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Human epithelial cells stimulated with *Vibrio cholerae* produce thymic stromal lymphopoietin and promote dendritic cell-mediated inflammatory Th2 response

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ABSTRACT

Vibrio cholerae induces acute inflammatory response at intestinal epithelial surface; the underlying cellular immune mechanisms for such effects are largely unexplored. Mucosal immune response is controlled by crosstalk between the intestinal epithelial cells (ECs) and dendritic cells (DCs). An EC-derived cytokine thymic stromal lymphopoietin (TSLP) has been found a critical regulator of several human inflammatory conditions. TSLP is highly elevated in ECs stimulated with *V. cholerae* and its recombinant flagellin (rFlaA). *V. cholerae* treated human ECs produce DC-attracting chemokine MIP-3α (CCL20). Flagellin, a potent *V. cholerae* factor was responsible for maximum stimulation of epithelial CCL20 production and subsequent DC activation. Activated DCs express high levels of costimulatory molecules and secrete inflammatory cytokines TNF-α, IL-6 and IL-1β. Bacteria stimulated ECs conditioned DCs to produce Th2 cell-attracting chemokines CCL17 and CCL22. TSLP and other mediators present in the *V. cholerae* stimulated EC-culture filtrate potently activated DCs, which subsequently primed $CD4^+$T cells to differentiate into T helper type 2 (Th2) cells that produce high amounts of IL-4, IL-13 and TNF-α and low IFN-γ. TSLP-induced proinflammatory response in DCs involved the transcriptional mechanisms, MAPKs (ERK1/2, p38 and JNK) and STAT3 activation. This study suggests TSLP and other mediators released from ECs in response to *V. cholerae* colonization actively influence DCs in initiating inflammatory response.

1. Introduction

Diarrheal diseases, including cholera, are the leading cause of morbidity and the second most common cause of death among children under 5 years of age globally. *Vibrio cholerae*, a highly motile non-invasive Gram negative bacteria is the etiological agent of cholera which affects several million people in the world each year (Nelson et al., 2009). The pathology of cholera has traditionally been considered noninflammatory, however, substantial clinical reports and laboratory investigations provide evidence for an inflammatory response in the disease (Qadri et al., 2002; Fullner et al., 2002; Bandyopadhaya et al., 2007; McGee et al., 1993; Silva et al., 1996; Speelman et al., 1985). There are no safe vaccines till date, the existing vaccines generate significant side-effects (noncholeric diarrhea and abdominal cramps), often referred to as vaccine "reactogenicity" in human volunteers (Levine et al., 1988; Stokes et al., 2004). The molecular basis of reactogenicity is unknown; this could be due to the presence of some other toxins and/or virulence factors in the bacteria or due to the induction of a proinflammatory response by the host. Th2 cell responses were reported in experimental and clinical cholera (Gagliardi et al., 2000; Xu-Amano et al., 1993; Bhuiyan et al., 2009). However, the roles of cellular immune responses in this disease are unclear.

Intestinal epithelial cells (ECs) in addition to provide primary physical barriers against commensal and pathogenic microorganisms act through multiple mechanisms to actively modulate the immune response in the gastrointestinal tract. Earlier reports have shown the responses of ECs to *V. choreae* or its different components (Bandyopadhaya et al., 2007). However, ECs *in vivo* do not act in isolation but in concert with several immune and nonimmune cells present in the intestinal lumen. Various subpopulations of dendritic cells (DCs) are present in the organized lymphoid structures of the intestinal immune system, including the Peyer's patches, mesenteric lymph nodes and throughout the small intestinal and colonic lamina propria (Coombes and Powrie, 2008). DCs are uniquely potent inducers of immune responses and it is increasingly clear that epithelium has the capacity to modulate the function of DCs and *vice versa*, through production of a diverse array of mediators. ECs were found to actively influence DC functions in bacterial

Abbreviations: ECs, epithelial cells; DCs, dendritic cells; TSLP, thymic stromal lymphopoietin; TSLPR, TSLP receptor; MOI, multiplicity of infection; CT, cholera toxin; LPSs, lipopolysaccharides; rFlaA, recombinant flagellin; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IgG, immunoglobulin G; TER, transepithelial resistance; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; ERK1/2, extracellular signal-regulated kinases; MAPK, p44/42 mitogen-activated protein kinases; SAPK/JNKs, the stress activated protein kinase/c-Jun NH2-terminal kinases.

