Modulation of host chemokine response by *Vibrio cholerae* OmpU

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Certificate of Examination

This is to certify that the dissertation titled **"Modulation of host chemokine response by *Vibrio cholerae* OmpU"** submitted by **Mr. Anurag Kanaujia** (Reg. No. MS07006) for the partial fulfillment of BS-MS dual degree programme of the institute, has been examined by the thesis committee duly appointed by the institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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(Supervisor)

Dated: May 8, 2012

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Arunika Mukhopadhaya at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Anurag Kanaujia
Dated: May 8, 2012

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

Dr. Arunika Mukhopadhaya
(Supervisor)

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All my batch-mates deserve special mention as great friends who made the five year stay in college memorable.

Finally, I also want to express my gratitude towards my parents for always supporting me.

Anurag Kanaujia

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Abstract

The human disease cholera is caused by the gram negative bacteria *Vibrio cholerae* (*V. cholerae*). *V. cholerae* enters into the body through oral route and colonize in the intestine. Main symptoms of the disease are caused by an exotoxin named cholera toxin released by the pathogen upon entering into the host system. OmpU, a major porin protein of *V. cholerae* helps the bacteria for well survival in the gut and provides the resistance against the first line of host defense in terms of bile resistance and antibacterial peptide resistance. In our laboratory we observed that OmpU down-regulates pro-inflammatory cytokines like TNF-α and IL-6. My project was aimed at elucidating the role of OmpU in modulation of host chemokine responses such as, CCL2 and CCR2 which are important for monocyte and macrophage recruitment at the site of infection. We showed that in RAW 264.7 mouse macrophage cell line, OmpU down-regulates the chemokine CCL2 which is involved in monocyte recruitment from blood to the site of infection. Hence, it confirms the anti-inflammatory role of OmpU and suggests its involvement in pathogenesis by down-regulating the host responses.

Chapter 1
Introduction

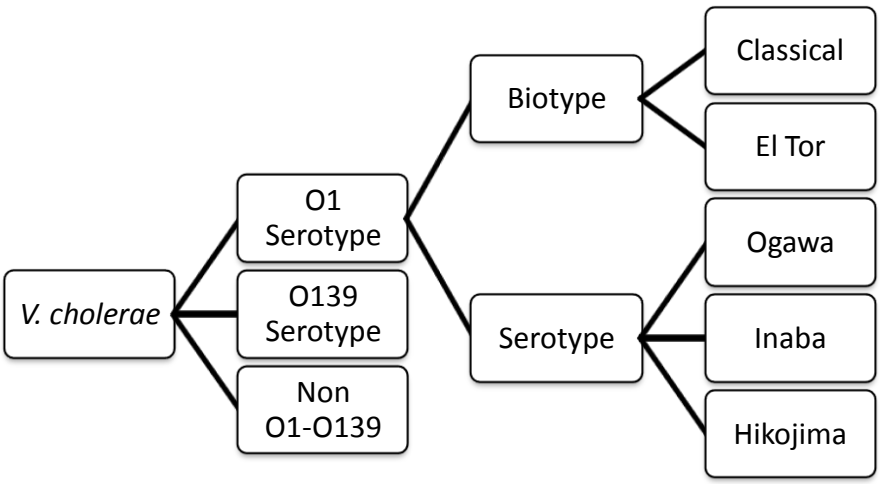
1. Introduction

1.1. *Vibrio cholerae*

Reports about instances of cholera exist since 1800's. The first cholera pandemic occurred in 1817 in the Indian subcontinent. There were five more pandemics of cholera (1829-1991) which affected Asia, Europe, Russia and North America. In 1883, Robert Koch, a German physician identified a comma shaped bacteria and established that it was responsible for causing cholera [1]. This was later named as *Vibrio cholerae.*

1.2. Biodiversity and early study

Vibrio cholerae (V. cholerae) the causative agent of cholera is a gram negative bacteria. It is capable of both respiration and fermentation based metabolism. It is a widely studied organism in terms of its pathological effects on humans. Serological classification of *V. cholerae* is based on the sugar composition of a somatic surface antigen 'O'. This classification of *V. cholerae* was described by Gardner & Venkatraman in 1935. On the basis of agglutinatination to "O" antiserum, *V. cholerae* strains were subdivided into two groups, the O1 serotype and Non-O1 serotype (Ramamurthy *et al.*, 1993a). There are close to 200 known serogroups of O type *V. cholerae* but only O1 are known to have the ability to infect humans. The main causes of cholera are the strains of O1 *V. cholerae* while the non-O1 serotypes are rarely associated with extra intestinal infections of ear, urine, septum and cerebrospinal fluid (Morris & Black, 1985) [2]. O1 Strains are further divided into two biotypes, Classical and El Tor; or three serotypes Ogawa, Inaba and a very rare and unstable Hikojima (Scheme 1).



Scheme1: ***V. cholerae strain variations***

After Koch's discovery, the fifth and sixth pandemics were associated with classical O1 serotype infection. There is no information available about the strains of *V. cholerae* which may have caused the initial four pandemics. However it is thought that these were also caused by classical O1 serotype. The seventh pandemic of cholera began in 1961; it affected most of the Asia, Middle East, Africa and South America and persisted for about 30 years. This cholera outbreak was caused by the El-Tor biotype which has displaced the classical biotype from all over the world [25]. A new serotype termed O139 evolved in 1992 in south-east Asia [26]. The O139 serotype is supposed to have emerged as a result of horizontal gene transfer from the O1 to non-O1 serotype [2]. In addition to this there have been reports that suggest conversion of non-toxic *Vibro* strains into toxic strain by phage transduction with cholera toxin (CT) encoding phage *ctxφ* [3].

1.3. Independent existence

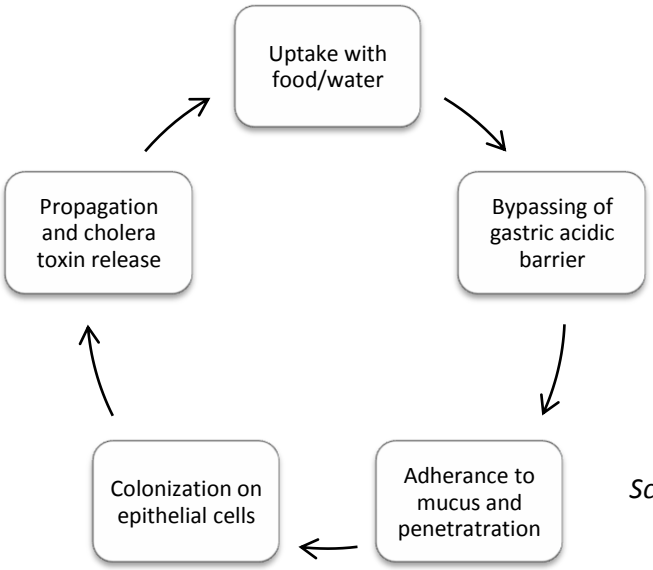
V. cholerae can be found in natural 'aquatic ecosystems' where they colonize on the surfaces of plants and chitin surfaces of marine insects. In fact studies suggest that specific strains (e.g. O1 strain) can acquire acid tolerance from chitin while they colonize on these surfaces [4]. The bacterial colonies can grow in three dimensions by formation of biofilms composed of secretory carbohydrates [6]. Species of *Vibro* expressing exopolysaccharide (VPS) encoded by *vps* gene have been shown to possess the ability to form three dimensional biofilms [6]. In *V. cholerae* strains of O1 El Tor and O139 express exopolysaccharide and exist in form of three dimensional biofilms. It is established that biofilms act as physical barriers for factors that can adversely affect the bacteria [6]. It also provides suitable microenvironment for bacterial growth. The proposed mechanism of bacterial biofilms in *Vibrio* suggests that there are three steps in the biofilm formation which are dependent on different characteristics of bacteria [6]. First step is the attachment of cells on the surface of plant or chitin by flagellum guided motion. Once the cells attach they must colonize and form small colonies. Colonization of bacteria is followed by exopolysaccharide (VPS) expression and biofilm synthesis. Colonization is assisted by mannose-sensitive haemagglutinin (MSHA) like pili.

In addition to the biofilm formation, in low availability of nutrients, *V. cholerae* can change its metabolic regulation and convert into a viable non-culturable strain (VNC). This allows it to persist in adverse environments and proliferate when sufficient nutrients

are available. The characterization of VNC strains is not completed yet and the existence of these stains is unclear as the underlined molecular processes of conversion are unknown [1].

1.4. Infectious life cycle

V. cholerae enters the human body with contaminated food or water and reach the small intestine after crossing the gastro-acidic barrier. In the intestine bacteria adhere to the epithelial cells and colonize, eventually producing the cholera toxin and causing cholera symptoms [1] (Scheme 2).



Scheme 2: ***Infectious life cycle of V. cholerae***

To establish the infection, the bacteria must pass through and survive the gastric acid barrier of the stomach and then penetrate the mucus lining that coats the intestinal epithelia. They also must survive in the intestinal environment, which contains growth inhibitory substances like bile salts and organic acids and also factors of the innate immune system, such as complement proteins of serum (secreted by liver cells), defensins and antimicrobial peptides produced by Paneth cells and the commensal bacteria present in the intestine. As the human gastrointestinal environment provides different salt concentration, temperature, nutritional content and acidity as compared to the aquatic environment, in response to these harsh environmental changes perhaps *V. cholerae* has developed the ability to survive, colonize, and express virulence factors and those acquired properties becomes responsible for the development of diarrhea.

