Study of signaling pathway underlying OmpU-mediated dendritic cell activation

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Notations

Abbreviation	Full form
PAMP	Pathogen associated molecular pattern
PRR	Pattern recognition receptor
OmpU	Outer membrane protein U
TLR	Toll like receptor
MAPK	Mitogen activated protein kinase
JNK	c-Jun N-terminal kinase
sh-RNA	Short hairpin ribonucleic acid
BMDC	Bone marrow derived dendritic cell

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Abstract

Vibrio cholerae, an enteric bacterium, is the causative agent of the disease cholera. Pathogenic strains of *V. cholerae* elicit its pathogenicity from ToxR regulon. A major outer membrane protein OmpU, which is encoded by ToxR regulon, plays a crucial role in host's immuno-modulation on infection. OmpU acts as a pathogen associated molecular pattern (PAMP) that is recognized by the pattern recognition receptors (PRRs) present on the host's innate immune cells' such as on the surface of macrophages and monocytes. Further, OmpU can activate dendritic cell as well in terms of production of pro-inflammatory Toll like receptors (TLRs), a major class of PRRs, on PAMP recognition induces downstream signaling cascades that lead to the production of pro-inflammatory molecules. In macrophages and monocytes TLR1/2 hetero-dimer is responsible for recognition of OmpU. In this study we investigated the TLRs involved in OmpU recognition in dendritic cells and underlying signaling molecules involved in immune responses.

Chapter 1

Introduction

1.1 Basic Theory

1.2 Experimental Method

1.1 Basic Theory:

Vibrio cholerae is a gram negative, rod shaped, facultative anaerobe found primarily in aquatic environments. *V. cholerae*, on the basis of its surface antigen, is divided into different serogroups. Antigen specific serogroups of *V. cholerae* can be further characterized by their capacity to produce cholera enterotoxin (Cholera toxin [CT]) (1). Cholera toxin producing serogroups O1 and O139 are associated with the cholera pandemics, while the biotype El Tor of serogroup O1 being the causative agent of the current cholera pandemic (2). The ToxR regulon of *V. cholerae* encodes for cholera toxin (CT), toxin co-regulated pili (TcpA) and accessory colonization factors (Acfs) (2). Cholera toxin (CT) and toxin co-regulated pili (TcpA) are critical for *V. cholerae* mediated pathogenicity in humans (3). These factors help the bacteria in colonizing and infecting the epithelial cells of the host's small intestine. Outer membrane protein U (OmpU), an outer membrane β-barrel porin, is also positively regulated by the ToxR regulon (4). Apart from its role as a porin, OmpU confers resistance against bile salts and antimicrobial peptides which helps the bacteria to thrive in hostile gastro-intestinal conditions (5, 6).

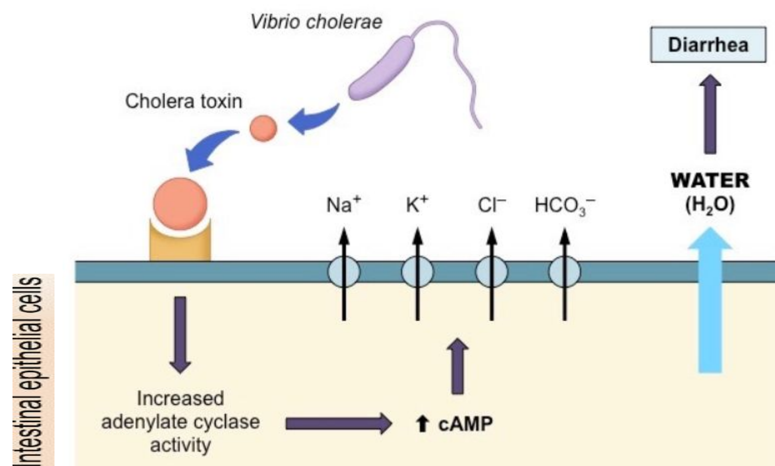

Figure 1: *Vibrio cholerae* mode of infection in intestine.

