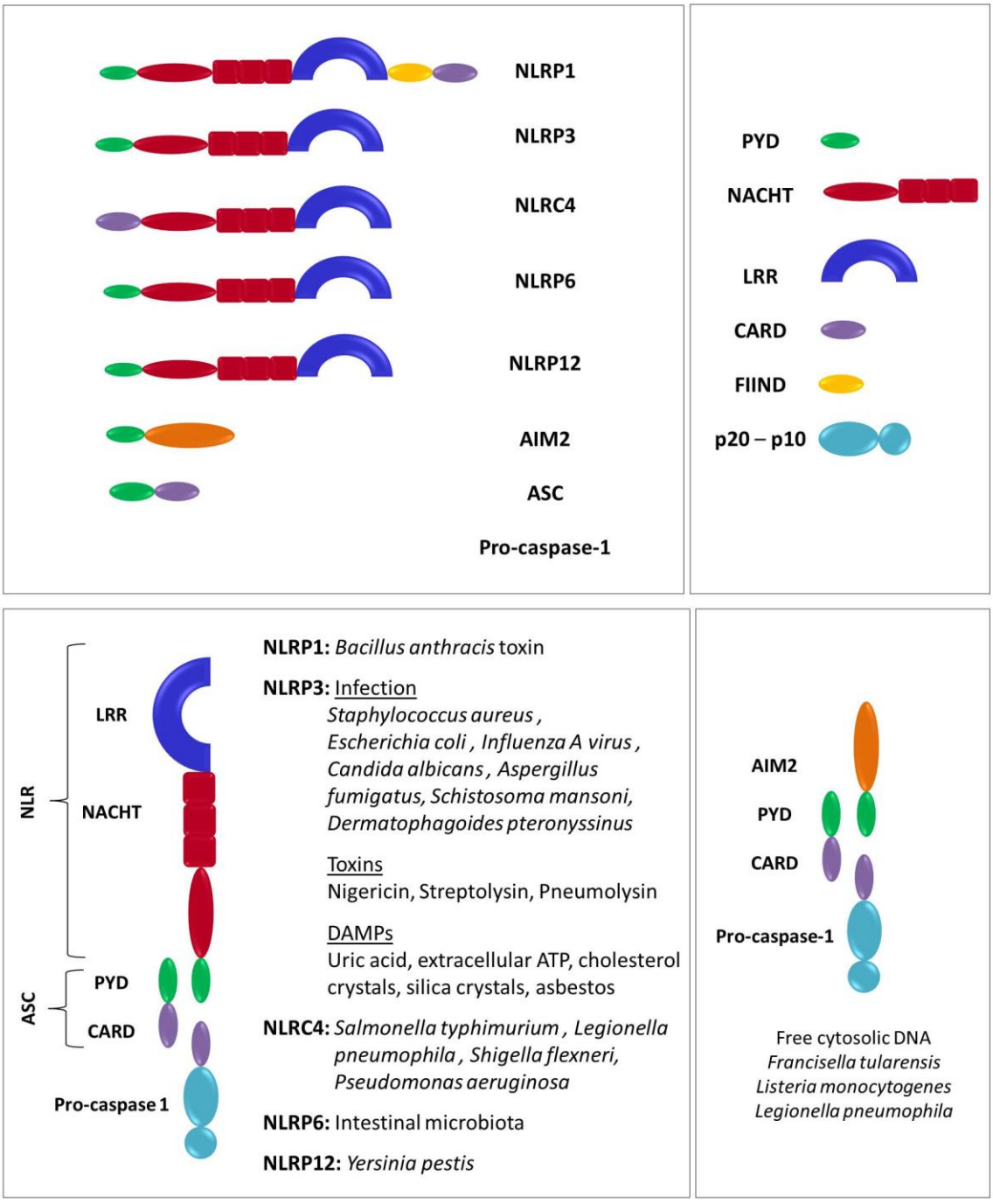


Illustration 17. General structure of inflammasomes and their activators [222,226,227]

A noncanonical inflammasome is mediated by caspase-11 in mice. It has been shown to promote NLRP3 activation by enhancing IL-18 processing. Further, it recognizes intra-cellular LPS and bacteria and induces IL-1α secretion but not that of IL-1β [228]. Human caspase-4 and caspase-5 perform similar function and can be involved in activation of NLRP1 and NLRP3 inflammasomes, respectively [229].

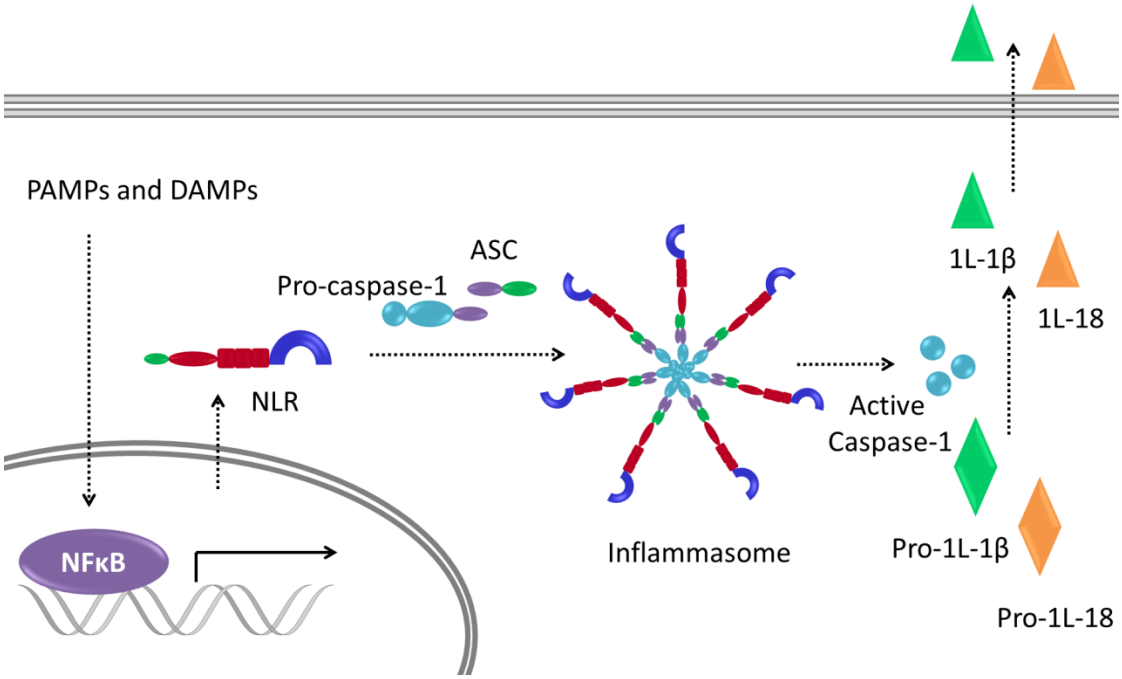


Illustration 18. Canonical activation of inflammasomes

5.2 Objectives

Our previous results indicated that OmpU behaves as a PAMP and induces pro-inflammatory mediator production in monocytes and macrophages [142]. We further wanted to probe whether OmpU can activate DCs, as they play a crucial role in both innate immunity and adaptive immunity. We employed murine BMDCs and splenic DCs to explore these aspects.

5.3 Results

5.3.1 Differentiated BMDCs and isolated splenic DCs express CD11c

The nature of BMDCs differentiated from hematopoietic cells was determined by analyzing surface expression of CD11c by flow cytometry. Similarly, purity of MACS-isolated splenic DCs from splenocytes was assessed in terms of CD11c expression. Both types of DCs expressed high levels of CD11c, indicative of cDCs (Fig. 16).

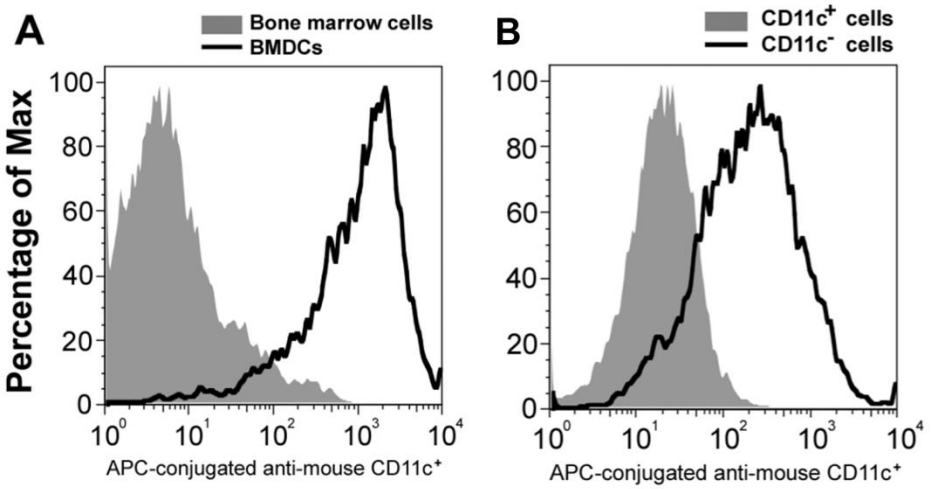


Fig. 16. CD11c expression by differentiated BMDCs and purified splenic DCs.

(A) Bone marrow cells were differentiated using 10 ng/ml murine GM-CSF for 7 days. Cells were harvested and analyzed for surface expression of CD11c by flow cytometry (B) CD11c expression of MACS-purified splenic DCs and negative fraction was assessed by flow cytometry.

5.3.2 OmpU induces pro-inflammatory mediator production in murine BMDCs

On recognition and internalization of microbes or microbial products, DCs become activated and secrete cytokines. We wanted to determine whether OmpU could activate immature murine BMDCs and induce pro-inflammatory mediator production. We observed that OmpU-treated BMDCs exhibited enhanced secretion of pro-inflammatory cytokines, such as, TNFα, IL-6, and IL-1β at 24 h (Fig. 17).

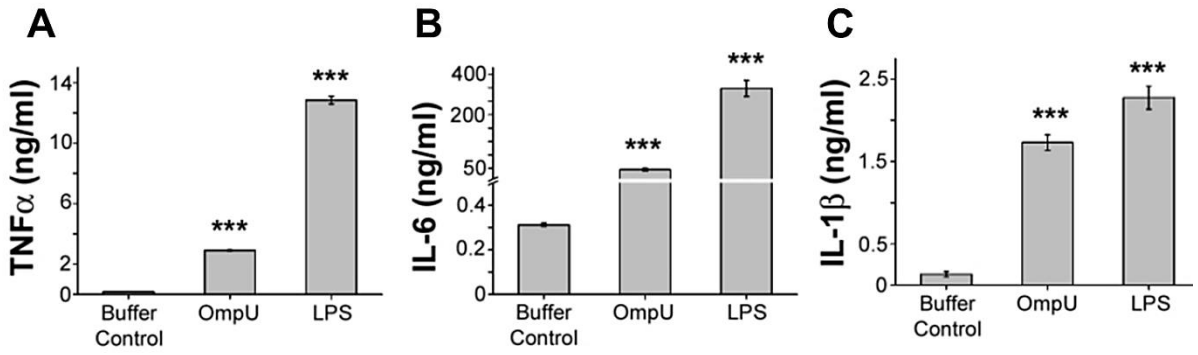


Fig. 17. OmpU induces pro-inflammatory cytokine production in murine BMDCs.

BMDCs were treated with 2 μg/ml OmpU for 24 h. Buffer-treated and LPS-treated (1 μg/ml) served as negative and positive controls, respectively. Levels of (A) TNFα, (B) IL-6, and (C) IL-1β were measured by ELISA in culture supernatants after 24 h. Results are expressed as mean ± SEM and represent the average of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus buffer control.

5.3.3 OmpU activation of DCs is independent of TLR2 and TLR4 signaling

DCs can recognize PAMPs via PRRs, such as TLRs, and become activated. One of the earlier reports from our laboratory showed that OmpU leads to pro-inflammatory mediator production in a TLR1/2-MyD88-dependent manner in monocytes and macrophages [177]. As OmpU-treated BMDCs secreted several pro-inflammatory cytokines, we wanted to determine whether this secretion was in response to OmpU-induced TLR-mediated signaling. As TLR2 heterodimerizes with either TLR1 or TLR6 on activation, we wanted to first explore whether TLR2 was involved in OmpU-mediated DC activation. In addition to TLR1, TLR2, and TLR6, TLR4 is also present on the surface of DCs. Hence, we also assessed the involvement of TLR4 in OmpU-mediated signaling. We observed that the use of neutralizing antibodies against TLR2 and TLR4 did not affect OmpU-mediated TNFα and IL-6 release in BMDCs (Fig. 18A, 18B). As BMDCs were differentiated *in vitro,* we assessed TLR2 and TLR4 involvement in splenic DCs. We observed a similar result in splenic DCs as well (Fig. 18C, 18D). As TLR1 and TLR6 heterodimerize with TLR2, their involvement in OmpU-mediated DC activation also can be ruled out. This suggests that OmpU activates DCs in a TLR2 (TLR1/TLR6)- and TLR4-independent manner. Therefore, the PRR involved in OmpU recognition remains elusive.