1.5. *V. cholerae* virulence factors

The pathogenesis of *V. cholerae* is dependent on its ability to colonize in small intestine after which these bacteria can metabolize and produce disease causing toxins. [5] The colonization of bacteria is dependent of various factors like cell motility [11], cell surface molecules, expression of virulence genes and local bacterial cell density [12]. *Vibrio* pili are responsible for adherence to intestinal mucosa [13]. Cholera is associated with symptoms like nausea, vomiting, profuse watery diarrhea, rapid loss of body fluids and food poisoning. The disease is caused by various toxins released by the bacteria.

Cholera toxin (CT) is the major mediator of disease [7]. It is composed of five CtxB subunits and one CtxA subunit. The CtxB subunits interact with ganglioside GM_1 cell surface receptors and toxin is internalized by receptor mediated endocytosis. After the endocytosis CT is translocated to the endoplasmic reticulum via the golgi apparatus. The A subunit dissociates into A1 and A2 subunits which are responsible for GTPase (G_{sa}) activation [23] and binding to endoplasmic reticulum degradation protein (ERD2) for retro-translocation of toxin to cytoplasm respectively. CT mimics characteristics of misfolded protein and enters the host cell cytosol by endoplasmic reticulum associated protein degradation pathway (ERAD). The ERAD pathway ensures that proteins transiting through the ER for secretion are properly folded [8]. The CtxA1 subunit of CT causes ADP-ribosylation of adenylate cyclase, leading to production of cyclic AMP (cAMP), which activates protein kinase A (PKA) which then phosphorylates cystic fibrosis transmembrane regulator (CFTR), leading to Cl^- secretion and water loss [9].

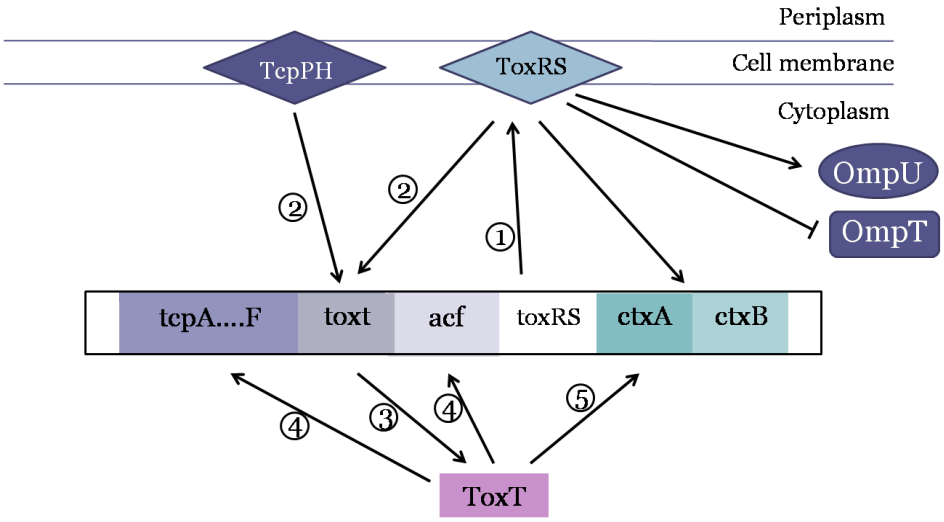
Apart from CT, *V. cholerae* secretes various other toxins such as accessory cholera enterotoxin (Ace) and zonula occludens toxin (Zot), accessory cytotoxins, *V. cholerae* repeats in toxin (VcRTX), hemagglutinin protease (HAP) [12] and hemolysin (HlyA). The predicted amino acid sequence of ace is similar to many ATPases like human plasma membrane calcium pump (CaPM) (56% similarity) and CFTR (42% similarity) and it is implicated to be able to increase the ion exchange and cause fluid secretion [29]. Zot is a tight junction loosening protein and increases the permeability of small intestinal mucosa [28]. VcRTX possess actin depolymerizing and cross linking activity when it is added to tissue culture cells and results in rounding up and increased permeability of cells. HAP is an exported zinc-metalloprotease that causes desquamation of tissues. Addition of HAP to

the cell monolayer disrupts the barrier function of the tight junction. HlyA is a pore forming toxin that causes necrosis or cell lysis depending upon cell type and toxin concentration. It had been observed that the disease causing ability in CT deleted strains (such as JBK70 and CVD101) is significantly decreased. However, due to the presence of zot, ace and VcRTX these strains have still posses the ability to cause less severe diarrhea [29].

1.5.1. *V. cholerae* outer membrane proteins

Outer membrane proteins (OMPs) present in gram negative bacteria can act as antigens to modulate hosts immune responses. These immune responses can be pro-inflammatory or anti-inflammatory [10]. Those OMPs that initiate pro-inflammatory responses are likely to be probed for their vaccine potential. On the other hand, if they are anti-inflammatory in nature, their role is further investigated in connection to bacterial pathogenesis. Porins are one of the major classes of outer-membrane proteins of gram-negative bacteria. They act as channels for the solute transport. Apart from their channel function they perform various other roles such as, they mediate antibiotic resistance and act as receptors for complement and phages [10]. In some cases, they also act as adhesins and help in the attachment with the host cell surface. Porins also can modulate immune responses of the host. Most of the reports suggest their pro-inflammatory nature. There are very few reports confirming their role as mediators of pathogenesis.

V. cholerae has six outer membrane proteins (OMP): OmpU, OmpT, OmpA, OmpX, OmpV and OmpS. Two of the porins, OmpU and OmpT are regulated by ToxR regulon which is the master regulator of *V. cholerae* virulence genes (Scheme 3). OmpU which is a 38kDa protein is positively regulated by the ToxR regulon whereas, OmpT is negatively regulated by the regulon (Scheme 3). Earlier studies suggest that OmpU is involved in bile and organic acid resistance [15, 16]. Antimicrobial peptides are produced by commensal bacteria present in the colon and these provide resistance to pathogenic colonization in gut. Researchers showed that OmpU also imparts resistance against anti bacterial peptides [15]. Therefore OmpU ensures the survival of the bacteria in the gut.



Scheme 3: **Tox R regulon**: *toxR* and *toxt* act together with their membrane bound partners ToxR/S and TcpP/H to modulate the expression of cholera toxin (*ctx*) and toxin co-regulated pili (*tcp*). It also affects OmpU expression positively and down-regulates OmpT [1, 2]. The virulence cassette expression is activated by the translation of ToxR/S proteins (1) which lead to translation of ToxT (2, 3) which regulates tcp, acf and ctx expression (4, 5).

1.6. Innate immune response against gram negative bacteria

Human gastrointestinal tract offers good protection against pathogenic invasion. It is covered with a thick lining of mucus and houses several immune components especially, gut associated lymphocyte tissues (GALT) which are important in host defense against bacteria, virus and other parasites [17]. The intestinal epithelia comprises of specialized cells called M cells that can transfer pathogenic organisms and markers from the lumen to Peyer's patches where they can be detected and processed by antigen presenting cells. Macrophages and dendritic cells (DCs) are also present in GALT. In addition, gut epithelial cells are also equipped with pattern recognition receptors (PRR) and can act as antigen presenting cells. In cases of bacterial invasion the macrophages and DCs are activated and an immune response is mounted. Macrophages and DCs produce immunomodulatory molecules (eg. NFkB, IL-6, IL-12 etc.) which induce inflammatory response and further activate the adaptive branch of the immune system.

The molecular surface characteristics of bacterial cells are responsible for their pathogenesis. They can aid in bacterial invasion or in adherence and colonization. In this way surface molecules direct the host response. Porins could play a crucial role in the

pathways associated with bacterial infections. There are numerous studies concerning the effect of porin on the host immune system which include cellular activation, cytokine release and other diverse immunological effects. A number of studies on the immunomodulatory functions of porins reveal involvement of toll-like receptors (TLRs) and downstream signaling pathways which culminate in the up-regulation of NFkB. NFkB up-regulation ensures production of cytokines such as TNFα, IL6, IL12 etc. which are important inflammatory mediators. One very crucial aspect during induction of pro-inflammatory response is recruitment of monocytes at the site of infection. These monocytes differentiate into macrophages in the tissues. Macrophages are phagocytes that initiate innate immune response in the host in the form of early response and the chronic phase response. Chemokines play an important role in recruitment of monocytes from bone marrow to the blood and from blood to the tissue. Two such chemokines which are important for monocyte recruitment are CCR2 and its ligand CCL2.

Chemokine receptor 2 (CCR2) regulates monocyte and macrophage recruitment and is necessary for macrophage-dependent inflammatory responses [18]. CCR2 is a 374 amino acid outer membrane monomeric protein expressed in both human and mouse almost identically. There are two major populations of monocytes. The CCR2 expressing cells ($Ly6C^{hi}$) are involved in pathogen clearance while CCR2 deficient cells contribute to wound healing and blood patrolling mechanisms [32]. CCR2 is involved in monocyte emigration for bone marrow to blood and monocyte recruitment from blood to the infected tissues. It is a receptor for chemokine CCL2.

