

Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD) signaling during *vibrio cholerae* infection

Abstract

Cholera remains a significant health problem in developing countries due to its ability to spread rapidly and kills a high proportion of those affected. The disease is produced by *Vibrio cholerae* that colonizes in the human intestine and causes inflammatory diarrheal diseases. The reactogenicity of vaccine strain causes a serious problem in clinical settings. Besides the study of organisms *V. cholerae*, a thorough understanding of the host response following *V. cholerae* infection is indispensable to combat the disease from newly emerging threats. Elucidation of molecular mechanisms of *V. cholerae* induced inflammatory response through Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD) will eventually help to design proper vaccine or drugs for appropriate targets.

Keywords: cholera, *vibrio cholera*, tlr, nod, cytokines, inflammation, lps, cholera toxin, reactogenicity, vaccine

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Abbreviations

TLR: Toll-Like Receptor; NOD: Nucleotide-Binding Oligomerization Domain; CT: Cholera Toxin; LPS: Lipopolysaccharide; CARDs: Caspase Activating and Recruitment Domains; APCs: Antigen Presenting Cells; MAPK: Mitogen Activated Protein Kinase; NLR: Nod Like Receptor; PYDs: Pyrin Domains; OMV: Outer Membrane Vesicle

Introduction

Vibrio cholerae is a highly motile non-invasive Gram-negative organism which colonizes the small intestine and produces a potent enterotoxin called cholera toxin (CT)—a major virulence determinant that causes massive intestinal fluid loss leading to profuse watery diarrheal syndrome associated with *V. cholerae* infection.^{1,2} There are more than 200 serogroups of *V. cholerae* recognized on the basis of their lipo polysaccharide (LPS) O side chain antigenic structures.³ There are two serogroups of *V. cholerae* O1 and O139 which can infect humans and cause epidemic and pandemic cholera. The serogroup *V. cholerae* O1 is subdivided into two biotypes, classical and El Tor depending on biochemical properties and phage sensitivity. Each biotype can be divided into three serotypes depending on expression of three O-antigens (A, B, and C): (1) Ogawa (A and B), (2) Inaba (A and C) and (3) Hikojima (A, B and C).^{4,5} The pathogenesis of cholera is a multi factorial process and involves several genes encoding virulence factors that aid the pathogen in its colonization, coordinated expression of virulence factors, and toxin action. The expression of virulence factors in *V. cholerae* is coordinately regulated by ToxR, an inner membrane protein which regulates the ctxAB structural gene and CT expression.^{6,7} ToxR also regulates on which requires another Trans membrane transcriptional activator TcpP to synergistically activate the expression of ToxT.^{8,9} ToxR acts as a master regulator and remains under the control of environmental factors.¹⁰ ToxR directly regulates the expression of the outer membrane porin proteins OmpU and OmpT in a separate branch of the ToxR cascade independent of TcpP and ToxT,^{11,12} which has been suggested to be involved in adherence during pathogenesis.^{7,13} Toxinogenicity is predominating pathogenic factor, but colonization is clearly an essential step in disease progression. The organism must colonize the small bowel to

release CT. No diarrhea is seen when volunteers are fed strains of *V. cholerae*, which is unable to colonize.¹⁴

Cholera has traditionally considered as a non-inflammatory diarrheal disease but some evidence point towards an inflammatory component in the pathogenesis of the disease.¹⁵⁻²² including increased infiltration of neutrophils, degranulation of mast cells and eosinophils, and production of some innate defense molecules during acute cholera patients.²⁰⁻²⁶ Cholera patients are often treated with antimicrobials or antibiotics.^{27,28} (Table 1), but the growing trend of antibiotics resistance or poorly designed vaccines is craving the right component of vaccine development which could lead to the effective immunization. However, after elimination of several toxin genes, including the CT from vaccine wild-type strains, mild to moderate diarrhea is inevitable in volunteers.²⁹⁻³¹ The reactogenicity of vaccine strains in volunteer studies points towards the presence of some other component besides CT that can elicit a host response. The adaptive immunity against *V. cholerae* and CT has been investigated intensely for development of effective vaccine.^{26,32,33} Little is known about the innate defense mechanisms during cholera that may be involved in the early defense against the infection and also in the initiation of the adaptive immune response. However, beyond the understanding of the mode of action of CT, we need an extensive knowledge regarding the response of the host to *V. cholerae* infection.

