Priming of CD4+ T cells with porin of *Shigella dysenteriae* activates the cells toward type 1 polarization

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Keywords: chemokines, IFN-γ, porin, T₄, cells, type 1 polarization

Abstract

Macrophages treated with porin of *Shigella dysenteriae* were potent stimulators of naive T₄ cells since the porin-pulsed macrophages strongly proliferated the T cells and up-regulated the activation molecules CD69 and CD25 on CD4+ T cells in allogeneic mixed leukocyte reaction. Immunization of C57BL/6 mice with porin selectively induced the intracellular expression and release of IFN-γ and had no effect on IL-4 expression. In parallel to the predominant release of the T₄ cytokine IFN-γ, up-regulation of CCR5 on the immune CD4+ T cells and messenger ribonucleic acid (mRNA) expression of the T cell chemokines macrophage inflammatory protein-1α and regulated on activation normal T cell expressed and secreted showed Th₁ bias. The porin-primed CD4+ T cells expressed the mRNA for T-box expressed in T cells, a Th₁-specific transcription factor confirming the adjuvant-induced transition of T cells to polarized effector T₄,1 cells. The immune CD4+ T cells proliferated and released IL-2 and IFN-γ profoundly in response to re-challenge with porin-pulsed macrophages but not to BSA-pulsed macrophages in vitro, which demonstrated the presence of porin-specific CD4+ T cells of T₄,1 phenotype. The study highlights that porin has the capacity of an adjuvant to unfold and maintain the T cell function and thereby to activate adaptive immunity.

Introduction

Porin, the major outer membrane protein with pore-forming ability, is of particular interest because it has been characterized as an immunoadjuvant when used as a component of outer membrane proteosome complexes (1) or as a recombinant protein (2). Porin was purified to homogeneity from the human pathogen *Shigella dysenteriae* type 1 (3) in view of a potential adjuvant to be incorporated in vaccine preparation against shigellosis, since drug-resistant strains of *Shigella* spp. are rapidly appearing (4), complicating the clinical management. Adjuvanticity of porin of *S. dysenteriae* has been described in peritoneal macrophages and B-1 cell populations (5, 6). Although our earlier studies have shown activation of the innate immune system by the protein, it is essential to know whether porin can afford to unlock the otherwise blunted adaptive immune system through activation and polarization of CD4+ T cells to be established as an adjuvant that can successfully bridge the innate and adaptive immunity.

Helper CD4+ T cells of the adaptive immune system play a central role in the regulation of immune responses. Identification of CD4+ T cell subsets has been a major step in understanding the intricate immune response to pathogens. When a naive CD4+ T cell interacts with major histocompatibility-peptide complex on antigen-presenting cell (APC), the consequent adaptive immune response starts taking shape. With this interaction, T₄,1 cells start to proliferate, express the required activation markers and differentiate to T₄,1 or T₄,2 effector cell subsets to produce specific set of cytokines (7) and chemokines. The differentiation path is determined by the environment in which naive T cells interact with APC. The effector T₄,1 cells characteristically release IL-2, IFN-γ and tumor necrosis factor (TNF)-β, whereas the effector T₄,2 cells release the signature cytokines, IL-4, IL-5 and IL-13 (8). Apart from the cytokines, polarized T cells are known to show differential chemokine profiles. Thus, while the T₄,1 cells express chemokines like macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation normal T cell expressed and secreted (RANTES) (9), lymphotactin and MIP-1γ, T₄,2 cells are characterized by MIP-2 (10) and macrophage-derived chemokine (MDC) (11). Chemokine
receptors have also served as useful markers in T<sub>1</sub>/T<sub>2</sub> response studies: CXCR3 and CCR5 are preferentially expressed in T<sub>1</sub> cells playing a pivotal role in positioning of the effector T<sub>1</sub> cells to the inflammatory sites in contrast to CCR3 and CCR4, which are associated with T<sub>2</sub> cells (12). Recent studies have shown that T-box expressed in T cells (T-box) is a transcription factor selectively expressed in T<sub>1</sub> cells. It shapes the T<sub>1</sub> immune response by inducing the T<sub>1</sub>-specific genes and simultaneously represses the T<sub>2</sub> cytokines (13). GATA-binding protein-3 (GATA-3), on the other hand, is a T<sub>2</sub>-specific transcription factor that controls the development of T<sub>2</sub> response (14). The T<sub>1</sub> cellular immune response is critical for anti-tumor and anti-microbial immunity (15); thus, powerful immunomodulatory adjuvants that induce T<sub>1</sub> polarization are an important tool of vaccination strategies (16, 17). The type of response induced by an antigen is dependent on a combination of host genetic factors, the local cytokine milieu and the nature and dose of antigen (18).

