ORIGINAL ARTICLE

Porins and lipopolysaccharide from *Salmonella typhimurium* regulate the expression of CD80 and CD86 molecules on B cells and macrophages but not CD28 and CD152 on T cells

M. Galdiero[1], *M. G. Pisciotta*[2], *E. Galdiero*[3], *and C. Romano Carratelli*[1]

[1]Dipartimento di Medicina Sperimentale, Facoltà di Medicina e Chirurgia, Seconda Università di Napoli, [2]Dipartimento di Patologia Generale, Facoltà di Medicina e Chirurgia, Seconda Università di Napoli, and [3]Dipartimento di Fisiologia Generale, Sezione di Igiene e Microbiologia, Università degli Studi di Napoli 'Federico II', Napoli, Italy

Objective The aim of this study was to evaluate the effect of porins from *Salmonella typhimurium* on costimulatory molecules such as CD80/CD86 and CD28/CD152. The interactions between these molecules are able to influence the immune response through the regulation of cytokines release which, on their own, are able to regulate the immunological response by a feedback mechanism.

Methods *S. typhimurium* strain SH5014 (a rough lipopolysaccharide (LPS) producing strain) was used as the source of porins and LPS. Peripheral blood mononuclear cells were obtained from healthy adult donors. THP1 cells were obtained from ATCC (Rockville, MD, USA). Immunofluorescence and flow cytometry were performed using a FACS IV (Becton–Dickinson, Mountain View, CA, USA).

Results Our results show that porins of *S. typhimurium* increase the expression of CD86 and the expression of CD80 both on B lymphocytes and macrophages, while the expression of CD28 and CD152 on T lymphocytes was unaltered. The expression of CD80 and CD86 is dose-dependent and starts after 24 h post treatment, peaks at 48 h and goes back to the basal value after 72 h.

Conclusions *S. typhimurium* porins are able to induce a high expression of costimulatory molecules (CD80 and CD86) on lymphocytes and macrophages.

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INTRODUCTION

CD80 and CD86 are expressed by antigen-presenting cells (APC) and have been shown to be capable of delivering a costimolatory signal [1–4]. CD28 and CD152 are molecules expressed on T cells that may be involved in APC-dependent T-cell activation [2,5]. Activation of T cells to cytokine production and proliferation requires at least two distinct signals [6,7]: the first signal is provided by TCR antigen in the context of the major histocompatibility complex (MHC), and the second signal is provided by a set of receptor–ligand interactions in which CD28 and CD152, CD80 and CD86 play a predominant role.

Some outer membrane proteins of Gram-negative bacteria act as porins and have an important role both in bacterial cell physiology and interaction with the host [8–11]. Porins are present in the outer membrane of Gram-negative bacteria as integral membrane proteins and are released together with the lipopolysaccharide (LPS) during cell growth by a mechanism known as blebbing [12] or by bacterial lysis [13].

Porins perfom several biological functions on inflammatory and immunological cell response

Corresponding author and reprint requests: Caterina Romano Carratelli, Dipartimento di Medicina Sperimentale, Facoltà di Medicina e Chirurgia, Seconda Università di Napoli.Via Costantinopoli, 16–80138 Napoli, Italy
Tel: +39 81 5665658
Fax: +39 81 5667578
E-mail: caterina.romano@unina2.it

[10,11,14–16]. The protective role of the outer membrane protein antibodies, among which are porins from *Salmonella* [17], *Neisseria* [18,19], *Haemophilus* [20] and *Vibrio* [21], has been previously demonstrated. Furthermore, antiporin antibodies have been demonstrated to be bactericidal and opsonic [18,22] and porin serovar-specific protection has been demonstrated to exist in prostitutes with a history of multiple gonococcal infections [23], and in patients with pelvic inflammatory disease who recover spontaneously and who have high levels of antiporin antibodies [10]. Moreover, the amount of porins and the type of LPS present on the surface of bacteria are correlated with virulence [24,25]. During a protective immune response several mechanisms are activated. When purified porins are experimentally inoculated, for example, during experimental oral infection with *S. typhimurium*, in survivor mice T-lymphocyte differentiation occurs, leading to a prevalence of the Th-1 response, while treatment with purified porins does not induce in vivo a similar pattern of differentiation [26,27].