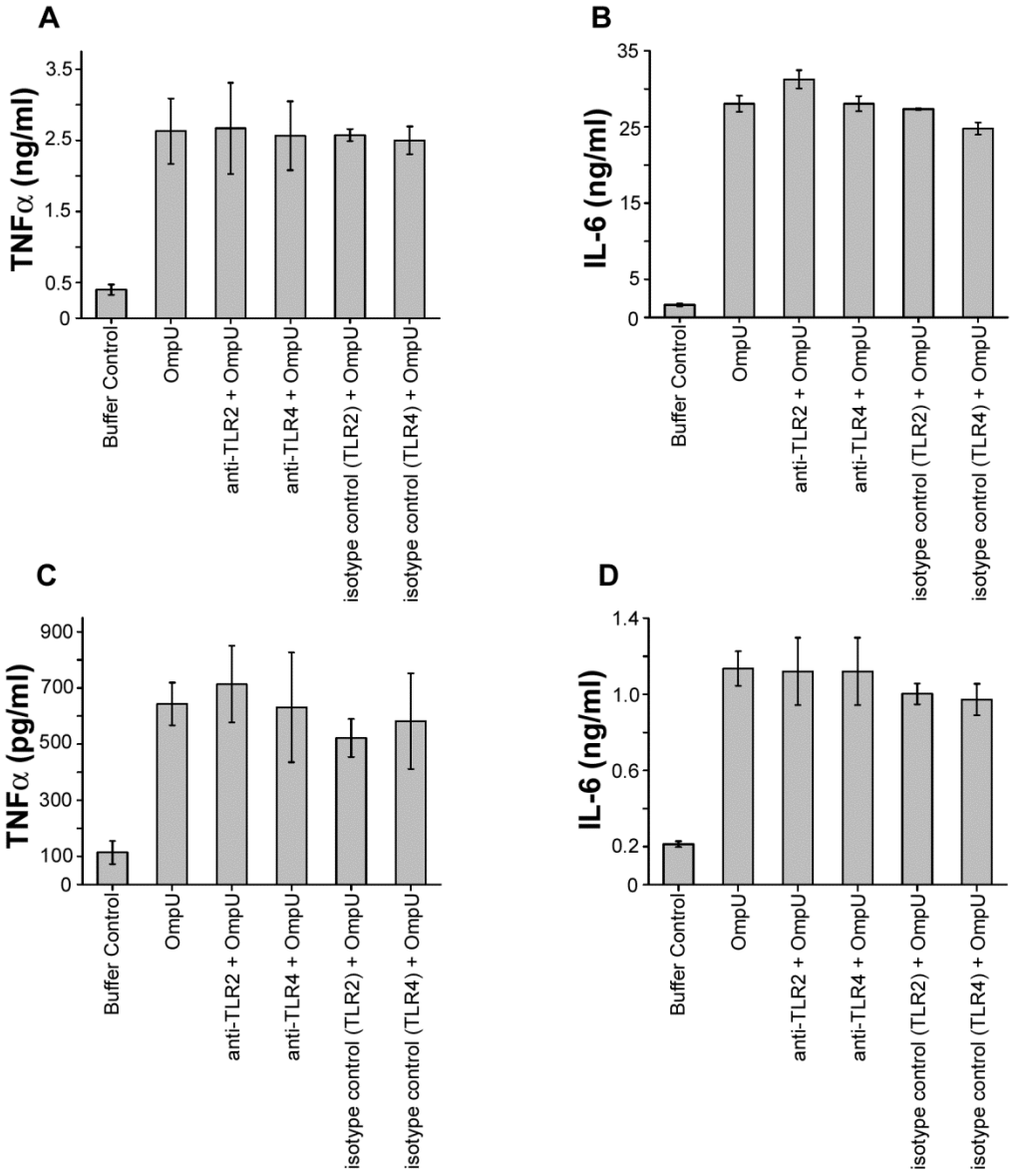


Fig. 18. OmpU induced pro-inflammatory cytokine production in DCs is TLR2 and TLR4 independent.

BMDCs (A,B) and splenic DCs (C,D) were treated anti-TLR2 and anti-TLR4 neutralizing antibody and after 1 h of incubation, cells were treated with 2 μg/ml OmpU and incubated for 24 h. Cells treated with buffer and isotype antibody served as controls. Collected supernatants were analyzed for TNFα (A,C) and IL-6, (B,D) levels by ELISA. Results are expressed as mean ± SEM and represent the average of three independent experiments.

5.3.4 OmpU-induced TNFα and IL-6 in BMDCs is mediated by MAPKs, p38 and JNK and transcription factors, NFκB and AP-1

The production of pro-inflammatory cytokines TNFα, IL-6, IL-1β, and IL-12, is initiated by two important transcription factors, NFκB and AP-1 [146]. These transcription factors can be activated by MAPKs, p38 and JNK [230]. Further, MAPKs and transcription factors can be activated in a TLR-dependent, as well as, TLR-independent manner [230].

As our data suggested that OmpU activates DCs probably in a TLR-independent manner, we further determined whether MAPKs and these transcription factors are involved in OmpU-mediated pro-inflammatory cytokine production. We determined the involvement of p38 in OmpU-mediated signaling treating BMDCs with p38 inhibitor VX-745. It competes with p38α for ATP and prevents phosphorylation of p38 which is required for its activation [231]. Further, to probe the involvement of JNK in OmpU-mediated DC activation, BMDCs were treated with JNK inhibitor JNKIN8. It forms a covalent adduct with conserved cysteine residue present in all JNK isoforms and prevents them from being in their active conformations [232].

NFκB remains in the cytoplasm in an inactive form bound to its inhibitor IκB. On phosphorylation, IκB is ubiquitinylated and degraded, leading to the release of NFκB. Released NFκB then translocates to nucleus. To probe whether NFκB is involved in OmpU-mediated signaling, BMDCs were treated with NFκB inhibitor MLN4924, which inhibits ubiquitinylation of IκB and prevents NFκB release [233]. In its active form, AP-1 transcription factor is a dimer of a Fos family member and a Jun family member. On phosphorylation c-jun (the most common family member involved in inflammatory responses) translocates to the nucleus and dimerizes with a Fos family member. Inhibitor SP600125 prevents phosphorylation of c-jun by competing with JNKs for ATP [234].

We observed that pretreatment with p38 and JNK inhibitors led to an 85% and 87% decrease in OmpU-mediated TNFα production and an 80% and 40% decrease in IL-6 production, respectively (Fig. 19A, 19B). Similarly, the use of NFκB and AP-1 inhibitors, led to a decrease in OmpU-mediated TNFα levels by 80% and 40% and IL-6 by 63% and 36%, respectively (Fig. 19D, 19E). To ensure that decreased cytokine production was not due to decreased cell health, we performed LDH-release assay. Results indicated that

OmpU and inhibitor treatments were not cytotoxic (Fig. 19C, 19F). These results indicate that OmpU-mediated cytokine production involved transcription factors, NFκB and AP-1, as well as, MAPKs, p38 and JNK.

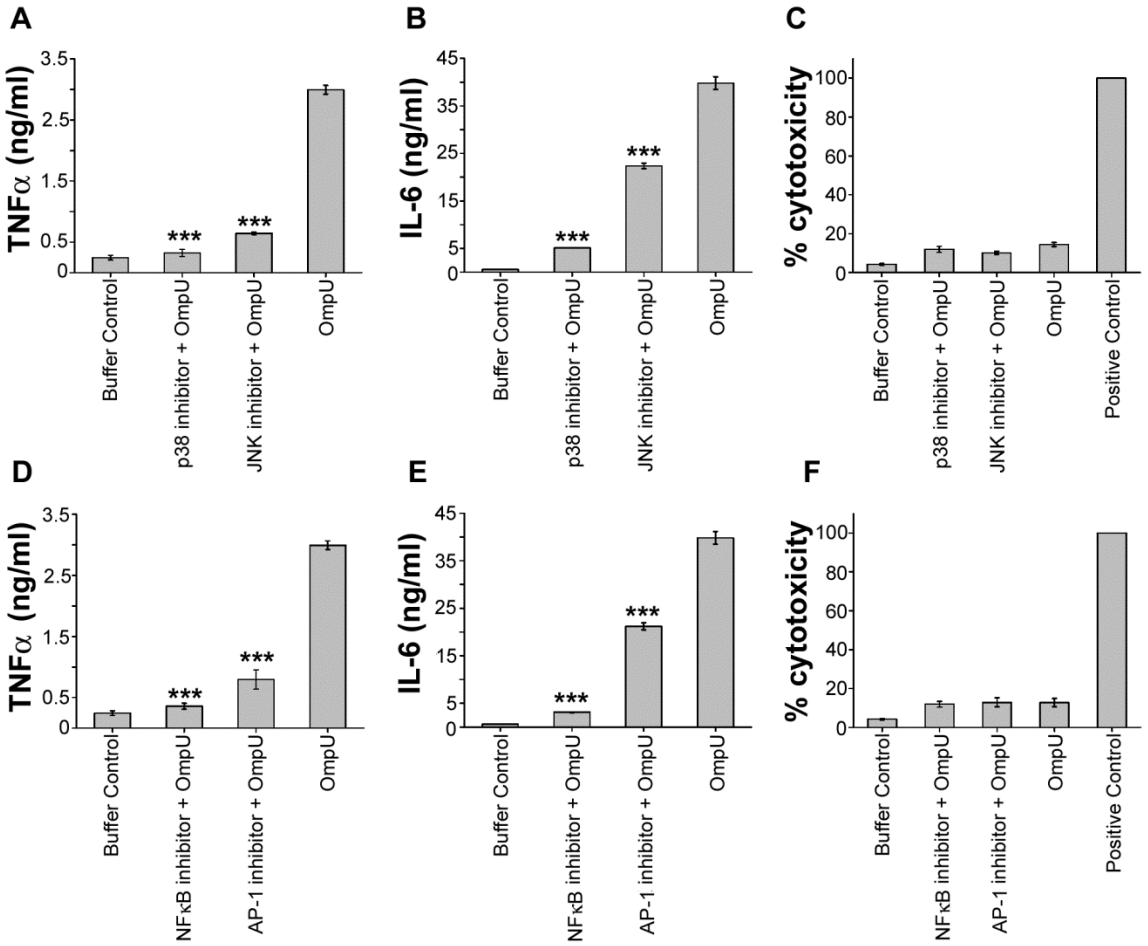


Fig. 19. OmpU-induced TNFα and IL-6 production in BMDCs is dependent on MAPKs, and NFκB and AP-1 transcription factors.

BMDCs were pretreated with p38 inhibitor VX745 (50 nM), JNK inhibitor JNKIN8 (250 nM), NFκB inhibitor MLN4924 (500 nM), and AP-1 inhibitor SP600125 (20 μM) for 1 h. After 1 h, cells were incubated with 2 μg/ml OmpU for 24 h. Collected supernatants were analyzed for TNFα (A,D) and IL-6, (B,E) production by ELISA. With the same treatments, cell viability (C,F) was assessed by LDH-release assay. Results are expressed as mean ± SEM and represent the average of three independent experiments. ***$p < 0.001$ versus OmpU.

5.3.5 OmpU-induced IL-1β in DCs is mediated by MAPKs, p38 and JNK and transcription factors, NFκB and AP-1

As OmpU-mediated TNFα and IL-6 production were dependent on MAPKs and transcription factors NFκB and AP-1, we further probed whether IL-1β production involved a similar pathway. BMDCs and splenic DCs were treated with chemical inhibitors against p38, JNK, NFκB, and AP-1.

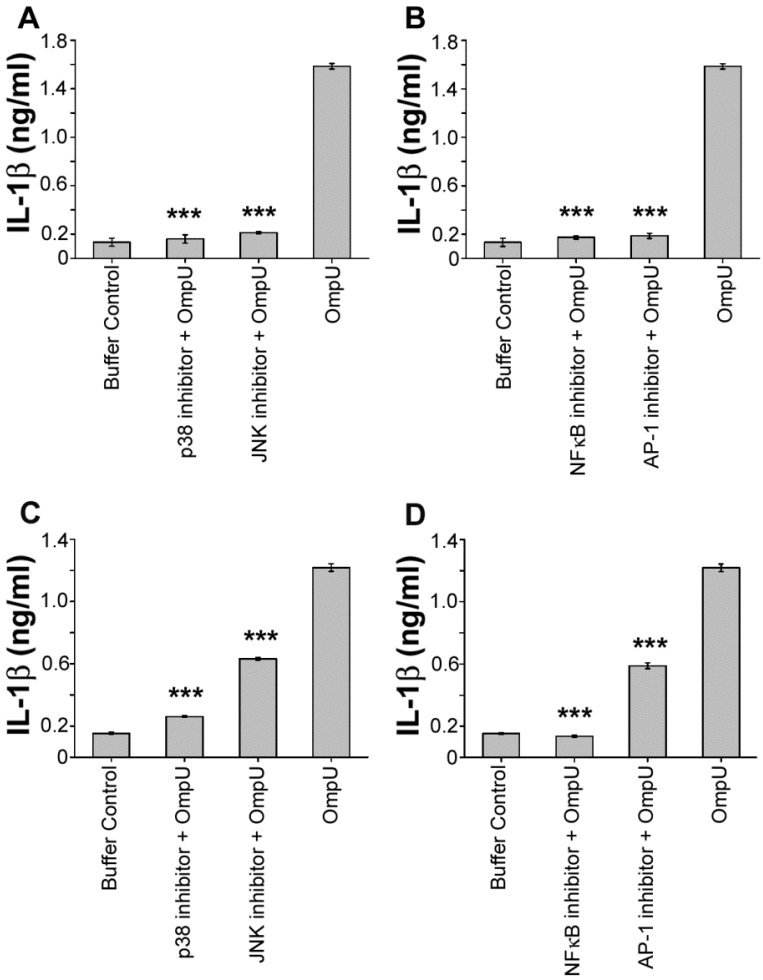


Fig. 20. OmpU induced IL-1β production in DCs is dependent on MAPKs, and NFκB and AP-1 transcription factors.