Chemokine ligand 2 (CCL2) is a chemokine mainly produced by the activated macrophages. It functions as chemotactic protein for monocytes which helps in their recruitment to specific sites of infection [24]. CCR2-CCL2 receptor ligand pair plays important role in monocyte recruitment to site of infection. There are two models proposed for the emigration of monocytes from bone marrow to blood. First model proposes that CCL2 production by mesanchymal stem cells (MSCs) and CXCL12-abundant reticular (CAR) cells increases monocyte chemokinesis thereby increasing the chance of monocyte to come in contact with the blood vessel followed by transmigration in the lumen in CCR2 independent manner. The second model proposes that CCL2 produced by MSC and CAR cells in proximity to vessel wall binds to glycosaminoglycans (GAGs) and forms chemokine gradient. This attracts monocytes

towards the vessel wall and then they are guided for transmigration through the vessel wall.

Recruitment of monocytes to the site of infection is very crucial for clearing the infection from the system. Macrophages express CCL2 in response to activation by stimulation of innate receptors such as TLRs and pro-inflammatory cytokines. Production of CCL2 by the activated macrophages guide the recruitment of CCR2 expressing monocytes from the blood to the site of infection.

Towards elucidating the role of these chemokines in *V. cholerae* pathogenesis, we had intended to explore the effect of purified OmpU on host chemokine responses which are particularly important for inducing inflammatory responses. Our study was aimed towards characterizing the effects of OmpU on the expression of this chemokine ligand receptor pair. Observation from our experiments would lead to development of a better understanding of host-pathogen interaction.

Chapter 2
Materials and Methods

2. Materials and Methods

In the following section I have enlisted all the materials and methods that were used during my dissertation work. Materials section contains information about the reagents and chemicals used for protein reagent preparation, bacterial and mammalian cell culture, real time polymerase chain reaction (RT PCR) and flow cytometry. Methods section contains description of methods and protocols followed in various experiments.

2.1. Material

For recombinant protein (rOmpU) preparation *Escherichia coli* Top10 (Novagen), and Origami strains (Novagen) were cultured in Luria Bertani (LB) broth (Hi Media). For wild type protein (wtOmpU) preparation *V. cholerae* El-Tor O1 (MTCC code. 3905) was cultured in Brain Heart Infusion (BHI) broth (Sigma). rOmpU preparation involved utilization of ampicillin, imidazole, and glycerol from Himedia, lysis buffer from B Biosciences and protease inhibitor cocktail, LDAO from Sigma and Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fermentas). For wtOmpU preparation in addition to lysis buffer N-octyl POE (Alexis biochemical), sodium sarcosinate (Sarkosyl NL-97) from Sigma and triton X-100 from Himedia were used. Murine macrophage cell lines RAW 264.7 and human monocytic cell line THP-1 were obtained from ATCC and NCCS Pune respectively. Cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (Invitrogen). Dexamethasone and LPS (Sigma) were used as positive controls for CCR2 and CCL2 expression respectively. Primers for mouse c*cl2* gene (forward: TTAAAAACCTGGATCGGAACCAA, rev.: GCATTAGCTTCAGATTTACG GGT); and human *ccl2* and *ccr2* genes (forward: CAGCCAGATGCAATCAATGCC, reverse: TGGAATCCTGAACCCACTTCT); (forward: TGTCCACATCTCGTTCTC GGT, reverse: CCGCTCTCGTTGGTATTTCTGA) respectively were selected from Primer Bank database [20] and were synthesized by Integrated DNA technologies. RNA isolation was carried out using Nucleopore total RNA isolation Kit^{TM} (Genetix) and used for cDNA preparation using Maxima first strand cDNA synthesis kit for RT-qPCR (Fermentas). Maxima SYBR Green qPCR Master Mix was obtained from Fermentas and used for qPCR to estimate specific mRNA expression levels. For flow cytometry analysis and western blot PE conjugated anti mouse/human CCL2 antibody was obtained from Abgent, anti mouse/human CCR2 antibody was obtained from Abcam.

For flow cytometry Golgi stop was obtained from BD biosciences. Saponin, sodium azide, and paraformaldehyde were obtained from Sigma.

2.2. Methods

2.2.1. Purification of wild type (wt) OmpU

Purification of wtOmpU was carried out using method suggested by Chakrabarti *et al.* There were slight modifications introduced to the protocol in order to get a better yield and decrease LPS contamination. Bacteria was cultured in BHI media in 37^{o}C in a shaking incubator (New Brunswick Incubator Shaker e25R) and cells were harvested at 2,057 x g for 15 min using an Eppendorf 5810R centrifuge. Pellet was resuspended in appropriate amount of 20 mM Tris-HCl buffer (pH 7.6) and protease inhibitor cocktail was added to prevent protease activity during cell lysis in following step. Cells were lysed by sonication (30 second pulses of 30 amplitude) to obtain a translucent lysate. The lysate was centrifuged at 12,857 x g for 1 hr to remove the debris and the supernatant was subjected to ultracentrifugation at 73,000 x g for 20 min (Hitachi himac C120GXII). After thawing the membrane suspension was subjected to ultra centrifugation at 73,000 x g for 20 min. Outer membrane was extracted from crude cell envelope by incubating with 1% sodium sarcosinate (Sarkosyl NL-97), at 37°C and the pellet was washed several times in Tris-HCl. The washed pellet was suspended in the Tris-HCl buffer containing 4% triton X-100, incubated at 37°C for 30 min, and centrifuged at 105,000 x *g* for 60 min at 4°C. The supernatant was passed through a manually packed DEAE-cellulose (70.65 ml) (Sigma) column equilibrated with Tris-HCl buffer containing 0.1% Triton X-100. The bound protein was eluted from the column by a continuous gradient of 0 to 0.2 M NaCl using fast protein liquid chromatography, FPLC (GE ÄKTA purifier) in Tris-HCl containing 0.1 M Triton X-100, and 1-ml fractions were collected.

Each fraction was examined by SDS-PAGE, and fractions containing OmpU were pooled and concentrated using a 10 kDa cut off membrane (Ultracel 10K, Millipore). The concentrated sample was loaded on to a Sephacryl S-200 gel filtration column (GE Healthcare) attached to FPLC and equilibrated with 10 mM Tris-HCl (pH7.6) containing 0.5% N-octyl POE. 2 ml fractions were collected and each was examined by SDS-PAGE. The fractions showing single 38-kDa band of OmpU were collected and

stored at 4°C. Following protein estimation with Bradford method protein was aliquoted and kept at -20^{o}C for further use.

2.2.2. Purification of recombinant (r) OmpU

2.2.2.1. Obtaining protein inclusion body from *E. coli* culture

E. coli Origami B cells (Novagen) were transformed with pET-14b (the vector carries an N-terminal His-Tag sequence followed by a thrombin site and three cloning sites, Novagen) vector containing recombinant *ompU* cassette. Recombinant OmpU over expressing Origami cells were cultured in 100 ml LB medium with ampicillin and incubated overnight at 37^{o}C in a shaking incubator. Protein expression was induced by adding IPTG, when optical density of culture reached 0.5-0.6 (at 600 nm) which is the range for exponentially growing bacterial culture. Cells were harvested by centrifugation at 2,057 x g for 20 min at 4^{o}C (Eppendorf 5810R centrifuge). Lysis buffer and protease Inhibitor cocktail were added to the pellet to obtain cell lysate. Genomic DNA was degraded by sonication of lysate (30 seconds pulses of amplitude 20) till the viscosity of mixture decreased. The lysate was centrifuged at 18,514 x g for 30 min, at 4^{o}C in an oakridge tube (Tarsons) to obtain inclusion body containing rOmpU.

2.2.2.2. Processing of inclusion body

Inclusion body from culture was washed twice with PBS containing 100 mM NaCl and was further sonicated in the same buffer. After sonication mixture was centrifuged at 18,514 x g for 15 minutes at 4^{o}C. Pellet was resuspended in 10 ml of buffer containing 8M urea and incubated at room temperature for 30-45 minute to allow solubilization of inclusion body. After solubilization mixture was again centrifuged at 18,514 x g for 20 min at 4^{o}C. The solubilized protein was present in the supernatant.

2.2.2.3. Purification of the denatured protein by Ni-NTA column

Supernatant containing solubilized protein was mixed with 20 mM imidazole. Ni-NTA Column (QIAGEN) (5ml volume) was washed with PBS and equilibrated with 8M urea. Supernatant containing the solubilized protein and imidazole was loaded on to the column. Column washed with PBS containing 8M Urea and 20 mM imidazole. (In this step protein binds to nickel with histidine-tag and buffer is washed away) Protein was eluted with PBS containing 8 M urea and 300 mM imidazole in 4 fractions of 6ml each.

Column was washed with 50ml PBS to remove remaining urea from the column. Fractions and flow through were checked for the protein by SDS PAGE.

