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Vibrio cholerae porin OmpU induces LPS tolerance by attenuating TLR-mediated signaling

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ABSTRACT

Porins can act as pathogen-associated molecular patterns, can be recognized by the host immune system and modulate immune responses. *Vibrio cholerae*porin OmpU aids in bacterial survival in the human gut by increasing resistance against bile acids and anti-microbial peptides. *V. cholerae*OmpU is pro-inflammatory in nature. However, interestingly, it can also down-regulate LPS-mediated pro-inflammatory responses. In this study, we have explored how OmpU-pretreatment affects LPS-mediated responses. Our study indicates that OmpU-pretreatment followed by LPS-activation does not induce M2-polarization of macrophages/monocytes. Further, OmpU attenuates LPS-mediated TLR2/TLR6 signaling by decreasing the association of TLRs along with recruitment of MyD88 and IRAKs to the receptor complex. This results in decreased translocation of NFκB in the nucleus. Additionally, OmpU-pretreatment up-regulates expression of IRAK-M, a negative regulator of TLR signaling, in RAW 264.7 mouse macrophage cells upon LPS-stimulation. Suppressor cytokine IL-10 is partially involved in OmpU-induced down-regulation of LPS-mediated TNFα production in human PBMCs. Furthermore, OmpU-pretreatment also affects macrophage function, by enhancing phagocytosis in LPS-treated RAW 264.7 cells, and down-regulates LPS-induced cell surface expression of co-stimulatory molecules. Altogether, OmpU causes suppression of LPS-mediated responses by attenuating the LPS-mediated TLR signaling pathway.

1. Introduction

Porins, a class of outer membrane proteins, form channels across the bacterial outer membrane and allow passage of ions and solutes. Porins have versatile functions, as they are present at the critical junction of the bacterial membrane and its environment. Apart from their channel function, porins can mediate antibiotic resistance and act as receptors for complement proteins and bacteriocins (Achouak et al., 2001). Further, porins can act as pathogen-associated molecular patterns (PAMPs), and can be recognized by the host immune cells. Porins may be used by bacteria to their advantage and mediate pathogenesis. For example, OmpU porin of *Vibrio splendidus* helps in bacterial invasion (Duperthuy et al., 2011) and that of *Vibrio vulnificus* (Goo et al., 2006) is an adherence factor, *Neisseria gonorrheae* porin PorB can induce apoptosis of host cells (Duperthuy et al., 2010; Goo et al., 2006; Holm et al., 2004; Kozjak-Pavlovic et al., 2009; Muller et al., 1999; Negm and Pistole, 1999). Conversely, porins can also elicit immune responses by inducing production of pro-inflammatory cytokines, a phenomenon that has been well documented for porins of *Salmonella*, *Shigella* and *Neisseria* species (Biswas et al., 2007; Biswas, 2000; Galdiero et al., 1993, 1990, 2001; Ingalls et al., 2001; Jorth, 2006; Ray et al., 2003; Stein et al., 2010).

The outer membrane of gram-negative bacterium *V. cholerae*, the causal organism of the human diarrheal disease cholera, harbors several porins that play crucial roles in bacterial survival and homeostasis. One of the *V. cholerae* porins, OmpU, has been implicated in anti-microbial peptide resistance and bile acid resistance (Duret and Delcour, 2006; Mathur et al., 2007; Mathur and Waldor, 2004; Merrell et al., 2001). These two key features suggest that *V. cholerae* OmpU helps in bacterial survival in the gut amidst harsh conditions. This concept is reinforced by the fact, that the expression of OmpU increases from 30% to 60%, in the gut by ToxR regulon, which controls the expression of a variety of virulence genes in *V. cholerae*, including that of cholera toxin (Crawford et al., 1998;

Abbreviations: CCL, cysteine cysteine ligand; CXCR, cysteine-x-cysteine receptor; ELISA, enzyme linked immunosorbent assay; IL, interleukin; IRAK, interleukin-1 receptor associated kinase; LPS, lipopolysaccharide; MKP-1, MAPK phosphatase 1; MyD88, myeloid differentiation factor 88; NFκB, nuclear factor kappa B; PAMP, pathogen associated molecular patterns; PRR, pattern recognition receptors; SHIP-1, SH2-containing 5′-inositol phosphatase; SOCS, suppressor of cytokine signaling; TF, tissue factor; TLR, toll like receptor; TNFα, tumor necrosis factor α; TOLLIP, toll interacting protein.

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DiRita and Mekalanos, 1991; Higgins and DiRita, 1994; Krukonis et al., 2000; Miller and Mekalanos, 1984 Provenzano and Klose, 2000). Recently, a study suggested that OmpU can be considered as one of the biomarkers of *V. cholerae* as its sequence is conserved among epidemic strains (Paauw et al., 2014).

Our previous work has shown that *V. cholerae* OmpU is pro-inflammatory in nature as it elicits production of inflammatory mediators such as NO, TNFα and IL-6 in different cell types of mouse and human origin (Sakharwade et al., 2013). Further, it induces M1-polarization of monocytes/macrophages via TLR1/TLR2-MyD88-NFκB-dependent pathway (Khan et al., 2015). Interestingly, we established that *V. cholerae* OmpU has the ability to attenuate host's immune response induced by LPS, a potent pro-inflammatory agent. Cells pretreated with OmpU, exhibit a dampened pro-inflammatory mediator production, upon secondary challenge with LPS, as compared to cells that were treated with LPS alone.