BMDCs (A), (B) and splenic DCs (C), (D) were pretreated with p38 inhibitor VX745 (50 nM), JNK inhibitor JNKIN8 (250 nM), NFκB inhibitor MLN4924 (500 nM), and AP-1 inhibitor SP600125 (20 μM) for 1 h. After 1 h, cells were incubated with 2 μg/ml OmpU for 24 h. Collected supernatants were analyzed for IL-1β production by ELISA. Results are expressed as mean ± SEM and represent the average of three independent experiments. ***$p < 0.001$ versus OmpU.

Results indicated that the use of MAPK-, as well as, transcription-factor inhibitors led to an approximate 85–90% decrease in OmpU-mediated IL-1β in BMDCs (Fig. 20A, 20B). In splenic DCs, p38 and JNK inhibitors decreased IL-1β by 75% and 50%, respectively (Fig. 20C). Further, OmpU-mediated IL-1β production decreased by 80% and 50% in response to NFκB and AP-1 inhibitors, respectively (Fig. 20D). These results indicate that similar to TNFα and IL-6, OmpU-dependent IL-1β production is also mediated by MAPKs, and transcription factors AP-1 and NFκB in BMDCs and splenic DCs.

5.3.6 OmpU-mediated IL-1β production in DCs is mediated by the NLRP3 inflammasome

As OmpU induced production of IL-1β in DCs, we wanted to probe whether caspase-1 and NLRP3 inflammasome were involved. BMDCs and splenic DCs were pretreated with caspase-1 inhibitor, parthenolide, which directly alkylates p20 subunit and also hampers NLRP3 inflammasome by interfering with its ATPase activity [235]. DCs were also treated with NLRP3 inflammasome inhibitor isoliquiritigenin, which prevents NLRP3-activated ASC-oligomerization [236].

Our data suggest that with the use of caspase-1 and NLRP3 inflammasome inhibitors OmpU-mediated IL-1β levels decreased by 87–89% in BMDCs (Fig. 21A) and by a similar extent in splenic DCs (Fig. 21B). LDH-release assay in BMDCs suggested that the concentrations of both these inhibitors were not cytotoxic to cells (Fig. 21C).

Furthermore, we wanted to assess whether levels of active caspase-1 increased in OmpU-treated DCs. OmpU-treated BMDCs (Fig. 21D) and CD11c-enriched fraction of splenocytes (Fig. 21E) exhibited conversion of pro-caspase-1 to active caspase-1 implicating caspase-1 in IL-1β production. Altogether, these results are indicative of NLRP3 inflammasome and caspase-1 involvement in OmpU-mediated IL-1β in DCs.

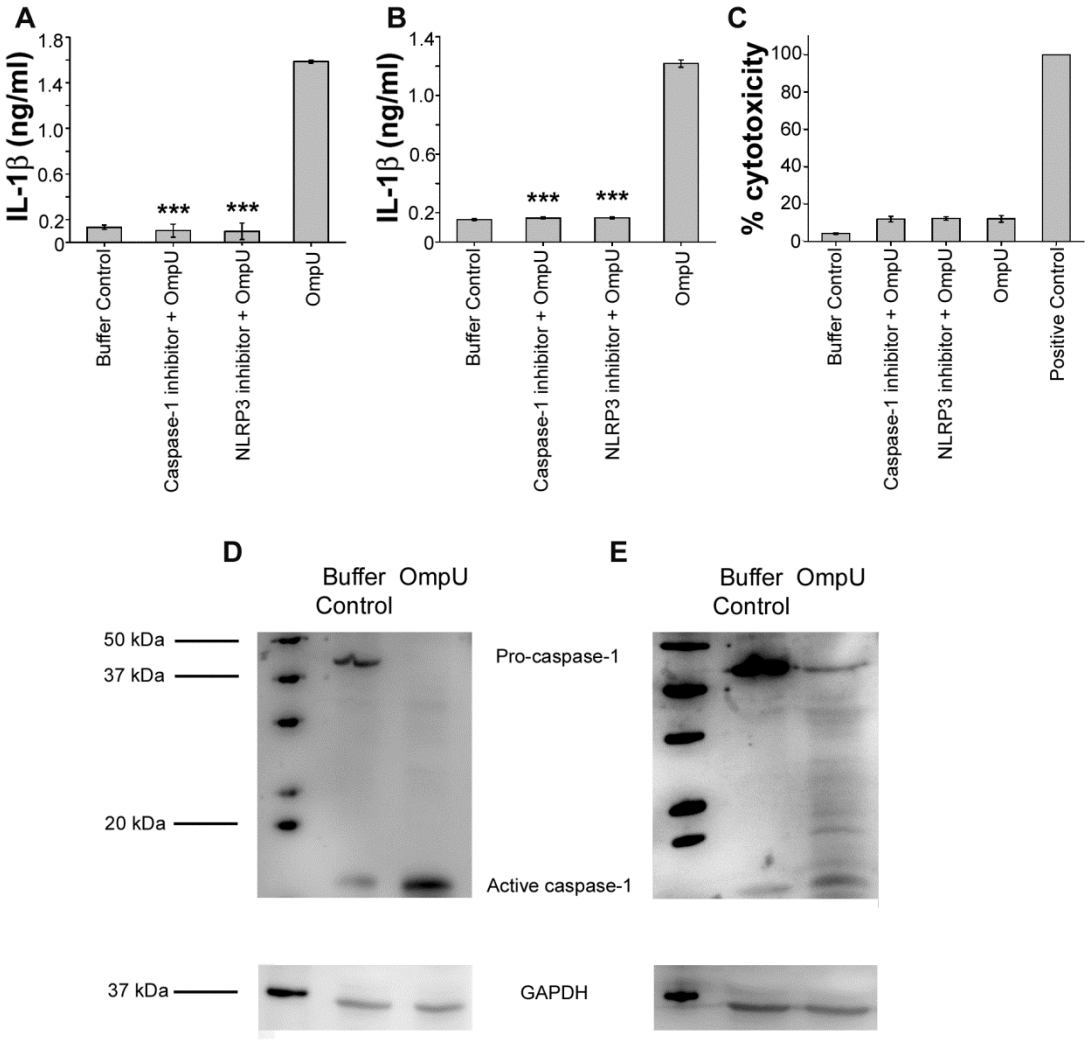


Fig. 21. OmpU induced IL-1β production in DCs is dependent on NLRP3 inflammasome and caspase-1 activation.

BMDCs (A) and splenic DCs (B) were pretreated with, caspase-1 inhibitor parthenolide (18 μM) for 30 min and NLRP3 inflammasome inhibitor isoliquiritigenin (80 μM) for 15 min. After respective time periods, cells were incubated with 2 μg/ml OmpU for 24 h. Buffer-treated and OmpU-treated cells served as controls. IL-1β levels were measured by ELISA in culture supernatants after 24 h. Cell viability (C) after incubation with caspase-1 and NLRP3 inflammasome inhibitor was assessed by LDH-release assay in BMDCs. Results are expressed as mean ± SEM and represent the average of three independent experiments. ***$p < 0.001$ versus OmpU. BMDCs (D) and CDd11c-enriched fraction of splenocytes (E) were treated with 2 μg/ml OmpU for 2 h. Whole cell lysates were prepared and analysed for caspase-1 by immunoblotting. Blots are representative for three independent experiments.

5.4 Conclusions

DCs are important innate immune-response mediators along with monocytes and macrophages, which together form the mononuclear phagocytic system. Our earlier results proved that OmpU induced pro-inflammatory mediator production in monocytes and macrophages. We further investigated whether OmpU activated DCs as well. We studied the effects of OmpU on murine BMDCs and splenic DCs and assessed their CD11c expression by flow cytometry (Fig. 16), which indicates that they were cDCs. OmpU-treated BMDCs secreted pro-inflammatory cytokines, TNFα, IL-6, and IL-1β (Fig. 17).

We further explored whether this activation was TLR2 or TLR4 dependent as OmpU mediates its responses via TLR2 in monocytes and macrophages [177]. With the use of neutralizing antibodies against TLR2 and TLR4, OmpU-mediated TNFα and IL-6 remain unaffected in BMDCs, as well as, splenic DCs (Fig. 17) suggesting OmpU does not mediate its signaling via TLR1/2/4/6 in DCs. We further explored whether MAPKs, p38 and JNK and transcription factors AP-1 and NFκB were involved in OmpU-mediated cytokine production by using chemical inhibitors. With the use of these inhibitors, TNFα and IL-6 levels reduced considerably in BMDCs (Fig. 19). Similarly, as in the case of TNFα and IL-6, MAPKs and transcription factors AP-1 and NFκB were also involved in IL-1β production in BMDCs (Fig. 20A, 20B) and splenic DCs (Fig. 20C, 20D).

As OmpU induced IL-1β production in BMDCs, we examined whether it was mediated by inflammasomes. We used chemical inhibitors against caspase-1 and NLRP3 inflammasome and observed that OmpU-mediated IL-1β secretion decreased substantially in BMDCs, as well as, splenic DCs (Fig. 21A, 21B). Immunoblotting for caspase-1 indicated that OmpU induced conversion of pro-caspase-1 to active caspase-1, facilitating IL-1β secretion in BMDCs and CD11c-enriched splenocyte fraction (Fig. 21D, 21E). We assessed cytotoxicity in response to various treatments with chemical inhibitors to rule out the possibility that decreased cytokine production was due to decrease cell viability by LDH release assay. Our results showed that cell viability was minimally affected (Fig. 20C, 20F; Fig. 21C).

Altogether, our results indicated that OmpU led to production of pro-inflammatory cytokines TNFα, IL-6, and IL-1β from DCs that is independent of TLR1/2/4/6. Further,

MAPKs, p38 and JNK and transcription factors, AP-1 and NFκB mediate this cytokine response. Additionally, OmpU-mediated IL-1β production is dependent on NLRP3 inflammasome and caspase-1.

Chapter 4: Modulation of adaptive responses by OmpU

6.1 DCs are professional antigen-presenting cells; bridging innate and adaptive responses

The innate immune system provides an immediate and critical anti-microbial response but it is non-specific and short-lived. Another crucial and vital role of the innate immune system involves alerting and activating the adaptive immune branch to initiate a specific, memory-generating, and long-lasting response against microbes and microbial products [96]. One of the ways by which the innate immune system does so is via the interaction of APCs and T cells. T cells are one of the critical mediators of the adaptive immunity. Both cell-mediated and humoral adaptive immune responses are dependent on T-cell activation. $CD8^+$ T cells or cytotoxic T cells (Tc) promote cell-mediated immunity, whereas, $CD4^+$ T cells or T helper cells (Th) shape both cell-mediated and antibody-mediated humoral immunity [237]. APCs usually display extrinsic antigens (as opposed to intrinsic or self-antigens) via MHC-II molecules to $CD4^+$ T cells. Out of the three APCs (macrophages, DCs, and B cells), DCs are highly potent in antigen presentation and are called professional APCs. In an immature condition, DCs can capture antigens but are poor in presenting them to T cells. However, in their mature state, DCs act as professional APCs [238]. This is due their greater capacity to recognize and phagocytose/endocytose microbes and PAMPs. PAMP-activated DCs usually undergo maturation and display enhanced co-stimulatory molecules on their surface. They migrate to lymph nodes and activate $CD4^+$ and $CD8^+$ T cells [219]. Thus, DC maturation is a prerequisite for shaping adaptive responses [219].

6.1.1 The interaction of DCs with $CD4^+$ T cells can lead to different immunological outcomes

Three interactions between DCs and $CD4^+$ T cells are essential for optimal Th-cell activation. The first entails the interaction of antigenic peptide-loaded MHC-II (MHC-II–Ag) with T-cell receptor (TCR) complex. The second includes interaction of co-stimulatory molecules. These two interactions usually lead to secretion of cytokines, which augments functions of both cells (Illustration 19) [239,240].

The interaction of co-stimulatory molecules is decisive for T-cell activation. Several co-stimulatory molecule interactions between DCs and T cells are possible, which determine the fate of T cells. If T cells receive the first signal alone, that is, MHC-II–Ag, they become anergic. Anergy can also be induced if co-stimulatory interaction is present but involves T-cell suppressive molecules, such as, CTLA-4 or PD-1 [241]. The interaction of T cell Fas ligand with Fas receptor present on APC, leads to yet another distinct outcome, which involves apoptosis of T cells [242].

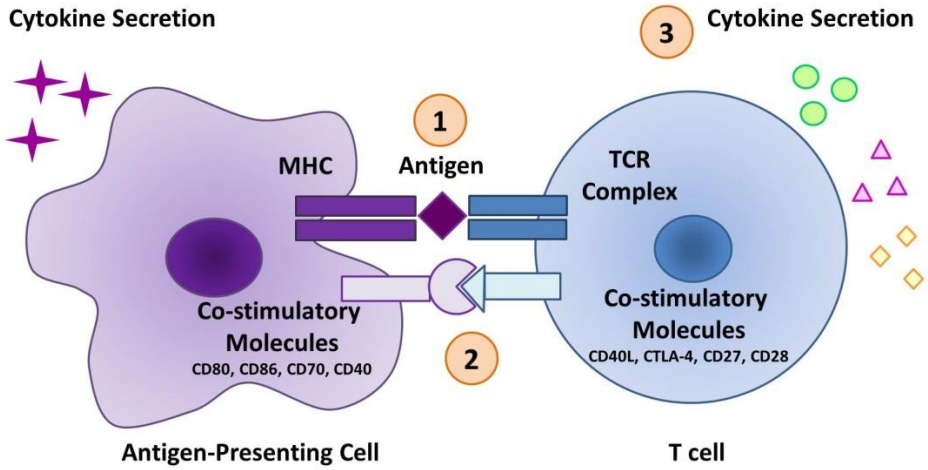


Illustration 19. APC–T cell interaction: MHC and co-stimulatory molecules anti-presenting cells

T cells therefore, require precise co-stimulatory molecule interaction in order to be optimally activated. These interactions usually involve CD80-86 with CD28, CD40 with CD40L, ICOSL with ICOS present on DCs and T cells, respectively [241]. Interaction of CD80-86 with CD28 provides the initial signal and has wide spread effects, such as, proliferation and enhanced effector functions. It provides a synergistic signal and activates transcription factors NFκB, AP-1, and NFAT that control T-cell proliferation and differentiation. CD28 signaling controls expression of T-cell cytokines and chemokines, as well as, downstream co-stimulatory molecules, such as, CD40L and ICOS [243]. This ensures the delivery of a ‘second wave’ of signals within hours or days of the initial CD28 interaction with CD80-86 in secondary lymphoid organs or after migration to tissues. These long-term effects shape effector T-cell functions and memory [243].