Taken from "Digestive Infections/Bioninja"(https://ib.bioninja.com.au/options/option-d-human-physiology/d2-digestion/digestive-infections.html#previous-photo)

Pathogen associated molecular patterns (PAMPs):

Pathogen associated molecular patterns are specific molecular motifs present on the microbial surface or genetic material that signals the host about an intrusion (7). These PAMPs comprise a wide range of molecules like lipopolysaccharides, lipoproteins, lipoteichoic acid, peptidoglycans, CpG DNA, single stranded DNA and double stranded RNA. Upon recognition by pattern recognition receptors of immune cells PAMPs can activate innate immune cell activation in the host and induce pro-inflammatory responses. OmpU can act as a PAMP and induce pro-inflammatory immune responses in macrophages, monocytes and human PBMCs (8). We observed that OmpU as a PAMP can induce immune responses in bone marrow derived dendritic cells (BMDCs).

Pattern recognition receptor (PRRs):

PRRs are the receptors present inside the cytoplasm or on the surface of immune cells. PRRs are germline receptors that on PAMP recognition induce signaling cascades that leads to the production of inflammatory mediators. There are four known classes of PRRs: Toll-like receptors (TLRs), C-type lectin like receptors (CLRs), RIG-I like receptors (RLRs) and NOD like receptors (NLRs) (9). TLRs is a major class of PPRs that is present on the surface of membrane, endosome or lysosome.

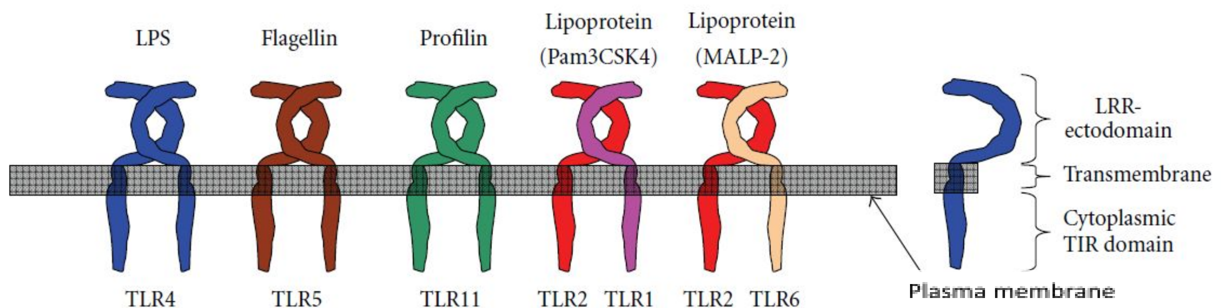

Figure 2. Membrane TLRs and the ligands they recognize. Taken from "Current views of toll like receptors pathways, Masahiro Yamamoto and Kiyoshi Takeda, Gastroenterology Research and practice", DOI: 10.1155/2010/240365

Toll like receptors (TLRs):

The membrane TLRs generally recognize the PAMPs present on the surface of the pathogens and alert immune cells about an infection. The leucine-rich extracellular domain of the TLR binds to the PAMP and triggers the downstream signaling pathway through a cytoplasmic TIR domain. 10 TLRs in humans and 12 in mice have been reported (9). Depending upon the type of PAMP and cell types, different TLRs come into play. They generally make hetero- or homo-dimers. TLR2 generally makes hetero-dimer with either TLR1 or TLR6 and recognizes mainly lipo-protein as PAMP, whereas, TLR4 generally makes homodimer and recognizes LPS as the PAMP. In macrophages and monocytes OmpU is recognized by TLR1/TLR2 hetero-dimer (10). Therefore, our speculation was that BMDCs also employ TLR1/TLR2 for recognition of OmpU.