Table I Using of Antibiotics in cholera treatment

Antibiotics used for cholera treatment

Doxycycline.¹¹⁹

Tetracycline.¹²⁰

Ciprofloxacin.¹²¹

Azithromycin.^{122,123}

Erythromycin.¹²⁴

Chloramphenicol.¹²⁵

Furazolidone.¹²⁶

Sulfaguanidine.¹²⁵

Orfloxacin.¹²⁰

Trimethoprim-sulfamethoxazole.¹²⁸

V. cholerae induced inflammatory response

Cell culture models were initiated in hopes of developing an in vitro system for the study of host–*V. cholerae* communication and adherence. The interaction of the host with *V. cholerae* and other Vibrio species has been examined in a number of intestinal epithelial cells models like HT29–18N2, Caco–2, T84, HeLa and Int407 cells.^{34–37} and documented the release of pro inflammatory cytokines upon *V. cholerae* infection in intestinal epithelial cells.³⁸ Recently another experimental model using H4 cells—non transformed human fetal primary small intestinal epithelial cells provided preliminary evidence that CT induces an enhanced secretion mediated in part by a developmental up-regulation of the cAMP response in immature versus mature human small intestine.³⁹ Rodriguez et al has shown that studies on delineating the factor responsible for reactogenicity of vaccine strains with the highly differentiated mucin-secreting cell line HT29–18N2.⁴⁰ Once *V. cholerae* has been detected by epithelial cells, a number of signal transduction pathways are activated within the infected cells and initiate defensive responses by the host.^{37,41–46} These signaling pathways usually result in the activation of nuclear factor kappa B (NF-κB) transcription factors important in driving expression of genes involved in the inflammatory response.^{38,43,47} Certain *V. cholerae* strains as well as CT may stimulate a modest intestinal inflammatory response.^{15,18,25} In a mouse pulmonary model of infection,⁴⁸ have shown the evidence of inflammation including infiltration of polymorpho nuclear leukocytes (PMNs), tissue damage, localized release of tumor necrosis factor (TNF)-α, interlukins (IL)-6 and the neutrophil chemo attractant protein macrophage inflammatory proteins (MIP)-2 by accessory toxins of *V. cholerae*. Recently, *V. cholerae* mediated host inflammation is also scored in the neonatal mouse model.⁴⁹ Similarly, *V. cholerae* vaccine strains promote symptoms consistent with inflammation in human volunteers.⁵⁰ In altogether, these evidences suggest the existence of an inflammatory component in the diarrhea of clinical cholera. But, CT has shown immune deviating properties in macrophages.^{51,52} However, little is known about the role of *V. cholerae* in initiating the innate inflammatory response and the potential contribution of individual *V. cholerae* components to cytokine induction through toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NODs) receptors.

TLR and NOD pathway

Recognition and uptake of microbes are based on germ line-encoded pattern recognition receptors (PRRs) like Toll-like receptors (TLR) and nucleotide-binding oligomerization domain (NOD) receptors.⁵³ These receptors detect conserved microbial structures that are not found in the host. A common aim of the innate immunity is to rapidly detect and stop the spread of a pathogen. In contrast to the innate immunity, adaptive or acquired immunity is specific to foreign antigens. Adaptive immunity offers pathogen-specific detection and targeted immune response, which is usually effective also against those microbes that may evade innate immune responses. Moreover, the immunological memory offers rapid, specific and efficient immune response upon re infection. The development of adaptive immunity requires a complex co-operation between antigen presenting cells (APCs) (macrophages, dendritic cells, and B cells) and T lymphocytes.^{54–56}

TLRs play a crucial role in microbial recognition, induction of antimicrobial genes, cytokines, chemokines and the control of adaptive immune responses.^{57–59} NOD (NOD1 and NOD2)-like receptors (NLRs) are a physically distinct family of intracellular PRRs, which is apparently in the context of microbial reorganization

and the induction of inflammatory response.^{58,60,61} TLRs and NODs are widely expressed in various cell types of the gastrointestinal mucosa and immune cells (monocytes/macrophages or dendritic cells) participating in host defense against microbes.

