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Cellular immune response induced by *Salmonella enterica* serotype Typhi iron-regulated outer-membrane proteins at peripheral and mucosal levels

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The role of purified iron-regulated outer-membrane proteins (IROMPs) from *Salmonella enterica* serotype Typhi in modulation of specific T-cell responses was studied. The cellular immune response induced by IROMPs was measured by assessing the delayed-type hypersensitivity (DTH) response, lymphocyte proliferation, T-cell phenotyping and cytokine-producing cells using lymphocytes isolated from the spleen and Peyer's patches of IROMPs-immunized, immunized-challenged, infected and control mice. IROMPs immunization resulted in an enhanced DTH response and exhibited a significant increase in the protein-specific proliferative response of lymphocyte from the spleen as well as Peyer's patches. A significant increase was also observed in the ratio of $CD4^+/CD8^+$ cells in the immunized mice as compared to the infected mice. Results of the cytokine analysis revealed that during the initial period there was increased production of interleukin (IL)-2- and interferon (IFN)-γ-producing cells in the spleen and Peyer's patches, indicating a Th1 type response, whereas in the later period of the study, increased production of IL-4-producing cells suggested a Th2 type response. The results of this study suggest a role for *S*. Typhi IROMPs in modulating the cellular immune response at peripheral and mucosal levels.

INTRODUCTION

Typhoid fever is an important public health problem in many developing countries. It is caused by *Salmonella enterica* serovar Typhi (*S*. Typhi), which replicates within the cells of the reticuloendothelial system. It is well documented in the literature that humans as well as experimental animals respond to *Salmonella* infection by activating not only humoral but also cell-mediated immune responses (McGhee & Kiyono, 1993). Attempts to identify the relevant protective antigens that induce effective immune responses are continuing. In addition to several attenuated *S*. Typhi strains, various subunit vaccines including Vi, LPS (alone or conjugated to proteins) and outer-membrane proteins (OMPs) have been evaluated.

Recent interest in the study of microbial pathogenesis has focused on interactions of bacteria with the diverse and ever-changing environment of the host (Smith, 1998). There is now much evidence that the growth environment largely determines the composition and other biological properties of the bacterial surface. Iron is one of the essential nutrients required for bacterial survival and invasion (Payne, 1988). *S*. Typhi has been reported to express iron-uptake systems characterized by secreted siderophores along with the expression of certain proteins on the cell surface referred to as iron-regulated outer-membrane proteins (IROMPs). The environment of the host has been reported to regulate the *in vivo* expression of IROMPs, and recently, these proteins have been shown to have inflammatory potential (Chanana *et al.*, 2005). These proteins have been considered to be promising vaccine candidates for a number of Gram-negative bacteria including *Haemophilus influenzae*, *Helicobactor pylori* and *Neisseria* species (Schryvers, 1989; Banerjee-Bhatnagar & Frasch, 1990; Worst *et al.*, 1996).

S. Typhi IROMPs have also been observed to have immunogenic potential (Fernandez Beros *et al.*, 1989) and are able to stimulate antibody-mediated protection at systemic and mucosal levels (Sood *et al.*, 2005). Therefore, the present study was carried out to assess the T-cell-specific cellular immune response in affording protection against *S*. Typhi infection in a murine model.

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Abbreviations: DTH, delayed-type hypersensitivity; IFN, interferon; IL, interleukin; IROMP, iron-regulated outer-membrane protein; OMP, outer-membrane protein; PP, Peyer's patch.

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METHODS

Animals. Female inbred BALB/c mice, 4–6 weeks old (15–20 g), were procured from the Central Animal House, PGIMER, Chandigarh, India. Animals were kept in well-aerated rooms and were given sterile water and feed *ad libitum* throughout. Mice were acclimatized to the laboratory conditions for 1 week before the experiments. Mice were handled and disposed of according to guidelines of the Institute Ethical Committee.

Bacterial strain and growth. In the present study, a standard strain of *S.* Typhi Ty2 (Vi+) was obtained from the Central Research Institute (CRI), Kasauli, India. The culture was checked for purity and was characterized biochemically as well as serologically. The strain was maintained in 10 % glycerol broth and also stored as lyophilized ampules. *S.* Typhi was grown under normal, i.e. iron non-deficient, conditions (without dipyridyl) as well as iron-deficient conditions (in the presence of 200 μM dipyridyl). The iron content of the medium was determined as described previously (Sood *et al.*, 1999).