2.2.2.4. Refolding of denatured protein

Protein refolding was done by slowly adding purified protein in refolding buffer (10% glycerol, 0.5% LDAO in PBS) and incubating at 4^{o}C while gently mixing the sample. Refolded protein was centrifuged at 15,000 x g for 15 min to remove the aggregates and particulate matter. The supernatant was passed through sephacryl S200 column. The eluted protein was estimated by Bradford method and checked by SDS-PAGE and stored at -20^{o}C.

2.2.3. Cell lines

Murine Macrophage cell line RAW 264.7 was used for characterization of CCL2 expression. Cell lines were maintained in RPMI supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin. The cells were kept in an incubator with 95% air and 5% CO_2 humidified atmosphere at 37^{o}C. Human acute monocytic leukemia cell line THP-1 (undifferentiated) was used to characterize CCR2 expression. These were also maintained in the same conditions as that of RAW 264.7 cells.

2.2.4. RNA isolation

RAW 264.7 and THP-1 cell were plated at 2x10^{6} cells/ml density in 2 ml complete medium and treated with LPS or protein or with LPS in addition to protein. After treatment cells were isolated by centrifugation and washed with ice cold PBS to remove culture media as it can significantly affect the RNA yield. The RNA isolation was done using Nucleo-pore RNASure Total RNA isolation kit. Cells were resuspended in 350 µl lysis buffer (LBA1) containing 3.5 µl β-mercaptoethanol and homogenized using 1 ml syringe with 30G needle (xtra-fine) to achieve complete lysis. Lysate was centrifuged at 11,000 x g for 1 minute. Supernatant was then transferred to a shredder column in a collection tube and centrifuged at 11,000 x g for 1 minute. Ethanol (70%, 350 µl) was added to the flow-through in collection tube and mixed. Mixture was transferred to column (RNASure Mini Column) and placed in a collection tube. Column was centrifuged at 11,000 x g for 30 seconds and flow through was discarded. Desalting buffer (DSB, 350µl) was added to the column and the column was centrifuged at

11,000 x g for 1 minute. DNase enzyme stock was prepared by adding DNase reaction mixture to an aliquot of rDNase. This mix was directly added to column matrix and incubated at room temperature for 15 minutes. Further, DNase was washed by adding 200µl of wash buffer (LBA2) to column and centrifuge at 11,000 x g for 30 seconds. Column was washed with 600µl of wash buffer (WBA3) and column was centrifuged at 11,000 x g for 30 seconds and flow-through discarded. Washing step was repeated with 200µl wash buffer (WBA3). This time column was centrifuged for 2-3 min to ensure complete removal of wash buffer. Column was placed in RNase free tube and 50µl water (DNase/RNase free) was added directly to the column matrix. Column was placed on an RNase free tube and was centrifuged at 11,000 x g for 30 seconds to elute the RNA from the column. The isolated RNA was either used immediately or stored at -80^{o}C.

2.2.5. Complementary DNA (cDNA) synthesis

cDNA synthesis was done using Fermentas cDNA synthesis kit. cDNA synthesis reaction mixture prepared by mixing reaction mix (4µl), maxima enzyme mix (2µl), template RNA (1µg), nuclease-free water to make total reaction volume 20µl. Mixture was then incubated consecutively at 25^{o}C for 10 min, 50^{o}C for 15 min and 85^{o}C for 5 min using thermal cycler (Thermo Scientific). Synthesized cDNA was used for PCR or stored at -20^{o}C for future use.

2.2.6. Quantitative polymerase chain reaction

The qPCR was done using Fermentas 'maxima SYBR green qPCR master mix (2X)' kit. Master mix was prepared by adding maxima SYBR green qPCR master mix (5µl), forward primer (0.125µM), reverse primer (0.125µM), nuclease free water to make the total reaction volume 10µl. The master mix was mixed thoroughly and dispensed appropriate volumes into PCR tubes or plates. Template DNA was added (~500 ng/reaction) to the individual PCR tubes or wells containing master mix. The reactions were gently mixed without creating bubbles and briefly centrifuged. The reactions were incubated at 95^{o}C for 10 min for initial denaturation and then subjected to 40 cycles of denaturation, annealing and extension consecutively at 95^{o}C for 15 sec, 60^{o}C for 30 sec and 72^{o}C for 30 sec using thermal cycler (Eppendorf Mastercycler eprealplex). The relative quantization of cDNA expression was done using ΔΔCt method as per the protocol mentioned in Schmidt and Livek *et al.*

2.2.7. Intracellular MCP-1 (CCL2) chemokine staining by flow cytometry in RAW264.7 cell line

RAW 264.7 cells were treated with buffer, LPS or protein. After 5 hours of stimulation golgi stop was added to stop CCL2 secretion from cells. Cells were further incubated for 9 to 10 hours with the golgi stop. The cells were harvested and washed with PBS and FACS buffer (PBS containing 1% FBS and 0.1% sodium azide) and were suspended in the FACS buffer. Cells were treated with Fc Block (BD Pharmingen) in 1:100 dilution for 20 min. After blocking of the Fc receptors, cells were fixed by adding 2% para-formaldehyde for 20 min. Fixed cells were washed with FACS buffer to remove para-formaldehyde and were permeablized by washing with saponin containing permeablizing buffer (FACS buffer containing 0.1% saponin). After permeabilization cells were incubated for 30 min with PE conjugated anti-mouse CCL2 antibody (at 1/50 dilution) to stain intracellular CCL2. Cells were washed with permeablizing buffer and PBS to remove excess antibody. Finally the cells were resuspended in PBS and acquired using a flowcytometer (BD FACS Calibur) and analyzed using FlowJo. Alternatively cells were stored at $4^{o}C$ in 2% para-formaldehyde for 2 to 3 days and washed with PBS before acquiring.

Chapter 3
Results and Discussion

3. Results and discussion

3.1. Probing of CCL2 expression in gene level and protein level in response to OmpU and LPS using RAW 264.7 cell line

CCL2 is very important in recruitment of monocytes from the bone marrow to blood and from blood to the infection site. Activated macrophages secrete CCL2 in response to pro-inflammatory mediators. That is why we have chosen RAW 264.7 murine macrophage cell line to probe CCL2 up-regulation and expression. We also used LPS as the positive control as LPS is a potent pro-inflammatory agent and various reports suggest its role in CCL2 up-regulation [21].

3.1.1. Effect of OmpU in up-regulation of *ccl2* gene

3.1.1.1. LPS up-regulates *ccl2* and maximum up-regulation is achieved after 12h and 24h of LPS stimulation

We have done a time course profile of *ccl2* up-regulation in response to LPS at 5 different time points 8h, 12h, 24h, 36h and 48h of LPS stimulation (Figure. 1). We observed that expression of *ccl2* peaks after 12 hours and 24 hours of LPS stimulation and level of up-regulation of the gene is similar at both the time points.

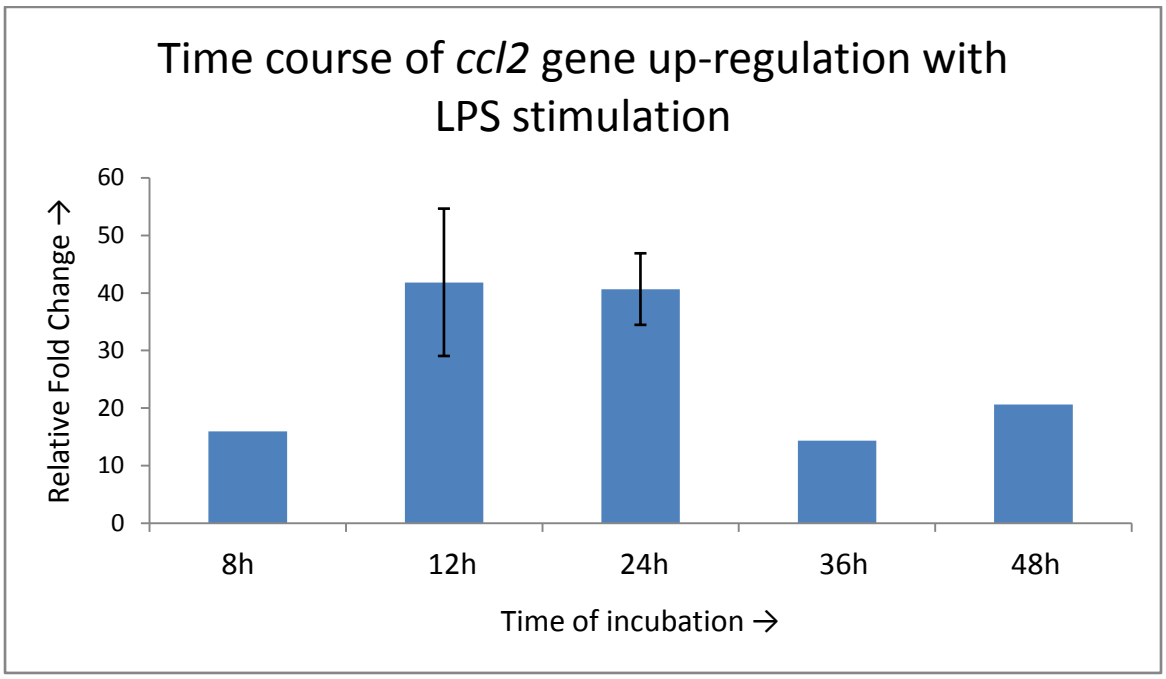


Fig.1. Time dependent profiling of *ccl2* gene up-regulation levels in RAW 264.7 cells in response to LPS stimulation was done using qPCR.
$1.5x10^6$ cells were treated with 1µg/ml of LPS for 8h, 12h, 24h, 36h, and 48h time points. 12 hours and 24 hours treatments were done in triplicates and variation has been shown as percentage of error.