It has been shown that LPS upregulates the CD80 expression in human monocytes. Among the different accessory molecules expressed on APC, CD80, CD86 and CD54 molecules were found to play major roles in T-cell activation by interacting with their counter receptors CD28, CD152 and CD18, respectively [5,28,29]. It is known that *Brucella abortus* [30] may augment the interaction of APC with T cells via a direct induction of CD54 and CD80, CD86 on the APC and indirectly through interleukin(IL)-12 induced CD18 on T cells. Porins from *S. typhimurium* are able to activate CD54 [31] and other cytokines that are involved in the regulation of the immune response [8,14].

Considering the activity of the porins in regulating the immune response and their ability to behave like antigens, we tested in vitro the effect of *S. typhimurium* porins in regulation of the costimulatory molecules CD80, CD86, CD28 and CD152.

MATERIALS AND METHODS

Porins preparation

S. typhimurium strain SH5014 (a rough LPS-producing strain), served as the source of porins. The porins were isolated as described by Nurminen [32]. Briefly, 1 g of envelopes was suspended in 2% Triton X-100 in 0.01 Tris-HCl (pH 7.5, containing 10 mM EDTA); after the addition of trypsin (10 mg/g of envelopes), the pellet was dissolved in sodium dodecyl sulfate buffer (SDS buffer, 4% w/v in 0.1 M sodium phosphate, pH 7.2), and applied to an Ultragel ACA 34 column equilibrated with 0.25% SDS buffer. The fraction containing proteins, identified by its absorbance at 280 nm (A_{280}), was extensively dialyzed and checked by SDS-polycrylamide gel electrophoresis in slabs (SDS-PAGE) according to Laemmli [33]. The protein content of the porin preparation was determined by the method of Lowry et al. [34]. All possible traces of LPS were revealed on SDS-PAGE stained with silver nitrate as described by Tsai and Frasch [35] and by *Lymulus* amoebocyte lysate assay [36]. The LPS concentration in the porin preparation was estimated to be $< 0.005\%$ w/w. In addition, in some experiments polymyxin-B (Sigma-Aldrich Co, Napoli, Italy) was mixed with porins in order to neutralize the biological activity of traces of LPS that could be present in the preparation. The porins were incubated with polymixin-B at room temperature for 1 h in a ratio of 1 : 10 (w : w). LPS was incubated with polymyxin-B in a ratio of 1 : 100 (w : w) [37]. LPS, porins and the mixture containing polymyxin-B were used in pyrogen-free phosphate-buffered saline (PBS).

Preparation of LPS

LPS was isolated from *S. typhimurium* SH5014 by the phenol-chloroform-ether method described by Galanos et al. [38]. Briefly, bacteria were washed sequentially with water, 95% ethanol, acetone, and diethyl ether at 4 °C. The dry bacterial powder was treated with a mixture of liquid phenol-chloroform-petroleum ether in a volume ratio of 2 : 5 : 8. After centrifugation, the acqueous layer was collected and dialyzed to remove phenol. Subsequently, the dialyzed material was concentrated in a rotary evaporator at 35–40 °C and centrifuged to remove any insoluble material. The supernatant was treated with RNase for 2 h at 55 °C. After centrifugation, the pellet was collected, resuspended in water, and lyophilized.

Preparation of cell suspensions

Peripheral blood mononuclear cells (PBMC) were obtained from a buffy coat of a blood sample from

healthy adult donors (courtesy of the Blood Bank, Second University of Naples, Italy). The buffy coat was diluted six-fold with RPMI-1640 (Labtek Laboratories, Eurobio, Paris, France) and washed twice. The pellet was resuspended in RPMI-1640, applied on Ficoll-Hypaque gradients (Pharmacia, Uppsala, Sweden) and centrifuged for 30 min at 900 g.

The cells were suspended in RPMI 1640 with 10% fetal calf serum (FCS) (Labtek Laboratories, Eurobio, Paris, France) and antibiotics and incubated for 1 h in 5% CO_2 at 37 °C. The adherent cells were cultured overnight in RPMI 1640 with 10% FCS. Cell viability was evaluated by the trypan blue exclusion test. At least 96% of the cells thus obtained were monocytes as determined with a FACS analyzer (Becton-Dickinson) with monoclonal antibody CD14 (Becton-Dickinson).