Extraction and purification of IROMPs. OMPs were extracted under normal and iron-limited conditions as described previously (Chander *et al.*, 2004). Briefly, bacterial growth was harvested by centrifugation at 10 000 r.p.m. for 20 min. The pellet obtained was suspended in 20 mM phenyl methyl sulphonyl fluoride (PMSF; Sigma). Cells were then disrupted by ultrasonication (Sonicator, Ultrasonic processor XL; Misonix) (10 cycles of 30 s with 1 min interval in between) and undisrupted material was removed by low-speed centrifugation (3000 ***g***, 4 °C, 20 min). The supernatant was then ultracentrifuged (Optima XL-100K Ultracentrifuge; Beckman Coulter) at 100 000 ***g*** for 60 min at 4 °C and the pellet was suspended in 1 % Sarkosyl (ICN) (in 20 mM Tris/HCl buffer, pH 7·6). After incubation for 2 h at 37 °C, the detergent-insoluble OMP fraction was collected by ultracentrifugation at the same speed. The recovered outer-membrane fraction was suspended in 20 mM Tris buffer containing 2 mM PMSF and stored at −20 °C. These proteins were resolved on SDS-PAGE. The IROMPs preparation showed three bands in the molecular mass range of 66–97 kDa as reported recently (Chanana *et al.*, 2005). IROMPs eluted from the gel (Hager & Burgess, 1980) were further purified by HPLC (Protein pack SW 125) as described earlier (Sood *et al.*, 2005). Thereafter, the proteins were analysed again by SDS-PAGE. The concentration of LPS in the IROMPs preparation as determined by *Limulus* amoebocyte lysate (LAL; Sigma) assay was found to be 0·02 %.

Immunization protocol. In the present study, for active immunization (Isibasi *et al.*, 1988), animals were divided into four groups of 10 mice each. (1) Control group: mice were injected with 0·5 ml normal saline intraperitoneally (i.p.). (2) Infected group: mice were injected with 0·5 ml *S.* Typhi (1×10^4 c.f.u. i.p.) in the presence of 5 % hog mucin. (3) IROMPs-immunized group: mice were immunized with 7·5 μg (Sood *et al.*, 2005) of purified IROMPs (the immunizing dose that gave the maximum protection) in 0·5 ml saline on days 0 and 14; no adjuvant was used while immunizing the animals. (4) IROMPs-immunized-challenged group: the mice were immunized as mentioned above and challenged intraperitoneally with $1{\cdot}40 \times 10^8$ c.f.u. (challenge dose) 7 days after the second dose of immunization.

Mice in each group were sacrificed at days 3, 7 and 15 after the final immunization/challenge dose as described earlier. The spleen and Peyer's patches lymphocytes were removed for immunological studies.

Isolation of spleen lymphocytes. For isolation of the splenic lymphocytes (Kiyono *et al.*, 1988), aseptically removed spleens were washed in PBS and teased in RPMI 1640. Splenic homogenates were kept in centrifuge tubes for 10 min to remove the tissue debris and then the cell suspensions were centrifuged at 1200 r.p.m. for 10 min at 4 °C. Lysing solution (Becton Dickinson Immunocytometry systems) was added to the pellets for 10 min at room temperature to lyse the red blood cells. Cells were then washed twice with RPMI 1640. Splenocytes were loaded onto the Ficoll-Isopaque density-gradient column and centrifuged at 1800 r.p.m. for 20 min at 4 °C. Mononuclear cell suspension recovered from the interface was subjected to two cycles of adherence in plastic tissue-culture dishes at 37 °C in a 5 % CO_2 atmosphere for 1 h each. Non-adherent cells (lymphocytes) were finally suspended in RPMI 1640 containing 10 % fetal calf serum (FCS) and an antibiotic cocktail (streptomycin and penicillin).

Isolation of Peyer's patches (PPs) lymphocytes. For isolation of the PP lymphocytes (Beagley *et al.*, 1988), the small intestines of mice were exposed on sterile gauze. PPs were identified, and in each case the serosal side of the patches was carefully dissected. The PPs were washed in normal saline and teased in RPMI 1640 and the homogenates were kept undisturbed for 5–10 min to settle cell clumps before the cell suspension was centrifuged at 1200 r.p.m. for 10 min at 4 °C. The pellets were washed three times with cold RPMI 1640. Macrophages were removed by two adherence cycles at 37 °C in 5 % CO_2 atmosphere for 1 h each. Non-adherent cells (lymphocytes) were suspended in RPMI 1640 containing 10 % FCS and an antibiotic cocktail (streptomycin and penicillin).