Mitogen activated protein (MAP) kinase:

Receptor activation initiates cascades of downstream signalling culminating in activation of transcription factors such as, NFκB and AP-1. AP-1 transcription factor activation requires upstream MAPkinase activation. MAP kinases are signal transduction molecules that are activated in response to external or internal stimuli such as microbial infection, stress etc. Phosphorylation of MAP kinase kinase kinase (MAP3K) leads to the activation of MAPkinase cascades. MAP3K activates MAPkinase kinase (MAP2K). MAP2K activates MAP kinases, such as, ERK, JNK and p38 (11). Among the 3 MAPKs p38 and JNK are known to activate AP-1.

Previously in our lab, TLR dependent MAP kinase activation has been observed in OmpU-treated macrophages and monocytes (12). We wanted to check whether in OmpU-activated BMDCs MAPkinase activation happens or not.

1.2 Experimental Methods:

Bacterial strain:

E. coli Top10 cells were used for all cloning based experiments.

Bacterial culture:

All cloning based bacterial cells were grown overnight in Luria bertani (LB) broth at 37°C.

Mammalian cell:

OmpU treatment and sh-RNA transfection was performed on BMDCs.

Culture condition:

BMDCs were maintained in RPMI 1640 media containing 10% FBS, 1% penicillin and streptomycin, 1% β-mercaptoethanol, 0.1 mM non-essential amino acids (NEAA) and 1mM sodium pyruvate. The culture was incubated in 5% CO_2 at 37°C.

Antibiotics used:

a) 50 μg/ml ampicillin was used as a selectable marker in bacterial cultures.

b) To prevent bacterial contamination 100 μg/ml penicillin and streptomycin was used in cell culture

Plasmid used:

GFP containing LRV1 vector (From Dr. Samarjit Bhattacharyya's lab, IISER Mohali) was used to clone TLR1 sh-RNA. The insert was cloned under an Ub-H1 promoter.

TLR1 sh-RNA construct:

mRNA sequence of the TLR1 was taken from NCBI. Dharmacon and reverse complement program was used to find sense fragment of sh-RNA

Whole cell lysate of BMDCs for probing MAPK levels:

Cells flushed from the bone marrow of mice were treated with ACK lysis buffer and then centrifuged at 3500 rpm and 4°C for 5 min. The pellet was re-suspended and plated at a density of 3×10^6 cells/plate in 2 ml media. The plated cells, on alternate days, were differentiated by GM-CSF with a concentration of 10 ng/ml. Fully differentiated BMDCs were observed on day 7. The differentiated cells were pre-treated with 10 µg/ml Polymixin B for 30 min to neutralize any LPS contamination in OmpU. 3 µg/ml of OmpU treatment was given and harvested at different time intervals such as, 30 min, 60 min and 120 min respectively. Following incubations cells were washed immediately with PBS and harvested in 1 ml buffer (PBS). Harvested cells were centrifuged at 3500 rpm and 4°C for 5 min. The pellet was re-suspended and lysed in 50 µl whole cell lysis buffer (0.1%Triton X, 150 NaCl, 0.1 SDS and 50Mm Tris-HCl, pH-8) and 2 µl of protease inhibitor cocktail (PI) was added to it. The cells were sonicated at 10 A for 15 seconds and centrifuged at 12000 rpm and 4°C for 30 min. The protein levels in the lysates were then probed through western blotting.

Antibodies Used for MAP kinase western blotting:

a) Primary antibodies of Total-JNK and p-JNK were used in 1:1000 dilution.

b) HRP conjugated anti-rabbit secondary antibody was used in 1:5000 dilution.

Co-immunoprecipitation (Co-IP) studies:

BMDCs were plated at a density of 6×10^6 cells/plate in 6 ml media. The cells were given 10 μg/ml Polymixin B treatment for 30 min. The two plates of BMDCs were treated with OmpU and buffer respectively for 10 min. 5 μg/ml of OmpU treatment was given, while equal volume of PBS + 0.5% LDAO was used as a buffer. Immediately after 10 min the cells were washed with PBS and resuspended in 2 ml PBS. The whole cell lysates of harvested cells were prepared in 200 μl lysis buffer as mentioned earlier. The lysates were then incubated with 3.5 μl of specific TLR/OmpU antibody and were kept in a rotor at 4°C for 3 h. 20 μl of protein A/G agarose beads (Santacruz) were added to the lysates and left overnight in the rotor at 4°C. The beads were washed thrice with whole cell lysis buffer at 4°C for 5 min and centrifuged at 6000 ×g in table top centrifuge. Finally, 50 μl of SDS-PAGE loading dye was added to the beads for elution. The samples were then heated at 60°C prior to loading the samples in SDS-PAGE and the protein levels were checked using western blotting.