Nonadherent cells (lymphocytes) were harvested, washed, and resuspended at 3×10^6 cells/mL. Flow cytometry analysis of stained cells with monoclonal antibody CD3 (Boerhringer-Roche Diagnostic S.p.A., Milano, Italy) demonstrated that more than 94% of the isolated cells were T lymphocytes.

Cells

A clone of the human monocytic cell line, THP-1 cells (ATCC TIB-202; ATCC) was grown at 37 °C in an atmosphere of 5% CO_2 in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin (Labtek Laboratories).

The human T lymphoma cell line Jurkat (ATCC TIB-152) was cultured at 37 °C in an atmosphere of 5% CO_2 in a complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL of penicillin, 100 μg/mL streptomycin, and 0.05 mM of 2-mercaptoethanol.

PBMC were activated by Phorbol 12-Myristate 13-Acetate (PMA) (Sigma-Aldrich Co) at a concentration of 250 ng/mL for 48 h at 37 °C.

Some experiments were performed on cells incubated with cycloheximide (10 μg/mL) (Sigma-Aldrich Co) for 15 min and then treated with porins for 48 h, to determine whether de novo protein synthesis was required for increase in CD80 and CD86 molecule expression.

Flow cytometry

Immunofluorescence and flow cytometry were performed using a FACS IV (Becton-Dickinson). Cells were stained at 4 °C and washed in Hank's balanced salt solution containing 1% bovine serum albumin (BSA) and 0.1% sodium azide. Macrophages (10^6/mL) were stained with Peridinin Chlorophyll protein (PrCP)-conjugated anti CD14 MAb (clone MΦP9) plus Phycoerythrin (PE)-conjugated anti CD86 MAb (clone IT2.2) and Fluorescein Isothiocyanate (FITC)-conjugated anti CD80 MAb (clone BB1), to determine the $CD80^+$ and $CD86^+$ cells; lymphocytic cells (10^6/mL) were stained with: (i) cy-chrome™- conjugated anti CD19 MAb (clone CD28.2) plus FITC-conjugated anti CD80 MAb (BB1) and PE-conjugated anti CD86 MAb (IT2.2) to examined $CD80^+$ and $CD86^+$ B cells; (ii) FITC-conjugated anti CD3 MAb (clone HIT3a) plus PE-conjugated anti CD28 MAb (CD28.2) and cy-chrome™-conjugated anti CD152 MAb (clone BNI 3.1) to determine $CD28^+$ and $CD152^+$ T cells.

All antibodies were used at saturating amounts for flow cytometric studies and were purchased from Pharmigen (San Diego, CA, USA).

Statistics

All experiments were carried out in triplicate; results were expressed as the mean ± standard deviation.

RESULTS

Porins and LPS preparations

The purification and contamination of porins by LPS have been addressed in previous studies [9,39]. The purity of the porin preparation, checked by SDS-PAGE, is shown in Figure 1, lane B. SDS-PAGE revealed two bands with molecular weights of 34 000 and 36 000.

The *Limulus test* showed the presence of LPS at a concentration < 0.005% w/w; these traces of LPS did not show any biological activity under our experimental conditions (data not shown). The patterns of the LPS preparations as revealed on SDS-PAGE by silver nitrate staining method showed that LPS from *S. typhimurium* SH5014 migrates as a rough LPS in comparison to the commercial preparation (Sigma-Aldrich Co).