DTH response. Delayed-type hypersensitivity (DTH) test was performed as described elsewhere (Desiderio & Campbell, 1985). Each animal received 7·5 μg IROMPs intradermally to the left hind footpad and the same volume of PBS to the right hind footpad. Footpad thickness was measured by vernier calipers calibrated to 0·01 mm before and 24, 48 and 72 h after injection. Readings were taken in triplicate from three different directions. Foot pad swelling (FPS), measured in mm, was taken as an indicator of DTH response and calculated as follows: FPS=footpad thickness after injection−footpad thickness before injection.

Lymphocyte-proliferation assay. T-cell reactivity to the pure IROMPs preparation was determined by *in vitro* stimulation. The lymphocytes isolated from spleens and PPs of mice from each group (on days 0, 3, 7 and 15) were cultured at a concentration of 1×10^6 cells ml^{-1} in RPMI 1640 containing 10 % heat-inactivated FCS in the presence of Con A (2 μg) and IROMPs (1 μg) at 37 °C in a humidified atmosphere with 5 % CO_2 for 66 h. After 66 h, ^{3}H-thymidine (1 μCi) was added to each well under sterile conditions (Singh *et al.*, 1999). The cultures were kept under similar conditions for a further 6 h and thereafter the cells were harvested and the radioactivity was measured by suspending the harvested cells in Bray's scintillation fluid and counting on a β-counter (Rackbeta, 1214). The results were expressed as counts per minute (c.p.m.).

Immunophenotypic study. Immunophenotyping of T cells ($CD4^+$ and $CD8^+$) isolated from spleen and PPs was done as described elsewhere (Landy & Muirhead, 1989). Lymphocytes (1×10^6 cells; the viability of the cells was checked by the 0·4 % trypan blue exclusion method) were incubated with anti-Lyt2 ($CD8^+$) and L_3T_4 ($CD4^+$) mAbs conjugated with fluorescein iso-thiocynate (FITC) (Pharmingen) for 30 min at room temperature in the dark. Subsequently, cells were washed in PBS and fixed in 0·05 % paraformaldehyde. Five thousand cells from each sample were analysed on CELLQUEST software of FACScan (Becton Dickinson).

Measurement of cytokine (IL-2, IFN-γ, IL-4)-producing cells. Cytokine-producing cells were assessed by *in vitro* culture of lymphocytes (Sander *et al.*, 1991; Jung *et al.*, 1993; Seder & Paul, 1994). The lymphocytes isolated from spleen and PPs from various test groups (on days 0, 3, 7 and 15) were cultured at a concentration of 1×10^6 cells ml^{-1} in RPMI 1640 with 10 % FCS, antibiotics and 1 μg IROMPs for 48 h at 37 °C in 5 % CO_2 with 95 % humidity. Then 10 μl monensin

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(2 mM) was added to each well, followed by further incubation for 6–8 h. The cells were harvested and fixed in 0·5 % paraformaldehyde for 20 min at 4 °C. Cells were then washed twice with PBS (pH 7·2–7·4) and suspended in 100 μl 0·1 % saponin in PBS. After 10 min, rat antimouse interleukin mAbs [2 μl (10^6 cells)$^{-1}$; Pharmingen] were added separately in each well and incubated at room temperature for 1 h. The cells were washed twice after incubation. Secondary antibodies (IgG, $F(ab)_2$ fragment, FITC-labelled) at 1 : 1000 dilution (ICN Pharmaceuticals) were added and incubated in the dark at room temperature for 30 min. After that, the cells were washed twice with PBS and then subjected to FACScan analysis.

Statistics. The statistical significance of results was determined by using the unpaired student's *t* test and two-way analysis of variance (ANOVA) with multiple comparisons. Data were considered significant at $P < 0·05$.

