Immunobiology 221 (2016) 1369–1373

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Pathogen-associated porin turns IL-10 competent B-1a cells toward proinflammatory cytokine response

Amlan Kanti Ghosh, Debolina Sinha, Ratna Biswas, Tapas Biswas*

Division of Immunology, National Institute of Cholera and Enteric Diseases, Kolkata, India

ARTICLE INFO

Article history:
Received 4 May 2016
Received in revised form 19 July 2016
Accepted 22 July 2016
Available online 29 July 2016

Keywords:
Activation molecules
B-1a cells
Cytokines
Porin
Siglec-G

ABSTRACT

Shigellosis is a major problem in the developing countries causing mortality and morbidity particularly among the children. *Shigella* spp. harbours the epithelial cells of the human colon to infect the host and spread the disease.

We analyzed the response of B-1a cells, the major component of the mucosal immune system to porin of *Shigella dysenteriae* type 1. We show that porin while proliferating B-1a cells, deplete Siglec-G, the inhibitory molecule present on B-1a cells. Adjuvanticity of porin has been shown to govern innate signaling for promoting host adaptive immune response. Up-regulation of CD69 and CD40 denotes activation of the cells parallel to abrogation of Siglec-G. As a result of cell activation, porin stimulated the inflammatory cytokines of $CD5^+$ B-1a cells, otherwise rich in IL-10. The work shows B-1a cell responses promote the immunopotentiating activity of porin.

1. Introduction

Shigellosis, which involves invading the epithelial cells of the colon in humans, remains a major cause of infant mortality in developing countries (Keusch, 1986). Rapidly emerging drug-resistant strains of *Shigella* spp. have threatened the clinical management of the disease (Replogle et al., 2000). Porin, the major outer membrane protein with pore-forming ability of Gram-negative bacteria has since been studied with great enthusiasm to establish its role as a potential adjuvant. These proteins are immunogenic on their own and do not require the addition of adjuvants to evoke an immune response (Wetzler et al., 1992). Also, the unique property of porin to augment the humoral response to poorly immunogenic substances like polysaccharides and peptides shows great promise as a novel component of vaccines (Donnelly et al., 1990; Livingston et al., 1993).

Porin was purified to homogeneity from the outer membrane of *Shigella dysenteriae* type 1 and was found to be strongly immunogenic, surface-exposed and cross-reactive within *Shigella* spp (Roy et al., 1994). Moreover, sera of patients convalescing from shigellosis show titres of anti-porin Abs to *Shigella dysenteriae* type 1 (Ray et al., 2003). These features make the protein all the more attractive as an adjuvant for use in vaccine formulation against shigellosis. Studies of adjuvanticity of porin including but not limited to, peritoneal cavity (PerC) B cells, dendritic cells (DCs) and macrophages (MΦs) (Ray and Biswas 2005; Ray et al., 2004; Biswas et al., 2007) have shown to primarily stimulate TLR2 for coordinating innate signaling with adaptive immune responses (Banerjee et al., 2008).

B cells represent an important link between adaptive and innate immune system as they express both Ag specific B-cell receptors (BCR) as well as toll-like receptor (TLR) and have various functions during development of immune responses to infection. In addition to serving as Ab producing cells, B cells also act as APC, participate in activation of T cells and secrete cytokines to control or exacerbate infection. There is growing evidences that support these functions are associated with variable subsets of B cells.

There exist phenotypically distinct populations of B cells in mice and humans, termed B-1 and B-2, which have been proposed to originate from entirely separate precursor B-cell lineages (Montecino-Rodriguez et al., 2006). Unlike B-2 cells, B-1 cells can operate independently of T cell help (Fagarasan and Honjo, 2000) by producing 'natural' IgM (Ehrenstein and Notley, 2010) and secreting IgA Ab without any antigenic encounter (Macpherson et al., 2000) or under the influence of commensal bacteria (Suzuki and Fagarasan, 2008), some food and self-Ags. B-1 cells are further divided into B-1a ($CD11b^+CD5^+$) and B-1b ($CD11b^+CD5^-$) "sister"

Abbreviations: APC, antigen-presenting cell; DC, dendritic cell; LPS, lipopolysaccharide; MΦ, macrophage; PerC, peritoneal cavity; Siglec-G, sialic-acid-binding immunoglobulin-like lectin; TLR, toll-like receptor.