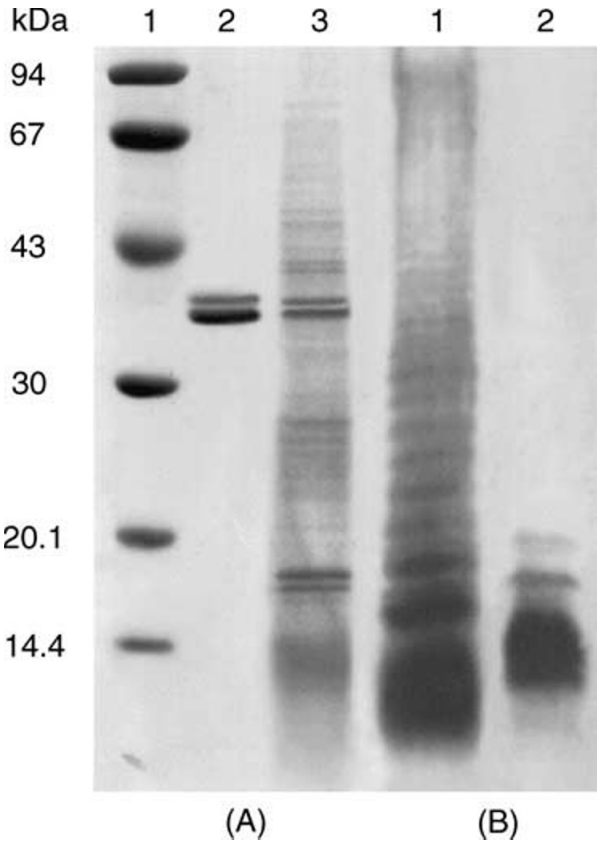

Figure 1 SDS-PAGE analysis of the outer membrane protein preparation, porin and lipopolysaccharide (LPS) extract from *S. typhimurium* SH5014. The gel was stained with Coomassie blue. (a) Lane 1, molecular mass standards (phosphorylase b, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anydrase, 30 kDa; trypsin inhibitor, 20 kDa; α-lactoalbumin, 14 kDa); lane 2, *S. typhimurium* porin (10 μg); lane 3, *S. typhimurium* total OMP (10 μg). (b) Lane 1: *S. typhimurium* smoth LPS (Sigma-Aldrich S, Italy) (20 μg); lane 2: SH5014 LPS (10 μg).

Expression of CD80 and CD86 by B cells

The expression of these molecules was examined on unstimulated B cells and on B cells stimulated with 0.04 μM, 0.2 μM and 0.4 μM of *S. typhimurium* porins. These concentrations have been chosen because of their biological activity shown in previous studies [9,27]. Figure 2(a) shows that when B lymphocytes are incubated with porins (0.2 μM) for 48 h there is a marked increase in the expression of CD86 (50% ± 7) and a lower increase in the expression of CD80 (37% ± 6). The control cells expressed low levels of CD86 and undetectable levels of CD80. The results show that incubating B lymphocythes with LPS (2.5 μM) for 48 h induced a light increase in the expression as well of CD86 (35% ± 7) as CD80 (25% ± 5) (Figure 2(b)). The dose-response plot showed the same profile whether LPS (0.5 μM, 2.5 μM and 5 μM) or porins (0.04 μM, 0.2 μM and 0.4 μM) were used as the stimulus. Porin concentrations of 0.02 μM did not induce any stimulation while concentrations of 0.8 μM were toxic for B lymphocythes in cultures (data not shown). After stimulation with porins for 24 h the expression of CD86 and CD80 increased, peaked at 48 h and started to decrease at 72 h (Figure 3). A transient increase in CD86 expression also occurred early on B cells that were cultured in vitro in the absence of porins. After 15 h culture the CD86 expression returned nearly to the level originally detected on freshly explanted B cells. The CD86 increase induced by porin stimulation was completely inhibited by cycloheximide (data not shown), pointing out that novel protein synthesis is required for an increased CD86 expression.

The cells stimulated with porins mixed with polymixin B (porin: polymyxin B, 1 : 10, w : w) to neutralize the effect of small amounts of LPS in the porin preparation, showed the same results as the porins on their own (data not shown). The cells stimulated with LPS mixed with polymixin B (LPS: polymixin B, 1 : 100 w : w) did not modify the expression levels of CD80 and CD86 (data not shown).

Expression of CD80 and CD86 by macrophages

The monocytes isolated from pheripheral blood were cultured for 5 days. Then they were stimulated with porins at concentrations of 0.04 μM, 0.2 μM, 0.4 μM or LPS at concentrations of 0.5 μM, 2.5 μM and 5 μM. Macrophages stimulated with porins 0.2 μM or LPS at concentration of 2.5 μM showed a higher expression of $CD86^+$ (48% ± 7 and 33% ± 6, respectively) and $CD80^+$ (32% ± 6 and 22% ± 5, respectively) compared to untreated controls (data not shown). Macrophages activated by PMA expressed higher amounts of both molecules; further stimulation of activated macrophages with porins or LPS did not increase CD80 and CD86 expression (data not shown).

We also examined THP1 (a monocyte immortalized line) for expression of CD80 and CD86. This cell line mainly expresses CD86. The porin stimulation did not cause any change in the fluorescence intensity after staining cells with antibodies CD80 FITC and anti CD86 PE (data not shown).