RESULTS AND DISCUSSION

In vivo environmental stresses including acid stress and iron limitation have been reported to influence bacterial pathogenicity by regulating gene expression and thereby affecting the production of virulence determinants (Bearson *et al.*, 1997; Finlay & Falkow, 1997; Smith, 1998; Eriksson *et al.*, 2003; Rishi *et al.*, 2004). Therefore, in the present study, OMPs expressed under iron-limited conditions were extracted and purified in order to evaluate their potential for inducing the cellular immune response (Fig. 1). Even if a small amount of contamination of LPS was present in the preparation, it should not influence the immune response, as reported elsewhere (Calderon *et al.*, 1986). However, an attempt will be made to carry out mass spectroscopic analysis to identify these proteins further in future.

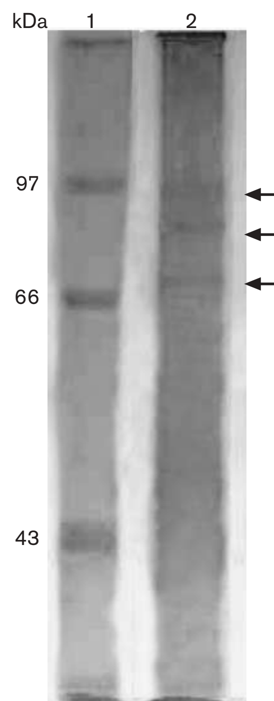

Fig. 1. Electrophoretic pattern of purified IROMPs on silver-stained gel. Lane 1, standard molecular mass markers; lane 2, three IROMPs.

DTH response

The DTH response indicated by footpad swelling was significantly higher ($P < 0·05$) in the infected and immunized-challenged mice compared to the control ones (Fig. 2) at 24, 48 and 72 h post-infection. However, no significant difference was seen between the two immunized groups. The potential of IROMPs as immunostimulatory molecules is evident since as little as 7·5 μg of the protein was observed to enhance the DTH response significantly. This is in contrast to a 50 μg dose of the same antigen as discussed recently (Chanana *et al.*, 2005) or of porins extracted under normal conditions (Singh *et al.*, 1999). Since organisms under environmental stresses have been reported to express more virulence determinants, lower concentrations of bacteria/bacterial products might be required. The lower concentration observed to be effective in the present study fits in with this hypothesis very well. The increase in DTH response following IROMPs immunization also highlights the importance of cellular factors for the development of protective immunity. Our results are in agreement with reports in which induction of the DTH reaction in response to porin immunization was shown to be responsible for protective immunity in *Salmonella* infections (Sharma-Rishi *et al.*, 1989; Tabarie *et al.*, 1994; Gupta *et al.*, 1996), where T_{DTH} cells were found to be T cells with surface phenotype of L_3T_4/$CD4^+$ (Mastroeni *et al.*, 1993).

Lymphocyte proliferation

The involvement of cell-mediated immunity in cases of enteric pathogens was described much earlier (Mackaness, 1971; Kauffman, 1995). It has been suggested that cellular responses could be either systemic, largely mediated by the liver as well as the spleen, or intestinal, mediated by gut-associated lymphoid tissues (GALT). *S.* Typhi is presumed to enter the body through specialized epithelial cells called M (microfold) cells located in PPs. After multiplying in the submucosal area, *S.* Typhi enters the blood stream and

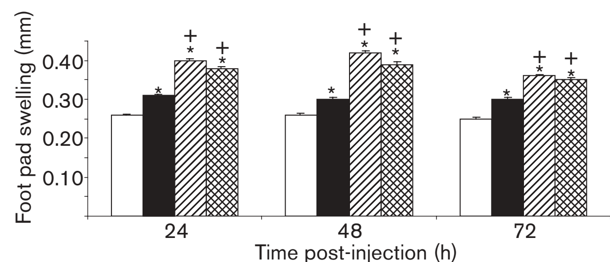

Fig. 2. DTH response. Data represent mean ± SEM of three readings of each experiment (mouse) done in duplicate. $^*P < 0·05$ as compared to control group. $^+P < 0·05$ as compared to infected group. Bars: open, control; filled, infected; striped, immunized; hatched, immunized-challenged.

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spreads throughout the body. The bacteria multiply in the spleen as well as the liver and are then released into the blood stream in large numbers.