Among the outer membrane components of Gram-negative bacteria, LPS mostly induces a response that involves T<sub>1</sub> cells (19). The immunogenic and protective roles of other cell-surface molecules like porin of Neisseria (20), Salmonella (21) and Shigella (22) have been extensively documented. We have reported that porin of S. dysenteriae induces the expression of IgG2a, a T<sub>1</sub>-specific IgG (23), on mouse peritoneal cavity (PerC) B-2 cells (24). The protein as an adjuvant also induced the PerC macrophages to release IL-12 and type 1 chemokines (5), which are important for determining the type of adaptive immune response to be initiated. In context of these previous findings of ours, in this paper, we show porin-induced activation and type 1 response of CD4<sup>+</sup> T cells both in vivo and in vitro.

**Methods**

**Bacterial strain**

*Shigella dysenteriae* type 1 strain A020332 used in this study was cultured as described elsewhere (25).

**Immunogen**

Porin was purified to homogeneity from *S. dysenteriae* type 1 (3) and made cell culture compatible as described elsewhere (6). The purified protein was free from LPS, which could not be detected by estimation of neutral sugar (26) and ester content (27). Absence of trace amount of LPS in the purified porin was confirmed by the *Limulus* amebocyte lysate assay (22).

**Immunization of mice**

C57BL/6 and BALB/c mice were obtained from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Jamai-Osmania, Hyderabad, India, and maintained as described elsewhere (24). The experiments with animals were conducted in accordance with the Animal Ethical Committee guidelines of National Institute of Cholera and Enteric Diseases, Kolkata, India. Six-week-old female mice were twice peritoneally immunized, 1 week apart, with 25 μg of porin per mouse. Two days after the second injection, mice were sacrificed and primed CD4<sup>+</sup> T cells were isolated from the spleen. Untreated groups each comprising five mice were kept as control.

**Purification of PerC macrophages**

PerC macrophages of 6-week-old C57BL/6 mice were purified as described elsewhere (28). Briefly, the peritoneal washing containing the macrophages was collected on sterile plastic Petri dishes and incubated at 37°C in 5% CO<sub>2</sub> for 2 h. The cells of the monocyte-macrophage lineage adhered on the surface of the Petri dishes to form a cell monolayer. The adhered PerC cells were collected by centrifugation at 400 × g for 8 min and suspended in RPMI-1640 (GIBCO, Invitrogen Corp., Grand Island, NY, USA) containing 5 U ml<sup>-1</sup> penicillin, 5 μg ml<sup>-1</sup> streptomycin, 0.1% gentamicin, 2% fetal bovine serum (FBS) and 0.1% insulin-transferrin-selenium.

**Purification of CD4<sup>+</sup> T cell subset**

Single-cell suspension containing CD4<sup>+</sup> T cells were harvested from the spleen after passing through 105-μm nylon mesh. The cells were devoid of erythrocytes by treating with 0.15 M NH<sub>4</sub>Cl containing 10 mM KHCO<sub>3</sub> and 0.1 M EDTA (pH 7.2) and then suspended in RPMI-1640 with 10% FBS. T cell-enriched splenocytes were obtained by two passages through nylon wool column (12 ml). More than 96% pure CD4<sup>+</sup> subset-enriched T cells was prepared by negative selection using Pan CD4<sup>+</sup> T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and autoMACS System (Miltenyi Biotec). Naive CD62L<sup>+</sup> CD4<sup>+</sup> T cells were purified from the splenic CD4<sup>+</sup> subset-enriched T cells by positive selection using anti-mouse CD62L microbeads and autoMACS System.