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handling across EC-monolayers (Rimoldi et al., 2005a,b). In human, an EC-derived cytokine thymic stromal lymphopoietin (TSLP) has been found to potently activate DCs to initiate inflammatory conditions (Soumelis et al., 2002; Coombes and Powrie, 2008). TSLP activates $CD11c^+$ myeloid DCs with the upregulation of major histocompatibility complex class II and co-stimulatory molecules and the production of Th2 cell-attracting chemokines CCL17 and CCL22 (Zhou et al., 2005; Ziegler, 2010). TSLP-activated DCs promote $CD4^+$ T cells to differentiate into inflammatory Th2 cells that produce IL-4, IL-5, IL-13, and TNF-α (Zhou et al., 2005; Ziegler, 2010). Thus, TSLP seems to be capable of initiating an activation cascade that could lead to inflammation and disease. Additionally, TSLP expression is associated with pathogenesis of many inflammatory diseases including atopic dermatitis and asthma (Soumelis et al., 2002; Ziegler, 2010). DCs are one of the several cellular targets of TSLP and myeloid DCs express the highest levels of TSLP receptor (TSLPR) at both the mRNA and protein levels (Liu et al., 2007). The mucosal environment in mammals is highly tolerogenic, however, after exposure to pathogens, it is able to shift toward an inflammatory response. DCs are highly responsible, through the secretion of cytokines and expression of surface markers for the outcome of such immune response. As TSLP is involved in the pathogenesis of inflammatory diseases, we hypothesize that the regulation of TSLP and its influence on DCs may provide the understanding of how we might modulate inflammatory responses induced by *V. cholerae*.

In this study, we analyzed the role of *V. cholerae* stimulated EC–DC crosstalk in generating inflammatory response. We used an *in vitro* coculture system to study EC–DC–*V. cholerae* interaction in a spatial distribution similar to that found *in vivo*.

2. Materials and methods

2.1. Bacterial strains

V. cholerae O395, O395FLAN (O395 insertion in *flaA* gene) and O395CTXAN (O395 insertion in *ctxA* gene) strains were maintained at −70 °C in Luria–Bertani (LB) medium containing 20% (v/v) glycerol. *V. cholerae* cells were grown in LB medium. Streptomycin and ampicillin (Sigma–Aldrich) concentrations were 1 mg/ml and 15 μg/ml, respectively, for *V. cholerae* wherever appropriate (Sarkar et al., 2005).

Bacteria were heat-killed by exposure to 60 °C for 1 h. Metabolically inactive bacteria was obtained by treating the bacterial cell suspension with 0.3% (w/v) sodium azide (Sigma–Aldrich) for 1 h.

2.2. EC culture, infection and stimulation

The cell lines Int407, HT29 and Caco-2 were brought from National Center for Cell Sciences, Pune, India and cultured in the defined media supplemented with 10% fetal bovine serum (GIBCO-BRL, Gaithersburg, MD) containing penicillin/streptomycin and gentamicin in the presence of 5% CO_2 at 37 °C.

Medium was put off from confluent cell layers, washed with PBS and fresh medium without antibiotic was added. Bacteria from overnight culture suspended in antibiotic-free medium were added at ∼100 multiplicity of infection (MOI) and incubated for the stipulated time periods. Cells designated as non-infected controls were also replenished with fresh media.