The way antigens are acquired by individual lymphatic tissue affects the outcome of the immune response. For example, the same antigen may produce qualitatively different immune responses in lymph nodes, the spleen and PPs (Anderson, 1990). Antigens in blood are filtered, trapped, processed and presented in blood/tissue interfaces in the spleen, which results in peripheral immunity, like the antigens trapped in lymph and processed by lymph nodes. However, the spleen microenvironment is somewhat more complicated because it also accommodates antigen-presenting cells and immunoreactive T and B cells from other tissues committed to peripheral or mucosal immunity. Antigens in the lumens of enteric organs are non-destructively endocytosed by M cells and are trans-cytosed on PPs. Therefore, in the present study the splenic as well as PPs lymphocytes were isolated from different groups of mice to determine the role of T cells in providing protection against *S.* Typhi challenge.

There was a significant increase ($P < 0{\cdot}001$) in the IROMPs-induced proliferative response in lymphocytes isolated from the spleens of the immunized group [$25291{\cdot}35 \pm 868{\cdot}32$ c.p.m. $(10^6\ \text{cells})^{-1}$] compared to the control group [$4388{\cdot}98 \pm 77{\cdot}80$ c.p.m. $(10^6\ \text{cells})^{-1}$]. The lack of significant differences in thymidine incorporation in the absence of antigen in control and protein-immunized mice indicated that immunization with IROMPs does not result in the appearance of non-specifically activated lymphocytes.

An increase was observed in the proliferation of splenic and PP lymphocytes after *S.* Typhi infection on days 3, 7 and 15 as compared to control mice (Fig. 3a, b). Immunization with purified IROMPs caused a further increase in proliferation ($P < 0{\cdot}001$) compared to the control and infected groups. After challenge of the immunized animals there was a sharp increase in the proliferative response in spleen lymphocytes at 3 days post-infection that decreased gradually but remained higher in comparison to control and infected mice during the study period. In PPs lymphocytes at 3 and 7 days post-infection there was no significant difference between the immunized groups. However, the counts decreased significantly at 15 days post-infection in the immunized-challenged group (Fig. 3b); this may be due to a change in the antigen expression on the surface of the antigen-presenting cells along with the major histocompatibility complex (MHC) molecules by that time. It was interesting to observe that a significantly lower amount of IROMPs (1 μg) was required for the transformation of lymphocytes in this study, in contrast to the porins (20 μg) used previously (Singh *et al.*, 1999). This points towards the sensitivity of OMPs regulated by iron availability.

Phenotypic characterization of T lymphocytes

In order to assess the role of T-cell subsets ($CD4^+$ and $CD8^+$) in conferring the protective immunity, we studied the $CD4^+/CD8^+$ ratio in spleen and PP lymphocytes. There was a decrease ($P < 0{\cdot}01$) in the $CD4^+/CD8^+$ ratio in the spleen as well as PP lymphocytes following infection (Fig. 4a, b). This decrease may be attributed to a proportion of the subpopulation of lymphocytes undergoing apoptosis following infection. In our earlier studies, we observed that macrophages that interact with *Salmonella enterica* serotype Typhimurium grown under iron stress undergo cell death by the process of apoptosis (Chanana *et al.*, 2004). Immunization with IROMPs evoked a significant increase in the ratio during

