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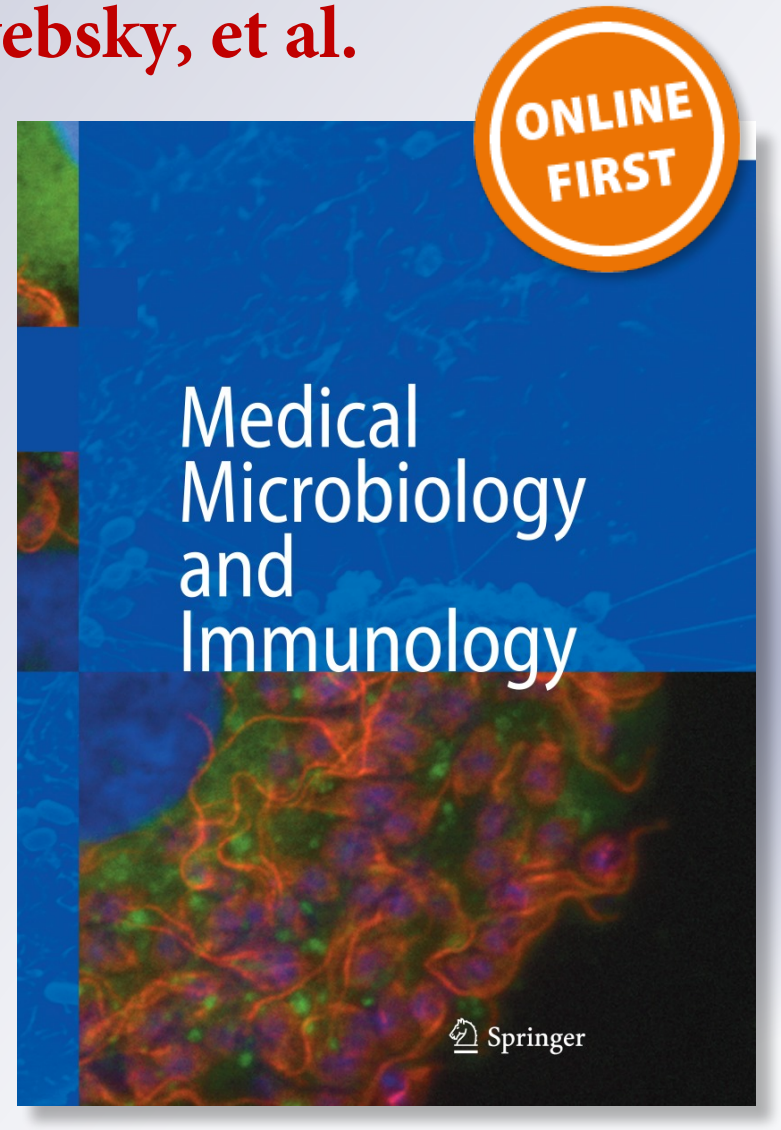

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ORIGINAL INVESTIGATION

Oral administration of Shiga toxin-producing *Escherichia coli* induces intestinal and systemic specific immune response in mice

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Abstract Hemolytic uremic syndrome (HUS) is the major complication of gastrointestinal infections with enterohemorrhagic *Escherichia coli* (EHEC) and is mediated by the production of Shiga toxins (Stx). Although it has been previously reported that not only HUS patients but healthy children have anti-Stx antibodies, very little is known about how these infections impact on mucosal immune system to generate a specific immune response. This work aimed to evaluate the immune responses elicited after a single oral dose of EHEC in a mouse model of HUS at weaning. We found sequential activation of T and B lymphocytes together with an increased percentage of IgA-bearing B cells in Peyer's patches and mesenteric lymph nodes. We also found fecal anti-EHEC IgA and serum anti-Stx2 IgG in EHEC-inoculated mice. Besides, these mice were partially protected against an intravenous challenge with Stx2. These data demonstrate that one episode of EHEC infection is enough to induce activation in the gut-associated lymphoid tissue, especially the B cell compartment, and lead to the production of specific IgA in mucosal tissue and the generation of systemic protection against Stx2 in a percentage of intragastrically inoculated mice. These data also support the epidemiologic observation that a second episode of HUS is very rare.