Activated T cells undergo clonal expansion on successful antigen presentation, which amplifies the response.

6.1.2 Interaction of DCs and Th cells shapes subsequent adaptive responses

Depending on the nature of PAMPs, DCs can secrete several cytokines. These cytokines determine the type of immune response generated by adaptive cells, both T and B cells. Initiator cytokines secreted by DCs act on T cells, which further secrete various effector cytokines. A combination of different initiator cytokines can polarize T cells toward different phenotypes (Table 6). Coffman, Mosmann, and Bottomly [244] during the 1980s proposed that two different types of T-cell polarizations, Th1 and Th2 that promote classical delayed-type hypersensitivity and allergic reactions, respectively. Additionally, they postulated that the development of one kind of polarization inhibits the other and vice versa. However, currently, there are about seven different types of T-cell polarizations known: Th1, Th2, Th9, Th17, Th22, Treg, and Tfh. These phenotypes perform a number of biological functions [245] (Table 6).

6.1.2.1 Th1 cells: Th1 cells secrete pro-inflammatory cytokines IFNγ and TNFα, which stimulate innate and T-cell responses. They promote IgG2 production by B cells that is important for opsonization responses. Th1 response is dominated by cell-mediated form of immunity including cytolytic activity [246]. Further, this response is crucial for elimination of intra-cellular pathogens, such as, *Lesihmania* and *Mycobacterium*, *S. typhimurium*, *Listeria monocytogenes* (bacteria), *C. neoformans* (fungus), herpes simplex and influenza virus. Th1 cells are also involved in anti-tumor responses [247].

6.1.2.2 Th2 cells: Th2 cells produce IL-4, IL-5, IL-9, IL-10, and IL-13 and promote IgG1 and IgE class switching of B cells, as well as, recruitment of eosinophils [248]. Unlike Th1 responses, Th2 responses are humoral-mediated. These responses are important in mucosal immunity and against extra-cellular pathogens, such as, helminths and nematodes [249].

6.1.2.3 Th17 cells: Th17 cells produce IL-17, IL-21, and IL-22. Th17 cells are important for clearance of extra-cellular and intra-cellular pathogens. IL-17 is involved in neutrophil recruitment [250]. IL-21 is important for IgE class switching and IL-22 plays a role in acute inflammation and immune-cell proliferation [251].

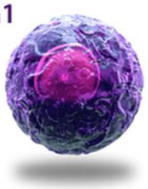
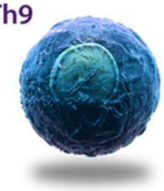
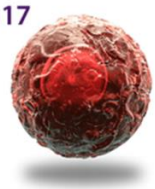
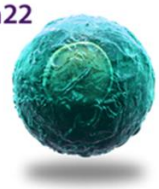
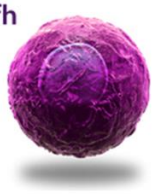
Initiators	Th type	Effectors	Processes
IL-12 IFNγ IL-18 IL-27	Th1 	IL-2 IFNγ TNFα	Macrophage activation Killing of intracellular pathogens
IL-2 IL-4 IL-33	Th2 	IL-3 IL-4 IL-5 IL-6 IL-10 IL-13 IL-25 IL-31	Antibody production by B cells against extracellular parasites Eosinophil activation and response against extracellular parasites
IL-4 TGFβ	Th9 	IL-9 IL-10	Immune response against extracellular parasites
TGFβ IL-6 IL-1α IL-1β IL-21 IL-23	Th17 	IL-17 IL-21 IL-22 IL-26	Immune response against extracellular bacteria and fungi
IL-6 TNFα IL-23	Th22 	IL-13 IL-22	Keratinocyte proliferation and epidermal hyperplasia
IL-2 TGFβ	Treg 	IL-10 TGFβ IL-35	Immunosuppression and tolerance
IL-6 IL-21 IL-27	Tfh 	IL-6 IL-10 IL-21	Promotion of germinal center responses and B cell class switching

Table 13. Different T-cell polarizations and their biological effects [252]

6.1.2.4 Treg cells: Treg (T regulatory) cells develop in the thymus and express CTLA-4 and GITR ligands, IL-10, and TGFβ important for immune-suppressive responses [182]. Their main role is to maintain tolerance to self-antigens, as well as, to restore immune homeostasis after pathogen clearance [253].

6.1.2.5 Tfh cells: Follicular helper T cells are present in germinal centers near B-cell zones. They promote antibody responses and memory B-cell generation [254].

6.1.2.6 Th9 cells: Th9 cells produce IL-9 and IL-10. Similar to Th2 cells, they are involved in intestinal response to helminths [255].

6.1.2.7 Th22 cells: Th22 cells are recruited to skin where they mediate tissue repair as well defense against skin pathogens [256].

6.2 Objectives

Our previous results indicated that OmpU behaves as a PAMP and activates pro-inflammatory responses in DCs although we could not determine the PRR responsible for DC activation. As PAMP–PRR interaction is not only important for DC activation but also for DC maturation, we further explored different aspects of DC maturation in context of antigen presentation and studied whether OmpU-primed DCs can shape T-cell responses. We assessed whether OmpU affected antigen-presenting characteristics of DCs, such as, co-stimulatory molecule expression and $CD4^+$ T-cell activation and polarization.

6.3 Results

6.3.1 OmpU induces maturation of DCs and macrophages

We determined whether OmpU affected surface expression of co-stimulatory molecules, CD80 and CD86 on APCs, such as, DCs and macrophages. We assessed expression of both these molecules in RAW 264.7 cells, peritoneal macrophages, and BMDCs. OmpU-treated macrophages displayed enhanced CD80 expression (Fig. 22A, 22B), whereas BMDCs expressed increased levels of CD80, as well as, CD86 molecules (Fig. 22C). OmpU thus, leads to APC maturation thereby, suggesting that OmpU may assist antigen presentation.

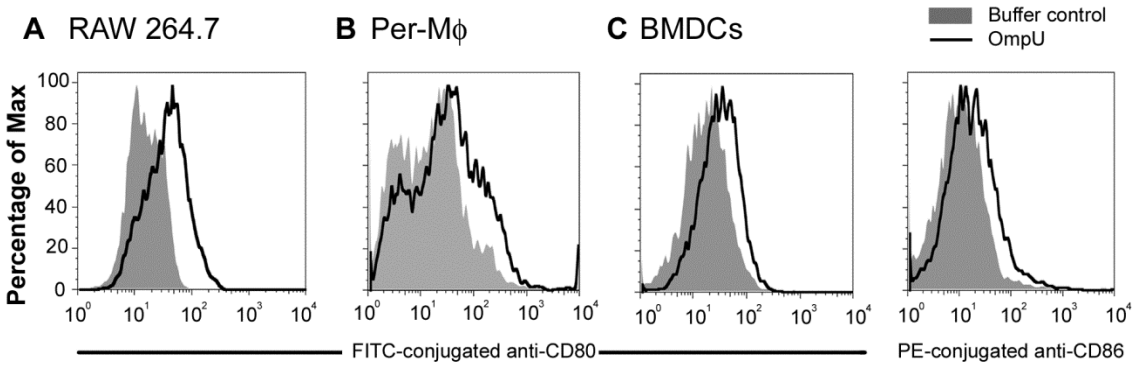


Fig. 22. OmpU enhances surface expression of co-stimulatory molecules in macrophages and BMDCs.

RAW 264.7 (A), mouse peritoneal macrophages (B), and BMDCs (C) were treated with 2 μg/ml OmpU for 24 h. Following incubation CD80- and CD86-surface expression was assessed by flow cytometry. Histograms are representative of three independent experiments.

6.3.2 OmpU induces a high IL-12/IL-10 production ratio in murine BMDCs

DCs can secrete initiator cytokines, such as, IL-12 or IL-10 that can polarize T cells toward Th1 or Th2, respectively. We determined levels of these two cytokines in response to OmpU in BMDCs. OmpU elicited higher IL-12 levels (9 ng/ml) than IL-10 (2.6 ng/ml) (Fig. 23) in BMDCs. IL-12 is important for Th1 type of responses that promote macrophage activation and cell-mediated killing. Thus, suggesting that OmpU might polarize T cells toward Th1 type.

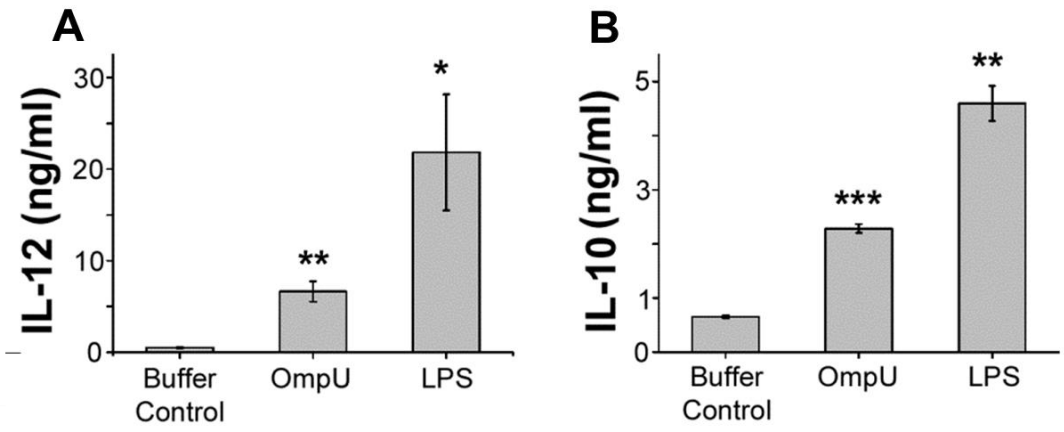


Fig. 23. OmpU induces high IL-12/IL-10 ratio in BMDCs.

BMDCs were treated with 2 μg/ml OmpU for 24 h. Buffer-treated and LPS-treated (1 μg/ml) cells were served as negative and positive controls, respectively. Levels of (A) IL-12 and (B) IL-10 were measured by ELISA in culture supernatants after 24 h. Results are expressed as mean ± SEM and represent the average of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus buffer control.

6.3.3 OmpU-primed splenic DCs polarize CD4$^+$ T cells toward Th1-type response in vitro

We further explored whether OmpU could shape T-cell polarization. To determine whether DCs can present OmpU-derived peptides to CD4$^+$ T cells and can activate them, we co-cultured murine OmpU-primed splenic DCs with murine CD4$^+$ T cells *in vitro*. We observed an increase in T-cell proliferation in terms of increased BrdU-incorporation (Fig. 24A). Thus, suggesting that OmpU possesses the ability to activate T cell-mediated immune responses. As OmpU induced a strong IL-12 signal in DCs, we probed whether OmpU could polarize T cells toward Th1 type. Therefore, we assessed IFNγ, TNFα, IL-10, and TGFβ levels in co-culture supernatants by ELISA. We observed that OmpU lead to the secretion of IFNγ, the hallmark of Th1 polarization responses (Fig. 24B). However, we did not observe production of other cytokines assessed. Therefore, OmpU might promote cell-mediated immunity *in vivo*.

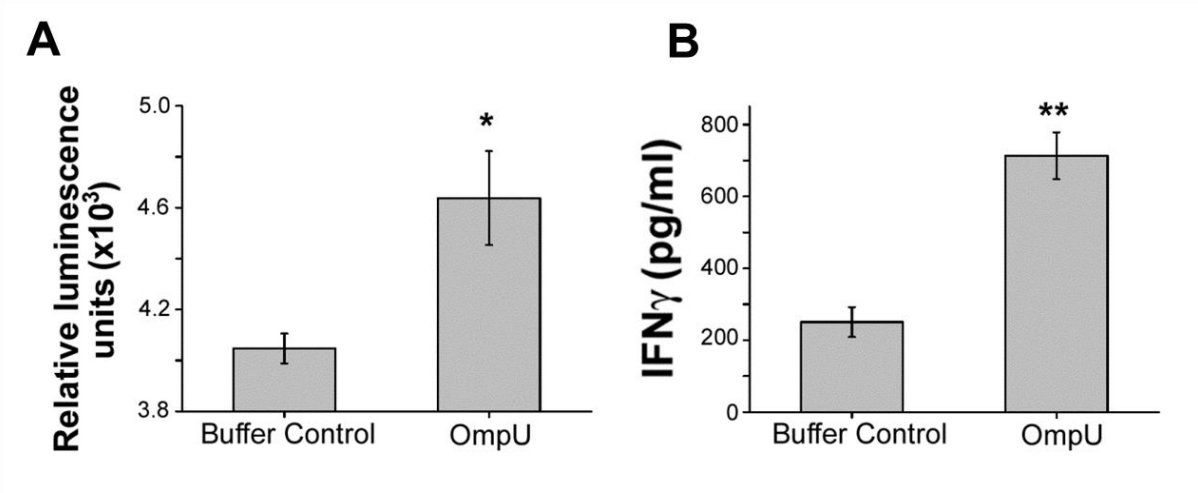


Fig. 24. OmpU-primed DCs increase CD4$^+$ T-cell proliferation and induce IFNγ secretion in vitro.