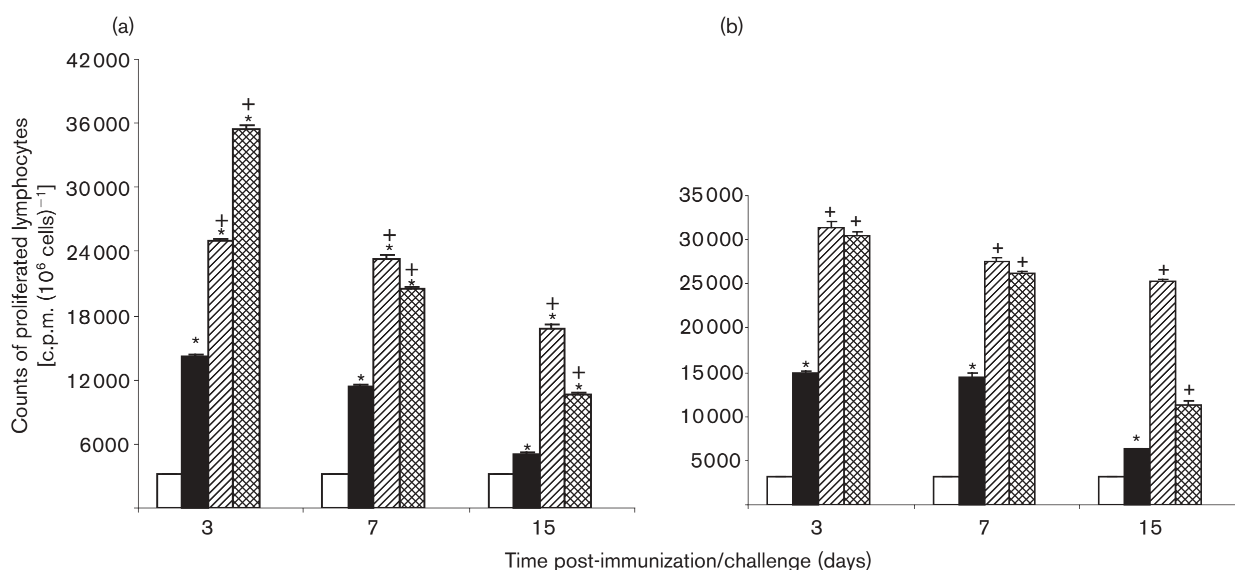

Fig. 3. T-cell proliferation in the spleen (a) and Peyer's patches (b). Data represent mean ± SEM of three experiments done in duplicate. $^*P < 0{\cdot}05$ as compared to control group; $^+P < 0{\cdot}05$ as compared to infected group. Bars: open, control; filled, infected; striped, immunized; hatched, immunized-challenged.

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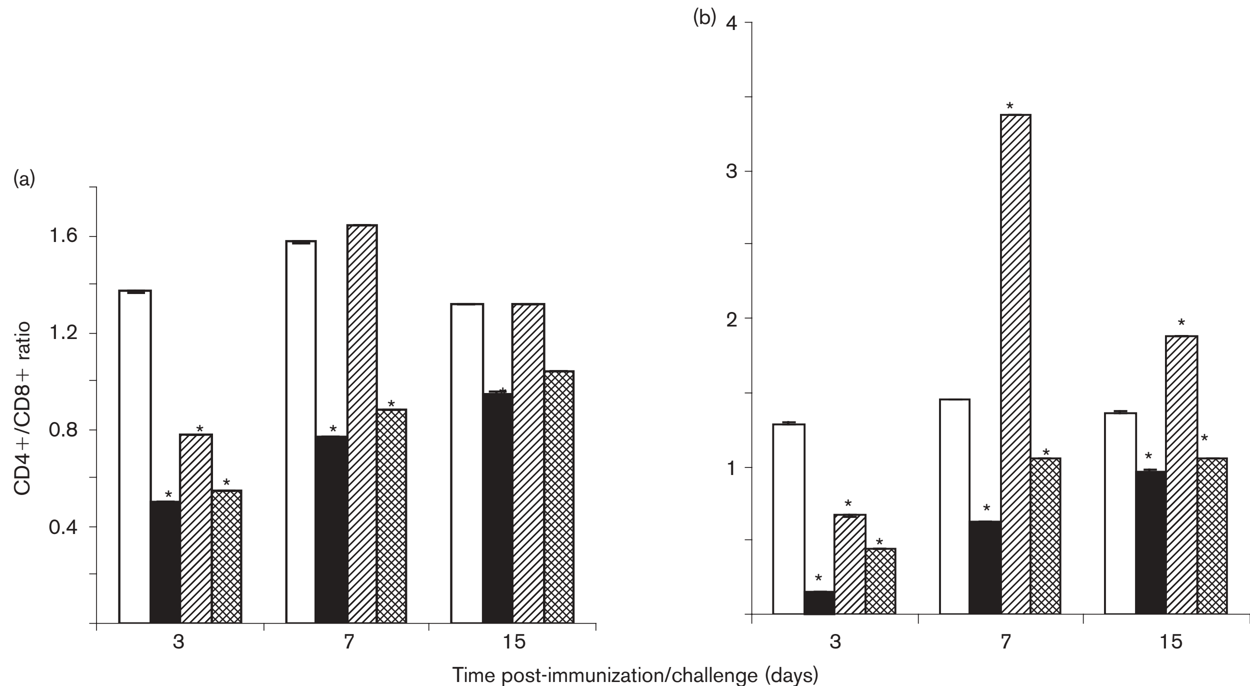

Fig. 4. $CD4^+/CD8^+$ lymphocyte ratio in the spleen (a) and Peyer's patches (b). Data represent mean ± SEM of three experiments done in duplicate. $^*P<0{\cdot}01$ as compared to control group. Bars: open, control; filled, infected; striped, immunized; hatched, immunized-challenged.