TLRs and NODs function initiate by following ways:

- Recognition of molecular patterns expressed on pathogens
- Activation of signaling pathways through cascade upon recognition of molecular patterns
- Initiation of secretion of pro/anti-inflammatory cyto- and chemokines that control adaptive immune system
- Induction of antimicrobial functions

TLRs signaling

The TLRs were found to be essential for anti-fungal and anti-bacterial defense in the Drosophila innate immunity system.^{62–64}

Mammalian TLRs are categorized by three common structural features:

- A ligand binding extracellular domain with leucine rich repeats (LRR)
- A short trans membrane region
- A highly homologous cytoplasmic Toll/interleukin 1 receptor (TIR) domain initiates downstream signaling cascades.^{65,66}

TLRs have emerged as the first-line innate immune surveillance systems to detect the presence of foreign pathogens and activate the cytokine responses that occur during infection, and to a large extent, shape the whole of the inflammatory response with all its consequences, both beneficial and harmful. To date, 11TLR homolog's have recognized and their Ligands are peptidoglycan,⁶⁷ LPS,⁶⁸ diacyl- or triacyl-lipopeptide,⁶⁹ dsRNA,⁷⁰ un methylated CpG DNA motifs,⁷¹ and flagellin.⁷² TLR Ligands could be a major focus of adjuvant research for modern vaccine combinations tailored to specific pathogens.⁷³

TLR signaling is activated by ligand binding, which initiates the dimerization or multimerization of TLRs. Subsequently, TIR-domain-containing adaptor molecules are recruited to the intracellular domain of TLRs. Until now, five adaptor molecules have been identified:

- Myeloid differentiation factor 88 (MyD88)
- TIR-domain containing adaptor inducing IFN-β (TRIF)
- TIR-domain containing adaptor protein (TIRAP)
- TRIF-related adaptor molecule (TRAM)
- Sterile alpha and HEAT/Armadillo motif protein (SARM).⁷⁴

The activation of downstream signaling is mediated by MyD88 and TRIF.⁷⁵ TLR signaling results in the activation and nuclear translocation of NF-κB, interferon regulatory factors (IRFs) and mitogen activated protein kinase (MAPK)-regulated transcription factors.⁶⁶ These transcription factors regulate the expression of pro-inflammatory cytokines, chemokines and IFNs.

NODs signaling

Nod1 and Nod2, two cytosolic mammalian proteins, have been recognized as intracellular peptidoglycan receptors.⁷⁶ Both contains caspase activating and recruitment domains (CARDs) at their NH2-

termini, a single CARD domain in Nod1 and two tandem CARD domains containing receptor-interacting proteins (Rip)2 in Nod2, followed by a nucleotide binding domain and a series of tandem LRRs. The interaction between Nod1 and Rip2 initiates CARD–CARD association. Rip2 binds to the IκB kinase (IKK) complex, which is the essential component in the pathway that leads to the activation of NF-κB. Rip2 is also involved in the TLR-mediated activation of NF-κB, suggesting a convergence of the signal transduction pathways that are activated by TLRs and Nod1.⁷⁷ Nod1 detects peptidoglycan from gram-negative bacteria, whereas Nod2 can detect peptidoglycan from both gram-negative and gram-positive bacteria.^{78,79} Nod-like receptor (NLRP) protein scan form inflammasomes in activated cells through interaction of their pyrin domains (PYDs) with other pyrin-containing proteins, namely the adaptor ASC, which allows the formation of the inflammasome complex, recruiting and activating caspase-1, resulting in the processing of the potent inflammatory cytokines.^{60,80}

Activation of TLR and NOD pathways by *V. cholerae* expressed molecular patterns LPS: LPS, one of the major components of the outer membrane of *V. cholerae* induces pro inflammatory cytokines through MyD88-dependent TLR4 signaling pathway in macrophages.^{81,82} LPS could not activate the TLR4 signaling efficiently in epithelial cells,⁸³ due to lack of CD14 and TLR4 on epithelial cells.⁸⁴ *V. cholerae* mediated pro inflammatory response is also attenuated in mice with mutation in TLR4 gene.⁸⁵ MsbB has functional lipid A acyltransferase which is prerequisite for transferring a 3-hydroxylaureate to the glucosamine disaccharide in *V. cholerae* LPS.⁸⁶ The under acylated LPS is generated in msbB mutant strain of *V. cholerae* which exhibits less endotoxicity due to reduced stimulation of TLR4.⁸⁷ During the acute stage of *V. cholerae* infection, LPS-TLR4 mediated inflammatory responses could be blocked by host derived LPLUNC1.⁸⁸ *V. cholerae* phosphatidylserine decarboxylase (PSD) is capable of activating host innate immunity through TLR4.⁸⁹ and it could have potential application for vaccine adjuvant.

