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Short Communication

Antigenic relatedness defines Toll-like receptor 2 is crafted on ligand blueprint

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ABSTRACT

Toll-like receptors are located particularly on mammalian immune cells to recognize pathogen-associated molecules. Toll-like receptors are categorized on the basis of ligand specificity that includes Toll-like receptor 2 with affinity for bacterial porin, the major outer membrane protein.

Here we show TLR2 antibody recognizes the monomer of porin, primarily a TLR2-ligand in Western blot, thus displaying relatedness of primary structures between the receptor and its ligand. Quantitative analysis revealed relatedness of the native porin molecule with TLR2 was as high as 71%, suggesting imprint of native porin trimer is mostly copied by the receptor crossing limits of primary structures. Flow cytometric analysis of TLR2 on HEK-293 cells shows the receptor and ligand also have common molecular patterns on surface, which is distinctively separate from regions assigned for putative TLR*ligand interaction.

Molecular mimetic and specificity of TLR will caution investigators targeting TLR–ligands to develop adjuvants and vaccines.

Introduction

Microbial organisms made their place in nature by invading all possible niches. Although most of these organisms overlook the presence of mammals and some are friendly called commensals (Lathrop et al. 2011), few are strongly infective causing diseases (Macdonald and Monteleone 2005). In order to rescue themselves from microbial infections, mice and human have developed a scanning mechanism of pathogenic structures and thus pathogens using pattern recognition receptors (Kawai and Akira 2010) particularly belonging to a group of leucine rich proteins called Toll-like receptors (TLRs) (Akira and Takeda 2004). There are 13 TLRs in mice and 10 in human (O'Neill et al. 2013) of which TLR2 is one of the TLRs that primarily recognizes porins and peptidoglycans of pathogenic bacteria (Massari et al. 2002; Michelsen et al. 2001). All Gram-negative bacteria have porins located in their outer membrane (Nikaido and Vaara 1985; Pagès et al. 2008). These major proteins are exposed on surface, exhibit pore-forming ability and are immunogenic (Massari et al. 2003; Roy et al. 1994). Shigellosis patients produce convalescent Abs to the protein (Ray et al. 2003), thus prioritizing it as a target for adjuvant development (Wetzler et al. 1996; Banerjee et al. 2008). Study of adjuvanticity of porin has shown it unifies innate signaling with adaptive immunity (Banerjee et al. 2008). In this study we analyzed relatedness of the receptor TLR2 with its ligand porin to establish existence of overlapping molecular contours demarcated from regions for receptor and ligand interaction. Since TLR–ligands are being analyzed for efficacy of adjuvants and vaccines, this work will be insightful for judging and locating molecular moieties for therapeutics.

Material and methods

Immunogen

Porin was purified to homogeneity from *Shigella dysenteriae* type 1(strain A020332) (Roy et al. 1994). In brief, bacteria were disrupted with an ultrasonic disintegrator and centrifuged at 100,000 × *g* for the envelope fraction, which was suspended in 1% sodium lauroyl sarcosine (Sigma–Aldrich). The preparation was centrifuged at 100,000 × *g* for 1 h to obtain the outer membrane proteins (OMP). The OMP fraction was suspended in 2% SDS in 10 mM Tris–HCl, pH 7.7 and ultracentrifuged. The pellet was twice extracted with

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50 mM Tris–HCl, pH 7.7 containing 0.4 M NaCl, 1% SDS, 5 mM EDTA and 3 mM NaN_3, ultracentrifuged and 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. Absence of trace amount of lipopolysaccharide in the purified porin was confirmed biochemically and by *Limulus* amebocyte lysate assay. Bovine serum albumin (BSA) (Sigma–Aldrich) was commercially obtained for the study.

Anti-porin Ab

C3H/OUJ mice were gifted by the Institute of Life Sciences, Bhubaneswar, Orissa, India. The mice were housed in groups of six and given food and water *ad libitum* and maintained in the animal care facility of National Institute of Cholera and Enteric Diseases, Kolkata, India. The mice were injected intraperitoneally with 30 μg of purified porin in Freund complete adjuvant (Gibco), followed by three booster doses. Pre-bleed and antisera were collected and stored at −80 °C. The experiments with animals were conducted in accordance with the Animal Ethical Committee guidelines of National Institute of Cholera and Enteric Diseases.

Cell culture

The stably transfected cell line TLR2/HEK-293 expressing full-length human TLR2 (IML-202; Imgenex) was maintained in DMEM supplemented with 10% FBS (Gibco) and 10 μg/ml blasticidin. Cells were seeded in 6-well plates (BD Falcon) and grown to 80% confluent monolayers for analysis.

