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Bacterial ligand stimulates TLR2-dependent chemokines of colon cell

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ABSTRACT

Shigella spp. are known to penetrate the colonic epithelial cells causing shigellosis, which results in production of convalescent antibodies against porin, the surface exposed major outer membrane protein. Porin has been categorized as primarily TLR2-ligand and here we validated its signaling procedure in colonic INT-407 cells simulating the host scenario.

Porin up-regulated TLR2 and -6 followed by TLR2·MYD88 complex formation suggesting direct involvement of MYD88 for downstream signaling. Translocation of NF-κB p65 and p50 subunits on to the nucleus indicates involvement of the transcription factor in signaling. Porin-induced TLR signaling specifically stimulated the pro-inflammatory chemokine panel comprising of MIP-1α, MCP-1 and IL-8. Inhibition studies of TLR2 and NF-κB led to abrogation of the pro-inflammatory chemokine response, showing TLR-dependent signaling through NF-κB regulate gut activity.

This work elucidates TLR2 not only scans pathogen-associated molecule but also has a direct role in maneuvering colon cell response.

Introduction

Porins are major outer membrane proteins with pore-forming ability found in all Gram-negative bacteria (Nikaido and Rosenberg 1983). Besides lipopolysaccharide (LPS), convalescent antibodies are generated against porin in patients (Ray et al. 2003). This surface exposed protein from several pathogenic bacterial sources has been established primarily as a Toll-like receptor (TLR)2 ligand (Massari et al. 2002; Nascimento et al. 2012) and is targeted as a promising adjuvant. Since porin has been located as an important antigen in shigellosis patients, focus on its adjuvanticity studies have shown bridging of innate signaling of antigen-presenting cells with adaptive immune response. *Shigella dysenteriae* type 1 is a Gram-negative bacillus that comes in direct contact with colonic epithelial cells, therefore interaction of colon cells with surface exposed porin is important to know because colonic epithelial cells although not part of the immune system is provided with TLR to initiate intricate signaling resulting in skewing of immune responses. TLR signaling of colon cells could be the key event to check microbial pathogenesis. The human colon has to deal with continuous flow of heavy load of bacteria (Ley et al. 2006). Most of the bacteria represent the commensal microbiota that offers nutrients to the host; maintain immune homeostasis and shields against incoming pathogenic microorganisms. Colonic epithelial cells secrete antimicrobial peptides, such as mucin, defensins and histones to control commensal bacteria but for the infringing pathogens they have a separate armament called the TLRs (Hecht 1999). After few hours of entry of pathogenic bacteria, colonic epithelial cells trigger an array of pro-inflammatory chemokine and cytokine production that stimulate the mucosal inflammatory responses (Kim et al. 1998). Thus, epithelial cell signaling cooperate with mucosal immune response culminating in the interplay to exclude the invading bacteria. In pursuit to explain the dual conduct of colonic cells toward self and non-self microbiota, it was revealed that down-regulation of pattern recognition receptor (PRR) signaling pathways are the principal mechanism adapted by colonic epithelial cells to prevent any dysregulated pro-inflammatory gene expression (Abreu et al. 2001). PRRs act by recognizing distinct molecular patterns on pathogens, known as pathogen-associated molecular patterns (PAMPs). TLRs are the most important members of pattern recognition receptor (PRR) family with the ability to recognize PAMPs (Janeway and Medzhitov 2002; Kawai and Akira 2010). Colonic epithelial cells were reported to express TLRs in response to stimulation by pathogen-associated molecules. Flagellin of *Vibrio cholerae* is known to be recognized by TLR5 and culminates in production of IL-1β in INT-407 cells (Bandyopadhaya et al. 2008), pathogenic bacterial DNA up-regulates TLR9 and porin has been found to up-regulate TLR1

Abbreviations: APC, antigen-presenting cell; LPS, lipopolysaccharide; MYD88, myeloid differentiation factor 88; SARM, sterile alpha and TIR motif containing 1; TICAM-1, toll-like receptor adaptor molecule 1; TICAM-2, toll-like receptor adaptor molecule 2; TIRAP, TIR domain containing adaptor protein; TLR2, toll-like receptor 2.

