Study of Modulation of chemokines important for Neutrophil recruitment by *Vibrio cholerae* porin OmpU

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A dissertation submitted for th e partial fulfilment of BS - MS dual degree in Science

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Certificate of Examination

This is to certify that the dissertation titled "Study of Modulation of chemokines important for neutrophil recruitment by *Vibrio cholerae* porin OmpU." submitted by Ms. Kasturi Banerjee (Reg. No.- MS08029) 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.

Dr. Arunika Mukhopadhaya (Supervisor) Dr. Kausik Chattopadhyay Dr. Samarjit Bhattacharya

Dated: April 26, 2013

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. When ever 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.

Kasturi Banerjee

Dated : April 26, 2013

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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This project wouldn't have been complete without the guidance and support of Dr. Arunika Mukhopadhaya. Her insight and timely help, helped me immensely to complete the project in stipulated time and produce significant results.

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ABSTRACT

Vibrio cholerae is a human pathogen that causes fatal disease, known as, cholera. *V. cholerae* have multiple virulence factors like accessory colonization factor (acf), Toxin co-regulated pilus (TCP), cholera toxin (CT) etc. that help in pathogenesis of *V. cholerae*. Gram negative bacteria have outer membrane proteins (Omp), most of them are porins which form transmembrane channels. OmpU is an outer membrane porin protein that had been reported to provide resistance against bile and anti-microbial peptides in *V. cholerae*, adherence in *V. vulnificas* and effector of pathogenesis in *V. splendidas*. *V. cholerae* colonizes intestinal epithelial cells using a complex interplay of colonization factors. Work by other people from our lab showed that OmpU have a characteristic dual nature of immune responses. OmpU up-regulates the expression of pro-inflammatory molecules in monocytes and macrophages. But it had also been observed that OmpU inhibits LPS (lipopolysaccharide) mediated effects. To study whether there is any effect of OmpU on the chemokines crucial for neutrophil recruitment, we have probed THP-1 human monocytic cell line with purified recombinant OmpU protein. We observed that OmpU, in THP-1 monocytes induces the expression of all the important chemokines (i.e. IL-8, CXCL-1, CXCL-2, CXCL-5 and CCL-5 (RANTES)), involved in neutrophil recruitment.

INTRODUCTION

Vibrio cholerae is a gram negative bacteria that causes fatal human disease, known as, cholera. Cholera is an infection of small intestine with symptoms like profuse watery diarrhea, dehydration including low blood pressure, wrinkled skin, sunken eyes and food poisoning. Cholera spreads by fecal contamination of food and water due to poor sanitation. It had effected regions across the globe like Africa, Asia, Russia, South America and United states of America. There are two serogroups of *V. cholerae* found in nature (El Tor): O1 and O139. Cholera in regions of South-East Asia, i.e. India, Bangladesh, Pakistan and Myanmar is caused by both O1 and O139 strain of *V. cholerae* (Howard-Jones, 1984).

The cholera bacteria enters human host through oral route and infects the small intestine. *V. cholerae* colonizes the small intestine through toxin coregulated pilus (TCP), a thin, flexible, filamentous appendage on the surface of bacterial cells. The disease is mediated via cholera toxin (CT) encoded by *ctxA* and *ctxB* genes. Cholera toxin is an oligomeric complex made up of six subnits: single copy of A subunit and five copies of B subunit. *V. cholerae* secrets cholera toxin after coloniztion the intestine. The B subunit ring of the cholera toxin binds to GM1 gangliosides on the surface of intestinal cells and the toxin is endocytosed by the cell. After endocytosis cholera toxin A1 (CTA1) chain is released by the reduction of a disulfide bridge. CTA1 binds to ADP-ribosylation factor 6 (Arf6) and exposes its active site via conformational changes. Active CTA1 fragment catalyses $G\alpha_s$ of G protein and causes $G\alpha_s$ unit to lose its catalytic activity. Increased $G\alpha_s$ subunits increases intercellular concentration of cAMP, that leads to activation of cytosolic PKA. Active PKA then phosphorylate the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel proteins, which leads to ATP-mediated efflux of chloride ions and leads to secretion of H_2O,Na^+,K^+, and HCO_3^- into the intestinal lumen (source from Wikipedia-http://en.wikipedia.org/wiki/Cholera_toxin).

Vibrio species also have other effectors of pathogenesis that actively modulates the immune responses in host's body. Outer membrane protein present on the surface of *Vibrio* species had been reported to elicit immune responses. Porins a major category of outer membrane proteins found on gram-negative bacteria, are transmembrane proteins that act as channels for transport across the bilayer membrane. Porins had been observed to have other functions apart from transport of solutes like they act as receptors, imparts resistance and induce immune responses.

OmpU porin is one of the six outer membrane proteins of *V. cholerae* and had been

reported to provide resistance against bile (Wibbenmeyer *et.al*, 2002) and intestinal antimicrobial peptides (Mathur & Waldor M.K., 2004). OmpU porin is positively regulated by ToxR Regulon, the master regulator of *V. cholerae* virulence gene (Skorupski & Taylor, 1997). It had been observed in our lab that OmpU have a dual nature of immune response. OmpU induced expression of pro-inflammatory mediators like NO (Nitric Oxide), TNF-α, IL-6, when RAW 264.7 murine macrophages and THP-1 human monocytes and human PBMCs were treated with recombinant OmpU (rOmpU) protein. When cells were pretreated with rOmpU for a specific time duration and then activated by LPS stimulation, it had been observed that the expression of pro-inflammatory mediators like NO, TNF-α, IL-6 etc in response to LPS were down-regulated. Based on this background we wanted to study and understand the effect of OmpU on the chemokines involved in neutrophil recruitment.

Neutrophils are one of the first responder of innate immune responses. They have the highest phagocytotic potential than macrophages and dendritic cells. Maturity of neutrophils is strictly controlled inside the bone marrow. During bacterial infection, macrophages recruit neutrophils from the bone marrow to the site of infection for scavanging of the pathogen. Neutrophils get recruited from bone marrow to blood and from the blood to the site of infection by following chemokine gradient (chemotaxis). These chemokines are secreted by macrophages and monocytes when they recognize pathogen associated molecular patterns (PAMPs) like LPS etc. present on the surface of bacteria, by their Pattern Recognition Receptors (PRRs). In the recruitment process, neutrophils mature in blood circulation. Once neutrophils reach the site of infection, it enters the infected tissue via diapedesis and takes over the phagocytotic activity of macrophages and kills the bacteria. Evidences from literature had showed that free-flowing mature neutrophils could cause auto-immune diseases. Therefore, neutrophil recruitment is a tightly regulated process controlled by interplay of chemokines secreted by macrophages and monocytes (Sadik, Kim & Luster, 2011).