Antibodies used for co-immunoprecipitation and immunoblotting studies:

a) Primary antibodies against OmpU, TLR1 and TLR2 were used in 1:1000 dilution.

B) Anti-mouse and anti-rabbit secondary antibodies were used in 1:5000 dilution.

shRNA mediated gene knockdown:

The top and the bottom strand of shRNA were treated with CIP for 1 h and then pooled together and annealed overnight in gradually cooling water with 95°C as initial temperature. Empty LRV1 vector was double digested for 1hr at 37°C and then ligated with the TLR1-shRNA insert for 16 h at 16°C.The ligation mixture was transformed into chemically competent cells via heat shock method, and the cells were plated in amp - LB agar plate and left overnight at 37°C in the incubator. The observed colonies were then screened using colony PCR and the plasmids from positive colonies were isolated and sequenced. Cells for transfection were plated in 24-well plate at a density of 0.25×10^6 cells/well in 500 µl media and the cells were differentiated by 10 ng/ml IL-4 and 10 ng/ml GM-CSF. The cells were transfected with plasmid using TransIT 2020 transfection reagent (MIRUS) in OptiMEM media. The transfected cells were incubated at 37°C and 5% CO_2 for 48 h. After 48 h of transfection the cells were washed with PBS and harvested. As there is GFP in the vector, the transfection efficiency of the plasmid was checked in the FL-1 channel of the flow cytometer. To check the knockdown, RNA was isolated from the transfected cells and cDNA was prepared and RT-PCR analysis was done. We treated the knock down cells with OmpU for 24 h and checked the IL6 levels in them using ELISA.

Chapter 2

Results, conclusion and future outlook

2.1 Results

2.2 Conclusion and Future Outlook

2.1 Results:

OmpU involves JNK in activation of BMDCs:

Previous work done by Ms Vinica Dhar showed that OmpU can activate BMDCs in terms of production of pro-inflammatory responses in terms of IL-6 and TNF-α production and the activation involves transcription factors AP1 and NF-κB. As AP-1 is involved we speculated that JNK MAPK might be involved in OmpU-mediated proinflammatory immune responses. Towards this we activated BMDCs with OmpU and incubated for different time intervals. Following incubation the whole cell lysates were prepared and p-JNK levels were probed using western blotting

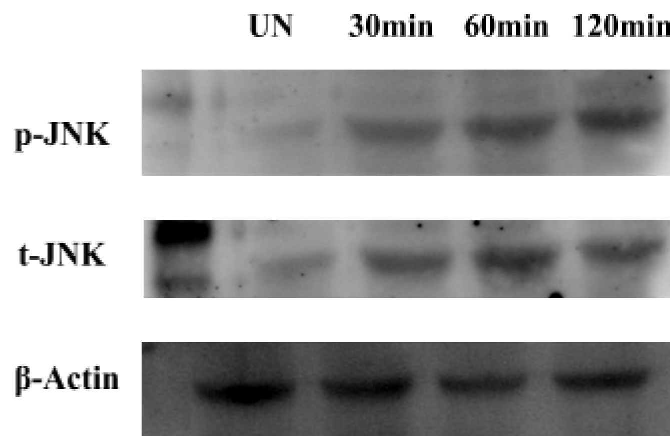

Figure 3. Western blots showing increase in JNK and p-JNK levels in the OmpU-treated BMDCs at different time points.

β-Actin was used as a loading control.

Inference:

From the above blots we observed that there is a marked increase in the p-JNK levels in the OmpU-treated BMDCs. Hence, it implies that JNK gets activated in OmpU treated BMDCs.