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and -4 in TLR2 down-regulated HT-29 cells (Ewaschuk et al. 2007; Mukherjee et al. 2013). In IEC-18 and HT29-CL19A, TLR2 is involved in binding and uptake of peptidoglycan (Bu et al. 2010).

We used INT-407 colon cells expressing basal level TLR2 (Mukherjee et al. 2013) to delineate the signaling mechanism of porin, primarily a TLR2-ligand and pathogen-associated product. Here we show TLR2 signaling was mediated through MYD88. The signaling check point resulted in stimulation of a specific panel of pro-inflammatory chemokines putatively to alert the immune cells for countering bacterial pathogenesis.

Materials and methods

Immunogen

Porin was purified from *S. dysenteriae* type 1 (strain A020332) (Roy et al. 1994). Briefly, bacteria were centrifuged at 100,000 × *g* following disruption with an ultrasonic disintegrator for the envelope fraction and suspended in 1% sodium lauroyl sarcosine (Sigma–Aldrich, St. Louis, MO, USA). The outer membrane protein (OMP) was isolated by centrifugation at 100,000 × *g* for 1 h and was reconstituted in 2% SDS in 10 mM Tris–HCl, pH 7.7 and ultracentrifuged. The supernatant was applied to a pre-equilibrated Sephacryl S-200 HR column. The fractions containing protein were assayed by the liposome-swelling method to show pore-forming activity of porin (Nikaido and Rosenberg 1983). Absence of trace amount of lipopolysaccharide in the purified porin was confirmed biochemically and by *Limulus* amebocyte lysate assay (Ray et al. 2003).

Cell culture

INT-407 (American Type Culture Collection) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS (Gibco, Grand Island, NY, USA) and antibiotics. Cells (1.5 million/ml/well) were seeded in 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and at 60% confluent monolayer, the cells were treated with porin (10 μg/ml/well) in comparison to untreated controls. For inhibition studies, cells were pre-incubated for 30 min with anti-TLR2 functional grade purified (neutralizing) antibody (eBioscience) to deplete TLR2 effect. Similarly, cells were pre-treated for 1 h with 30 μM/ml BAY-11-7082 IκB phosphorylation inhibitor (Santa Cruz Biotechnology) as per manufacturer's protocol to inhibit nuclear translocation of NF-κB.

Immunoprecipitation and immunoblot

Porin treated INT-407 cells were lysed in cold lysis buffer (Cell Signaling Technology). Supernatant containing the proteins were incubated overnight with mouse anti-human TLR2 (eBioscience) MAb-bound Protein A Sepharose™ CL-4B (Pharmacia, Uppsala, USA). Ag–Ab bound beads were boiled in Laemmli sample buffer for 5 min and the immunoprecipitates were applied to 10% SDS–PAGE. The electrophoresed proteins were transferred to nitrocellulose membrane and probed with anti-human MYD88 Ab or anti-human TLR2 MAb for detection of the respective molecules in TLR2·MYD88 complex. The Western blot imaging was made by incubation with HRP conjugated anti-mouse IgG (Cell Signaling Technology) and Super Signal® West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA).

Flow cytometry

Cells were stained at 4 °C in dark for 20 min with PE-conjugated anti-human TLR2 (eBioscience), MIP-1α, MIP-1β, RANTES, IL-8, FITC-conjugated anti-human IL-6 (BD Bioscience), APC-conjugated anti-human MCP-1, biotin-conjugated anti-human TLR6 (eBioscience), MCP-2 (R&D Systems, Minneapolis, MN, USA) and MCP-3 (BD Bioscience). Biotinylated MAbs were recognized by streptavidin–FITC/APC (BD Bioscience). Intracellular chemokines and cytokines were detected in the cells by fixing and permeabilizing with Cytofix/Cytoperm Kit (BD Pharmingen, San Diego, CA,