**Syngeneic and allogeneic mixed leukocyte reactions**

Syngeneic and allogeneic mixed leukocyte reactions (MLRs) were performed by culturing the untreated or porin-pulsed macrophages of C57BL/6 and BALB/c mice, respectively, with naive CD4<sup>+</sup> T cells of C57BL/6 mice. PerC macrophages were cultured in RPMI-1640 with 2% FBS in absence and presence of porin for 6 h followed by treatment with 40 μg ml<sup>-1</sup> of mitomycin-C (Sigma, St Louis, MO, USA) for 1 h at 37°C to inhibit cell proliferation. The cells were thoroughly washed and seeded at varying concentrations in 96-well flat-bottomed tissue culture plates to serve as stimulator cells. Purified naive CD4<sup>+</sup> T cells were added at a concentration of 0.2 × 10<sup>5</sup> cells per well and incubated at 37°C in 5% CO<sub>2</sub> for 3 days. Proliferative response of the T cells was determined by 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenylyl tetrazolium bromide (MTT) assay as described elsewhere (22). Expression of the activation molecules, CD69 and CD25, on the surface of the cells was monitored by flow cytometry on a FACSCalibur using CELLQuest software (Becton Dickinson, San Jose, CA, USA) after staining at 4°C in the dark for 20 min with FITC-conjugated anti-mouse CD69 (BD Biosciences PharMingen, San Diego, CA, USA) or CD25 mAb. Voltage settings for FITC parameters were done with isotype control. Statistical analysis was done by using isotype-matched controls as a reference (M1).
In vivo effect of porin on T cell

Six-week-old C57BL/6 mice were immunized with porin. Two days after the final immunization, splenocytes were harvested and CD4+ T cells purified using magnetic beads. CD4+ T cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 10 ng ml⁻¹) (Sigma), ionomycin (500 ng ml⁻¹) and Brefeldin A (10 μg ml⁻¹) for 5 h in 24-well-flat-bottomed tissue culture plates. Following activation, cells were stained with FITC-conjugated anti-mouse IFN-γ (BD Biosciences Pharmingen) and APC-conjugated anti-mouse IL-4 mAb and analyzed on a FACSCalibur using CELLQuest software for determination of intracellular cytokine expression.

Up-regulation of the chemokine receptors on CD4+ T cells obtained from non-immunized and immunized mice was analyzed on a FACSCalibur after staining with PerCP-conjugated anti-mouse CD4 (BD Biosciences Pharmingen) and FITC-conjugated anti-mouse CCR3, CCR5 or CXCR3 (Zymed Laboratories Inc., San Francisco, CA, USA).

In vitro macrophage:T cell co-culture

Purified CD4+ T cells from non-immunized and immunized mice were co-cultured with macrophages in 24-well plate at a ratio of 1:5 (macrophage:T cell). Macrophages were pulsed with porin, BSA or left untreated for 4 h before adding T cells. Supernatants from these cultures were collected after 4 days for cytokine ELISA. For proliferation assay using MTT (Sigma), macrophages were treated with mitomycin-C and CD4+ T cells purified using magnetic beads.

Statistical analysis

Results were expressed as the mean ± SEM, where applicable, of three independent experiments. The data were analyzed by Student’s t-test using a web-based program (www.statpages.net). A P-value of <0.05 was considered significant, and a P-value of <0.005 was considered highly significant.

Results

Stimulatory function of porin-pulsed macrophages in MLR

We examined the capability of porin-pulsed macrophages to stimulate naïve CD4+ T cells in MLR. Analysis of data revealed that porin-pulsed macrophages were potent stimulators of naïve, allogeneic CD4+ T cells as evident from the T cell proliferation assay and cell-surface expression of the activation molecules. Porin-pulsed macrophages induced 6.4-fold increase in T cell proliferation at the maximum stimulation of CD4+ T cells as evident from the T cell proliferation assay and cell-surface expression of the activation molecules. Porin-pulsed macrophages induced 6.4-fold increase in T cell proliferation at the maximum stimulator concentration of 50 × 10⁶ cells in allogeneic MLR (Fig. 1A). Flow cytometric analysis showed that a higher percentage of CD4+ T cells have an activated phenotype following culture with porin-pulsed macrophages as 46 and 36% of the cells expressed CD25 and CD69, respectively (Fig. 1B).

Intracellular expression and release of IFN-γ by immune CD4+ T cells

The pattern of intracellular cytokine expression of CD4+ T cells was tested following immunization of mice with porin. Flow cytometric analysis of the cells in vitro stimulation with PMA and ionomycin revealed that there was 9.4-fold increase in percentage of CD4+ T cells producing IFN-γ, the Th1 cytokine, due to immunization with porin (Fig. 2A). However, there was no change in percentage of CD4+ T cells expressing IL-4, the Th2 cytokine, compared with the non-immune cells. The cytokines released by immune CD4+ T cells were quantified by ELISA. The CD4+ T cells were
stimulated in vitro on anti-mouse CD3e-coated plates in the presence of soluble anti-mouse CD28 for 2 days. Re-stimulation of the T cells in vitro released 250 pg ml⁻¹ of IFN-γ (250 ± 34 SEM, \( P < 0.005 \)) (Fig. 2B). The data suggest that porin has an intrinsic ability to stimulate T cells to predominantly produce Th1 cytokine.