Literature review suggested that there are five highly specific chemokines involved in the neutrophil recruitment process (Sadik, Kim & Luster, 2011). They are CCL-5 (RANTES), IL-8, CXCL-1, CXCL-2, CXCL-5. CXCL1, CXCL-2, CXCL-5 and IL-8 (Sarkar & Bhowmik, 2012). CXCL-1, CXCL-2, CXCL-5 and IL-8 could bind to the same receptor, CXCR-2 present on the surface of matured neutrophils. Immature neutrophils have CXCR4 receptor on the surface that binds to the ligands (SDF-1) present in the bone marrow. These ligands prevent immature neutrophils from leaving the bone marrow

environment. Neutrophils switch their surface receptor from CXCR4 to CXCR2 for the mobilization of neutrophils from the bone marrow. This switching of surface receptors leads to maturation of neutrophils. The switching of surface receptors takes place under the induction of chemokine CXCL-1. CXCL-1 binds to CXCR-2 and mobilizes neutrophils from bone marrow to the blood. CCL-5 and IL-8 provides the chemokine gradient to mature neutrophils to migrate in the blood circulation and reach the site of infection. CXCL-2 recruits mature neutrophils from the blood to the proximity of the periphery of the infected tissue. Mature neutrophils start rolling on the surface of infected tissue and adhere. CXCL-5 polarizes the epithelium in baso-lateral direction of the neutrophils migration. Neutrophils trans-migrate and enter the infected tissue via diapedesis in between the polarized epithelium cells. Once inside the infected tissue, IL-8 induces the phagocytotic activity of neutrophils, thus enabling neutrophils to take over the phagocytotic activity of the tissue macrophages and kill the bacteria.

LPS had been used as the positive control for the study. It is a potent inducer of pro-inflammatory responses in monocytes and macrophages. From the literature we found that LPS could up-regulate the expression of these five important genes. Thus we used LPS to probe whether the chemokines get expressed in our model cell line: THP-1 monocytes.

In this study, we tried to explore whether OmpU induces the expression of all the five important chemokines (IL-8, CXCL-1, CXCL-2, CXCL-5 and CCL-5) involved in neutrophil recruitment, using THP-1 human monocytic cell line. We did the study by analyzing the expression profile of the aforesaid chemokines at mRNA level using semi-quantitative PCR techniques. We observed that purified rOmpU induces the expression of these chemokines but the fold change of expression and the time of maximum expression varied for different chemokines giving us an insight for the probable interplay occuring between these chemokines for a proper and effective neutrophil recruitment to the site of infection.

MATERIALS and METHODS

1. Refolding and purification of denatured recombinant OmpU (rOmpU) protein

Reagents used

1. Refoldng Buffer (50mL) – 1X PBS (Sterilized and autoclaved)
   10% glycerol
   0.5% LDAO (Sigma)
2. Running Buffer - for column chromatography (500mL)
   10mM Tris
   10mM NaCl
   0.5% LDAO (Sigma)
3. Protease Inhibitor cocktail (Sigma)

Materials used

1. 50 mL centrifuge tubes (BD Falcon)
2. Ice bucket
3. SEPHACRYL S200 resin (Wipro GE) for Size Exclusion Chromatography
4. Ultracentrifuge machine (Hitachi)

Method

E.coli containing *V. cholerae* OmpU casette was cultured and induced for protein expression. Inclusion bodies were denatured in presence of 8 Urea. Denatured rOmpU protein in 8M Urea was put on ice. 10mL of refolding buffer was taken in a separate tube and put into ice. 50µL of protease inhibitor cocktail was added to the refolding buffer. 1mL of denatured protein was added to 10mL of refolding buffer slowly in smaller alliquotes of 50µL and with constant shaking. The denatured protein in refolding buffer was incubated for overnight at 4ºC.

The tubings of Chromatography machine was washed with Mili-Q water and the baseline was checked to be close to zero. The column packed homogenously and tightly with SEPACRYL S200 resin and was washed with 120mL of Tris-NaCl-LDAO buffer by keeping the flow rate at 1mL/min and pressure of not more than 0.25MPa. Refolded denatured-protein in refolding buffer kept overnight was taken out and put in ice. Refolded protein mixture in buffer was transferred to ultracentrifuge tubes and ultracentrifuged at 15,000 rpm, 4ºC for 20mins. Aggregates and refolded protein gets separated by ultracentrfugation. The supernatant is isolated and stored in centrifuge tubes and the pellet is discarded. After the size-exclusion chromatography column had been washed by Tris-NaCl-LDAO buffer, the supernatant was passed through the column through a 10mL syringe. All the samples showing the characteristic peak of the protein were collected and stored in 2mL cetrifuged tubes at -20ºC.

2. Esitmtion and Quantification of purified rOmpU protein

Materials Used

1. UV-Vis spectrophotometer (PerkinElmer)
2. Quartz cuvette

Method

The collected purified protein was thawed from -20ºC and kept on ice. In one of the cuvette protein was taken and the concentration of protein was measured at 280nm wavelength (Molar exclusion coefficient of rOmpU – 1.068). Tris-NaCl-LDAO buffer was used as blank and the UV-Vis Spectrophotometer was standardized against the blank.

3. Validation of purified rOmpU protein

Reagents used

1. 10X PBS - Na_2HPO_4 14.4gm (Sigma)
   KH_2PO_4 2.4gm (Sigma)
   NaCl 80gm
   KCl 2gm

2. TAE Buffer - Tris 242gm (Sigma)
   Glacial Acetic Acid 57.1mL
   EDTA (0.5M, pH-8) 18.6gm

3. SDS PAGE buffers - Sol A – 29.2gm Acrylamide + 0.8gm Bisacrylamide (100mL)
   Sol B - 1.5M Tris-HCl (pH 8.8) + 0.4% SDS
   Sol C - 0.5M tris-HCl (pH 6.8) + 0.4% SDS

4. Running Buffer - 0.025M Tris
   0.192M Glycine
   0.1% SDS

5. Composition of Stacking Gel – Sol A (450µL) + Sol C (750µL) + Ammonium persulfate (APS) (0.03µL) + TEMED (10µL) + Mili-Q water 1.8mL

6. Composition of Resolving Gel (10%) - Sol A (3mL) + Sol B (2.25mL) +TEMED (8-10µL) + Mili-Q water (3.75mL)

7. Staining Solution - Comassie Blue 0.1%
   Acetic Acid 5%
   Metahnol 50%

8. Destaining Solution - Acetic Acid 10mL
   Methaol 10mL

Milli-Q water 80mL

Materials Used

1. Gel Casting Apparatus
2. Horizontal Shaker
3. SDS Apparatus
4. White light source

Method

The resolving gel was casted using gel apparatus. After the resolving gel has polymerized, the stacking gel was casted on top of the resolving gel and a 10 well comb was put on top of the stacking gel. After the gel was casted, running buffer was prepared. Running buffer was poured over the gel and the apparatus's tank was filled with the buffer. The protein samples mixed with loading buffer after heating for 5 mins was loaded in the wells. The gel was run at 100V, till the protein crosses 80% of the gel length. After the run is complete, gel is stained with staining solution and shake for 2 hours. After the staining, gel was washed with mili-Q water and then kept in destaining buffer for the overnight. The gel was kept over an white light source and the protein size and purity was confirmed (size of OmpU- 38kDa) from the position of the protein band.