OmpU probably induces activation via TLR2 and TLR1 could be the hetero-dimerizing partner

Ms Vinica Dhar observed that there was a decrease in the level of pro-inflammatory cytokines production upon OmpU treatment in BMDCs differentiated from TLR2$^{-/-}$ mice as compared to the BMDCs from wild type mice. As TLR2 makes hetero-dimer with either TLR1 and TLR6, further investigation was towards finding out the hetero dimerizing partner. Towards finding out if TLR1 is the hetero-fimerizing partner we plated BMDCs and treated with OmpU or buffer incubated for 10 min. Following incubation the lysates were prepared and pulled down with anti-TLR2 antibody and protein A/G agarose beads. The samples were then probed for TLR2, OmpU and TLR1 using Immuno-blotting.

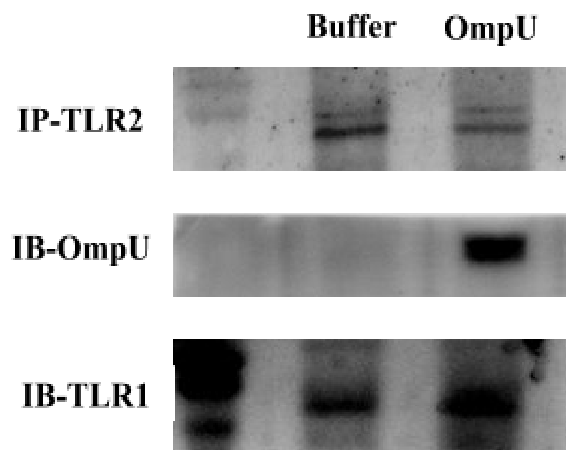

Figure 4. OmpU co-immunoprecipitates with TLR2 and TLR1

Inference:

TLR2 here serves as the loading control and we can observe from the blots that though there is less load of the pulled down lysate in the OmpU-treated lane, still there is an apparent increase in TLR1 levels in OmpU-treated cells. Further, as TLR2, OmpU and TLR1 co-immunoprecipitated together it suggests that TLR1 might be involved in OmpU-mediated immune responses and TLR1 may be the hetero-dimerizing partner of TLR2

TLR1 knock down was achieved by TLR1 shRNA

To confirm whether TLR1 is involved in OmpU-mediated activation of DCs we wanted to knock down the TLR1 gene with shRNA. Towards that, TLR1-shRNA were designed and inserted into LRV1 vector under the UB-H1 promoter region. Colonies were screened by colony PCR using promoter specific primers and the plasmid was isolated from the positive clones. The TLR1-shRNA containing plasmid was further transfected into BMDCs using MIRUS transfection reagent in optiMEM media. As the plasmid contains GFP, the transfection was visualized by flow cytometry in FL-1 channel.