the study period compared to the ratio observed in the infected mice. Subsequent challenge with *S.* Typhi resulted in a decrease ($P<0{\cdot}05$) in the $CD4^+/CD8^+$ ratio compared to that in the immunized group. However, the ratio in this group remained higher compared to that in infected animals. IROMPs immunization showed a preferential increase in the $CD4^+$ T-cell subset in spleens as well as in PPs, whereas $CD8^+$ cells showed an increase as a result of infection. The DTH cells have been shown to be $CD4^+$ (primarily of the Th1 subtype) in the majority of the cases (Kuby, 1997). An increased DTH response, proliferation of T cells (both in the spleen and PPs) and an increased $CD4^+/CD8^+$ ratio in mice following IROMPs immunization compared to infection may fit in with this hypothesis.

Cytokine-producing cells

The cytokine profile contributes to pronounced differences in Th cell function. A Th1 response is characterized by the production of interleukin (IL)-2 and interferon (IFN)-γ, and Th2 by the production of IL-4, IL-5, IL-6, IL-10 and IL-13, whereas Th0 is characterized by the production of cytokines that do not fall into Th1 and Th2 patterns. Therefore, to explore the possible involvement of Th1 and Th2 type cells, we analysed IL-2-, IFN-γ- and IL-4-producing cells in spleen and PP lymphocytes taken from all the test groups. Following infection with *S.* Typhi, increased proportions of IL-2- and IFN-γ-producing cells were observed on days 3, 7 and 15 compared to the control group (Fig. 5). Immunization with IROMPs further increased the proportions of IL-2- and IFN-γ-cytokine-producing cells, with the increase being more significant for IFN-γ producing cells at day 15 in spleen lymphocytes. Challenge with *S.* Typhi led to a smaller increase in the proportions of these cells compared to the immunized group. However, the number of IL-2-producing PP cells in the immunized animals remained higher throughout the study period in comparison to the other test groups. A smaller increase in IFN-γ-producing cells in the spleen was observed in the challenged mice as compared to immunized ones. However, in PP cells, challenge with *S.* Typhi resulted in a higher number of IFN-γ-producing cells compared to the immunized group.

In comparison to the control group, an increase was observed in the production of IL-4-producing cells in the spleen and PP for all the groups throughout the study period (Fig. 6a, b). In the spleen, immunized and infected groups did not show much change in the proportion of IL-4-producing cells with respect to one other on days 3 and 7, but on day 15, the immunized and immunized-challenged groups showed an increase in IL-4-producing cells compared to the infected group ($P<0{\cdot}05$).

Significantly higher numbers of IL2-, IFN-γ- and IL4-producing cells in the IROMPs-immunized mice suggested that immunization with these proteins elicits both Th1 and Th2 type responses. This type of Th response would be expected to play a critical role in resistance to *S.* Typhi