4. Isolation of RNA using Nucleopore Total RNA isolation RNASure® Mini Kit (Genetix)

Reagents Used

1. Cell Culture Media - RPMI1640 (Invitrogen)
   10% FBS (Invitrogen)
   Penicillin-Streptomycin
2. Lysis Buffer (LBA1) (Nucleopore)
3. β-mercaptoethanol
4. 70% ethanol
5. Desalting Buffer (DSB) (Nucleopore)
6. rDNAse (Nucleopore)
7. Reaction Buffer (Nucleopore)
8. LBA2 buffer (Nucleopore)
9. Wash buffer (WBA3) (Nucleopore)
10. RNAse-free Water (Nucleopore)

Materials Used

1. 6 well culture plates
2. 3mL syringes

3. RNASure® Shredder Column (provided in the kit) (Nucleopore)
4. RNASure® Mini Column (provided in the kit) (Nuclepore)
5. Centrifuge machine

Method

$2x10^6$ cells per 2mL of the media in two wells of a 6 well plate were plated and stimulated with either LPS or rOmpU. The cells were harvested at required time points in labelled 1.5mL centrifuge tubes. Treated cells were isolated by centrifugation. RNA isolation from the treated cells was done by using Nucleopore Total RNA isolation RNASure® Mini Kit (Genetix). Supernatant was discarded and 350µL of lysis buffer (LBA1) containing 3.5µL of β-mercaptoethanol was added to the pellet. The cellular suspension was vortexed vigorously and homogenized using 2mL syringe to completely lyse the cells. Lysate was transferred to a shredder column in a 2mL colletcion tube and centrifuged at 11,000g for 1 min. Shredder column was discarded and the filtrate was transferred to a 2mL centrifuge tube and 350µL of 70% ethanol was added and mixed thoroughly. Mixture was transferred to column (RNASure Mini Column) and placed in a collection tube (2mL). The column was centrifuged at 11,000g for 30 secs. The flow-through was discarded and 350µL of desalting buffer (DSB) was added to the mini column. The column was centrifuged at 11,000g for 1 minute. DNAse enzyme stock was prepared by adding 90µL of DNAse reaction mixture to 10µL of aliquotted r.DNAse. 95µL of the mixture was directly added to the membrane of the mini column and the column was kept for 30mins incubation. After incubation, 200µL of wash buffer (LBA2) was added to the column and the column was centrifuged at 11,000g for 30 secs to stop the reation. The mini column was placed in a new collection tube and 600µL of wash buffer (WBA3) was added to the column. The column was centirfuged at 11,000g for 30 secs. The flow-through was discarded and 200µL of WBA3 was again added to the column. The column was then centrifuged at 11,000g for 3 mins. The mini-column was placed in RNAse free tube and 60µL of RNAse-free water was added directly to the membrane. The column was centrifuged at 11,000g for 1 min to elute the RNA from the column. Isolated RNA was then stored at -80ºC.

5. Complementary DNA (cDNA) Synthesis:

Reagents Used

1. RNAse free water
2. Isolated RNA
3. Maxima Enzyme Mix (Fermentas cDNA synthesis Kit)
4. Reaction Mix (Fermentas cDNA synthesis Kit)

Materials Used

1. Thermal cycler machine (Therma Scientific)
2. Nanodrop

Method

The amount of isolated RNA was determined by using Nanodrop machine. Nanodrop scans the absorbance by RNA from about 200nm upto 350 nm, which is the relevant region for determining RNA concerntration and purity. RNA concentration was measured at 260 nm (Absorbance = 1 at 260 nm equivalent to 40µg/mL of RNA). In a 1.5mL centrifuge tube, mixture was prepared by using reaction mix (4µl), Maxima enzyme mix (2µl), template RNA (1µg), Nuclease-free water (remaining volume) to make total reaction volume 20µl. Mixture was then incubated and run consecutively in program pattern of 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.

6. Semi quantitative PCR (qPCR)

Reagents Used

1. RNAse Free water
2. Synthesized cDNA
3. Fermentas 'maxima SYBR green qPCR master mix (2X)' kit

Materials Used

Eppendorf Realplex Thermo Cycler

Primers Used (Primer Bank)

(HuCCL5(RANTES)	forward primer	CCAGCAGTCGTCTTTGTCAC	20
	reverse primer	CTCTGGGTTGGCACACACTT	20
HuIL8	forward primer	ACTGAGAGTGATTGAGAGTGGAC	23
	reverse primer	AACCCTCTGCACCCAGTTTTC	21
HuCXCL1	forward primer	GAAAGCTTGCCTCAATCCTG	20
	reverse primer	CTTCCTCCTCCCTTCTGGTC	20
HuCXCL2	forward primer	GGGCAGAAAGCTTGTCTCAA	20
	reverse primer	GCTTCCTCCTTCCTTCTGGT	20
HuCXCL5(ENA78)	forward primer	AGCTGCGTTGCGTTTGTTTAC	21
	reverse primer	TGGCGAACACTTGCAGATTAC	21

Method

Master mix was prepared by adding maxima SYBR green qPCR master mix (5µL), forward primer and reverse primer (1µL), nuclease-free water (3µL) to make the total

reaction volume 10µl. The master mix was mixed thoroughly and in aliquottes of 9µL was dispensed into PCR tubes or plates. Template DNA were added (1µL) to the individual PCR tubes or wells containing master mix. The reactions were gently mixed without creating bubbles and centrifuged for 5 mins at 3000rpm. The qPCR reaction was ran in Eppendorf Realplex Thermo Cycler and the program pattern was: incubation at 95^{o}C for 10min 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. The relative quantization of cDNA expression was done using ΔΔCt method as per the protocol mentioned in Schmidt and Livek *et al.*

RESULTS

1. Probing of CCL-5 expression in gene level by OmpU and LPS in THP-1 human monocyte cell line.

CCL-5 chemokine is a global recruiter of leukocytes from blood to the inflammatory sites. Monocytes secrete CCL-5 in response to pro-inflammatory mediators. We therefore, probed THP-1 monocytes with recombinant OmpU (rOmpU) protein and did a time course study at the gene level. We also probed the THP-1 monocytes with LPS as a control and did a time course study. LPS is present in the outer membrane of gram-negative bacteria and it is a potential inducer of pro-inlammatory responses. LPS had been reported to have role in CCL-5 upregulation (Sadik *et. al*,2011)

i) *CCL-5* mRNA expression was upregulated by LPS and the expression was maximum at 24 hours.

We have probed THP-1 monocytes with LPS at 5 different time points of 2 hours, 4 hours, 8 hours, 12 hours and 24 hours. The mRNA expression of *CCL-5* gene was studied across the time points (Figure 1).