Splenic DCs were treated with 2 μg/ml OmpU for 24 h and co-cultured with splenic T cells *in vitro* for 5 days. (A) T-cell proliferation was measured by BrdU-incorporation assay. (B) IFNγ levels in culture supernatants were measured by ELISA. Results are expressed as mean ± SEM and represent the average of three independent experiments. *$p < 0.5$, **$p < 0.01$ versus buffer control.

6.4 Conclusions

Our previous results highlighted the innate immune responses in terms of pro-inflammatory effects of OmpU in monocytes, macrophages, as well as, DCs. We wanted to explore whether OmpU can modulate adaptive responses as well. We first studied whether OmpU affected co-stimulatory molecule expression in macrophages and DCs. Our results indicated that OmpU enhanced surface expression of CD80 in macrophages (RAW 264.7 and peritoneal macrophages) (Fig. 22A, 22B) and that of CD80-86 in BMDCs (Fig. 22C). We further assessed whether OmpU secreted high levels of IL-12 or IL-10 in BMDCs. We observed that OmpU induced higher levels of IL-12 than IL-10 suggesting a Th1 type of response (Fig. 23). This prompted us to determine whether OmpU could affect CD4$^+$ T-cell responses. Co-culture of OmpU-primed splenic DCs with splenic CD4$^+$ T cells, led to T-cell proliferation (Fig. 24A) and increased levels of IFNγ in culture supernatants (Fig. 24B). Altogether, these results suggest that OmpU assists antigen presentation and might shape Th1 polarization *in vivo* as well.

IV

Discussion

7.1 Discussion

Porins are crucial for maintaining bacterial homeostasis as they mediate solute transport. They are present at the interface of bacteria and its environment and hence perform several roles related to bacterial protection. Apart from their channel property, porins can mediate bacterial survival and pathogenesis by aiding in adherence, invasion, and apoptosis of host cells. OmpC and OmpF of *E. coli* and *S. typhimurium*, *V. alginolyticus* OmpU, and *V. harveyi* OmpU are involved in antibiotic resistance [61,66]. *S. typhimurium* OmpC and *V. vulnificus* OmpU mediate adherence to host cells [70,73]. *V. splendidus* OmpU, *S. typhimurium* OmpD, *K. pneumoniae* OmpK36, and *S. flexneri* OmpC aid in invasion of host cells [108,257,258]. *N. gonorrhoeae* PorB and *P. aeruginosa* porin induce apoptosis in target cells [259,260].

Further, porins can behave as PAMPs, as sequences of most porins are identical to each other by atleast 50% [261]. All gram-negative bacterial porins share approximately 60% β-sheet content. Enterobacterial porins contain a characteristic PEFGGD amino acid signature sequence in their L3 loop. The OmpC and PhoE porins share 80–90% sequence similarity across different genera, whereas, OmpC, PhoE, and OmpK36 show 60% sequence similarity [262]. *Vibrio* falls under the γ-proteobacteria class. Porins from γ-proteobacteria show strong similarity to each other and contain short (10-residue) extensions at their N termini (*Vibrio cholerae* OmpU is an exception). When multiple porins from this class are aligned on the basis of average hydrophobicity and hydrophobic moment (by Gibbs motif sampling program), the *Vibrio–Photobacterium* group is unusual as they contain exceptionally long L3 loops [68]. OmpU shares around 50–70% sequence similarity across *Vibrio* species.

Porins can be recognized by PRRs present on host immune cells and can hence modulate immune responses. Porins from several gram-negative bacteria, such as, *Brucella abortus*, *Fusobacterium nucleatum*, *Haemophilus influenzae*, *H. pylori*, *K. pneumoniae*, *Neisseria*, *Pasteurella*, *Pseudomonas*, *Salmonella*, and *Shigella* species activate mouse and human macrophages and IECs [69,75,76,78,79,81,263-276]. These porins induce a pro-inflammatory response in terms cytokine and chemokine production, such as, TNFα, IL-6, IL-1β, IL-8, MIP-1α, MIP-1β, and RANTES. Furthermore, *Salmonella* porins, although lead to secretion of pro-inflammatory mediators, can also induce an immuno-suppressive cytokine, IL-10 [87].

Vibrio porin OmpU is an important mediator of host–pathogen interactions across several species as mentioned above. *V. cholerae* OmpU plays multiple roles in pathogenesis. It confers resistance against bile acids and anti-microbial peptides in the gut [105-107,139,277-280]. Its expression is positively regulated by the master virulence-gene controller, ToxR [39,50,59,281,282]. Furthermore, OmpU is expressed by pathogenic strains only [111] and its sequence is conserved among epidemic strains [113]. Apart from being present in the *V. cholerae* outer membrane, OmpU is also a dominant protein in naturally secreted vesicles. Moreover, vesicles administered in mice lead to protective responses [114,115]. Antisera of cholera patients contain antibodies against multiple OMPs including OmpU [116]. Inspite of multiple roles of OmpU in pathogenesis, it has not been characterized in terms of host immune responses and thus, is an important candidate for immunological study.

We initially wanted to determine whether *V. cholerae* porin OmpU could affect inflammation, which is one of the primary responses of the innate immune system. We assessed whether OmpU could activate the critical mediators of inflammation, monocytes and macrophages, in terms of pro-inflammatory mediator production. Our data indicated that OmpU classically activates monocytes and macrophages as it induced secretion of inflammatory mediators, such as, NO (Fig. 1), TNFα (Fig. 2), and IL-6 (Fig. 3) by increasing the mRNA expression of nitric oxide synthase 2 (enzyme that catalyzes NO production), TNFα, and IL-6 [142]. Thus, apart from its role in bacterial survival by conferring resistance against bile acids and anti-microbial peptides, we for the first time have characterized the pro-inflammatory role of a *V. cholerae* porin and establish that it behaves similarly to other gram-negative bacterial porins in terms of its ability to activate monocytes and macrophages. Further, OmpU up-regulates gene expression of important granulocyte-activating and neutrophil-recruiting chemokines, such as, MIP-1α, MIP-1β, and RANTES in monocytes and macrophages [177]. The production of these pro-inflammatory effectors is mediated by TLR1/2 heterodimer [177], suggesting that OmpU behaves as a PAMP. Furthermore, Δ*ompU V. cholerae* strains exhibit decreased pro-inflammatory cytokines and chemokines in human IEC line [117-120,283,284]. Recently, OmpU has been reported to induce caspase-independent programmed cell death by translocating to the mitochondria of host cells [285]. All these findings suggest that OmpU is an important pathogenic mediator of *V. cholerae*.

Although PAMPs can induce pro-inflammatory responses in monocytes and macrophages, certain PAMPs can also induce tolerance in these cells. Tolerance is defined as a refractory state of monocytes and macrophages due to subsequent challenges by pro-inflammatory stimuli [157]. Tolerant cells exhibit decreased pro-inflammatory responses and was first reported by Paul Beeson. He observed that the fever induced by typhoid vaccines in rabbits reduced on repeated injections [286]. A similar phenomenon was observed when humans recovering from typhoid and malaria were challenged with LPS, a common PAMP present in the gram-negative bacterial outer membrane [287,288]. Studies in mice were the first to implicate monocytes and macrophages in this response [289,290]. Several other PAMPs, apart from LPS, have been reported to induce tolerance, which include cell-surface PAMPs, such as, peptidoglycan (PGN), lipoarabinomannans (LAM), lipoteichoic acid (LTA), and intra-cellular PAMPs, such as, double-stranded RNA and DNA [202,291-296].

Interestingly, we observed that OmpU-pretreated cells showed suppressed pro-inflammatory mediator production when stimulated with LPS [142]. OmpU-pretreated RAW 264.7, THP-1, and human PBMCs exhibited decreased NO, TNFα, IL-6, and IL-12 production when challenged with LPS (Fig. 5) with the exception of IL-6 in case of human PBMCs. Furthermore, this phenomenon also occurred in human IEC line, HT29 in terms of TNFα and IL-8 (Fig. 6). During infection, OmpU is present, along with LPS, on the bacterial surface, as well as, in naturally secreted vesicles. Our study therefore, suggests that *in vivo* OmpU might aid in pathogenesis by decreasing the inflammatory response. This could involve decreased respiratory burst, inefficient recruitment of leukocytes, and reduced acute phase-response initiation. Further, down-regulation of IL-12 suggests that OmpU might prevent Th1 responses that mediate phagocyte-dependent inflammatory processes.

Toward probing the mechanism responsible for this phenomenon, we had two hypotheses, that either OmpU was polarizing cells toward an anti-inflammatory phenotype (M2 polarization/alternate activation) or OmpU was inducing tolerance in these cells. Macrophage tolerance and alternate polarization are important mechanisms by which the damaging effects of inflammation are curbed *in vivo*. Inflammation leads to production of pro-inflammatory mediators that create a toxic environment for pathogens. However, unchecked inflammation harms the host tissues as well. Tolerant and

alternatively activated macrophages ensure that the process remains measured and optimal. Subsequently, they help in tissue remodeling and repair of host tissues after an insult [157,158,164]. Although, tolerant and alternatively activated macrophages exhibit a similar phenotype, they differ in their induction and transcriptional gene programming. While these mechanisms are protective responses initiated by the host, pathogens can exploit them to prevent the cytotoxic inflammatory response. *L. donovani*, *M. tuberculosis*, African trypanosomes, *C. burnetti*, Dengue virus and Ross River virus, and *C. neoformans* induce alternate activation of macrophages [168-170,297-299]. Further, *Francisella tularesis* and parasites, such as, *L. major* and *Toxoplasma gondii* induce macrophage tolerance [292,300-302]. Both these parasites infect macrophages, within which, they develop and multiply. Macrophage tolerance thus, helps in their survival.

Tolerance can also be induced by several host mediators as well. Adiponectin is a protein hormone that regulates glucose and fatty acid breakdown. It is a soluble defense collagen secreted by adipocytes in bone marrow stromal cells. It acts as a negative regulator of myelomonocytic progenitor cells (macrophage progenitors) and inhibits functions of macrophages. By inducing tolerance in these cells, it prevents atherosclerosis, a metabolic pro-inflammatory disease [303-306]. Another host molecule involved in tolerance is heat shock protein 70 (Hsp70). Though, Hsp70 was initially identified as a chaperone protein, levels of Hsp70 increase in the cytosol during a stress response leading to tolerance. This could possibly affect inflammatory responses initiated after trauma [291]. Furthermore, apart from these two host molecules, hyaluronic acid, a component of the extra-cellular matrix, is released during tissue remodeling and induces monocyte and macrophage tolerance. It mediates tolerance via CD44 and is important for the resolution of inflammation at the site of tissue repair. [307]. Tolerance can also be induced by non-cellular components, such as alcohol. Acute alcohol exposure induces endotoxin tolerance and may prevent progression of alcoholic liver disease, whereas chronic alcohol consumption leads to the disease [308]. All these findings suggest that PAMPs, as well as, host proteins can employ macrophage tolerance for their benefit.

In some studies of endotoxin-mediated tolerance, researchers have reported overlapping features of M2 polarization and tolerance phenomena. But, Rajaiah *et al*. suggested that tolerance can be established in absence of IL-4, an important inducer of M2 polarization [175]. However, Pena *et al*. [173] and Porta *et al*. [174] have reported that M2-associated

chemokines, such as, CCL17, CCL22, and CCL24 are up-regulated in LPS-tolerant macrophages/monocytes, whereas, in our study we did not observe an increase in gene expression of any M2-associated chemokines including those of CCL17, CCL22, or CCL24 in OmpU/LPS-treated cells as compared to buffer/LPS-treated cells (Fig. 8). Therefore, our results pointed out that tolerance may be induced independently of M2 polarization.

Immune suppressive cytokines, IL-10 and TGFβ, have been associated with M2 polarization, but this association is debatable [309,310]. Even though these cytokines share overlapping features with M2-activated macrophages, the orchestration varies in many ways. Furthermore, monocytes pretreated with IL-1, IL-10, and TGFβ become tolerant to subsequent challenges with LPS [311,312]. Thus, cytokines secreted by macrophages in response to a tolerizing agent, help in reprogramming of macrophages, rendering them tolerant. Increased levels of IL-10 and TGFβ have been implicated in endotoxin- [174,313-315] and adiponectin-mediated tolerance [304]. Our results indicated that OmpU-induced IL-10 secretion plays a partial role in the down-regulation of LPS-mediated TNFα production in human PBMCs (Fig. 9). However, considering these results together, we cannot state whether this IL-10 involvement can indeed be justified as a M2 marker in our case, as other typical M2 markers and TGFβ showed no involvement (Fig. 8).

Tolerizing agents usually alter TLR signaling. Endotoxin-tolerant macrophages display attenuated TLR signaling, which leads to reduction in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines [157,158,316,317]. Apart from endotoxin, other cell surface PAMPs, such as, PGN, LAM, and LTA induce tolerance by attenuating TLR2 signaling [294,295]. Intra-cellular PAMPs, such as, double-stranded RNA, imidazoquinolines (synthetic TLR7–8 agonists) and CpG DNA alter TLR3, TLR7-8, and TLR9 signaling, respectively [202,293,296]. Furthermore, host protein Hsp70 activates innate immunity via TLR4 [291]. In endotoxin tolerance, decreased MyD88 recruitment, IRAK activity, and gene expression have been observed [152,191,293,318,319]. In other cases of tolerance, decreased IRAK-1/MyD88 association, IRAK-1 expression [295,320], as well as, decreased recruitment of TRAF6 to IRAK-1 have been documented [294].