To stimulate with supernatants, filter sterilized bacterial cell supernatants equivalent to an MOI of 100 bacteria/cell and 5× 100 MOI were incubated with ECs for 3.5 h. In some experiments, supernatants were heat treated (30 min at 95 °C), trypsin treated (2 h, 40 μg/ml; Invitrogen) and proteinase K treated (2 h, 200 μg/ml; Invitrogen), before incubation. Commercial cholera toxin (CT) (Sigma–Aldrich) (4.5 ng/ml for 3.5 h), lipopolysaccharide (LPS) (isolated from *V. cholerae* O139AP-1, 1 μg/ml for 8 h) and recombinant flagellin protein (rFlaA) (1 μg/ml for 3.5 h) of *V. cholerae* were used as other cell stimulants (Bandyopadhaya et al., 2007).

2.3. Construction of V. cholerae mutant strains

O395FLAN and O395CTXAN mutants of *V. cholerae* were constructed as described earlier (Sarkar et al., 2005). Internal fragments of *flaA*, and *ctxA* genes of *V. cholerae* O395 were amplified from genomic DNA using the respective oligonucleotide pairs FlaAF5′-CCATGGAACGCCTCTCATCA-3′ and FlaAR5′-CGTTGATGTAAGTGGCCAGCT-3′; ctxAF5′-TCAGACGGGATTTGTTAGGC-3′, ctxAR5′-CCTGCCAATCCATAACCATC-3′. The fragments were then cloned into a T-vector-pTZ57R/T of InsT/Aclone™ PCR Product Cloning Kit (MBI Fermentas) to obtain the recombinant plasmids. The plasmids were restriction digested with appropriate enzymes and the inserts were cloned into pGP704 to get the respective recombinant plasmids. These recombinant plasmids were then transformed into a λ pir lysogen of *Escherichia coli* SM10. Ampicillin-resistant transformants containing recombinant plasmids were selected and were conjugally transferred from *E. coli* SM10 to *V. cholerae* O395. Transconjugants resistant to both ampicillin and streptomycin were selected. The mutants were verified by PCR and Southern blotting (data not shown).

2.4. RNA extraction and cDNA preparation

ECs were washed with PBS and total RNA was extracted from each group by Rneasy Mini Kit (Qiagen Inc.). For cDNA preparation, 5 μg RNA was treated with Rnase-free DNase (GIBCO-BRL) in a 10-μl volume according to the manufacturer's protocol. Two microliters of DNase-treated RNA were reverse transcribed using the SUPERSCRIPT™ First-Strand Synthesis System (Invitrogen) with 0.50 μg oligo (dT) 12–18 in a total volume of 20 μl. The cDNA was synthesized at 42 °C for 50 min.

For isolation of RNA from DCs, cells were incubated for 24 h in HT29 supernatants with or without *V. cholerae* or rFlaA treatment. Total RNA and cDNA was obtained by the methods detailed above.

2.5. Semiquantitative RT-PCR

Two microliters of cDNA was PCR amplified in a 30 μl reaction volume containing 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5–2.5 mM $MgCl_2$, 0.26 mM of each dNTPs and 25 pmol of each primer as described earlier (Bandyopadhaya et al., 2007). The annealing temperature and product size of all the genes are listed in Table 1. For each chemokine gene, the primers were designed using primer3 software. To determine the chemokine gene expression, reactions were heat denatured for 5 min at 95 °C and then were amplified with 35 PCR cycles each comprising successive incubations at 95 °C for 30 s, annealing (52–57 °C, given for respective gene in Table 1) for 1 min and at 72 °C for 30 s. A further extension step was done at 72 °C for 7 min. PCR experiments for human TSLP, TSLPR, IL-7Rα and OX40L were followed as described (Rimoldi et al., 2005b; Ito et al., 2005). All PCR reactions were normalized by expression of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Amplicons were identified by ethidium bromide staining of agarose gels and scanned in gel documentation system (BioRad, Hercules, USA) and finally quantitated by software ImageJ (http://rsb.info.nih.gov/ij/index.html). The integrated density of each band was normalized to the corresponding G3PDH band.