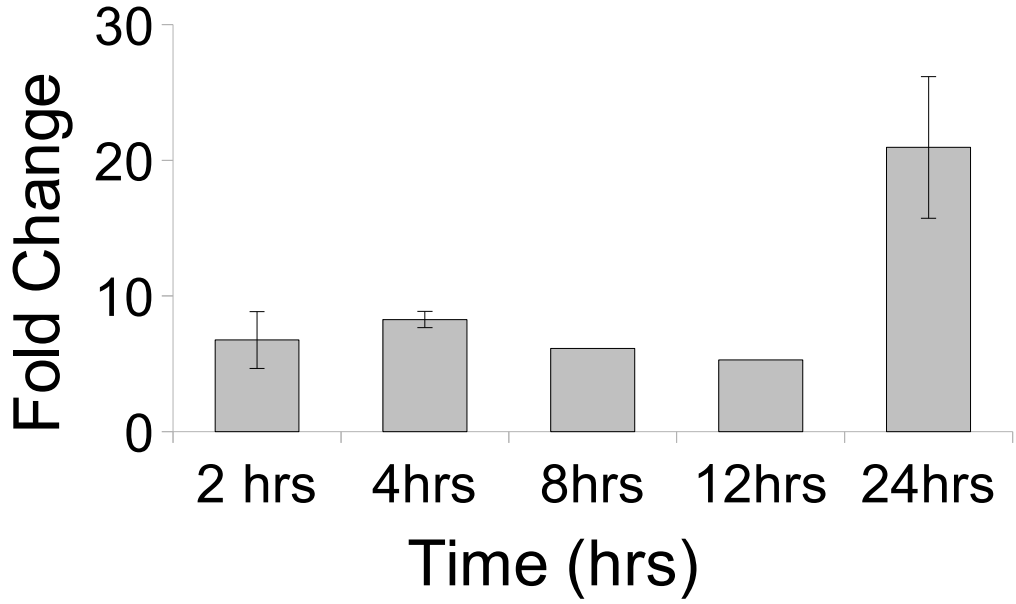

Figure 1. Time dependent expression profile of CCL-5 gene upregulation in THP-1 monocytes in response to LPS, measured by semi-quantitative PCR. 4x10^6 cells were treated with 1μg/mL of LPS at 2h, 4h, 8h, 12h and 24h. All the data had been done in triplicates (except 8h and 12h are in duplicates) and normalized with housekeeping genes (hHprt, 18sRNA and Rpl13) expression.

ii) *CCL-5* mRNA expression is upregulated by OmpU and the expression was maximum at 8 hours.

We have probed THP-1 monocytes with OmpU at 5 different time points of 2 hours, 4 hours, 8 hours,12 hours and 24 hours. The mRNA expression of *CCL-5* gene was studied across the time points (Figure 2).

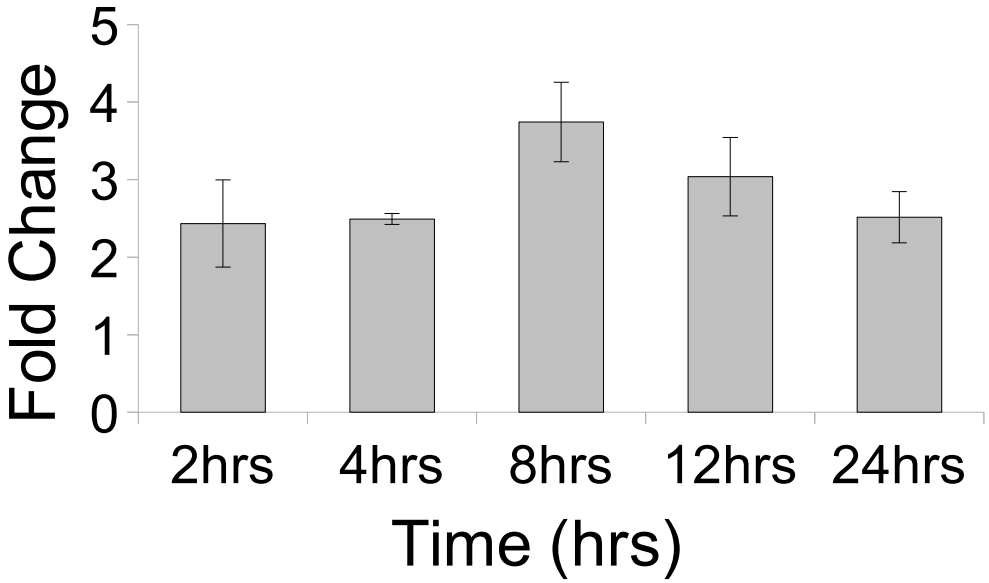

Figure 2. Time dependent expression profile of CCL-5 gene upregulation in THP-1 monocytes in response to OmpU, measured by semi-quantitative PCR. 4x10^6 cells were treated with 1.5µg/mL of OmpU at 2h, 4h, 8h, 12h and 24h. All the data had been done in triplicates and normalized with housekeeping genes (hHprt, 18sRNA and Rpl13) expression.

2. Probing of CXCL-1 expression in gene level by OmpU and LPS in THP-1 monocyte cell line.

CXCL-1 chemokine recruits neutrophils from bone marrow to blood circulation by inducing switching of CXCR4 to CXCR2 receptor on the surface of neutrophils. Monocytes secrete CXCL-1 in response to pro-inflammatory mediators. We therefore, probed THP-1 monocytes with recombinant OmpU (rOmpU) protein and did a time course study at the gene level. We also probed the THP-1 monocytes with LPS as a control and did a time course study. LPS had been reported to have role in CXCL-1 upregulation (Sadik *et. al*, 2011)

i) *CXCL-1* mRNA expression was upregualted by LPS and maximum expression was at 2 hours.

We have probed THP-1 monocytes with LPS at 5 different time points of 2 hours, 4 hours, 8 hours, 12 hours and 24 hours. The mRNA expression of *CXCL-1* gene was studied across the time points (Figure 3).

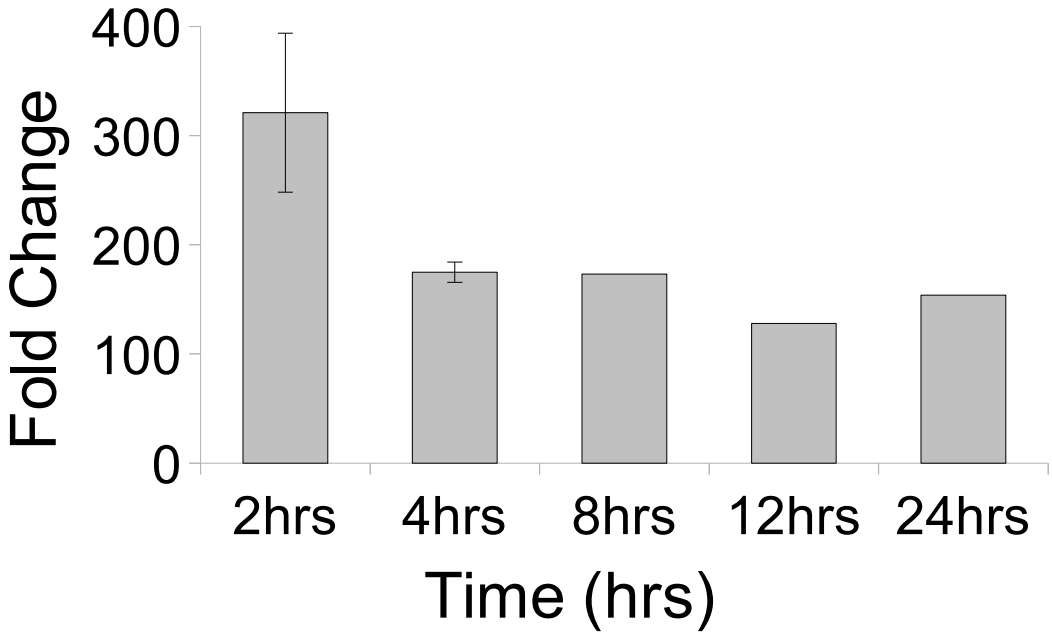

Figure 3. Time dependent expression profile of CXCL-1 gene upregulation in THP-1 monocytes in response to LPS, measured by semi-quantitative PCR. 4x10^6 cells were treated with 1μg/mL of LPS at 2h, 4h, 8h, 12h and 24h. All the data had been done in triplicates (except 8h, 12h & 24h is in duplicates) and normalized with housekeeping genes (hHprt, 18sRNA and Rpl13) expression.

ii) *CXCL-1* mRNA expression is upregulated by OmpU and the expression was maximum at 2 hours.