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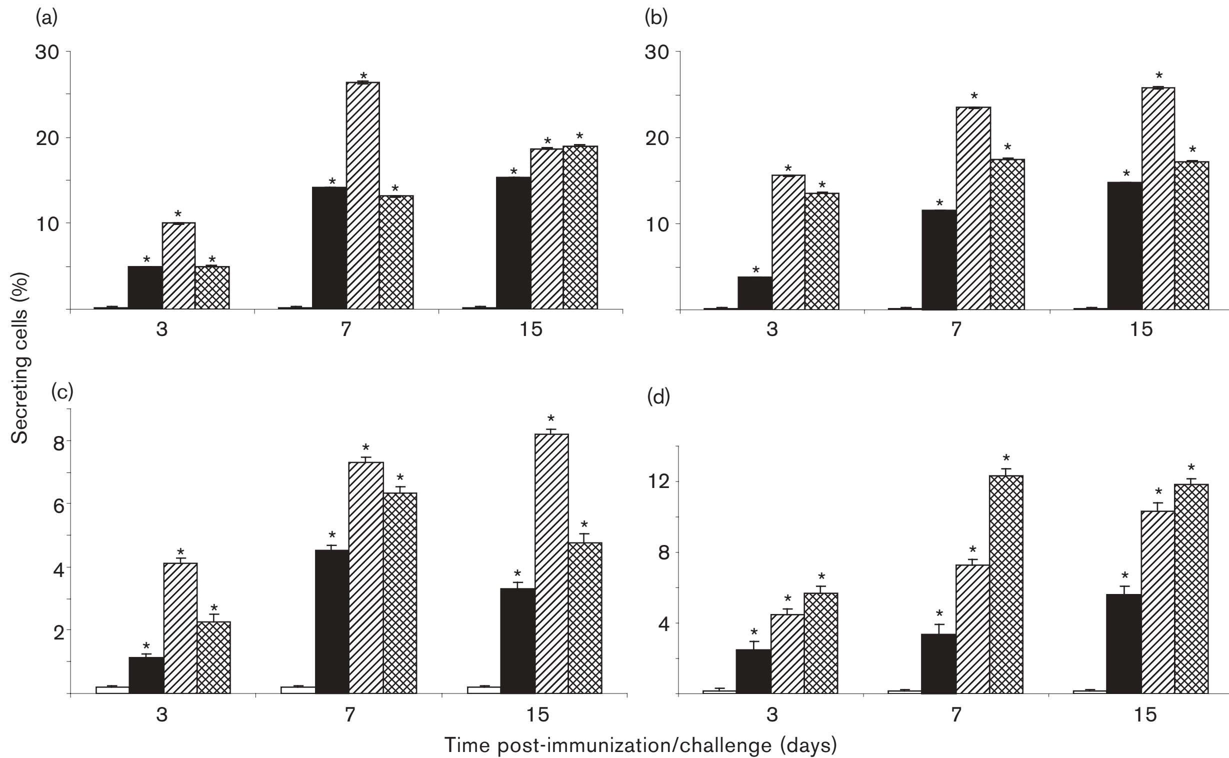

Fig. 5. Th1 cytokine-producing cells. (a, b) IL-2- and (c, d) IFN-γ-producing cells in the spleen (a, c) and Peyer's patches (b, d). Data represent mean ± SEM of three experiments done in duplicate. *$P<0.01$ as compared to control group. Bars: open, control; filled, infected; striped, immunized; hatched, immunized-challenged.

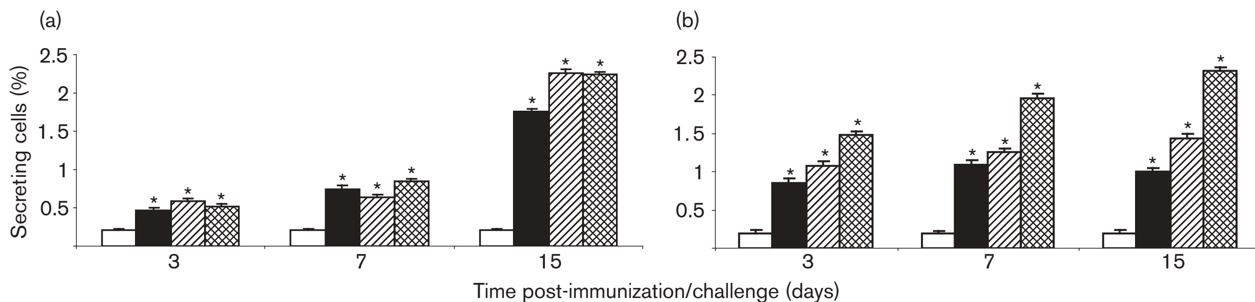

Fig. 6. IL-4-producing cells in the spleen (a) and Peyer's patches (b). Data represent mean ± SEM of three experiments done in duplicate. *$P<0.01$ as compared to control group. Bars: open, control; filled, infected; striped, immunized; hatched, immunized-challenged.

infection at the systemic level by contributing to the elimination of *S*. Typhi in localized macrophages of the reticuloendothelial system and in other cells.

In short, this study indicates that, in addition to porins, non-porin proteins like IROMPs expressed by *S*. Typhi induce a cellular immune response against infection through Th1 and Th2 type cells. The response to antigen presentation to lymphoid cells on PPs triggers commitment to mucosal immunity characterized by the secretion of specific IgA (Anderson, 1990). The increase in the lymphocytes in PPs might have caused the increase in the sIgA observed in our earlier study. Therefore, it is speculated that immunization with IROMPs may evoke peripheral as well as mucosal immunity against *S*. Typhi infection. As these antigens are expressed on the cell surface, they are likely to come in contact with the immune system in the infection process. This may be particularly true for humans, where proteins expressed under iron-limited conditions may act as good immunogens against typhoid fever.

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