* Corresponding author at: Division of Immunology, National Institute of Cholera and Enteric Diseases, P-33, CIT Road, Scheme-XM, Kolkata 700 010, West Bengal, India.

E-mail address: shigellaporin@yahoo.com (T. Biswas).

http://dx.doi.org/10.1016/j.imbio.2016.07.010
0171-2985/

cell populations (Gardby et al., 1998). B-1a cells are primarily found at the mucosal surfaces of the peritoneal cavity and are the major B-cell subset of the innate immune system that forms the first line of defense by producing natural Abs. Peritoneal B-1a cells are generally rich in IL-10 level and thus can play a regulatory role during infection. Previous studies have shown that porin activates B-1a and B-1b subsets gated from unsorted B-1 cell population for mounting responses (Ray et al., 2004).

As shigellosis involves infection of the gut, which harbours B-1 cells, the capacity of porin to activate purified PerC B-1a cells was examined. Here, we describe the interactions between porin and B-1a cells that resulted in up-regulation of the activation molecules and ablation of the inhibitory receptor on surface of the cells. This effect is accompanied by production of pro-inflammatory cytokines in a TLR2-dependent manner. We show that porin activates as well as influences the B-1a cell towards type 1 polarization. Thus, we propose that recognition of porin by TLR2 and induction of the activation markers culminating in the synthesis of pro-inflammatory cytokines could be a significant mechanism behind the immunopotentiating activity of porin.

2. Material and methods

2.1. Immunogen

Porin was purified to homogeneity from *S. dysenteriae* type 1 for cell culture as described elsewhere (Roy et al., 1994; Ray et al., 2004). The purified protein was free from lipopolysaccharide (LPS), which could not be detected by estimation of neutral sugar (Dubois et al., 1956) and ester content (Entenman, 1957). Absence of trace amounts of LPS in the purified porin was confirmed by *Limulus* amebocyte lysate assay (Roy and Biswas, 1996).

2.2. Mice

C57BL/6 mice, obtained from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Jamai-Osmania, Hyderabad, India, were bred and reared in the animal care facility of National Institute of Cholera and Enteric Diseases, Kolkata. The mice were housed in groups of six and given food and water *ad libitum*. Six-to-seven week old mice of both sexes were used for isolation of B-1a cells from the PerC. The experiments with animals were conducted in accordance with the Animal Ethical Committee guidelines of National Institute of Cholera and Enteric Diseases, Kolkata, India.

2.3. Purification and culture of PerC $CD19^+CD11b^+CD5^+$ B-1a cells

B-1a cells were purified from C57BL/6 mice**,** cell viability was assessed by Trypan blue exclusion and proliferation analyzed by MTT assay as described elsewhere (Ghosh et al., 2016). Briefly, the cells were sorted using PerCP-conjugated anti-mouse CD19 mAb (BD Pharmingen, San Diego, CA, USA), PE-conjugated anti-mouse CD11b mAb and APC-conjugated anti-mouse CD5 mAb (eBioscience, San Diego, CA, USA) on a FACSAria II using FACS-Diva software (Becton Dickinson, San Jose, CA, USA). The B-1a cell population obtained after sorting was 98% pure.