We have probed THP-1 monocytes with OmpU at 5 different time points of 2 hours, 4 hours, 8 hours,12 hours and 24 hours. The mRNA expression of *CXCL-1* gene was studied across the time points (Figure 4).

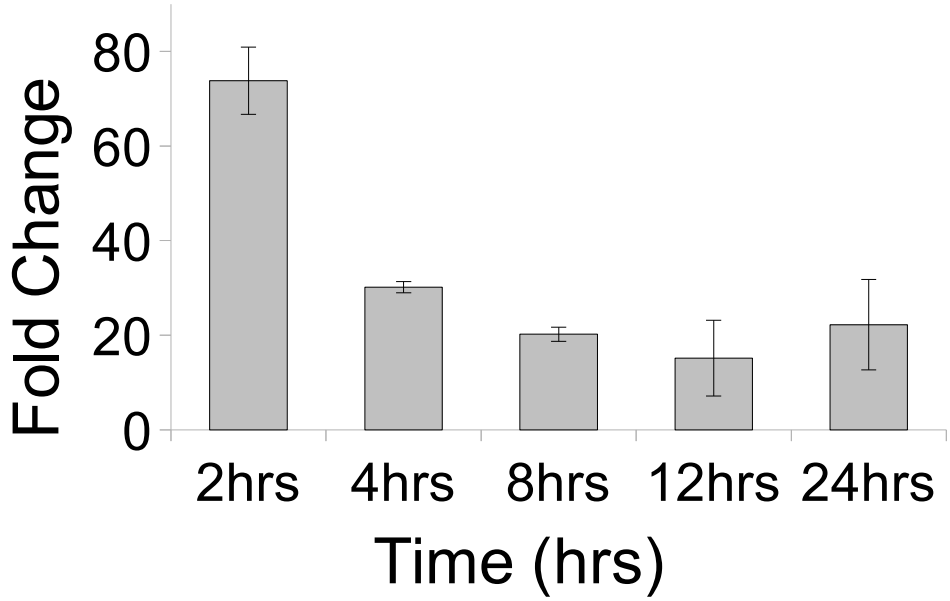

Figure 4. Time dependent expression profile of CXCL-1 gene upregulation in THP-1 monocytes in response to OmpU, measured by semi-quantitative PCR. 4x10^6 cells were treated with 1.5µg/mL of OmpU at 2h, 4h, 8h, 12h and 24h. All the data had been done in triplicates and normalized with housekeeping genes (hHprt, 18sRNA and Rpl13) expression.

3. Probing of CXCL-2 expression in gene level by OmpU and LPS in THP-1 monocyte cell line.

CXCL-2 chemokine recruits neutrophils from blood circulation to the site of infection. Monocytes secrete CXCL-2 in response to pro-inflammatory mediators. CXCL-2 binds to the CXCR2 receptor present on the surface of neutrophils and provides a chemotactic gradient for the neutrophils to follow to the site of infection. We therefore, probed THP-1 monocytes with recombinant OmpU (rOmpU) protein and did a time course study at the gene level. We also probed the THP-1 monocytes with LPS as a control and did a time

course study.

i) *CXCL-2* mRNA expression was upregulated by LPS and expression maximum was at 2 hours.

We have probed THP-1 monocytes with LPS at 5 different time points of 2 hours, 4 hours, 8 hours, 12 hours and 24 hours. The mRNA expression of *CXCL-2* gene was studied across the time points (Figure 5).

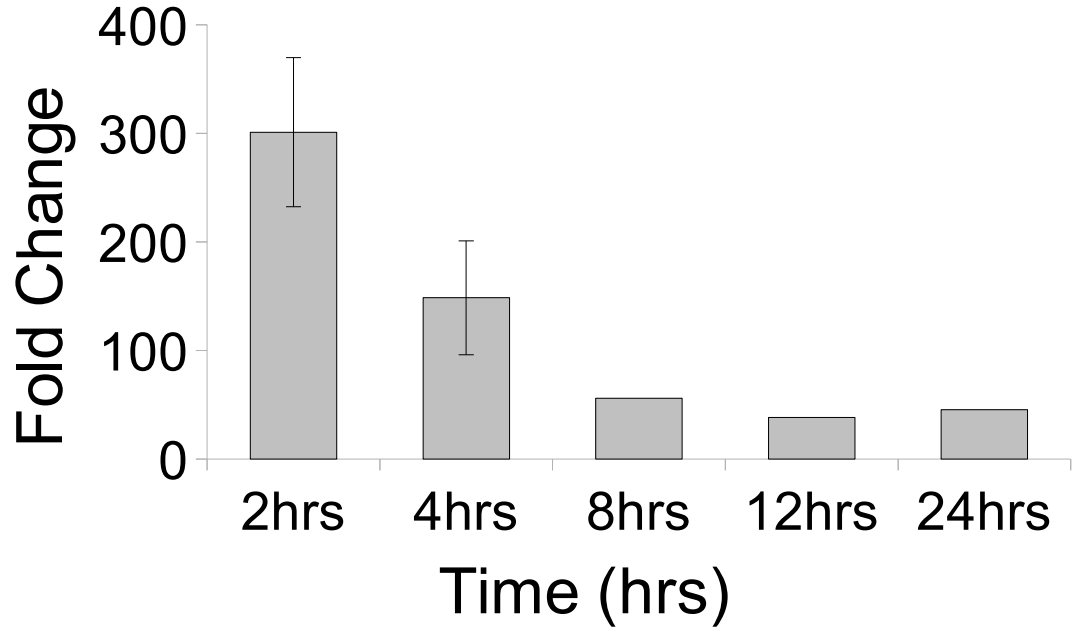

Figure 5. Time dependent expression profile of CXCL-2 gene upregulation in THP-1 monocytes in response to LPS, measured by semi-quantitative PCR. 4x10^6 cells were treated with 1μg/mL of LPS at 2h, 4h, 8h, 12h and 24h. All the data had been done in triplicates (except 8h, 12h & 24h is in duplicate) and normalized with housekeeping genes (hHprt, 18sRNA and Rpl13) expression.

ii) *CXCL-2* mRNA expression is upregulated by OmpU and the expression was maximum at 2 hours.

We have probed THP-1 monocytes with OmpU at 5 different time points of 2 hours, 4 hours, 8 hours,12 hours and 24 hours. The mRNA expression of *CXCL-2* gene was

studied across the time points (Figure 6).