3.1.1.2. Comparative analysis of *ccl2* gene up-regulation in response to LPS and OmpU at 24 hours

After the completion of the time course profile with LPS we checked the level of *ccl2* gene up-regulation in RAW 264.7 cells in response to OmpU. We compared the up-regulation of *ccl2* expression in RAW 264.7 cells following stimulation with LPS and OmpU for 24 hours. Treatment with OmpU showed significant up-regulation of *ccl2* gene but compared to LPS the response is much less (Figure 2). This observation suggests that compared to LPS, OmpU has less pro-inflammatory effect in terms of monocyte recruitment.

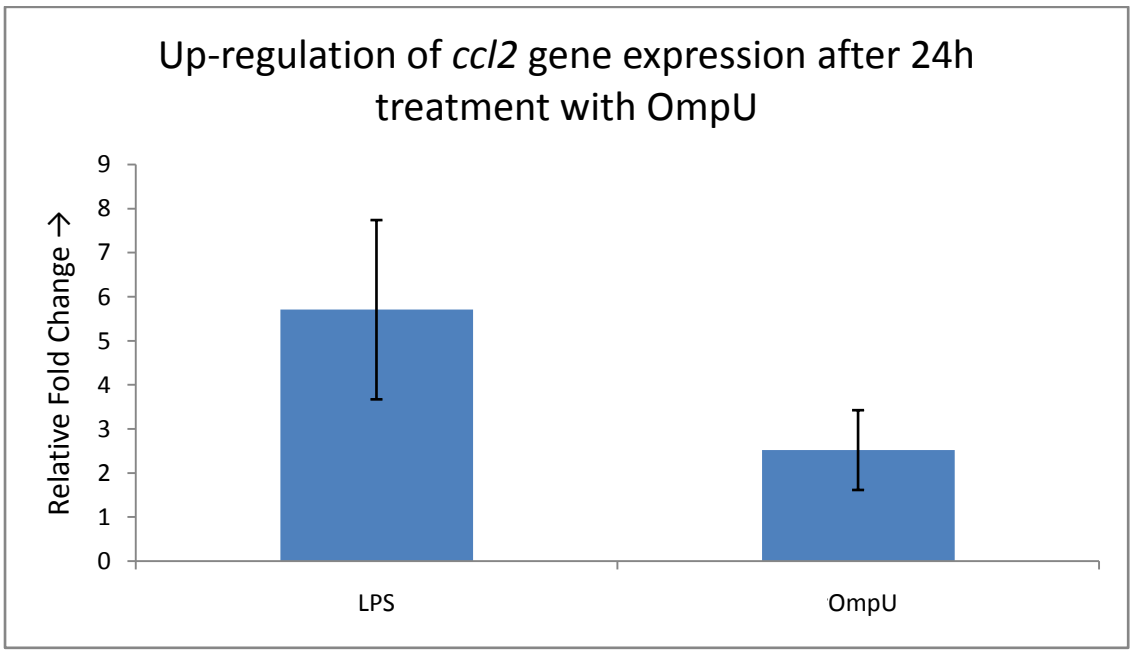


Fig.2. Comparative analysis of *ccl2* gene up-regulation after same time of stimulation in RAW264.7 cells in response to LPS and OmpU treatment.
We treated $1.5x10^6$ cells with OmpU (1.5mg/ml) and LPS (1mg/ml) for 24 hours. We observed that stimulation with OmpU causes up-regulation in *ccl2* gene expression (as assessed by qPCR) which is approximately half the amount of up-regulation caused by LPS.

3.1.1.3. Comparative analysis of *ccl2* gene up-regulation in response to LPS and OmpU at 24 hours

Observation of less up-regulation of *ccl2* in response to OmpU treatment compared to LPS treated cells led us to probe the effect of OmpU on LPS treatment. We wanted to see whether pretreatment with OmpU can down-regulate the pro-inflammatory response of LPS. We indeed observed down-regulation of *ccl2* in OmpU pretreated cells when further stimulated with LPS (Figure 3). This suggests that OmpU is probably playing an instrumental role in suppressing inflammation at the site of inflammation by preventing monocyte recruitment.

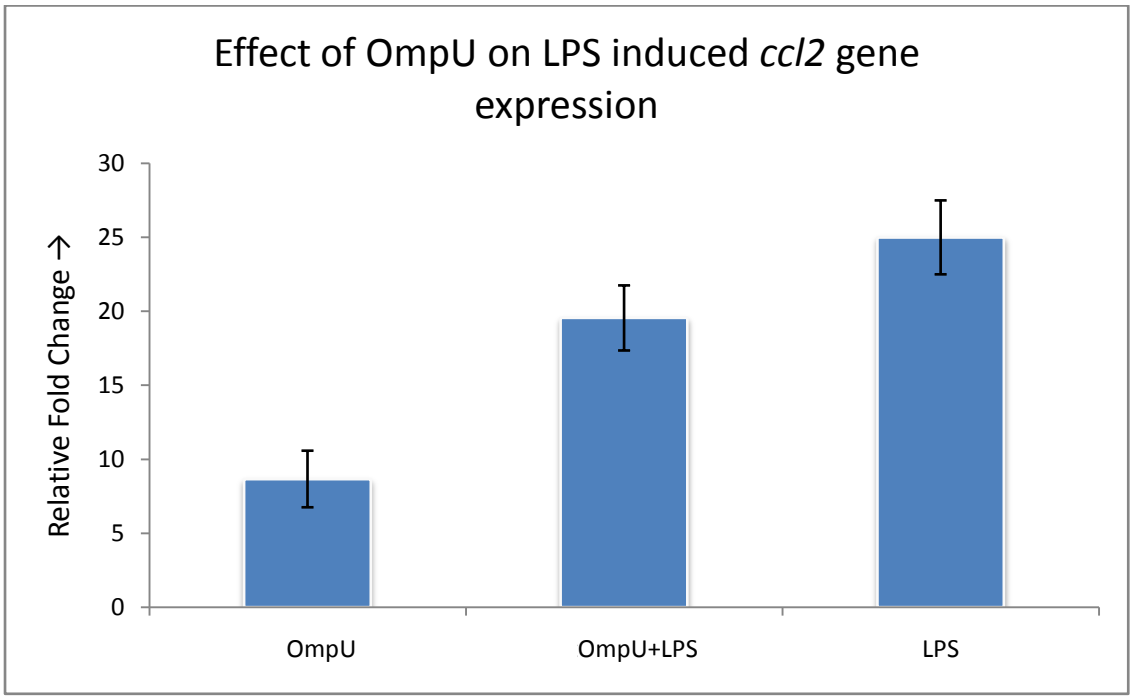


Fig.3. 1.5x10^6 cells were treated with 1.5mg/ml OmpU and incubated for 24 hours. These cells were further stimulated with LPS after removing the protein.
OmpU pretreated RAW 264.7 cells showed less up-regulation of *ccl2* gene when further stimulated with LPS in comparison to cells treated with LPS alone.

3.1.2. Effect of OmpU on CCL2 production

3.1.2.1. Probing of CCL2 expression in protein level in response to OmpU and LPS using RAW 264.7 cell line

To further confirm the down-regulatory effect of OmpU on CCL2 we wanted to probe it in the protein expression level. This we have done by flow cytometry analysis of the intracellular CCL2. Intracellular cytokine staining is used to detect the presence of antigens which are not expressed on cell surface or localized in a certain region of cell and cannot be correctly detected/estimated by direct staining methods. CCL2 being a secretory protein, is not localized in cells. Intracellular Staining utilizes the ability of certain molecules/compounds to stop the transport of protein from the cell organelles like golgi apparatus and endoplasmic reticulum (ER). Brefeldin A and monensin are the most commonly used compounds which restrict the movement of proteins from golgi apparatus. We have used BD GolgiStop^{TM} containing monensin. When a golgi protein transport blocker is used all the protein that is produced get accumulated in the golgi bodies and it can be targeted by using intracellular staining by using a suitable antibody targeted against the antigen of interest. To probe intracellular CCL2 we have used PE-conjugated anti-CCL2 antibody.

We observed huge amount of CCL2 production in response to LPS (Figure 4). Further, when we wanted to see the production of CCL2 in response to OmpU, it was expected that the production of CCL2 in response to OmpU treatment would be half of that in response to LPS treatment. We have performed two consecutive experiments (Figure 5 and Figure 6) and in both the cases we observed comparable amount of chemokine production in response to LPS and OmpU. For one experiment (Figure 5) buffer treated cells showed a stimulation of CCL2 though less than protein or LPS suggesting that the buffer was contaminated. In the second experiment (Figure 6) we observed that LPS caused only 25% increase in CCL2 production (Figure 6), suggesting that the LPS stock used for this experiment was not good. Both comparable and more expression of CCL2 in response to OmpU suggests that the protein may be contaminated with LPS.