B-1a cells were suspended in RPMI 1640 (Gibco, Grand Island, NY, USA) and the integrity of the cells, monitored by inverted light microscopy, was 97% viable as determined by Trypan blue exclusion. Cells were seeded in 96 well round-bottomed tissue culture plates (Becton Dickinson Labware, Franklin lakes, NJ, USA) at 1×10^5 cells/well/200 μl in RPMI 1640 containing 5 U/ml penicillin, 5 μg/ml streptomycin, 0.1% gentamicin, 2% fetal bovine serum (Gibco, Grand Island, NY, USA) and 0.1% insulin–transferrin–selenium (Gibco, Grand Island, NY, USA). Cells were incubated at 37 °C in 5% CO_2 for the indicated time periods in presence and absence of porin (10 μg/ml) or goat $F(ab')_2$ anti-mouse IgM (10 μg/ml) (Jackson Immunoresearch, West Grove, PA, USA).

2.4. Intracellular staining of cytokines

After incubation of untreated and porin or goat $F(ab')_2$ anti-mouse IgM treated cells, monensin (0.33 μl/ml) (BD Pharmingen, San Diego, CA, USA) was added 10 h prior to completion of incubation for blocking of IL-10 or IL-12. Blocked cells were fixed and permeabilized using Cytofix/Cytoperm kit (BD Pharmingen, San Diego, CA, USA) and stained with FITC-conjugated anti-mouse IL-10 or IL-12 mAb (BD Pharmingen, San Diego, CA, USA). Statistical analysis was done by using isotype-matched controls as references.

2.5. siRNA transfection of B-1a cells for knockdown of TLR2

Cells were transfected with 50 pmol siRNA directed against TLR2 (antisense: 5′-AUUCCGGAGACUUAAUGCCtt-3′) (Ambion, Grand Island, NY). Lipofectamine® 2000 transfection reagent (Invitrogen, Carlsbad, CA) containing siRNA was added to OptIMEM Glutamax (Life Technologies, Grand Island, NY), mixed together and kept for 20 min and finally added to the cells with 1 ml complete medium to be kept for 36 h. Silencer containing media was washed and fresh complete medium with the immunogen was added. 90% of the cells remained viable 36 h after transfection to which porin was added.

2.6. Flow cytometry

Cells were cultured in complete medium or in presence of porin or goat $F(ab')_2$ anti-mouse IgM and then incubated with purified unconjugated anti-mouse CD16/CD32 mAb (Fc Block™, BD Pharmingen, San Diego, CA, USA). The cells were washed with 20-fold dilution of FACSFlow™ and stained at 4 °C in the dark for 20 min with any one of FITC-conjugated anti-mouse CD25 (BD Pharmingen, San Diego, CA, USA), CD40, APC-conjugated anti-mouse CD5 (eBioscience, San Diego, CA, USA) or biotin-conjugated anti-mouse CD69 (BD Pharmingen, San Diego, CA, USA). Biotinylated mAb was recognized by streptavidin-FITC. Sialic-acid-binding immunoglobulin-like lectin (Siglec)-G was detected by staining with purified anti-mouse Siglec-G Ab (Santa Cruz Biotechnology, Dallas, TX, USA) followed by FITC-conjugated anti-goat IgG Ab. Parallel sets of cells were incubated with monoclonal immunoglobulin isotypes to serve as non-specific negative controls. The cells were fixed in 1% paraformaldehyde and equal number of cells was analyzed on a FACSCalibur using CELLQuest software (Becton Dickinson, San Jose, CA, USA).

2.7. Enzyme-linked immunosorbent assay (ELISA)

TNF-α, IFN-γ or IL-12 p70 present in the 5-day-old B-1a cell culture supernatants was measured by sandwich ELISA with pairs of Abs for binding and detection using the BD OptEIA ELISA Set according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA).

2.8. Statistical analysis

Results were expressed as the mean ± SEM, where applicable, of three independent experiments. Data were analyzed by the two-tailed paired Student's *t*-test and one-way ANOVA using ezANOVA statistical software. A p value of <0.05 was considered significant, and a p value of <0.005 was considered highly significant.