Expression of chemokine receptor and chemokines by immune CD4⁺ T cells

Next, we examined the expression of chemokine receptors and chemokines to know whether they show Th1 bias in parallel to the cytokine profile. Total RNA was isolated from CD4⁺ T cells and the change at mRNA level of the chemokine receptors was analyzed. CCR5 got solely expressed both at RNA and protein levels (Fig. 3A and B). No change could be detected in the expression of CCR3, CCR4, CCR7 and CXCR3. After immunization with porin, there was 3-fold (3.27 ± 0.23 SEM, \( P < 0.05 \)) increase in the mRNA of CCR5 compared with the non-immunized control, which was supported by 2.9-fold (2.85 ± 0.44 SEM, \( P < 0.05 \)) increase in the expression of the receptor on cell surface.

MIP-1α and RANTES were significantly up-regulated among the T cell chemokines, after immunization with porin. RT-PCR analysis showed 2- (2.12 ± 0.07 SEM, \( P < 0.05 \)) and 3.5-fold (3.46 ± 0.21 SEM, \( P < 0.005 \)) increase in the expression of MIP-1α and RANTES, respectively (Fig. 3C).
Porin priming of T cells for type 1 response

The expression of MIP-1β, MIP-2, MDC and lymphotactin remained unaltered after immunization with porin.

Expression of T-bet by immune CD4+ T cells

Immunization with porin expressed the mRNA of T-bet, a Th1-specific transcription factor in CD4+ T cells, which was 3-fold (2.99 ± 0.31 SEM, P < 0.005) more than the non-immunized control (Fig. 4). In contrast, the expression of the Th2-specific transcription factor GATA-3 was depleted in response to porin immunization compared with control.

Immunized CD4+ T cells proliferate in response to porin-pulsed macrophages

We studied whether CD4+ T cells of immunized mice could recognize porin presented by macrophages and were immune to re-challenge. The CD4+ T cells of immunized mice proliferated profoundly in response to porin-pulsed macrophages, which the cells from non-immunized mice were not capable of (Fig. 5A). Although the CD4+ T cells of immunized mice marginally responded to BSA-pulsed macrophages, the response to porin-pulsed macrophages was 3.2-fold (3.24 ± 0.37 SEM, P < 0.005) higher than that to BSA-pulsed macrophages. The significant difference in proliferation (P < 0.005) induced by porin-pulsed macrophages demonstrates the presence of porin-specific T cells, which solely recognized porin when re-challenged in vitro. The data indicate that porin has the specificity of an adjuvant to unfold and maintain the adaptive immunity.

Discussion

Bacterial surface antigens may induce protective immune response in host during an interaction between bacteria and host immune cells in the mucosal interface. The microbial antigen is taken up by APC, processed and presented to T cells. Interaction with T cells involves signaling through the T cell receptor and co-stimulatory molecules and release of cytokines, the nature of which dictates the CD4+ T cells to choose their Th careers, thereby initiating the induction of acquired immunity and also generation of immune memory. Porin, the surface exposed component of Gram-negative bacteria, has been pointed as a good adjuvant because it is known to augment the humoral response to otherwise poorly immunogenic substances like polysaccharides (PSs) and peptides (29, 30). Our previous data show that porin of S. dysenteriae could augment the release of the two pro-inflammatory mediators, nitric oxide and IL-1 by macrophages.
In response to LPS and PS moiety of LPS (31). We also reported that interaction of porin of *S. dysenteriae* with macrophages up-regulated the activation molecule CD40 with MHC class II (I-A^β); the co-stimulatory molecule CD80 and chemokines MIP-1α, MIP-1β and RANTES and produced the essential T₃,1-driving cytokines TNF-α and IL-12 (5). This led us to presume that the adjuvant effects of porin are mediated via activation of APC. Patients convalescing from shigellosis generate antibody against porin (25) and the protein expressed IgG2a on murine B-2 cells (24), indicating that porin has a role in the development of cellular immunity that likely involves T cell participation. Thus, the objective of this study was to determine whether the adjuvant effects of porin have greater functional implication in promoting proliferation and activation of CD4^+^ T cells.

We could demonstrate that interaction of porin-pulsed macrophages with CD4^+^ T cells was able to induce naive T cell proliferation in an allogeneic and to a small extent in syngeneic MLR, which leads to functional and phenotypic maturation of T cells evident from the expression of the activation molecules CD69 and CD25. Porin thus proves its adjuvanticity through activation of T cells, which paves the way for an intricate adaptive immune response.