In light of these studies, we assessed TLR and IRAK levels in whole cell lysates by western blotting and did not see any differences in their expression levels in response to OmpU/LPS versus LPS treatments. This prompted us to study whether the association of signaling mediators was being affected. We observed that OmpU-pretreatment attenuated LPS-mediated TLR signaling by decreasing association of TLR6, MyD88, and IRAK1/4 to TLR2 (Fig. 11). One of our earlier reports established that OmpU binds to TLR2 and mediates its signaling via TLR1/2-MyD88-dependent pathway [177]. Therefore, in order to rule out the possibility that the binding of OmpU to TLR1/2 led to decreased availability of TLR2 to bind to LPS (as TLR2 is shared in both cases), we demonstrated by co-immunoprecipitation assay that there was no change in TLR1 association with TLR2 in both buffer/LPS-treated and OmpU/LPS-treated cells (Fig. 11). This suggested that when LPS-activation occurred after OmpU-pretreatment, OmpU does not remain bound with TLR1/2 and TLR2 is free to heterodimerize with TLR6.

As OmpU-pretreatment attenuated LPS-mediated TLR signaling, we probed gene expression of several negative regulators, such as, SOCS-1, SOCS-3, MKP-1, TOLLIP, SHIP-1, and IRAK-M. IRAK-M is a known mediator of tolerance induced by endotoxin, adiponectin, LAM, and alcohol [163,188,189,191,206,294,295,306,308]. Our data indicated that OmpU-induced IRAK-M may probably play a role in the down-regulation of LPS-mediated responses in RAW 264.7 cells (Fig. 12). However, we did not observe involvement of other negative regulators that have been implicated in endotoxin-mediated tolerance [193-206].

TLR signaling usually culminates with NFκB activation, a well-known transcription factor involved in pro-inflammatory responses. NFκB activity is attenuated in LPS-mediated tolerant monocytes and macrophages [192,312,319]. Similarly, NFκB activity is altered in *Lesihmania*-, LAM-, PGN-, alcohol-, and Hsp70-mediated tolerance [171,291,292,295,308]. Our data showed that the translocation of NFκB members, RelA and c-Rel decreases in OmpU/LPS-treated cells as compared to buffer/LPS-treated cells (Fig. 13). Further, AP-1 involvement in tolerance has not been reported in other models of tolerance studied till now. Our results indicated that OmpU enhanced LPS-mediated JunD and Fra-1 members of the AP-1 family (Fig. 13A), which can be involved in the down-regulation in pro-inflammatory responses [214].

After determining the signaling events in OmpU-induced LPS tolerance, we further explored whether certain aspects of macrophage function are affected. Tolerant macrophages exhibit enhanced phagocytic capacity that ensures the removal of pro-inflammatory stimuli, as well as, intact bacteria and helps in resolution of inflammation. Although pathogens may exploit tolerance to prevent pro-inflammatory responses, a non-desired side effect may involve an increase in phagocytic capacity of macrophages. This response was initially observed in case of endotoxin-mediated tolerance [160-163,216]. Our findings showed that OmpU-pretreatment enhanced phagocytosis of RAW 264.7 cells on LPS-stimulation as compared to that of LPS-activated cells (Fig. 14). Enhanced phagocytosis can be attributed to increased surface expression of other surface receptors, such as scavenging receptors, despite attenuated LPS-mediated TLR signaling. This is indeed the case in endotoxin tolerance, in which expression of scavenging receptors, such as, MARCO and SR-A that are responsible for enhanced phagocytosis [192]. Probably, in OmpU-mediated tolerance, scavenging receptors may play a role in this phenomenon.

Another important feature of macrophages is the presentation of phagocytosed antigenic peptides to T cells via MHC molecules, which is important for initiation of adaptive responses. Interaction of CD80–86 co-stimulatory molecules with CD28 present on T cells is crucial in this regard. Decreased expression of co-stimulatory molecule leads to inefficient T-cell activation and reduced pro-inflammatory responses. This helps tolerant macrophages in resolving inflammation. Similar to endotoxin tolerant macrophages [215], OmpU/LPS-treated murine macrophages display decreased co-stimulatory molecule expression (Fig. 15).

Our study demonstrates for the first time that a bacterial porin can induce monocyte and macrophage tolerance. Although OmpU-mediated tolerance induction shares some features with endotoxin tolerance, such as, IRAK-M and IL-10 involvement, it also displays some unique features that are not documented in any other cases of tolerance induction, such as, decreased association of TLRs to form heterodimer, reduced nuclear translocation of NFκB members, increased translocation of suppressive AP-1 family members, and additionally, no association of M2-polarization markers (Illustration 19) [217].

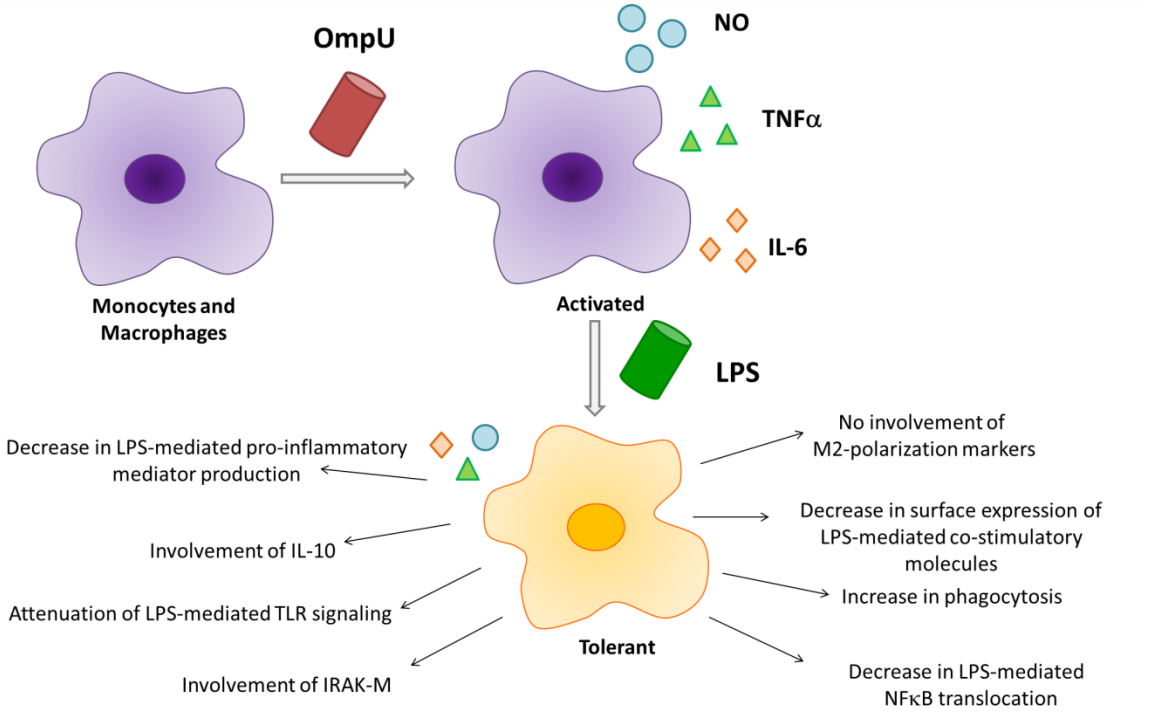


Illustration 19. Summary of OmpU-induced responses in monocytes and macrophages

After studying OmpU-mediated responses in monocytes and macrophages, we further explored its effects on another important immune cell—the DCs. DCs along with monocytes and macrophages form the mononuclear phagocyte system. Moreover, DCs form an important bridge between the innate and adaptive immunity. The functions of DCs can be summarized in two terms: sensors and sentinels. They act as sensors as they respond to a variety of environmental stimuli and undergo differentiation and maturation. As sentinels, they capture and process diverse antigens and migrate to lymphoid tissues, where they present these antigens to T cells. We assessed whether OmpU led to DC activation and maturation. Further, we determined whether OmpU-primed DCs affected T-cell responses.

Our findings indicated that OmpU induced secretion of pro-inflammatory cytokines: TNFα, IL-6, IL-1β, and IL-12 in DCs (Fig. 17, 23A). The production of these cytokines occurred in a TLR1/2/4/6-independent manner (Fig. 18) but involved MAPKs, p38 and JNK (Fig. 19A, 19B, 20A, 20C), and transcription factors, NFκB and AP-1 (Fig. 19D, 19E, 20B, 20D). We have planned future experiments to assess the role of OmpU in eliciting pro-inflammatory cytokine production in MyD88$^{-/-}$ murine DCs and appropriate

TLR2 and TLR4 positive controls. The resulting observations will enable us to confirm or refute the involvement of TLRs completely. Further, we are interested in determing possible PRR(s) that recognize OmpU. The intra-cellular MAPK cascade can be activated in a TLR-dependent or TLR-independent manner. MAPKs can be activated by a variety of stimuli including oxidative stress, osmotic stress, DNA damage, ultraviolet rays, growth factors, mitogens, pro-inflammatory cytokines, PAMP-recognition, etc. [230]. As ROS generated during phagocytosis can activate MAPKs, we examined whether ROS generation plays a role in OmpU-mediated cytokine secretion. On use of N-acetyl cysteine, a ROS scavenger, OmpU-mediated cytokine production was unaffected. Thereby, suggesting that ROS may probably not be responsible for MAPK activation. Our pursuit for the PRR responsible may help us understand whether the MAPK pathway is activated on PAMP recognition or whether other factors are involved.

As our results indicated that OmpU induced IL-1β production in DCs (Fig. 20), we explored the role of inflammasomes in this regard. Inflammasomes are multimeric protein complexes containing NLRs/AIM2, pro-caspase-1, and/or ASC that play a role in IL-1β and IL-18 secretion. IL-1β is transcribed into its pro-form on NFκB activation and is converted into active IL-1β by caspase-1 in a majority of cases. Oligomerization of inflammasome converts pro-caspase-1 into its active form and subsequently leads to IL-1β secretion [227]. Host cells can respond to bacterial infections by activating inflammasomes [220]. The NLRP3 inflammasome is activated by toxins, such as, *Staphylococcus aureus* hemolysins in a TLR-independent manner and *Streptococcus pneumoniae* pneumolysin in a TLR4-independent manner [321,322]. Further, bacterial infections caused by *M. tuberculosis* and *Burkholderia cenocepacia* activate NLRP3 inflammasome as well [323,324]. Moreover, more than one type of inflammasomes can be activated in response to infections. *Aeromonas veronii* aerolysin and type 3 secretion system (T3SS) mediators activate NLRP3 and NLRC4 inflammasomes [325]. Infection with *Listeria monocytogenes* leads to activation of AIM2, NLRC4, and NLRP3 inflammasomes [326]. Therefore, the presence of assorted PAMPs on one microbe may lead to activation of multiple types of inflammasomes.

Our results demonstrated that OmpU-mediated IL-1β was dependent on NLRP3 inflammasome and caspase-1 activation in BMDCs and splenic DCs (Fig. 21). Moreover, OmpU-mediated IL-1β production was dependent on both NFκB and AP-1 transcription

factors (Fig. 20). We are currently confirming involvement of NLRP3 inflammasome by immunoblotting. Further, we will be determining whether OmpU exclusively activates NLRP3 or whether other inflammasomes are involved. Interestingly, one of the reports from our lab proves that OmpU translocates to the mitochondria of host cells [285]. As changes in mitochondria can lead to inflammasome oligomerization [221,327], we will be further probing whether translocation of OmpU to the mitochondria might play a role in this regard. Our results for the first time indicate that apart from toxins, cytolysins, and T3SS effectors, bacterial porins can also activate inflammasomes.

Inflammasome activation in response to several *Vibrio* species has also been studied. *V. parahemolyticus* thermostable direct hemolysin and T3SS molecules trigger NLRP3 and NLRC4 inflammasomes; but two T3SS1 effector proteins, VopQ and VopS induce autophagy to prevent NLRC4 activation [328]. Therefore, pathogens can interfere with inflammasome activation to avoid anti-microbial responses. Further, *V. vulnificus* infection activates the NLRP3 inflammasome in a TLR and NOD1/2-dependent manner that involves NFκB [329]. *V. fluvalis* hemolysin also activates the NLRP3 inflammasome [330]. *V. cholerae* infection involves NFκB-dependent NLRP3 inflammasome activation but is TLR-independent [329]. This activation has been attributed to hemolysin, which is produced by El Tor strains but not by classical strains. Classical strains also lead to IL-1β secretion, albeit in a NLRP3-independent manner [331]. Our results indicate that OmpU may also assist NLRP3 inflammasome formation during *V. cholerae* infection.

So far, we have discussed the importance of DCs in innate immunity and how PAMP recognition activates DCs. This activation of innate receptors also affects another aspect of DC function; PAMP-recognition changes immature antigen-capturing DCs to mature antigen-presenting DCs. Mature DCs migrate to lymph nodes where they shape adaptive responses. Mature DCs are characterized by enhanced expression of co-stimulatory molecules on their surface. They interact with T cells and determine their effector functions and thus, play a critical role in influencing adaptive responses [219].

We observed that OmpU enhanced co-stimulatory molecule expression in BMDCs and macrophages, thereby suggesting APC maturation (Fig. 22). The effect of several porins have been studied in DCs. *Salmonella* porins induce pro-inflammatory cytokines and augment co-stimulatory molecule expression [332,333]. In contrast, *N. meningitidis* PorA induces DC maturation and triggers chemokine expression but not that of

pro-inflammatory cytokines [86]. This response may signify that it does not activate T cells toward Th1 phenotype but rather, helps in recruitment of immune cells. *Acinetobacter baumannii* OmpA enhances co-stimulatory molecule expression, which is independent of TLR2–TLR4 [334]. However, *N. meningitidis* PorB induces co-stimulatory molecule expression and IL-6 production in a TLR2-dependent manner [88]. *Shigella* porins also induce chemokines via TLR2 activation [335]. As mentioned earlier, the conserved sequences present in porins may contribute toward their PAMP nature, which leads to pro-inflammatory responses by monocytes and macrophages. However, the differences in their structure may be responsible for varied responses in DCs as epitopes of porins may differ drastically. Furthermore, macrophages and DCs may respond dissimlarly to the same PAMP [336]. Thus, not all porins may behave in the same way with respect to DC function, even though most porins induce a pro-inflammatory responses in monocytes and macrophages.