Outer membrane vesicle (OMV): OMV, which is considered as an alternative vaccine candidate for cholera infection also contains a relative large amount of LPS, peptidoglycan and CT.⁹⁰⁻⁹³ OMV interacts with epithelial cells and leads to inflammatory response.⁹⁴ TLR2 signaling pathway is activated by OMV of *V. cholerae* in human embryonic kidney cell line.⁸⁸ The quorum sensing regulator hapR regulates OMVs which might influence immune response through NOD1 and NOD2 pathway.⁹⁵ *V. cholerae* OMV activates pro inflammatory response through NOD1 pathway and promotes T cell polarization for adaptive immunity.⁹⁴

Flagellins: *V. cholerae* possesses a single polar flagellum which contains a flagellar core protein enclosed by a membrane sheath that appears to be contiguous with the outer membrane.⁹⁶ The flagellar filament itself is comprised of multiple flagellin subunits, which are highly related and conserved. The flagellin genes are organized in two unlinked chromosomal loci, flaAC and flaEDB.⁹⁷ The flaA gene is indispensable for assembly and function of the flagellum since a mutation in flaA but not in the remaining four flaBCDE genes, abolished flagella production and motility.⁹⁷ Motility of *V. cholerae* plays a crucial role in colonization of the bacteria to the small intestinal mucosa.⁹⁸ FlaA, FlaB and FlaD are recognized by the innate immune system through TLR5 and induces pro inflammatory cytokines through NF-κB and MAPK pathway.^{47,99} The inflammation induced by flagellins could be a major determinant for the *V. cholerae* vaccine reactogenicity.

Outer membrane proteins (Omp): The outer membrane protects Gram-negative bacteria against a harsh environment. The embedded

proteins are critical to the bacterial cell, such as translocation of solute or protein, as well as signal transduction. Expression of the OmpU outer membrane protein of *V. cholerae* is positively regulated by master regulator toxR.^{11,100} OmpU has adhesive properties which could play an important role in the pathogenesis of cholera.¹³ OmpU could potentially activate pro inflammatory response in epithelial cells.^{43,46} and macrophages. However, pre-exposure of OmpU could potentially block the TLR4-LPS induced cytokines.¹⁰¹

Accessory toxins: The main concern with non-CT-producing *V. cholerae* is that these strains are often sufficiently virulent to make them unsafe for use as live attenuated vaccines.^{15,102,103} The actions of accessory toxins of *V. cholerae* are responsible for the increased inflammation and contribute to the reactogenicity of live attenuated vaccine strains.^{29,85,104,105}

V. cholerae Hemolysin (HlyA) is an extracellular membrane-damaging and water-soluble cytolytic exotoxin with a molecular mass of 65 kDa.^{4,106,107} which contains two contiguous lectin domains, a β-trefoil domain homologous to the galactose-binding site of ricin and a β-prism domain homologous to the carbohydrate-binding site of the plant lectin jacalin.¹⁰⁸ The C terminus β-prism lectin domain, considered as the only functional sugar-binding site of HlyA, is susceptible to proteolytic deletion.¹⁰⁹ HlyA protein exhibits a dual effect on macrophage function. TLR4-Myd88 signaling is activated by β-prism lectin domain of HlyA whereas TLR2 signaling is up-regulated by HlyA cytolytic protein domain for continuity of macrophage activation.¹¹⁰ It induces apoptosis independent of TLR signaling like most of the toxin.¹¹¹ and activates the resting macrophages through TLR.¹¹⁰ *V. cholerae* hemolysine plus NOD1/NOD2 ligand induces NLPR3 inflammasome regulated caspae1 activation through NF-κB pathway.¹¹²