CCL2 production by RAW 264.7 cell line in response to LPS stimulation

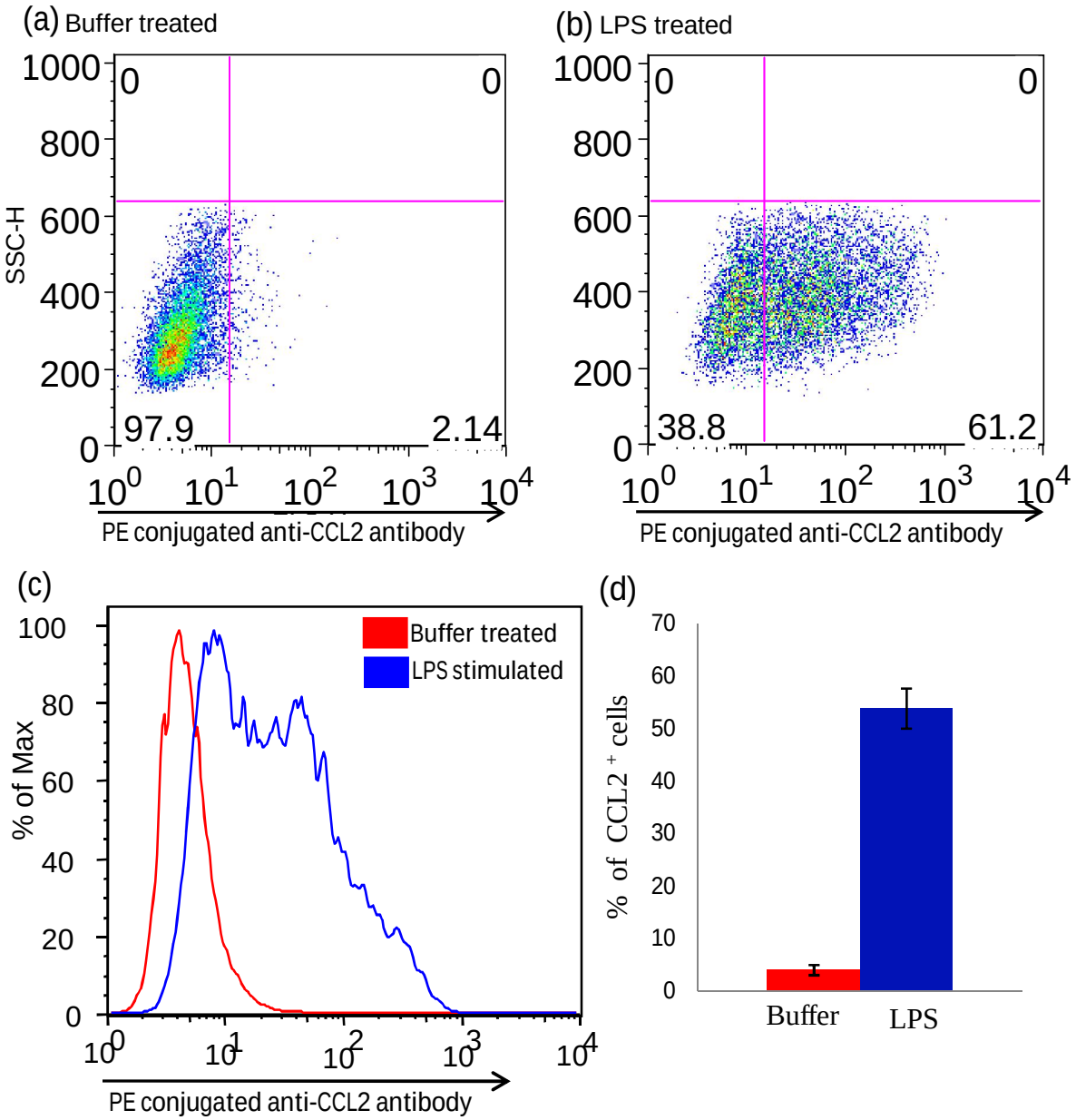


Fig.4. Flowcytometry analysis of CCL2 expression in RAW264.7 in response to LPS treatment

1.5x10^6 cells were stimulated with 1 mg/ml LPS for 6h and further added 0.7 ml/ml of BDGolgi Stop and incubated for around 10h. After that cells were harvested and stained with anti-CCL2 antibody, after addition of the GolgiStop™ 8-10 hour incubation was done. Panel (a) and (b) show the cell populations stained with PE conjugated anti- CCL2 antibody with PBS and LPS stimulated cells respectively. Panel (c) shows the difference between cell populations is about 60% in LPS treated cells (d) The histogram shows the variation of LPS stimulated of expression of CCL2 (40%-60%) in three different experiments.

CCL2 production by RAW 264.7 cell line in response to protein stimulation

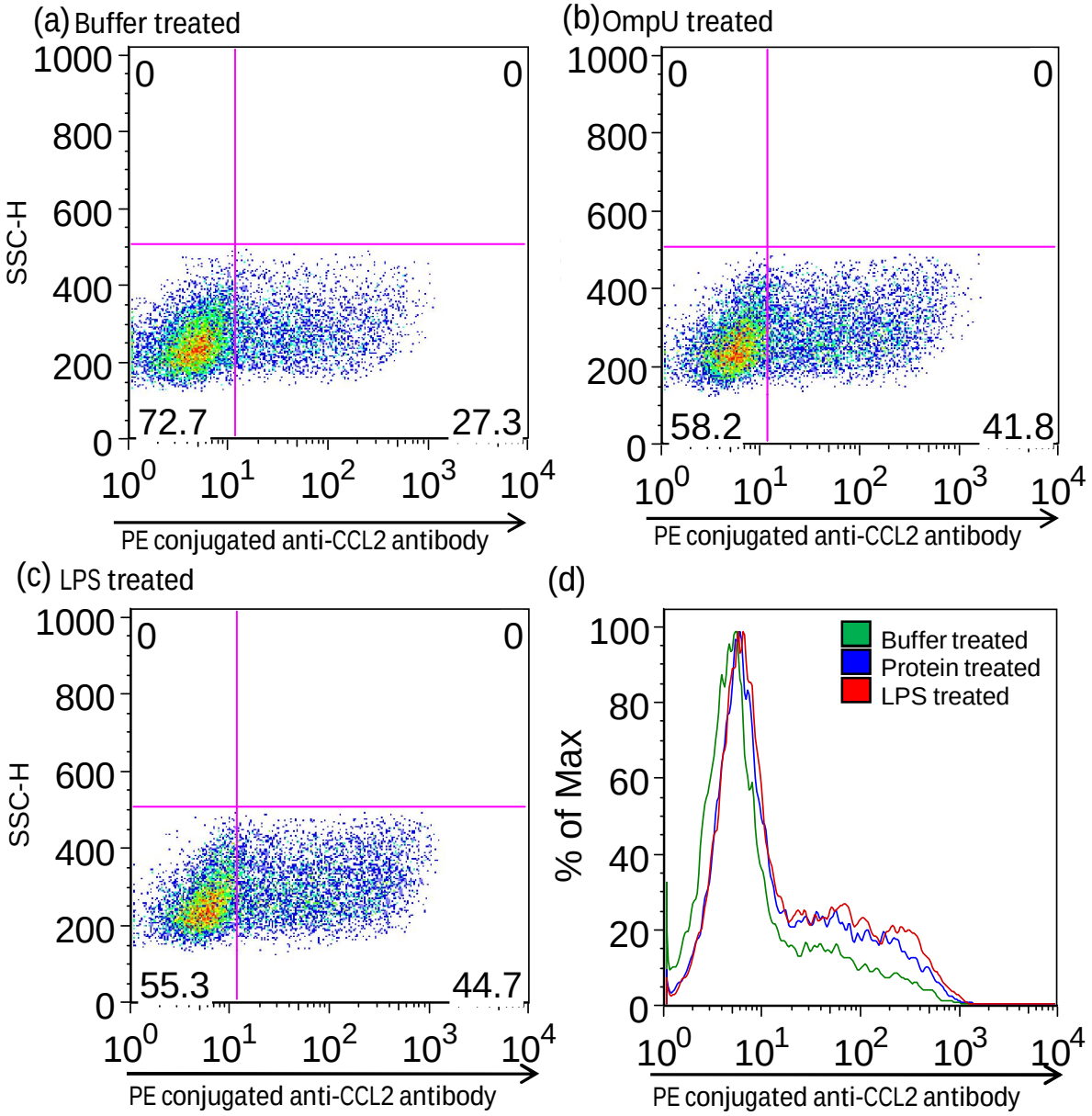


Fig.5. Flowcytometry analysis of CCL2 expression in RAW264.7 cells in response to LPS and OmpU treatment

$1.5x10^6$ cells were stimulated with 1mg/ml LPS and 1.5mg/ml OmpU for 6h and further added 0.7ml/ml of BD GolgiStop and incubated for around 10h. After that cells were harvested and stained with anti-CCL2 antibody. No significant difference in CCL2 expression can be observed in LPS and OmpU stimulated cells. Perhaps, the reason for this is the LPS contamination in OmpU which was not neutralized by 2.5mg/ml Polymyxin B treatment. Panels (a), (b) and (c) show the cell populations stained with PE conjugated anti- CCL2 antibody with PBS, Protein and LPS stimulated cells respectively. Panel (d) shows the difference between cell populations is about the same in all cells.