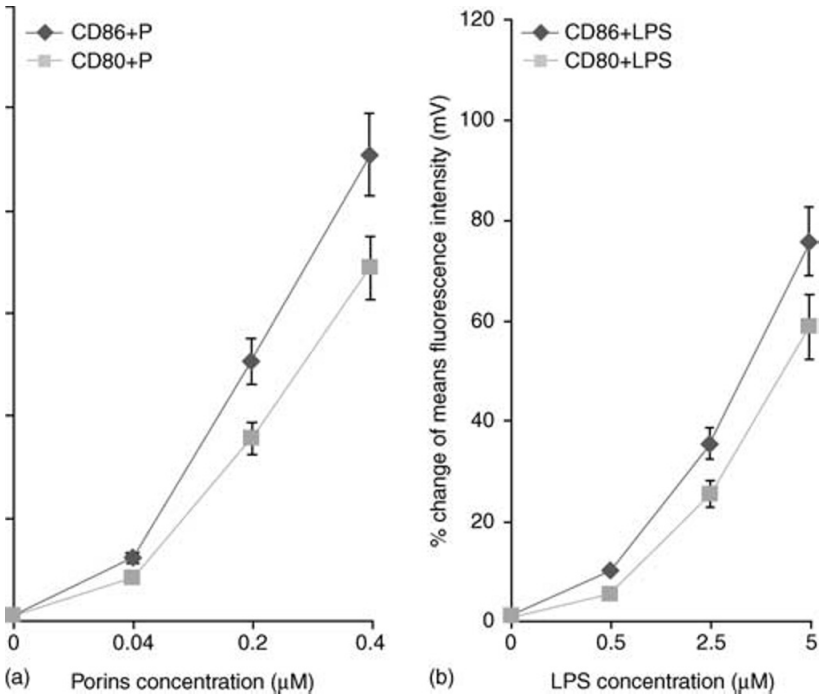

Figure 2 Expression of CD80 and CD86 molecules on B lymphocytes stimulated for 48 h with porins (a) or LPS (b) of *S. typhimurium*. The percent change in fluorescence was calculated using the following formula: control (mV) – cultured with porins or LPS (mV)/control (mV) X 100. The results are the means of three experiments ± SD.

Expression of CD28 and CD152 on T cells

$CD3^+$ T cells stimulated with *S. typhimurium* porins at a concentration of 0.2 μM and 0.4 μM for 24 h did not show any modification in the expression of CD28 and CD152. The percentages of $CD28^+$ T cells were not modified significantly by stimulation with porins, while the percentages of $CD152^+$ cells were considerably low, both in treated and untreated cells. Jurkat cells treated with porins were tested in order to gain deeper information on the expression of CD28 and $CD152^+$. This cell line showed higher percentages of CD28 and $CD152^+$ cells compared to freshly cultivated T lymphocytes (Figure 4). Longer incubation times did not modify the amount of CD28 and CD152 expression (data not shown). Porins did not modify the mean fluorescence of the treated cells compared to the untreated cells.

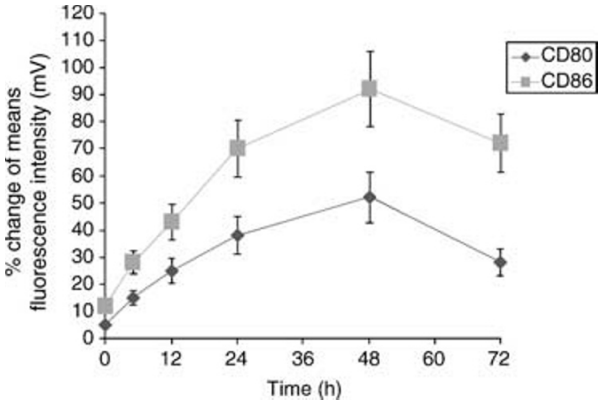

Figure 3 Expression of CD80 and CD86 molecules on B lymphocytes stimulated with porins of *S. typhimurium*. B lymphocytes were incubated with medium (control) and 0.2 μM of porins. The percent change in fluorescence was calculated as in Figure 2. The results are the means of three experiments ± SD.