V. cholerae secreted cytolysin (VCC) is another β-barrel group pore forming toxin of 79kDa molecular mass. Under proteolytic activation it forms oligomers in the membrane bound stage only.¹¹³ VCC acts as TLR2 Ligands in mast cells and activates adaptive immune response.¹¹⁴ A recent study has demonstrated that VCC is recognized by the host epithelial cells/macrophages and induces pro inflammatory response through TLR2/TLR6 dependent signaling pathway.¹¹⁵

The multifunctional-auto processing repeats-in-toxin (MARTX) bacterial protein toxins modulates the virulence Vibrio species and serve as delivery platforms for cytotoxic effect or domains. This family of toxins is defined by the presence of a cysteine protease domain (CPD), which proteolytically activates the *V. cholerae* MARTX toxin.¹¹⁶ *V. cholerae* MARTX also induces NLRP3-dependent caspase-1 activation.¹¹² but the detailed mechanism needs further investigation. These accessory toxins likely damage the cell membrane to transport the PAMPs into the cytosol activates the inflammatory response in the infected sites through formation of inflammasome. *V. cholerae* accessory toxins induce inflammatory responses through the infection of intestinal cells, which do not express TLRs or are insensitive to TLR signals (TLR2/4) to evoke tissue damage that may promote bacterial colonization.

Crosstalk between epithelial cells and immune cells during *v. cholerae* infection

V. cholerae colonizes in the intestinal epithelial layer and is transcytosed by specialized micro folded (M) cells, which is responsible for mucosal immune response.¹¹⁷ *V. cholerae* associated molecular patterns are recognized by the PRR in the intestinal epithelial cells and produces host defense molecules like cytokines

and chemokines. The coordinated release of cytokines/chemokines by intestinal epithelial cells is crucial for activating intestinal mucosal inflammatory responses as well as mucosal innate and adaptive immune responses. *V. cholerae* or *V. cholerae* flagellin activated epithelial cells secretes intestinal dendritic cells (DCs) inducing chemokines and subsequently stimulates immature DC cells.¹¹⁸ Therefore, DCs undergo maturation, which is associated with high surface expression of co-stimulatory molecules as well as different secreted immuno modulatory cytokines through MAPK and Signal Transducer and Activator of Transcription (STAT) pathway that drives the naïve T cells into T-helper (Th) 1 or 2 cells.¹¹⁸ A recent study has shown that *V. cholerae* produced OMV also activates DCs and primes a distinct population of CD4+ T cells that secrete IL-17.⁹⁴ In addition to Th1 or Th2 cells, DCs have also been implicated in the promotion of Th17 cells. Therefore, epithelial cells secreted inflammatory cytokines induce complex cross-talk between epithelial cells and immune cells and promote the pro inflammatory response by polarizing Th2/Th17 response during acute stage of cholera.^{26,94,118} To understand the reactogenicity vaccine strain, it is necessary to address the involvement of TLRs/NODs and *V. cholerae* associated molecular patterns in crosstalk between epithelial cells and immune cells.

Conclusion

V. cholerae coordinates the regulation of virulence gene functions and modulates the host innate immune response during the acute stage of infection. The progression of the diseases is extremely complex and multi factorial in nature. The recent studies demonstrate that *V. cholerae* induces nuclear responses through signal transduction pathway and subsequently activation of pro inflammatory cytokines modulated by *V. cholerae* secretary factors, virulence and motility may explain some of its reacotogenic mechanisms. Reactogenicity is a major concern with *V. cholerae* vaccine in the present scenario. Therefore, deletion of the accessory toxins and flagellin genes from the vaccine strain could be a way to develop the live attenuated vaccine for *V. cholerae* as well as other enteric pathogens. However, there is a significant gap in understanding the *V. cholerae* mediated inflammation through TLR or NOD pathways which needs further investigation. The interaction of *V. cholerae* expressed factors with TLR/NOD could be an emerging field in mucosal innate immunity and the future studies revealing the role of PAMPs in *V. cholerae* pathogenesis will be helpful to generate safe, live-attenuated and non-reactogenic *V. cholerae* vaccine strain.

Acknowledgment

None.

Conflict of Interest

None.

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