Antigen-presenting DCs interact with T cells via two signals that involve MHC-loaded–antigen and co-stimulatory molecules by APCs. After receiving successful signals, T cells release IL-2 which helps in their proliferation [337]. T cells also increase expression of IL-2 receptor on their surface, which enables IL-2 to bind and further activate proliferative pathways. IL-2 can act in an autocrine or paracrine manner and drive the clonal expansion process [338]. Thus, successful recognition of antigen can be assessed by determining T-cell proliferation. As OmpU-activated DCs exhibited increased surface expression of CD80–86 (Fig. 22), we further assessed whether OmpU could affect T-cell proliferation. We observed that co-culture of OmpU-primed murine splenic DCs with murine splenic $CD4^+$ T cells *in vitro* led to an increase in T-cell proliferation (Fig. 24A). This suggests that certain T-cell clone(s) from unimmunized mice can recognize OmpU-generated peptides and lead to clonal expansion *in vitro*. This may be attributed to certain epitopes being conserved among bacterial porins.

Furthermore, OmpU-treated BMDCs secreted high levels of IL-12, which is an important initiator cytokine for Th1 polarization (Fig. 23A). We assessed several cytokines, TNFα, IFNγ, IL-4, IL-10, and TGFβ in supernatants of co-cultured splenic DCs–$CD4^+$ T cells to determine what type of T-cell polarization occurred. OmpU-primed DCs induced secretion of IFNγ from T cells, a hallmark of Th1 polarization (Fig. 24B) suggesting OmpU may promote cell-mediated immunity *in vivo*.

Several studies regarding bacterial porins with respect to T-cell function have been carried out. *N. meningitidis* PorA, is suggested to bias T cells toward Th2 type as it inhibits production of IL-12 in mouse DCs [86]. In contrast, *A. baumanii* OmpA induces IFNγ in mixed lymphocyte reaction and triggers DCs to produce IL-12 [334]. Similarly, *S. dysenteriae* MOMP and *H. pylori* 30 kDa porin induce IFNγ and TNFα from T cells *in vitro* [69,92]. Incubation of human lymphocytes with *S. typhimurium* porins *in vitro* leads to secretion of IFNγ and IL-4 cytokines [263]. However, $CD4^+$ T cells from mice immunized with *S. typhimurium* porins exhibited enhanced IL-4 responses [339]. These results suggest that there might be variation in T-cell responses based on whether porins are administered *in vitro* or *in vivo.* Therefore, administration of OmpU *in vivo* may lead to a different T-cell phenotype.

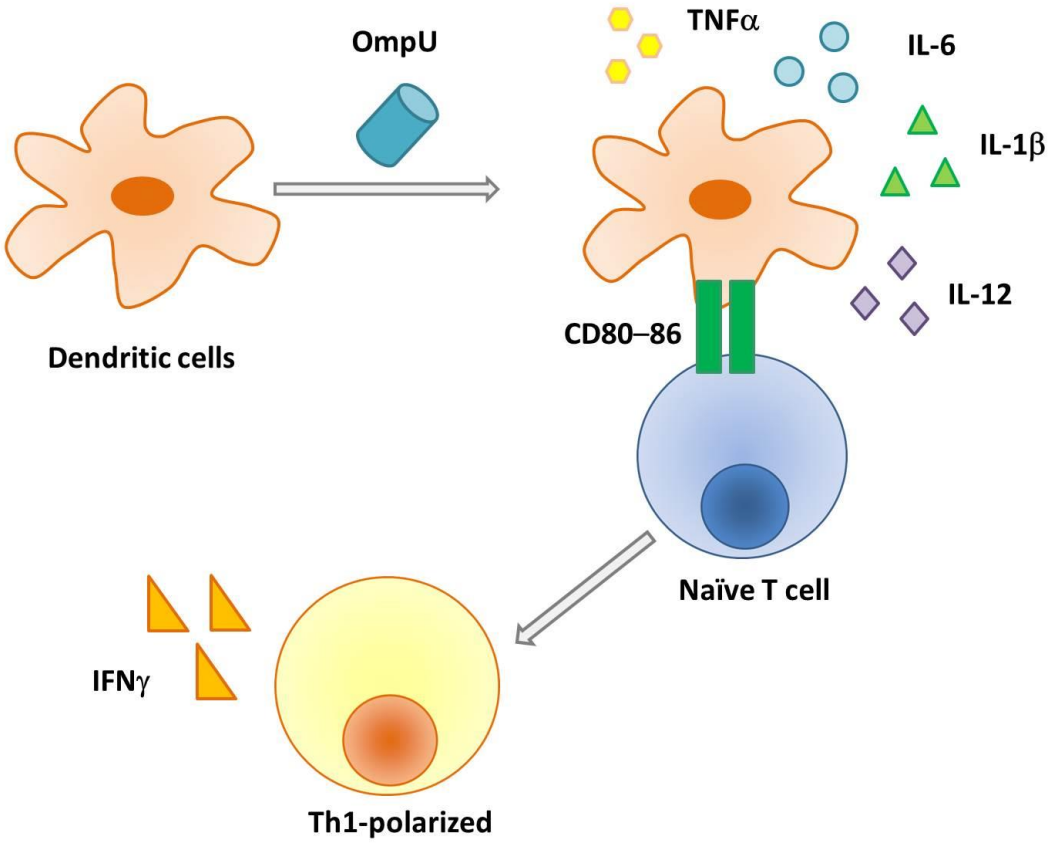


Illustration 20. Summary of OmpU-induced responses in DCs and T cells

Altogether our studies indicate that *V. cholerae* OmpU modulates host immunity by inducing pro-inflammatory responses in monocytes, macrophages, and DCs and polarizes T cells toward Th1, an inflammatory phenotype (Illustration 19–20). However, OmpU

also induces monocyte and macrophage tolerance on subsequent challenge with LPS. Our preliminary studies involving immunization of mice with OmpU have led to interesting results. We immunized mice with OmpU alone and OmpU with complete Freud's adjuvant (CFA) (via intra-peritoneal route and footpad, respectively). Control mice were treated with buffer and/or CFA similarly. After ten days, $CD4^+$ T cells were isolated from inguinal and popliteal lymph nodes and further stimulated with phorbol mystistate acetate (PMA) and ionomycin. We observed that in OmpU/CFA-treated $CD4^+$ T cells there was considerably less IFNγ and IL-4 secretion compared to buffer/CFA-treated mice. CFA contains heat killed *M. tuberculosis* that activates immune cells as it contains multiple PAMPs. As OmpU down-regulated pro-inflammatory responses of another PAMP, LPS in monocytes and macrophages, we hypothesized that OmpU is perhaps down-regulating T-cell functions in presence of other PAMPs as well. To determine if this was the case indeed, we performed a similar experiment but did not administer CFA via footpad, instead administered OmpU alone intra-peritoneally in mice. After similar stimulation of $CD4^+$ T cells isolated from inguinal and popliteal lymph nodes, we observed OmpU completely down-regulated basal levels of IFNγ but had no effect on IL-4 secretion. These initial results suggest that although OmpU-primed DCs induce IFNγ secretion in T cells *in vitro*, OmpU seems to down-regulate IFNγ even in absence of other PAMPs *in vivo*. We are currently exploring these aspects of OmpU on immuno-modulation *in vivo*. Our results pose a question on whether OmpU can be used as a vaccine candidate for cholera or whether cholera vaccine containing heat killed bacterial cells should have the OmpU gene deleted for better protection. As it seems that OmpU interferes with pro-inflammatory responses in presence of other PAMPs and may prevent Th1 responses *in vivo*.

So far, we have discussed the effects of recombinantly purified OmpU on several host-immune cells. During infection, OmpU is a dominant protein present in naturally secreted vesicles, as well as, on the surface of *V. cholerae*. Toward determining the role of OmpU in a physiological setting, we isolated naturally secreted vesicles from the late log phase of *V. cholerae* culture and studied its effects on IECs, the HT29 cell line. Our initial results indicate that these vesicles induce pro-inflammatory cytokine production and also down-regulate LPS-mediated effects; as in the case of OmpU. These effects can be attributed to several PAMPs present in vesicles. To identify the role of OmpU in vesicle-induced responses we intend to study the immuno-modulatory properties of naturally

secreted vesicles from Δ*ompU V. cholerae* and OmpU-incorporated proteoliposomes in IECs. Further, we are interested in investigating the role of OmpU during infection by administering naturally secreted vesicles from Δ*ompU V. cholerae* and OmpU-incorporated proteoliposomes *in vivo*. These studies will increase the current understanding of how OmpU, an important porin involved in host–pathogen interaction across the *Vibrio* species, modulates immune responses.

V

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VI

Synopsis

Synopsis: Study of modulation of host innate and adaptive immune responses by *Vibrio cholerae* porin OmpU

Sanica C. Sakharwade

Introduction

Vibrio cholerae is a gram-negative comma-shaped, facultative anaerobic bacterium that colonizes in the small intestine of the human host and causes an acute diarrheal disease cholera [1]. *V. cholerae*, being a gram-negative bacterium, has an outer membrane comprised of lipopolysaccharides (LPS) and protein complexes [2]. A dominant class of outer membrane proteins is porins, which form β-barrel channels across the outer membrane, are involved in solute transport, and are crucial for maintaining bacterial homeostasis.

OmpU is an important porin present across all *Vibrio* species and, in some of the cases, they are involved in multiple host–pathogen interactions, such as, adherence to host cells, anti-microbial peptide resistance, and antibiotic resistance and few have been proposed as vaccine candidates [3-8]. Out of the various porins present on the surface of *V. cholerae*, the expression of OmpU increases from 30% to 60% in the gut environment. Its expression is positively regulated by the ToxR regulon, which is a master regulator of almost all the virulence genes important for *V. cholerae* pathogenesis [9]. *V. cholerae* OmpU helps in bacterial survival in the gut by conferring resistance against bile acids and anti-microbial peptides [10,11]. The OmpU gene is present in environmental, as well as, clinical isolates [12]. Interestingly, the gene for OmpU is not present in non-pathogenic strains, that is, non-O1 and non-O139 strains [13] and is conserved among epidemic strains [14].

In spite of multiple reports of *V. cholerae* OmpU in host–pathogen interactions, it is poorly characterized in terms of its ability to evoke cellular responses, particularly in the context of the host immune system. Therefore, we wanted to explore whether OmpU could modulate the different aspects of the immune responses. Toward this aspect, we have following two specific aims:

Specific aim I: Whether OmpU possesses the ability to modulate the host's innate immune responses

Specific aim II: Whether OmpU can modulate adaptive immune responses of the host

Specific aim I: To probe whether OmpU possesses the ability to modulate the host's innate immune responses

Innate immune responses are the body's first line of defense against microbial infection. Innate immune cells orchestrate an enormous response together that manifests as swelling, redness, and pain; mainly in the invasion area and is associated with increase in body temperature. This type of innate immune response is known as inflammation. Among the various innate immune cells, macrophages and their precursor, monocytes, are important mediators of inflammation. Monocytes circulating in the blood stream differentiate to become macrophages at sites of inflamed tissues. Macrophages are phagocytic in nature and kill ingested microbes. The phagocytic process involves a respiratory burst that leads to generation of reactive oxygen and nitrogen intermediates (ROI/RNI) that are cytotoxic in nature. One important RNI is nitric oxide (NO), which also mediates vascular permeability. Further, both macrophages and monocytes recognize various pathogen-associated molecular patterns (PAMPs) by specialized pattern-recognition receptors (PRRs). The PAMP–PRR interaction can activate a signaling cascade that leads to release of pro-inflammatory mediators, such as, TNFα (tumor necrosis factor α), IL-6, IL-12, and IL-1β. TNFα production is enhanced by NO. Along with NO, TNFα and IL-1β increase endothelial permeability. TNFα is also an important activator of phagocytosis. TNFα, IL-1β, and IL-6 together mediate induction of fever and the acute phase response from liver. IL-12 activates natural killer (NK) cells, monocytes, and macrophages and is important for their phagocytic function. Further, IL-12 and IL-6 shape adaptive immune responses.

Therefore, toward exploring whether OmpU has the ability to induce pro-inflammatory responses we have under taken the following objectives:

Objective 1: To probe whether OmpU can activate innate immune cells, such as, monocytes and macrophages

We wanted to determine whether OmpU can lead to the production of pro-inflammatory mediators from monocytes and macrophages. We assessed the effect of OmpU in RAW 264.7-murine macrophage cell line, THP-1-human monocytic cell line,

and human peripheral blood mononuclear cells (PBMCs). Cells were treated with recombinantly purified OmpU (rOmpU) for different time periods and with different doses. Cell culture supernatants were collected and levels of three important mediators of the pro-inflammatory responses, NO, TNFα, and IL-6 were determined by Griess reaction and ELISA. Our data indicate that *V. cholerae* porin OmpU can activate monocytes and macrophages as it induces secretion of inflammatory mediators, such as, NO, TNFα, and IL-6 [15].

Therefore, our observations establish that a *V. cholerae* porin, OmpU may behave as a PAMP that induce pro-inflammatory mediator production in monocytes and macrophages.