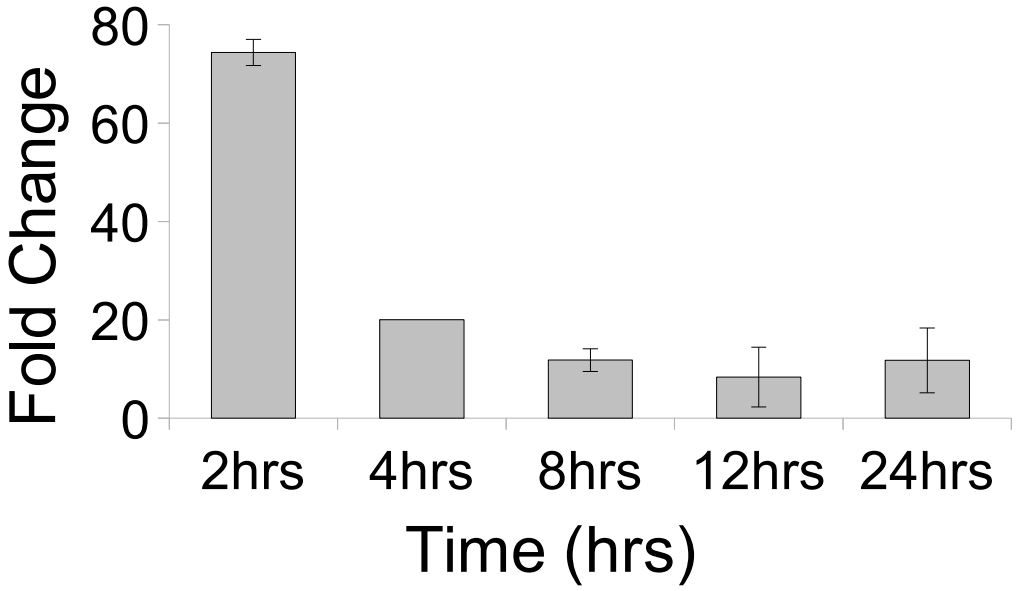

Figure 6. Time dependent expression profile of CXCL-2 gene upregulation in THP-1 monocytes in response to OmpU, measured by semi-quantitative PCR. 4x10^6 cells were treated with 1.5µg/mL of OmpU at 2h, 4h, 8h, 12h and 24h. All the data had been done in triplicates (except 4 hours is in duplicate) and normalized with housekeeping genes (hHprt, 18sRNA and Rpl13) expression.

4. Probing of CXCL-5 expression in gene level by OmpU and LPS in THP-1 monocyte cell line.

CXCL-5 chemokine recruits neutrophils by binding with CXCR2 receptor present on the surface of neutrophils, from blood circulation onto the surface of infected tissue by baso-laterally polarizing the epithelium cells. This polarization helps in the diapedesis of neutrophils in the infected tissue. Monocytes secrete CXCL-5 in response to pro-inflammatory mediators.. We therefore, probed THP-1 monocytes with recombinant OmpU (rOmpU) protein and did a time course study at the gene level. We also probed the THP-1 monocytes with LPS as a control and did a time course study. LPS had been reported to have role in CXCL-2 upregulation (Sadik *et. al*,2011).

i) *CXCL-5* mRNA expression was upregulated by LPS and maximum expression was at 4 hours.

We have probed THP-1 monocytes with LPS at 5 different time points of 2 hours, 4 hours, 8 hours, 12 hours and 24 hours. The mRNA expression of *CXCL-5* gene was studied across the time points (Figure 7).

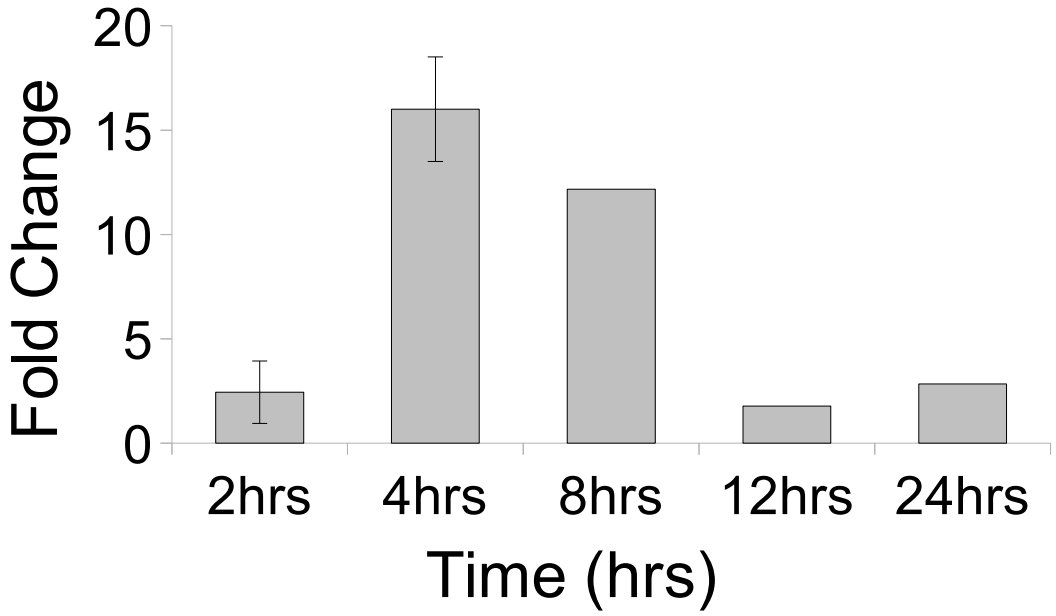

Figure 7. Time dependent expression profile of CXCL-5 gene upregulation in THP-1 monocytes in response to LPS, measured by semi-quantitative PCR. $4x10^6$ cells were treated with 1μg/mL of LPS at 2h, 4h, 8h, 12h and 24h. All the data had been done in triplicates (except 8h, 12h & 24h are in duplicates) and normalized with housekeeping genes (hHprt, 18sRNA and Rpl13) expression.

ii) *CXCL-5* mRNA expression is upregulated by OmpU and the expression was maximum at 4 hours and continued high expression was observed at 8 hours.

We have probed THP-1 monocytes with OmpU at 5 different time points of 2 hours, 4 hours, 8 hours,12 hours and 24 hours. The mRNA expression of *CXCL-5* gene was studied across the time points (Figure 8).

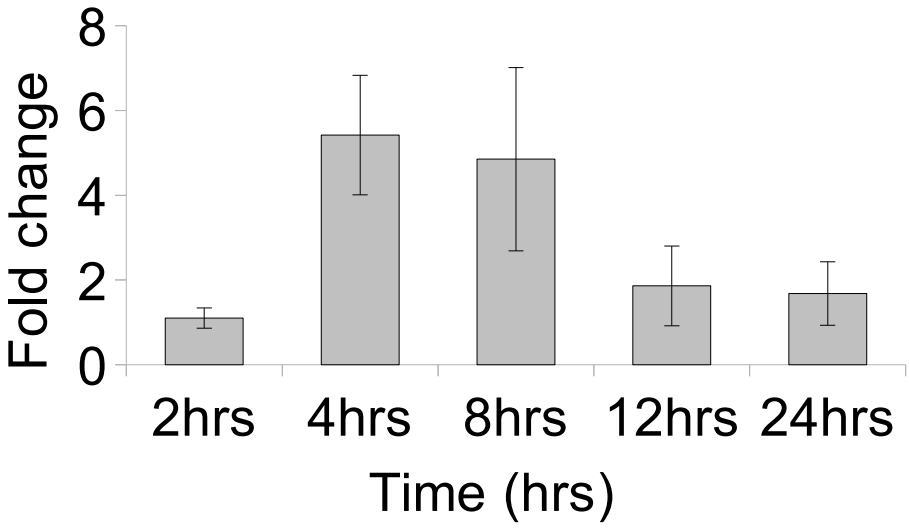

Figure 8. Time dependent expression profile of CXCL-5 gene upregulation in THP-1 monocytes in response to OmpU, measured by semi-quantitative PCR. 4x10^6 cells were treated with 1.5µg/mL of OmpU at 2h, 4h, 8h, 12h and 24h. All the data had been done in triplicates and normalized with housekeeping genes (hHprt, 18sRNA and Rpl13) expression.