SDS-PAGE and immunoblot

25 μg of porin was boiled in Laemmli sample buffer for 15 min and separated on 10% SDS-polyacrylamide gel along side Prestained Protein Marker (Cell Signaling Technology). The proteins were stained with Coomassie brilliant blue. The electrophoresed protein was transferred onto nitrocellulose membrane (Bio-Rad). The membrane was blocked with 0.4% non-fat dry milk (BD Difco™) for 1 h and washed three times in Tris-buffered saline (TBS) with 0.1% (v/v) Tween-20 for 10 min, followed by incubation with mouse anti-porin Ab (Roy et al. 1994), rabbit anti-mouse/human TLR2 Ab (Santa Cruz Biotechnology) or anti-mouse/human TLR4 Ab for 2 h. The membrane was washed and incubated with HRP conjugated horse anti-mouse IgG (Cell Signaling Technology) or goat anti-rabbit IgG. Ag–Ab complex was detected with Super Signal® West Dura Extended Duration Substrate (Thermo Scientific).

ELISA

96-well ELISA plates (Nunc) were coated with 4 μg/well of porin or BSA in 0.1 M PBS, pH 8.0. After overnight incubation at 4 °C, the wells were blocked with 0.4% non-fat dry milk (BD Difco™). The plates were incubated for 2 h at RT. After blocking, mouse anti-porin Ab, rat anti-mouse TLR2 Ab (eBioscience) or anti-mouse TLR4 Ab was added to the wells at 1:500 dilution and incubated for 1 h at RT. After incubation, the wells were washed with PBS containing 0.05% (v/v) Tween-20. Next, HRP conjugated horse anti-mouse IgG (Cell Signaling Technology) or goat anti-rat IgG at 1:1000 dilution was added and incubated for 45 min at RT. The wells were washed and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added. After 15 min of incubation, the reaction was stopped by Stop Solution (BD Biosciences). The plates were measured at A_{450} in a Varioskan Flash Multimode Reader (Thermo Scientific™).

Fig. 1. Analysis to demonstrate relatedness of primary structures of porin and TLR2. Native porin was boiled at 100 °C for 15 min and electrophoresed on 10% SDS-PAGE. The 38 kDa monomer of porin was identified by Coomassie brilliant blue as indicated. After electrophoresis the protein was transferred onto a nitrocellulose membrane and recognized by anti-porin Ab. The blot was reprobed with anti-mouse/human TLR2 or TLR4 Ab as indicated. The data given were obtained in one representative experiment out of three.

Flow cytometry

Cells were incubated at 4 °C in the dark for 20 min with F_c-block followed by rabbit anti-mouse/human TLR2 Ab (Santa Cruz Biotechnology), functional grade purified mouse anti-human/mouse TLR2 MAb (eBioscience) or mouse anti-porin Ab followed by FITC conjugated goat anti-rabbit IgG/horse anti-mouse IgG. The cells were fixed in 1% paraformaldehyde and analyzed on a FACSCalibur using CELLQuest software (Becton Dickinson).

Statistical analysis

Results were expressed as the mean ± SEM of three independent experiments. Data were analyzed by Student's *t* test and one-way ANOVA using ezANOVA statistical software. A *p*-value of <0.05 was considered significant.

Results and discussion

Western blot analysis shows 38 kDa monomer of porin is robustly recognized by commercially available anti-TLR2 Ab after stripping of anti-porin Ab that detected the immunogen (Fig. 1). The data denotes both the ligand and its receptor are constituted of related primary structures. Quantification and thereby considering detection of native porin trimer by anti-porin Ab as 100% (Fig. 2A), recognition of porin by anti-TLR2 Ab was 71% confirming molecular homology between porin and TLR2 crossing beyond the relatedness of primary structures. However, preincubation of porin with neutralizing anti-TLR2 MAb could not inhibit detection of porin by anti-porin Ab. Similarly, preincubation of porin with anti-porin Ab did not deplete detection of the protein by neutralizing anti-TLR2 MAb, indicating specificity of the receptor binding components of the ligand. Next, flow cytometric analysis of TLR2 on stably transfected cell line TLR2/HEK-293, expressing full-length human TLR2 showed expression of the receptor over isotype control both in the presence of anti-porin Ab and anti-TLR2 Ab (Fig. 2B), indicating relatedness of TLR2 surface-exposed structures with its ligand. In HEK-293 cells, detection of TLR2 by anti-porin Ab was although identical to that of anti-TLR2 Ab (Fig. 3A), incubation with neutralizing anti-TLR2 MAb revealed an absolutely separate profile (Fig. 3B) indicating specificity of ligand binding components of the receptor.

Since microbial organisms evolved earlier than mammals to occupy nature, it is obvious designing and shaping of TLR backbone in mammals have taken place based on molecular patterns located on pathogenic microorganisms categorically keeping apart the ligand binding domains. Our data of molecular relatedness