Furthermore, we observed a dual nature of the *V. cholerae* OmpU in terms of host cell activation. Interestingly, cells pretreated with OmpU and subsequently challenged with another pro-inflammatory agent, LPS, exhibited reduced pro-inflammatory mediator production. Cells were pretreated with OmpU for 24 h, replated in fresh media, and challenged with LPS for different time periods. We observed that OmpU-pretreated LPS-stimulated cells (i.e., OmpU/LPS-treated cells) exhibited decreased NO, TNFα, IL-6, and IL-12 production compared to buffer-pretreated LPS-activated cells (i.e., buffer/LPS-treated cells). Additionally, we established that cell health deterioration was not the cause of the down-regulation phenomenon by performing MTT assay.

Therefore, our observations indicat that OmpU is able to differentially regulate pro-inflammatory mediators.

Objective 2: To determine whether OmpU induces a state of tolerance in monocytes and macrophages

We further wanted to explore the mechanism by which OmpU was attenuating LPS-mediated responses. Toward this, we had two hypotheses, that OmpU-pretreatment followed by LPS-activation (OmpU/LPS-treated cells) was either polarizing macrophages toward M2 state, which exhibits an anti-inflammatory phenotype or was inducing a state of tolerance in monocytes or macrophages. As OmpU was classically activating (M1 polarization) monocytes and macrophages [15,16], we assessed whether subsequent LPS stimulation led to a transition from M1 to M2 phenotype. Alternatively activated macrophages (M2 polarized) are involved in wound repair and regeneration after an

inflammatory insult or can be exploited by pathogens for evasion from anti-microbial responses. Our second hypothesis was whether OmpU/LPS-treatment induces a phenomenon known as tolerance. Macrophage tolerance is defined as the reduced capacity of host (*in vivo*) or of cultured monocytes/macrophages (*in vitro*) to optimally respond to reexposure of an inflammatory stimulus [17]. Both of the phenomena, tolerance and M2 polarization prevent excess inflammation *in vivo* and protect the host from its detrimental effects. However, there are contrasting reports whether M2 polarization occurs during tolerance or not, and whether these two processes are mechanistically different [18,19].

By gene-expression analysis of a panel of M2 markers we observed that OmpU/LPS-treated cells did not significantly enhance mRNA levels of any of the M2 markers as compared to those cells treated with buffer/LPS. Therefore, our results indicated that M2 polarization is not involved in OmpU-mediated down-regulation process.

Suppressor cytokines, such as, IL-10 and TGFβ have been associated with M2 polarization, as well as, tolerance [18,20]. Our results show that neutralization of IL-10 with neutralizing antibody could partially rescue TNFα production in OmpU/LPS-treated PBMCs. However, considering these results together, we cannot state whether IL-10 involvement can indeed be justified as a M2 marker in our case, as other typical M2 markers and TGFβ showed no involvement.

As our above results indicated that OmpU does not induce M2 polarization in LPS-activated cells, we further assessed whether OmpU-pretreatment induces a state of tolerance in LPS-activated cells. As most of the well documented tolerance models involve alteration in the TLR signaling pathway [21], we explored whether TLR signaling is affected in OmpU/LPS-treated cells. Our data indicated that OmpU-pretreatment decreased LPS-induced association of TLR dimer, as well as, the recruitment of the adaptor molecule MyD88 to the receptor complex as compared to buffer/LPS treatment. Further, association of kinases IRAK1 and IRAK4 with MyD88 was also decreased in OmpU/LPS-treated cells compared to buffer/LPS-treated cells. Hence, our observations suggest that OmpU-pretreatment attenuates LPS-mediated TLR-signaling at multiple steps.

Tolerant monocytes and macrophages also exhibit enhanced levels of several negative regulators of the TLR-signaling cascade [22]. We determined gene expression of different negative regulators of TLR signaling but did not observe increased mRNA levels of any such regulators in OmpU/LPS-treated cells as compared to buffer/LPS-treated cells, except for IRAK-M in RAW 264.7 cells. This was further confirmed by western blotting. However, IRAK-M overexpression in THP-1, human PBMCs, and mouse peritoneal macrophages did not occur. Therefore, our observation suggests that IRAK-M may contribute toward attenuated TLR signaling in RAW 264.7 cells.

TLR signaling usually culminates in the activation of the transcription factor NFκB, which is one of the key regulators of pro-inflammatory responses. We assessed translocation of two members of NFκB family, by western blotting, that is, RelA (p65) and c-Rel, which are commonly involved in pro-inflammatory responses. We observed that the translocation of both RelA and c-Rel to the nucleus was reduced on OmpU/LPS treatment, as compared to buffer/LPS treatment. Thus, our observations suggest that OmpU modulates LPS-induced TLR signaling pathway and affects transcription factor levels in the nucleus, ultimately resulting in decreased pro-inflammatory mediator production by LPS.

As all the above results indicated that OmpU-pretreatment probably induces tolerance in LPS-activated cells, we further confirmed it by probing other features of tolerance. Endotoxin-tolerant macrophages demonstrate certain features, such as, enhanced phagocytosis and decreased costimulatory molecule expression that are important for resolution of inflammation [23]. We observed that phagocytic capacity of OmpU/LPS-activated RAW 264.7 cells was enhanced, as compared to buffer/LPS-treated cells. Further, OmpU-pretreatment decreased LPS-mediated surface expression of CD80 in RAW 264.7 cells. Similarly, in murine peritoneal macrophages, CD86 surface expression was reduced in OmpU/LPS-treated cells as compared to buffer/LPS-treated cells.

Altogether, our results proved that a bacterial porin, OmpU is able to induce macrophage tolerance.

Specific aim II: To probe whether OmpU can modulate adaptive immune responses of the host

The innate immune cells interact with adaptive immune cells to initiate a specific, memory-generating, and long-lasting response against microbes and microbial products. Out of the three antigen-presenting cells (APCs), that is, macrophages, DCs, and B cells, DCs are highly potent and are called professional APCs. They act as sensors and respond to a variety of environmental stimuli and undergo differentiation and maturation. When immature DCs, (which have not encountered an antigen) sense PAMPs via PRRs, they secrete cytokines and chemokines in response to the recognition and can mediate inflammatory responses. This process of signaling via innate receptors is important for DC maturation. Therefore, we have under taken the following objectives under this specific aim to determine how OmpU modulates DCs and whether by modulating APCs, OmpU could also modulate T-cell function.

Objective 1: To probe whether OmpU can activate DCs

To explore whether and how OmpU could modulate DC function, murine bone marrow dendritic cells (BMDCs) and splenic DCs were treated with OmpU and cytokine levels were assesed by ELISA. We observed that OmpU-treated DCs produce pro-inflammatory cytokines, such as, TNFα, IL-6, and IL-1β. As mentioned earlier, TNFα and IL-1β are mainly involved in vasodilation. Further, IL-6 is involved in acute phase response. TNFα, IL-1β, and IL-6 also induce hyperthermia. IL-1β and IL-6 increase proliferation and differentiation of lymphocytes.

Further, we probed whether OmpU-induced activation of DCs is mediated via TLRs. One of the earlier report from our laboratory showed that OmpU mediates its signaling via TLR1/2-MyD88-dependent pathway in monocytes and macrophages [16]. However, our findings with neutralizing antibody against TLR2 indicated that TLR2 is not involved in OmpU-mediated TNFα and IL-6 production from DCs. As TLR2 heterodimerizes either with TLR1 or TLR6 and the fact that TLR1 and TLR6 cannot signal without TLR2, our observation therefore, suggests that TLR1 and TLR6 are also not involved in OmpU-mediated activation of DCs. Similarly, with the use of neutralizing antibody against TLR4, we observed that even TLR4 is not involved in OmpU-mediated

activation of DCs. Therefore, which PRR or TLR recognizes OmpU and activates DCs remains elusive.

PAMP–PRR signaling can activate transcription factors, such as, NFκB and AP-1. Activation of MAPK cascade can activate these transcription factors as well. MAPK activation can be dependent or independent of TLR signaling. Therefore, we further explored whether MAPKs, p38 and JNK and transcription factors, NFκB and AP-1 were involved in OmpU-mediated cytokine production by the use of chemical inhibitors. We observed a marked decrease in TNFα, IL-6, and IL-1β production with the use of MAPK, as well as, transcription factor inhibitors. We are further interested in how OmpU activates the MAPK signaling in DCs.

Additionally, we probed whether any inflammasome is involved in OmpU-mediated activation of DCs. IL-1β is generally produced as pro-IL-1β and is converted into its mature form by activated caspase-1. Inflammasomes convert pro-caspase-1 into active caspase-1 [24]. Inflammasomes are multi-protein heptameric complexes consisting of caspase-1, PYCARD, and intra-cellular PRRs, such as, NLRs (NOD-like receptors). NLRP1, NLRP3, or NLRC4 containing inflammasomes are commonly activated in bacterial infections. Certain inflammasomes also contain AIM2 and pyrin proteins that are not part of the NLR family. With the use of chemical inhibitors for caspase-1 and NLRP3, we observed a substantial decrease in OmpU-mediated IL-1β production in BMDCs and splenic DCs. In addition, we observed increased production of active caspase-1 on OmpU treatment of DCs. Thus, our data suggest that OmpU-induced IL-1β production is mediated via NLRP3 inflammasome-dependent caspase-1 activation.

Objective 2: To explore whether OmpU possesses the ability to shape adaptive responses by polarizing T helper cells toward a certain phenotype

T cells are one of the critical mediators of the adaptive immunity. Both cell-mediated and humoral adaptive immune responses are dependent on T-cell activation. $CD8^+$ T cells or cytotoxic T cells are important for cell-mediated immunity, whereas, $CD4^+$ T cells or T helper cells (Th) are important for shaping both cell-mediated and antibody-mediated humoral immunity. In response to different cytokine stimuli, T helper cells can be polarized toward different type of Th cells, such as, Th1, Th2, etc. Th1 cells

are important for cell-mediated, as well as, humoral immunity, whereas, Th2 cells are biased toward humoral immunity and allergy. Therefore, T-cell activation and differentiation are vital prerequisites for shaping adaptive immunity. DCs capture and process diverse antigens and migrate to lymphoid tissues, where they present these antigens to T cells. DCs then, undergo maturation and display enhanced co-stimulatory molecules on their surface, which is critical for T-cell activation. In the immature condition, DCs can capture antigens but are poor in presenting them to T cells. However, in their mature state, DCs act as professional APCs. PAMP–PRR signaling is important for DC maturation. Our previous results prove that OmpU is able to activate DCs, though we could not determine the PRR responsible for DC activation. We further explored different aspects of DC maturation in context of antigen presentation and studied whether OmpU-primed DCs can shape T-cell responses.

We observed that OmpU induced a high IL-12/IL-10 cytokine ratio in BMDCs. IL-12 promotes Th1 and IL-10 promotes Th2 polarization, respectively. Therefore, high IL-12 production and low IL-10 production by DCs in response to OmpU suggested that OmpU possesses the ability to initiate cell-mediated immunity and phagocyte-dependent inflammation.

For effective antigen presentation and T-cell responses, co-stimulatory molecules play a crucial role. Therefore, we wanted to determine whether OmpU can affect surface expression of CD80 and CD86 on BMDCs, as well as, macrophages by flow cytometry. We observed that OmpU increased surface expression of CD80 and CD86 in BMDCs and CD80 in RAW 264.7 and murine peritoneal macrophages. This suggests that OmpU leads to maturation of APCs, such as, macrophages and DCs.

To determine whether DCs can present OmpU-derived peptides to $CD4^+$ T cells and can activate them, we co-cultured OmpU-primed splenic DC with $CD4^+$ T cells and observed an increase in T-cell proliferation in mixed lymphocyte reactions suggesting that OmpU possesses the ability to activate T-cell mediated immune responses. As OmpU-induced a strong IL-12 signal in DCs, we probed whether OmpU could polarize T cells toward Th1 type. Therefore, we assessed IFNγ levels in co-culture supernatants and we observed that OmpU led to the secretion of IFNγ, the hallmark of Th1-polarization responses. Therefore, our data strongly suggest that the OmpU might promote cell-mediated immunity *in vivo*.

Conclusions

Altogether, *V. cholerae* porin OmpU activates innate immunity and induces the production of pro-inflammatory mediators, such as, NO, TNFα, IL-6, IL-1β, and IL-12 from monocytes, macrophages, and DCs. Moreover, OmpU induces endotoxin tolerance in monocytes and macrophages and thus, differentially modulates inflammatory mediator production. Our study reveals that none of the important M2-polarizing markers are involved in OmpU-mediated tolerance induction in LPS-activated cells. Though OmpU-mediated tolerance shares some features of endotoxin tolerance, such as, IRAK-M and IL-10 involvement, it also displays some unique features that are not documented in any other cases of tolerance, such as, decreased association of TLRs to form heterodimer, reduced nuclear translocation of NFκB members, and no involvement of M2-polarization markers [25].

Further, our findings suggested that TLR may not be involved in OmpU-mediated DC activation. MAPKs, p38 and JNK and transcription factors, NFκB and AP-1 are involved in OmpU-mediated signaling in DCs. Moreover, OmpU-induced IL-1β production involves NLRP3 inflammasome-dependent caspase-1 activation.

OmpU also shapes adaptive responses by enhancing surface expression of co-stimulatory molecules on macrophages and DCs. Further, OmpU induces a high IL-12/IL-10 ratio and OmpU-primed DCs leads to T-cell proliferation and Th1 differentiation *in vitro*.

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Publications

1. Sakharwade SC, Sharma PK, Mukhopadhaya A (2013) *Vibrio cholerae* Porin OmpU Induces Pro-Inflammatory Responses, but Down-Regulates LPS-Mediated Effects in RAW 264.7, THP-1 and Human PBMCs. PLoS One 8:e76583.

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