CCL2 production by RAW 264.7 cell line in response to protein stimulation

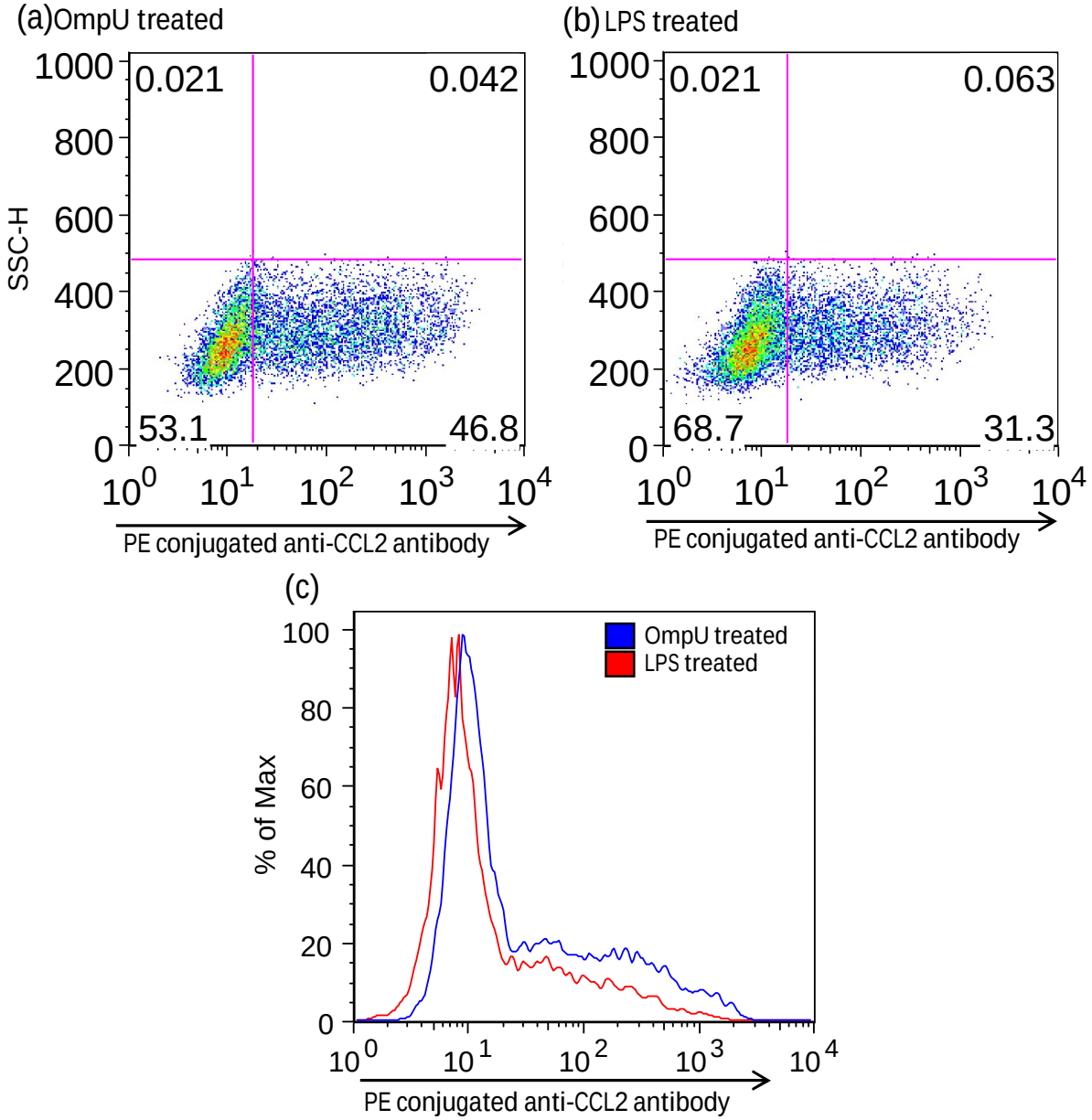


Fig.6. Flowcytometry analysis of CCL2 expression in RAW264.7 cells in response to LPS and OmpU treatment.

Panel (a) and (b) show the cell populations stained with PE conjugated anti-MCP1. (c) The CCL2 expression in LPS treated cells was lower than OmpU treated cells suggesting contamination of protein stock with LPS.

3.2. Probing of CCR2 expression in gene level and protein level in response to OmpU and dexamethasone using THP-1 cell line

CCR2 is expressed by monocytes. For our study we have chosen THP-1 human monocytic cell line to probe CCR2 expression. From the literature it is known that LPS down-regulates CCR2 expression both *in vitro and in vivo* which is why it was ruled out as a control for the study of CCR2 [27]. Dexamethasone was used as positive control for the CCR2 expression in different studies [22]. We have done a time course for *ccr2* with 10^{-7}M dexamethasone treatment for 2, 4 and 8 hour of incubation (Figure 7). The dose was chosen after consulting the literature [22].

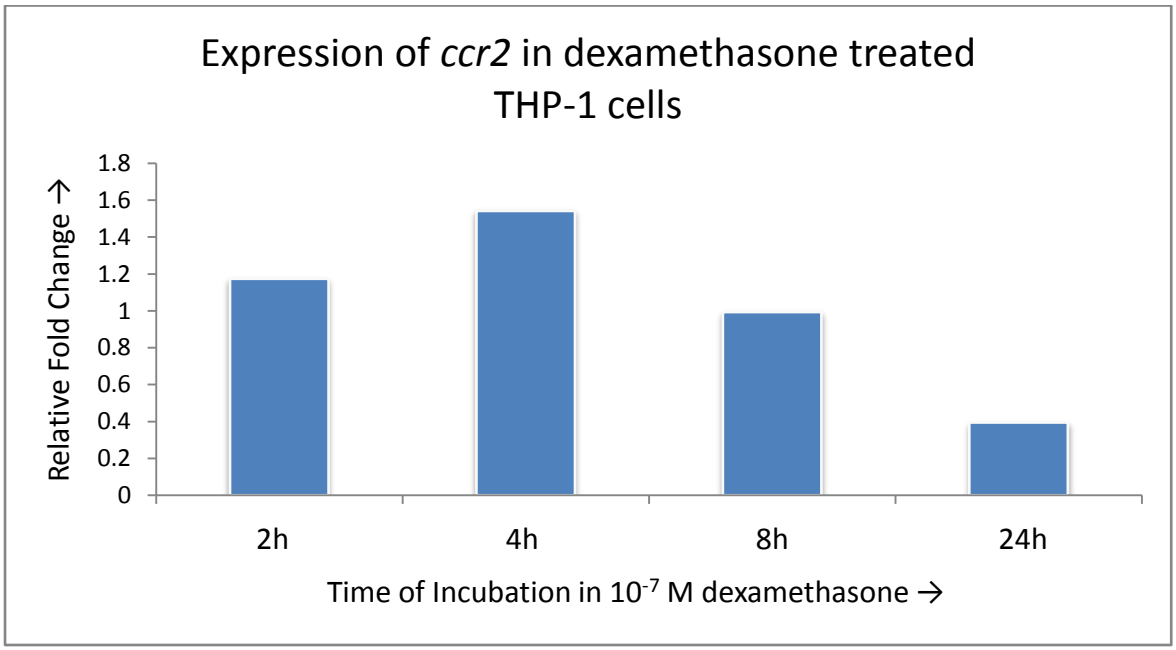


Fig.7. Time dependent profiling of levels of *ccr2* gene expression in undifferentiated THP-1 cells in response to 10^{-7}M dexamethasone stimulation.
$2x10^6$ cells/ml were treated with 10^{-7}M of Dexamethasone for 2h, 4h, 8h and 24h. Up-regulation profile shows that *ccr2* gene level was maximum after 4 hours of stimulation.

When very less up-regulation of *ccr2* expression (~1.5 fold) was observed after 4 hours dexamethasone treatment. We treated the cells with different dosage of dexamethasone ranging from 10^{-9} to 10^{-5} M and checked at 4 hours to observe up-regulation of *ccr2* gene (Figure 8). This time we observed 1.2 fold maximum up-regulation in response to 10^{-9} M dexamethasone.