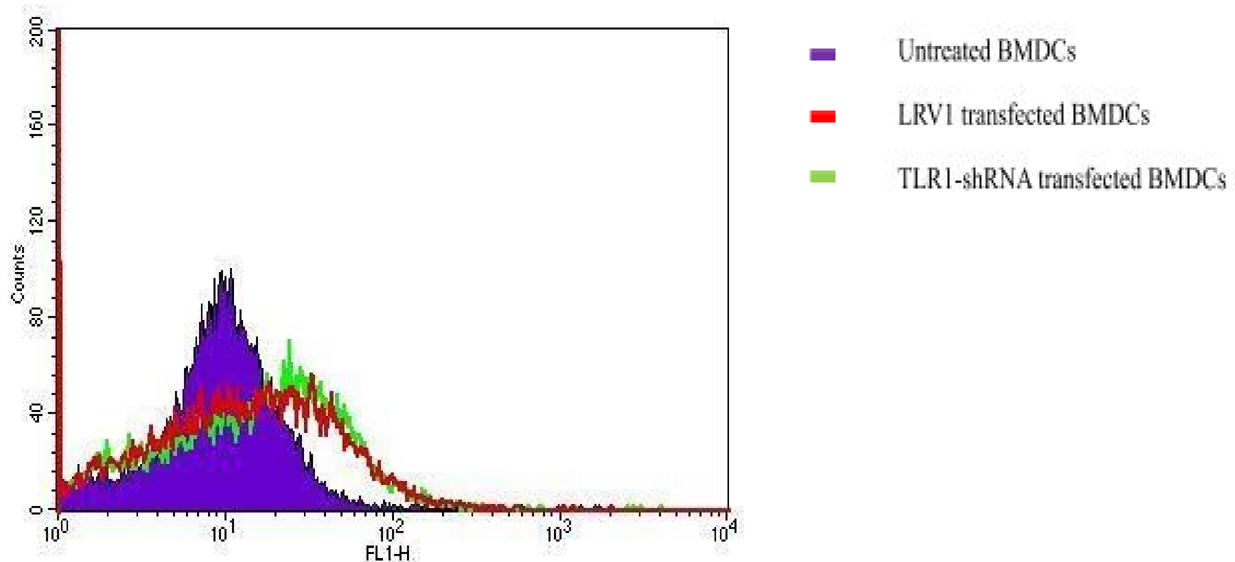

Figure5. Histogram plot of the untransfected and transfected BMDCs showing the relative cell count and fluorescence

Inference:

We can observe from the above histogram plot that there is a peak shift towards higher fluorescence in the FL1 channel in case of the transfected BMDCs in comparison to un-transfected cells. Thus, it implies that a certain population has taken up the vector carrying the TLR1-shRNA insert. We further checked the extent of TLR1 knockdown in the transfected cells using RT-PCR.

To check whether TLR1-shRNA can knock down TLR1 we have isolated RNA from the LRV1-transfected and TLR1-shRNA-transfected BMDCs performed RT-PCR. 18srna and rpl13 was used as a housekeeping gene control. Gene expression level of TLR1 was measured in terms of fold change over TLR1 gene level in un-transfected BMDCs.

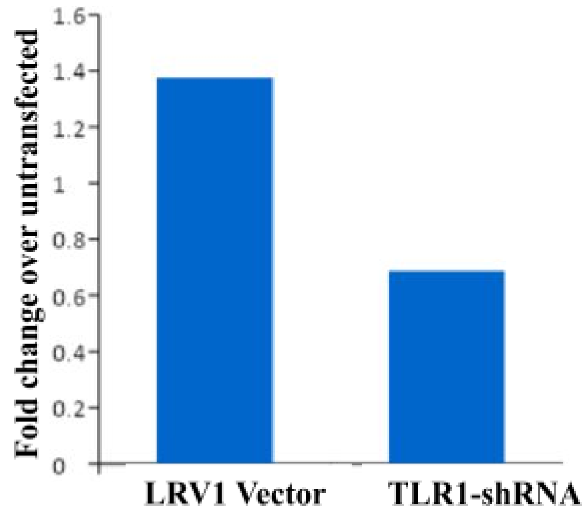

Figure6. Bar graph showing fold change in TLR1 levels in LRV1 and TLR1-shRNA transfected BMDCs

Inference:

The above graph shows that there is a decrease in the TLR1 level in the TLR1-shRNA transfected BMDCs compared to the control. This implies that there is a TLR1 knockdown at the gene expression level in the TLR1-shRNA transfected BMDCs. To further confirm the role of TLR1 in OmpU recognition in BMDCs, the levels of pro-inflammatory cytokine produced in TLR1 knock down BMDCs is to be checked.