5. Probing of IL-8 (CXCL-8) expression in gene level by OmpU and LPS in THP-1 monocyte cell line.

IL-8 (CXCL-8) chemokine is a well studied recruiter of neutrophils. It binds to CXCR2 receptor present on the surface of neutrophils, and provides a strong chemotactic gradient to neutrophils to migrate from bone-marrow to blood to site of infection. Monocytes secrete CXCL-5 in response to pro-inflammatory mediators.. We therefore, probed THP-1 monocytes with recombinant OmpU (rOmpU) protein and did a time course study at the gene level. We also probed the THP-1 monocytes with LPS as a control and did a time

course study. LPS had been reported to have role in IL-8 (CXCL-8) upregulation (Sadik *et. al,* 2011).

i) *IL-8 (CXCL-8)* mRNA expression was upregulated by LPS and maximum expression was at 24 hours.

We have probed THP-1 monocytes with LPS at 5 different time points of 2 hours, 4 hours, 8 hours, 12 hours and 24 hours. The mRNA expression of *IL-8 (CXCL-8)* gene was studied across the time points (Figure 9).

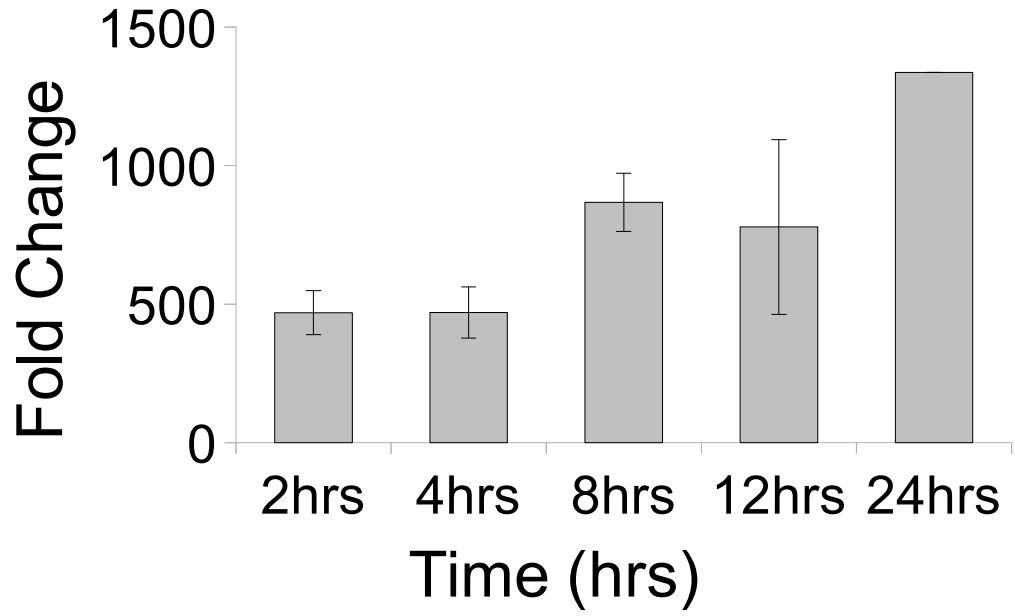

Figure 9. Time dependent expression profile of IL-8 (CXCL-8) gene upregulation in THP-1 monocytes in response to LPS, measured by semi-quantitative PCR. 4x10^6 cells were treated with 1μg/mL of LPS at 2h, 4h, 8h, 12h and 24h. All the data had been done in triplicates (except 24h is in duplicate) and normalized with housekeeping genes (hHprt, 18sRNA and Rpl13) expression.

ii) *IL-8 (CXCL-8)* mRNA expression is upregulated by OmpU and the expression was maximum at 2 hours and recurrent high expression was observed at 8 hours.

We have probed THP-1 monocytes with OmpU at 5 different time points of 2 hours, 4 hours, 8 hours,12 hours and 24 hours. The mRNA expression of *IL-8 (CXCL-8)* gene was studied across the time points (Figure 10).

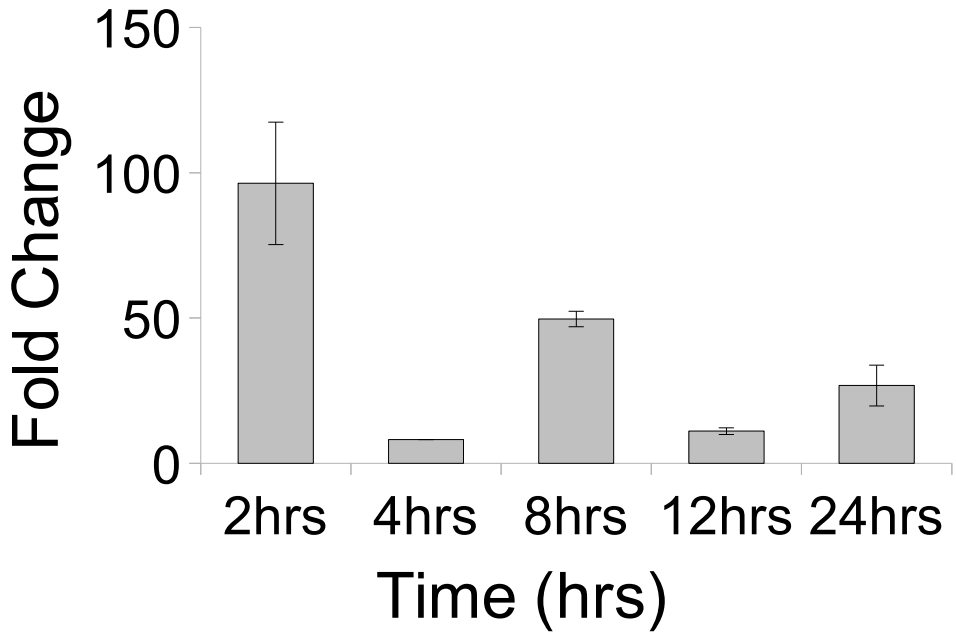

Figure 10. Time dependent expression profile of IL-8 (CXCL-8) gene upregulation in THP-1 monocytes in response to OmpU, measured by semi-quantitative PCR. 4x10^6 cells were treated with 1.5μg/mL of OmpU at 2h, 4h, 8h, 12h and 24h. All the data had been done in triplicates (except 4 hours is in duplicate) and normalized with housekeeping genes (hHprt, 18sRNA and Rpl13) expression.

DISCUSSION

Neutrophils are the most potent phagocytic cells and they take over the phagocytotic activity of macrophages in an infected tissue. Neutrophils are recruited by monocytes and macrophages to the site of infection to kill the bacteria causing the infection. The five chemokines crucial for neutrophil recruitment are CCL-5 (RANTES), CXCL-1, CXCL-2, CXCL-5 (ENA-78) and IL-8 (Sadik, Kim & Luster 2011). These chemokines are strictly regulated by the monocytes or macrophage thus preventing any matured neutrophils to migrate in the blood circulation without any stimulation. If the recruitment process is not regulated, such wandering matured neutrophils could attack the host cells recognizing them as non-self entities, such cases leads to auto-immune diseases like arthritis etc. The recruitment process is an important part of the innate immune response. Therefore we tried to address a question, whether OmpU gets immunologically recognized by THP-1 monocytes, to induce the expression of chemokines involved in and initiate neutrophil recruitment.