Porin was also able to trigger the CD4^+^ T cells for a polarized immune response *in vivo*. Immune CD4^+^ T cells when re-stimulated with PMA and ionomycin *in vitro* up-regulated the intracellular expression of IFN-γ, the signature cytokine of T₃,1 cells (8), which was supported by significant increase in release of the cytokine upon re-stimulation of the immune cells with anti-CD3e and anti-CD28 mAbs. This suggests that porin selectively triggers the T₃,1 response as no change in either intracellular expression or release of the T₃,2 cytokine, IL-4, could be found in response to immunization with porin.

Chemokines and their receptors play an important role in the migration of leukocytes from vascular compartments to sites of inflammation (32). The pattern of expression of the chemokines and their receptors also reflect the T₃,1/T₃,2 intent of the effector T₃,1 cells (9–12). Thus, parallel to the cytokine profile, the T₃,1 bias of porin was highlighted in the up-regulation of mRNA expression for the type 1 chemokines MIP-1α and RANTES and induction of the chemokine receptor CCR5 on surface of the immune CD4^+^ T cells. Although both CCR5 and CXCR3 are known to be preferentially expressed by T₃,1 cells, porin failed to induce the expression of CXCR3 on immune CD4^+^ T cells. This observation is in correlation to our previous finding in which porin had failed to induce the up-regulation of the CXCR3 ligands, IP 10 and MIG in PerC macrophages (5). While other reports have documented the preferential expression of lymphotactin, RANTES, MIP-1α and MIP-1β by T₃,1 cells (9, 10), porin could induce only RANTES and MIP-1α expression, with no change in the other T₃,1 chemokines.

Among the two transcriptional factors T-bet and GATA-3, porin selectively induced the expression of T-bet and...
down-regulated GATA-3, confirming skewing of the immune system for a polarized type 1 response as T-bet is known to be specifically expressed in Th1 and not in Th2 cells (13). T-bet expression shown by porin-immunized CD4+ T cells is in correlation with expression of the type 1 chemokines, chemokine receptor and IFN-γ release by these cells, thus further strengthening the type 1 preference of porin.

To establish itself as a successful vaccine component, an adjuvant should be capable of generating T cells specific to it, which would initiate an elaborate acquired immune response on next encounter with the molecule. Our data indicate the generation of porin-specific Th1 cells following immunization, as CD4+ T cells isolated from immunized mice showed proliferation and produced IL-2 and IFN-γ when re-challenged with porin-pulsed macrophages, but not with BSA-pulsed macrophages. This essentially proves that T cell proliferation and IFN-γ production in culture was induced specifically by porin showing the presence of porin-recognizing immune controller of adaptive immune system. The activated Th cells is dependent on stat6 but not stat4.


Previously, we have demonstrated that porin of S. dysenteriae influences the outcome of immunological responses through the release of several cytokines by target APC (5), and this study shows porin to proliferate Th1 cells, the major controller of adaptive immune system. The activated Th1 cells respond by expressing activation molecules CD69 and CD25, transcription factor and type 1 chemokines and initiate the release of specific cytokines to get transformed from naive to polarized effector Th1,1 cells. The data suggest that porin has specificity of an adjuvant to communicate with T cells by suc-cytopilization of effector Th1 cells. The data suggest that porin has specificity of an adjuvant to communicate with T cells by suc-

Polarized effector T cells is dependent on stat6 but not stat4.

Funding
Council of Scientific and Industrial Research [National Eligibility Test Fellowship No. 9/482(37)/2004-EMR-I to A.B.], New Delhi, India; Department of Science and Technology, Government of India, New Delhi, India.

Abbreviations
- APC: antigen-presenting cell
- FBS: fetal bovine serum
- GAPDH: glyceraldehyde-3-phosphate dehydrogenase
- GATA-3: GATA-binding protein-3
- MDC: macrophage-derived chemokine
- MIP: macrophage inflammatory protein
- MLR: mixed leukocyte reaction
- mRNA: messenger ribonucleic acid
- MTT: 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide
- PMA: phorbol 12-myristate 13-acetate
- PS: polysaccharide
- PerC: peritoneal cavity
- RT: reverse transcription
- RANTES: regulated on activation normal T cell expressed and secreted
- T-bet: T-box expressed in T cells
- TNF: tumor necrosis factor

References