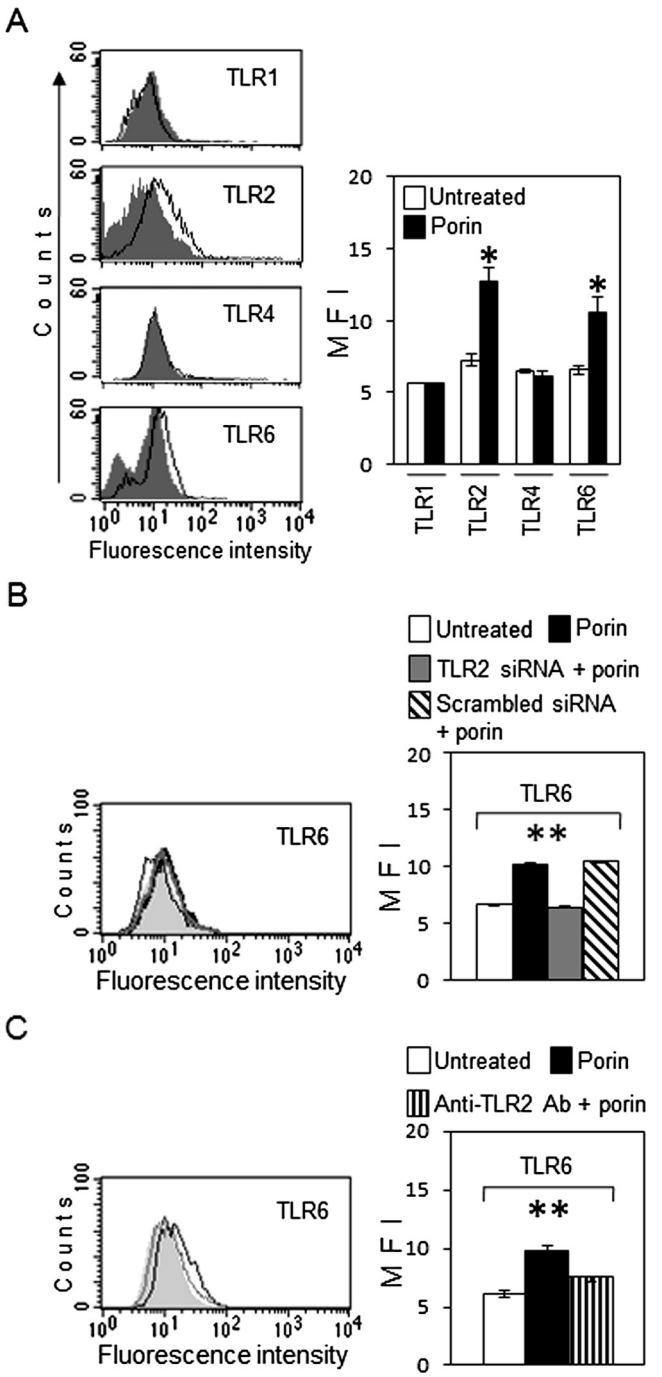

Fig. 1. Porin up-regulates TLR2 on INT-407 cells which regulates TLR6 expression. (A) Flow cytometric analysis shows expression of TLR2 and TLR6 on porin treated cells (black line) over untreated controls (shaded). (B) TLR2 siRNA (thin black line) treatment resulted in complete abrogation of porin-induced TLR6 expression compared to porin treated (thick black line) and scrambled siRNA plus porin treated cells (thick gray line). Shaded profile indicates untreated control. (C) Similarly, anti-TLR2 neutralizing Ab resulted in 66% depletion of porin-induced TLR6 expression (thin gray line) with that of porin treated cells (thin black line). Shaded profile indicates untreated control. Bar diagrams show mean ± SEM of three independent experiments. * indicates $p < 0.05$; ** indicates $p < 0.005$.