LPS is a well known potent inducer of pro-inflammatory responses. LPS had also been observed to induce expression of the five chemokines: CCL-5, CXCL-1, CXCL-2, CXCL-5 and IL-8 in monocytes and macrophages. We have used LPS as our control and observed that the THP-1 monocytes expressed these chemokines when treated with LPS (as reported in literature). Time dependent experiment showed the expression profile of these five chemokines at gene level in response to LPS. The expression profile of each chemokine was different. CCL-5 and IL-8 chemokines that provides chemotactic gradient for the migration of neutrophils from bone marrow to the site of infection had the maximum expression at 24 hours. CXCL-1 and CXCL-2 chemokines involved in maturation of neutrophils and migration of neutrophils from bone marrow to blood and from blood to site of infection, had the maximum expression at 2 hours. CXCL-5 chemokine involved in attachment and entry of neutrophils via diapedesis at the site of infection had the maximum expression at 4 hours. These studies showed that LPS up-regulatedinduces monocytes to recruit neutrophils from bone-marrow to the site of infection as reported in literature (Sadik *et. al*, 2011; Starr *et. al*, 2012; Heit *et. al,* 2008). These findings helped to further study the effect of OmpU on the expression of these five important chemokines.

OmpU is one of the outer membrane proteins of *V. cholerae* and had been reported to play a major role in survival of the pathogen in the gut (Sarkar *et. al*, 2012; Mathur *et. al*, 2004; Simonet *et. al*, 2003; Provenzano *et. al*, 2001; Wibbenmeyer *et. al*, 2002). OmpU had been observed to provide resistance against bile salts and anti-microbial peptides. In

our lab previously it was observed that OmpU up-regulatesss pro-inflammatory responses like IL-6, TNF-α, NO etc. in OmpU treated THP-1 monocytes. It was also observed that OmpU down-regulated the expression of these pro-inflammatory responses in LPS-treated THP-1 monocytes that were pre-incubated with OmpU. These observations suggested that OmpU might have a dual nature of immune responses. Therefore, we decided to study the effect of this dual nature of OmpU in modulation of crucial chemokines involved in neutrophil recruitment.

We probed THP-1 monocytes with 1.5μg of purified rOmpU protein and did a time dependent study at time points of 2h, 4h, 8h, 12h and 24h. Our results showed that rOmpU was able to upregulate the expression of CCL-5, CXCL-1, CXCL-2, CXCL-5 and IL-8 expression at gene level. But the expression profile of each of these chemokines was different suggesting the possible expression pattern involved in complex interplay of chemokines in neutrophils recruitment.

CCL-5 expression was almost expressed equally with differences in fold change between time points being small (Figure 2). Such an expression profile suggests that probably CCL-5 chemokine gradient is required throughout the recruitment process, since CCL-5 helps in migration of neutrophils and other leukocytes in blood circulation.

CXCL-1 and CXCL-2 expression profile was similar and both have their maximum expression at 2 hours (Figure 4 & 6). CXCL-1 induces switching of surface receptor of immature neutrophils from CXCR4 to CXCR2 thus enabling neutrophils to leave the bone marrow and mature in that process. CXCL-2 binds to CXCR2 receptor and induces these mature neutrophils to leave the bone marrow environment and flow in the blood circulation. CXCL-2 recruits mature neutrophils from blood circulation to the site of infection. The expression peak at 2 hours for both of these chemokines suggests that probably these are the first chemokines secreted by monocytes when *V. cholerae* OmpU is immunologically recognized. Due to this the neutrophils start getting matured and migrate from bone marrow to the site of infection.

CXCL-5 expression was maximum at 4 hours and reasonably high at 8 hours (Figure 8). CXCL-5 helps the mature neutrophils in the vicinity of infected tissue to attach and enter the tissue via diapedesis (Starr *et. al*, 2012). CXCL-5 polarizes the epithelium cells on the baso-lateral membrane in the direction of blood flow to allow maximum attachment of neutrophils to the tissue. After attachment neutrophils enters the epithelium via diapedesis. This whole process is strictly regulated by CXCL-5. The expression peak at 4 hours and 8 hours suggests that probably; after neutrophils have reached the vicinity of

infected tissue (that might take minmum of 2 hours in blood circulation), monocytes secrete the chemokine so that epithelium cells get polarized facilitating neutrophil binding to the epithelium and entry into the tissue via diapedesis.

IL-8 (CXCL-8) expression was maximum at 2 hours and reasonable high expression at 8 hours (Figure 10). IL-8 is a potential chemokine primarily involved in chemotaxis of neutrophils. IL-8 recruits migrating mature neutrophils in blood to reach the site of infection by providing a strong chemokine gradient. IL-8 also helps neutrophils to take over the phagocytic activity of macrophages once the neutrophils have entered the infected tissue via diapedesis. The initial expression peak at 2 hours suggests that probably monocytes secrete IL-8 along with CXCL-2 to make the recruitment of migrating neutrophils from bone marrow to the infected site. The second peak at 8 hours suggests that monocytes secrete IL-8 after the neutrophils have entered the infected tissue via diapedesis facilitated by CXCL-5. IL-8 helps the neutrophils that had entered the infected tissue to take over the phagocytic activity of macrophages and kill the bacteria by phagocytosis.

Our study indicates that OmpU was able to induce monocytes to recruit neutrophils from bone marrow to the infected site. OmpU gets immunologically recognized by THP-1 monocytes and thus OmpU acts as a signaling molecule and induces the expression of CCL-5, CXCL-1, CXCL-2, CXCL-5 and IL-8 at mRNA level. THP-1 monocytes expressed these five chemokines in response to LPS. The next study will be to examine whether expression of these chemokines in OmpU pre-treated cells gets affected in response to LPS. The mRNA expression of these five genes shows that the mRNA might translate and form the chemokines at protein level and get secreted by the monocytes. Therefore we need to study the expression of the five chemokines at protein level to justify our proposition that OmpU could stimulate monocytes to recruit neutrophils from bone marrow to the site of infection via the synthesis of these chemokines.

CONCLUSION

In the current study we were able to observe that rOmpU upregulated the expression of CCL-5, CXCL-1, CXCL-2, CXCL-5 and IL-8 in gene level. Thus, suggesting that OmpU could act as a PAMP for immunological recognition by monocytes, activating them to secrete chemokines for neutrophil recruitment. The secretion of the aforementioned chemokines facilitates monocytes to recruit neutrophils from bone marrow to the site of infection. Further experiments needs to be done to check whether there is any change of these chemokines at gene level in OmpU pre-incubated cells stimulated by LPS. This experiment will give us information that whether OmpU could down-regulate the expression of these genes when stimulated by a more potent inducer of immune responses.

Results from these gene level studies indicate that those five chemokines are probably made at protein level because the mRNA expression is up-regulated. Therefore, the production of CCL-5, IL-8, CXCL-1, CXCL-2 and CXCL-5 had to be studied at the protein level. Protein level study will help us to understand the modulation done by OmpU at the protein level.

These experiments need to be replicated in an *in-vivo* system. *In-vivo* studies will provide us with more information regarding the modulation of OmpU on the whole process of neutrophils recruitment. Cumulative *in-vitro* and *in-vivo* studies will help us to answer the role of OmpU in *V. cholerae*'s pathogenesis.

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