DISCUSSION

It is of considerable interest to identify the biologically relevant signals, mediated either by

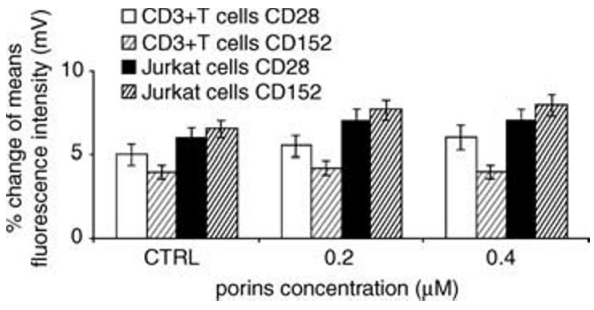

Figure 4 Expression at 24 h of CD28 and CD152 molecules on $CD3^+$ T and Jurkat cells stimulated with porins of *S. typhimurium* at concentrations of 0.2 μM and 0.4 μM. The results are the means of three experiments ± SD.

bacterial cells or by their components, that regulate costimulatory molecules.

The potential for signaling through the CD80/CD86, CD28, CD152 costimulatory pathway is very complex. T cells can express at least two receptors for costimulation, CD28 and CD152 [3,5,7,40] and APC can express two or more molecules capable of interacting with one or both of these receptors on T cells [41–43]. Bacterial cells and their components modify the cytokine release, and these molecules in turn are able to regulate the expression of costimulatory molecules. IL-5, interferon (INF)-γ and GM-CSF [44] differentially regulate the CD80 and CD86 expression. Therefore, cytokine release owing to bacterial components or whole bacterial cells can indirectly activate APC with a different expression of CD80 and CD86.

Our results showed that among the Gram-negative bacterial components, *S. typhimurium* porins were able to activate B-lymphocytes and macrophages inducing a higher expression of CD86 (50% ± 7 and 48% ± 7, respectively). The kinetics of the highest expression of these costimulatory molecules (CD80 and CD86) on lymphocytes and macrophages owing to porins is similar to the kinetics already demonstrated as a result of LPS [44]. Therefore, it seems that different stimuli induce the same kinetics of expression.

Using PBMC instead of purified monocytes [45] we demonstrated that LPS has a negative effect on T-cell activation by decreasing already induced $CD80^+$ macrophages; the induction of the CD80 expression by LPS was not significant, no changes in the CD86 expression were detected.

From our results we were unable to observe an increase in the expression of CD28 and CD152. CD28 is a membrane glycoprotein expressed on most thymocytes and peripheral T lymphocytes [46,47], CD152 is expressed by activation but not by resting T lymphocytes [5]. The CD152/CD80 interaction is not clearly defined but may serve to amplify a T-cell-immune response initiated by CD28. Binding of CD80/CD86 with both CD28/CD152 which are present on T cells seems to be critical in the induction and maintenance of an efficient cell-mediated response which strongly contributes to the control of bacterial infections. The interactions between APC and T-lymphocytes represent the mechanism that stimulates the induction of these responses. In our results there is a modulation of CD80/CD86 expression on APC owing to porins, while on T lymphocytes a modulation of the expression of CD28 and CD152 is absent. Therefore it is probable that during the in vivo response a wider expression of CD80 molecules on APC may represent one of the initial stimuli to activate the cell proliferation, cytokine production and cytotoxicity. It was reported that CD80 and CD86 can equally costimulate IL-2 and IFN-γ production, but that CD86 induces significantly more IL-4 than CD80 [48]. These data provided evidence that CD80 and CD86 may not deliver an identical costimulatory signal to Th-1 or Th-2 cells and may have selective effects on the differentiation of Th0 cells into Th-1 or Th-2 phenotypes. It has previously been demonstrated [27] that isolated porins induce a Th-2 response, while whole *Salmonella* cells mainly induce a Th-1 response. From our results CD86 is more stimulated than CD80 by porins, therefore contributing to a Th-2 response. The involvement of Th-1–Th-2 cell subsets is also probably modulated in vivo by effects of the cytokine network; Gupta *et al*. [49–51] detected a Th-2 response in the spleen of mice after infection with *S. typhimurium*, whereas this response became Th-1 if the mice were pre-immunized with *Salmonella* porins. Therefore it is likely that porins and LPS provide a microenviroment in which a well-balanced accessory molecule cytokine network is established.

In conclusion, during in vivo infection, the route of the immunological response may depend considerably on the interaction of the *Salmonella* with the host, for example by invasion of target cells or the extracellular release of its components by blebbing or bacterial lysis.

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