2.2 Conclusion and Future Outlook:

Reports have shown TLR and MAPK play a crucial role in OmpU mediated immune responses in monocytes and macrophages (12). In this report we have investigated whether TLR1/TLR2 heterodimer acted as PRR in OmpU recognition in BMDCs and probed the activated JNK MAPK levels in OmpU treated BMDCs. We observed that:

a) There is an increase in activated JNK levels upon longer exposure of OmpU

b). TLR1 is co-immunoprecipitated with TLR2 and there is an increase in TLR1 levels in OmpU treated cells as compared to the buffer treated cells.

c) TLR1 knock down was achieved in TLR1-shRNA transfected BMDCs (TLR1 gene expression level is decreased in TLR1-shRNA transfected BMDCs

Our results showed that TLR1 probably interacts with TLR2 to form a heterodimer and JNK activation takes place in OmpU treated BMDCs. To further elucidate the role of TLR1/TLR2 heterodimer as a PRR in OmpU treated BMDCs, the cytokine levels in TLR1 knocked down BMDCs is to be checked. The effect should be assessed with a proper control i.e., scrambled shRNA that needs to be designed. The role of JNK in OmpU mediated immune responses in BMDCs can be better understood by finding the transcription factors they activate.

Bibliography

1) James B. Kaper, J. Glenn Morris, Jr., and Myron M. Levine, Clinical Microbiology Reviews, Vol.8, No.1, Jan. 1995, p. 48-86, 0893-8512/95/$04.0010 doi: 10.1128/CMR.8.1.48

2) Brandon M. Childers and Karl E. Klose, Regulation of virulence in *Vibrio cholerae*: the ToxR regulon, Future Microbiology, July 2007, p. 335-344, doi:10.2217/17460913.2.3.335

3) Deirdre A. Herrington et al., Toxin, Toxin co-regulated pili, and the toxR regulon are essential for *Vibrio cholera* pathogenesis in humans, J. Exp. Med., Vol.168, October 1988, p.1487-1492, 0022-1007/88/10/1487/06 $2 .00

4) Huanyu Li, Weijiao Zhang and Changjiang Dong (Jan. 2018),Crystal structure of the outer membrane protein OmpU from Vibrio cholerae at 2.2 Å resolution, Acta crystallographica, Section D, Structural biology, 1;74(Pt 1):21-29, doi: 10.1107/S2059798317017697

5) Daniele Provenzano and Karl E. Klose, Altered expression of the ToxR-regulated porins OmpU and OmpT diminishes *Vibrio cholerae* bile resistance, virulence factor expression, and intestinal colonization, PNAS, August 29, 2000, 97 (18), 10220-10224 (https://doi.org/10.1073/pnas.170219997)

6) Jyoti Mathur and Matthew K. Waldor, The *Vibrio cholerae* ToxR-Regulated Porin OmpU Confers Resistance to Antimicrobial Peptides, Infection and Immunity, Vol.72, No.6, June 2004, p. 3577-3583, 0019-9567/04/$08.00_0 doi: 10.1128/IAI.72.6.3577–3583.2004

7) Ruslan Medzhitov, Chapter 17: The Innate Immune System. In: Fundamental Immunology (William E. Paul, ed), 5th edition, p. 315-328, Philadelphia: Lippincott Williams & Wilkins, c2003

8) Sanica C. Sakharwade, Praveen K. Sharma, Arunika Mukhopadhaya (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(9): e76583, doi:10.1371/journal.pone.0076583

9) Osamu Takeuchi and Shizuo Akira, Pattern Recognition Receptors and Inflammation, Cell 140, 805–820, March 19, 2010, doi: 10.1016/j.cell.2010.01.022

10) Junaid Khan, Praveen K. Sharma, Arunika Mukhopadhaya (2015), *Vibrio cholerae* porin OmpU mediates M1-polarization of macrophages/monocytes via TLR1/TLR2 activation, Immunobiology, Vol. 220, Issue 11, p. 1199-1209,https://doi.org/10.1016/j.imbio.2015.06.009

11) Yongliang Zhang and Chen Dong, MAP Kinases in Immune Responses, Cellular and Molecular Immunology, Feb. 2005, Vol. 2, No.1, p. 20-27

12) Aakanksha Gulati, Ranjai Kumar, Arunika Mukhopadhaya,Differential Recognition of Vibrio parahaemolyticus OmpU by Toll-Like Receptors in Monocytes and Macrophages for the Induction of Proinflammatory Responses, Infection and Immunity, May 2019, Vol. 87, Issue 5, e00809-18, DOI**:** 10.1128/IAI.00809-18.