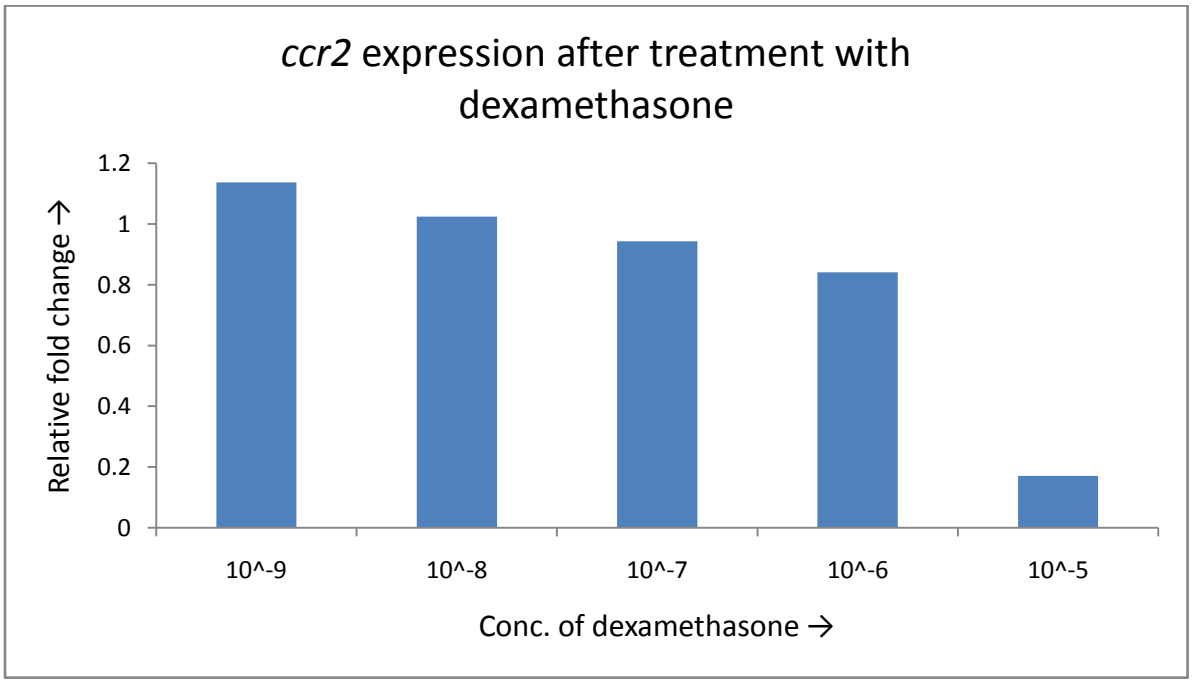


Fig.8. Comparative regulation of levels of *ccr2* gene at similar time point in undifferentiated THP-1 cells in response to different dosage concentrations of dexamethasone.
$2x10^6$ cells/ml were treated with 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M dexamethasone for 4 hours and gene expression levels were analyzed.

Chapter 4
Conclusions

4. Conclusions

CCR2 and CCL2 receptor ligand pair is very crucial for inducing host innate (inflammatory) response. Ly6C^{hi} monocytes emigrate from bone marrow to blood in CCR2-CCL2 dependent way and get recruited at the site of infection, again in CCR2-CCL2 dependent manner [24]. The sources of CCL2 in bone marrow are the MSC cells and CAR cells while the source of CCL2 at the site of infection are activated macrophages. In our study we showed that OmpU inhibits LPS induced *ccl*2 expression in mouse macrophage RAW 264.7 cell line. Owing to the involvement of CCL2 in monocyte recruitment to site of infection, these observations suggest that OmpU may have an anti-inflammatory effect. We have observed similar effect with rOmpU and wtOmpU. For the confirmation of quantitative PCR observations, we further probed CCL2 production in response to LPS at protein level using intracellular chemokine staining, however, this did not confirm the data because the protein reagent had LPS contamination which instead of down-regulating showed more or comparative CCL2 production as compared to LPS.

Standardization experiments of concentration and treatment time of dexamethasone did not show significant *ccr2* expression levels. The regulation of *ccr2* in dexamethasone treated THP-1 cells showed marginal increase as compared to constitutive *ccr2* levels. From the literature it is known that maximum up-regulation of *ccr2* in cell lines is around 5 fold [22]. Since the expression of *ccr2* is low in cell lines, it will be prudent to consider using monocytes derived from peripheral blood mononuclear cells (PBMC) as a system for this study.

5. References

[1] Reidl, J., Klose, K. E.; *FEMS microbiology review* (2002); *Vibrio cholerae* and cholera: out of the water and into the host.

[2] Review: *Vibrio cholerae*, G.B. Nair, J. Bartram, A.H. Havelaar, J. Hueb, J. Jacob.

[3] Faruque, S.M., Albert, M.J. and Mekalanos, J.J.; *Microbiol. Mol. Biol. Rev.* (1998); Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*.

[4] Nalin, D.R., Daya, V., Reid, A., Levine, M.M. and Cisneros, L.; *Infect. Immun.* (1979); Adsorption and growth of *Vibrio cholerae* on chitin.

[5] Levine, M. M., Kaper J. B., R. E. Black, and M. L. Clements.; *Microbiol. Rev.* (1983); New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development.

[6] Watnick, P. and Kolter, R.; *Mol. Microbiol.* (1999); Steps in the development of a *Vibrio cholerae* El Tor biofilm.

[7] Review: Holmgren, J. and Svennerholm, A. M.; *J. Infect. Dis.* (1977); Mechanisms of disease and immunity in cholera: a review.

[8] Tsai B, Ye Y, Rapoport T. A.*; Nat Rev Mol Cell Biol.* 2002; Retro-translocation of proteins from the endoplasmic reticulum into the cytosol.

[9] Viswanathan V. K., Hodges Kim & Hecht Gail; *Nature Reviews Microbiology*, 2009; Enteric infection meets intestinal function: how bacterial pathogens cause diarrhea.

[10] Achouak W., Heulin T., Pagès J. M.; *FEMS Microbiology Letters 199*, 2001; Multiple facets of bacterial porins.

[11] Guentzel M. N. and Berry L. J.; *Infection and immunity*, May 1975; Motility as a virulence factor for *Vibrio cholerae*.

[12] Zhu J., Miller M. B., Vance R. E., Dziejman M., Bassler B. L., and Mekalanos J. J.; *PNAS 99 (5)*, 2002; Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*.

[13] Tacket C. O., Taylor R. K., Losonsky G., Lim Yu, Nataro J. P., Kaper J. B. and Levine M. M.; *Infect. Immun.,* 1998; Investigation of the Roles of Toxin-Coregulated Pili and Mannose-Sensitive Hemagglutinin Pili in the Pathogenesis of *Vibrio cholerae* O139 Infection.

[14] Duperthuy *et al*; *PNAS*, 2011; Use of OmpU porins for attachment and invasion of Crassostrea gigas immune cells by the oyster pathogen *Vibrio splendidus*.

[15] Mathur J. and Waldor M. K.; *Infect. Immun.* 2004; The *Vibrio cholerae* ToxR-Regulated Porin OmpU Confers Resistance to Antimicrobial Peptides.

[16] Merrell D. S., Bailey C., Kaper J. B. and Camilli A.; *J. Bacteriol.* 2001; The ToxR-Mediated Organic Acid Tolerance Response of *Vibrio cholerae* Requires OmpU.

[17] Klein, S., Cohn, S. M., & Alpers, D. H. (2006). *Alimentary tract in nutrition.* In M. E. Shils, M. Shike, C. A. Ross, B. Caballero, & R. J. Cousins (Eds.), Modern nutrition in health and disease (10th ed.). Phildelphia, PA: Lippincot Williams @ Wilkins.

[18] Tsou C. C. L., Peters W., Si Yue, Slaymaker S., Aslanian A. M., Weisberg S. P., Mack M., and Charo I. F.; *J. Clin. Invest.*, 2007; Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites.

[19] The UniProt Consortium; Reorganizing the protein space at the Universal Protein Resource (UniProt); *Nucleic Acids Res.*, 2012.

[20] Spandidos A., Wang X., Wang H. and Seed B.: PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification *Nucl. Acids Res. 2010*.

[21] Zoelena *et al; Molecular Immunology* (2011); Endogenous MCP-1 promotes lung inflammation induced by LPS and LTA.

[22] Rol G. P., Cota M., Polentarutti N., Luini W., Bernasconi S., Borsatti A., Sica A., LaRosa G. J., Sozzani S., Poli G., and Mantovani A.; *J Immunol* 1999; Up-Regulation of CCR2 Chemokine Receptor Expression and Increased Susceptibility to the Multitropic HIV Strain 89.6 in Monocytes Exposed to Glucocorticoid Hormones.

[23] Cassel D. and Selinger Z.; *PNAS*, 1977; Mechanism of adenylate cyclase activation by cholera toxin: Inhibition of GTP hydrolysis at the regulatory site.

[24] Shi C. and Pamer E. G.; *Nature Reviews Immunology*, 2011; Monocyte recruitment during infection and inflammation.

[25] Siddique A. K. *et al*; *Lancet* (1991). Survival of classic cholera in Bangladesh.

[26] Shimada T. E. *et al*; *Lancet* (1993). Outbreaks of *Vibrio cholerae* non-O1 in India and Bangladesh.

[27] Zhou Y., Yang Y., Warr G. and Bravo R.; *Journal of Leukocyte Biology* (1999); LPS down-regulates the expression of chemokine receptor CCR2 in mice and abolishes macrophage infiltration in acute inflammation.

[28] Uzzaua S., Cappuccinelli P., Fasano A.; (1999); Expression of *Vibrio cholerae* zonula occludens toxin and analysis of its subcellular localization.

[29] Trucksis M., Galen J. E., Michalski J., Fasano A. and. Kaper J. B.; *PNAS* (1993); Accessory cholera enterotoxin (Ace), the third toxin of a *Vibrio cholerae* virulence cassette.