Various studies have revealed that attenuation of inflammatory responses can occur via two phenomena: alternate macrophage activation (M2-polarization) or macrophage tolerance. Alternatively activated macrophages are involved in wound repair and regeneration after an inflammatory insult or can be exploited by pathogens for evasion from anti-microbial responses (Gordon, 2003; Mantovani et al., 2005). Macrophage tolerance is characterized by attenuated pro-inflammatory response upon consecutive encounters with pro-inflammatory stimuli. This phenomenon prevents excess inflammation in vivo and thus prevents the host from its detrimental effects. However, there are contrasting reports whether alternatively activated state occurs during tolerance or not, and whether are these two processes are mechanistically different (Porta et al., 2009; Rajaiah et al., 2013).

In the present study, we have probed the underlying mechanism by which OmpU is able to down-regulate LPS-mediated pro-inflammatory responses. Though it is already known that OmpU itself, classically activates monocytes and macrophages (induces M1-polarization); but in this study we have examined whether down-regulation of LPS-mediated pro-inflammatory responses in OmpU-pretreated cells involves switching of monocyte/macrophage polarization state towards M2-phenotype, or it involves tolerance induction, or whether both the mechanisms are involved.

2. Materials and methods

2.1. OmpU purification

Recombinant form of *V. cholerae* OmpU was prepared as described previously (Sakharwade et al., 2013). Briefly, *V. cholerae* OmpU was recombinantly over-expressed in *Escherichia coli* BL21 (DE3). The recombinant protein was purified under denaturing conditions from urea-solubilized inclusion bodies by Nickel-NTA affinity chromatography, and was subsequently refolded by rapid dilution method and subjected to further purification by size exclusion chromatography (Khan et al., 2012). LPS contamination in purified protein samples was checked by LAL assay as previously described (Sakharwade et al., 2013).

2.2. Cell lines, primary cells and culture condition

RAW 264.7 (murine macrophage), THP-1 (human monocytic leukemia) cells (National Centre of Cell Sciences, Pune, India) and human PBMCs (peripheral blood mononuclear cells) were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA), at 37 °C in a 5% CO_2 humidified incubator. Mouse peritoneal macrophages were cultured in DMEM with 10% fetal bovine serum, 100 U/ml penicillin-streptomycin (Invitrogen Life Technologies).

Peritoneal lavage from six to eight week old female BALB/c mice was obtained from Panacea Biotec (Mohali) (approved by the Institutional Animal Ethical Committee of Panacea Biotec, Mohali). Freshly isolated cells were supplied in ice cold DMEM media (with 10% FBS) in 50 ml conical tube. Immediately cells were plated and after 2 h, non-adherent cells were removed and adherent cells were counted and plated according to experiment.

Peripheral blood mononuclear cells (PBMCs) were isolated from human blood as previously described (Sakharwade et al., 2013). Briefly, blood was drawn from healthy donor and 1.5 mg EDTA (HiMedia, Mumbai) per ml blood was used to prevent coagulation. Blood was diluted with PBS at 1:1 ratio and layered over Histopaque-1077 (Sigma–Aldrich, St. Louis, MO, USA). The sample was centrifuged at 400 × *g* for 30 min at 22 °C without acceleration or deceleration. Buffy layer was collected, transferred to fresh conical tube and washed twice with PBS at 250 × *g* for 10 min at ambient temperature. Cells were resuspended in complete RPMI medium and plated for experiment. Work with human blood is approved by the Institutional Bioethics Committee. Informed consent of all participating subjects was obtained.

2.3. Experimental design

For down-regulation studies, cells were treated with 5 μg/ml Polymyxin B (PmB) (Sigma–Aldrich, St. Louis, MO, USA) for 30 min, after which recombinant OmpU (2 μg/ml) was added and cells were incubated for 24 h. Cells were re-plated in fresh media without PmB and OmpU and stimulated with LPS (1 μg/ml) (Sigma–Aldrich, St. Louis, MO, USA) for different time points depending on experiments. Separately, control cells were treated with PmB for 30 min followed by protein-buffer (Tris–HCl pH 7.6 diluted in PBS containing 0.5% LDAO) for 24 h, then re-plated in fresh media, activated with LPS and incubated for different time periods as required by individual experiments. In some of the experiments, OmpU-pretreated cells, further treated with PBS served as negative control.

For gene expression studies, cells were plated at a density of 1.5×10^6 cells/ml in a 6 well plate with 2 ml of complete media in each well and treated as above. Cells were harvested for RNA isolation at various time points following respective treatments.

Unless otherwise mentioned, for cell surface expression study or cytokine analysis, cells were plated as 1×10^6 cells/ml in a 6 well plate with 1.5 ml of complete media in each well. Cells were harvested or culture supernatants were collected at different time points following respective treatments and analysed by flow cytometry or ELISA.

2.4. Analysis of mRNA levels for various signaling mediators

RNA isolation was carried out using Nucleo-pore RNA sure mini kit (Genetix Biotech, New Delhi, India) according to manufacturer's instructions. cDNA was synthesized from total RNA obtained using Maxima First Strand cDNA Synthesis Kit (Thermo, Fisher Scientific, Waltham, MA, USA). Semi-quantitative real time PCR was performed using Power SYBR mix (Invitrogen Life Technologies, Carlsbad, CA, USA) on Eppendorf Mastercycler EP Realplex Thermal Cycler (Eppendorf, Hamburg, Germany) according to the manufacturer's protocol. Primer sequences for genes were sourced from Primer Bank (Spandidos et al., 2010). Primers for specified genes were synthesized by IDT Technologies (Integrated DNA Technologies, Coralville, IA, USA). The real-time PCR data was analyzed by comparative C_T method of Schmittgen and Livak (Livak and Schmittgen, 2001).