Keywords EHEC · Mucosal immune response · Specific IgA · Serum anti-Stx2 antibodies

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Introduction

Hemolytic uremic syndrome (HUS) is often caused by infection with enterohemorrhagic *Escherichia coli* (EHEC) and is mediated by the production of Shiga toxins (Stx), particularly type 2 (Stx2). Although disease is most frequently associated with the O157:H7 serotype [1, 2], other non-O157 serotypes are also associated [3].

In Argentina, HUS constitutes an endemic disease and is the leading cause of kidney failure in children. Approximately, 500 cases of HUS were annually reported during the last 10 years, with an incidence that ranged between 7.8 and 17/100,000 children <5 years of age [4]. In this regard, we have recently reported that 67 % of healthy Argentinean children showed anti-Stx2 antibodies [5]. This frequency is significantly higher than those reported in other countries for an age-matched healthy population [6, 7]. This finding could be ascribed to the high circulation of Stx2-producing strains in Argentina [2, 8, 9] and suggests that EHEC are able to trigger the immune system and to generate a specific antibody response even in non-symptomatic cases.

A specific antibody response against Stx in both systemic circulation and mucosa is necessary to prevent HUS secondary to EHEC infections. A key strategy to generate a noninflammatory immune protection in mucosal tissues is the production of immunoglobulin A (IgA) [10]. It is the main antibody isotype involved in defense against

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pathogens and toxins in the mucosal immune system [11], by several mechanisms such as the generation of a barrier that prevents antigens from attaching to and penetrating the mucosal epithelium and the formation of immune complexes with antigens within epithelial cells of the gut lamina propria [12]. Additionally, about 80 % of IgA-producing cells reside in the gastrointestinal mucosa [13]. Peyer's patches (PP) are the main secondary lymphoid organ where B lymphocytes differentiate to IgA-producing cells [14], thus they have high levels of specific markers of the IgA class switching, such as α germinal transcripts and circular transcripts (CTα) [15], and cytokines as TGF-β [16]. After differentiation, these IgA-producing cells migrate to the mesenteric lymph nodes (MLN) and via the thoracic duct into circulation. Finally, they home to effector sites, such as the lamina propria [14, 17]. In addition to IgA, orally administered antigens can also induce the production of systemic IgG when they reach MLN and/or the spleen, presumably via dendritic cell (DC)- or B lymphocyte-mediated mechanisms [18].

EHEC is a food-borne pathogen, and the infection is established by actively colonizing the luminal side of the gastrointestinal epithelia. Despite the comprehensive information describing molecular basis for EHEC adherence to intestinal epithelial cells [19], very little is known about how the host resolves the infection [20] and how these infections impact on immune system to generate resistance. The absence of a small animal model to study this human-specific pathogen has contributed to the scarce knowledge on the interplay between EHEC and host mucosal immunity. Making use of the mouse model of EHEC infection at weaning recently developed in our laboratory [21], this study aimed to analyze the local and systemic immune response elicited by EHEC and their protective potential.

To do this, we orally administered a human-isolated EHEC into weaned BALB/c mice and identified lymphocyte activation in the gut-associated lymphoid tissue (GALT), i.e., PP and MLN. We also found fecal anti-EHEC IgA and serum anti-Stx2 IgG in EHEC-inoculated mice. In addition, these mice were partially protected when they were challenged intravenously with a lethal dose of recombinant Stx2 (rStx2). The knowledge of the mechanism underlying the induction of the immune response would contribute to a better understanding of the physiological protection induced during EHEC infections.

Materials and methods

Bacterial strain and growth

The enterohaemorragic *Escherichia coli* O157:H7 Stx2-producing (referred as EHEC) was isolated from a fecal specimen of a child with HUS (125/99) and used in oral gavage. Bacterial cultures and characterization were performed as previously described [21]. Briefly, strains were cultured overnight at 37 °C in tryptic soy broth (TSB) (Difco, Le Point de Claix, France). A 250 µl volume was inoculated into five Erlenmeyer flasks (125 ml) containing 25 ml TSB and incubated at 37 °C for 18 h. Cultures were centrifuged, and bacterial pellets were washed twice in phosphate-buffered saline (PBS) (pH 7.2; 0.01 M) and then resuspended in 1 ml of PBS. Aliquots were diluted (10^2–10^4), plated onto plate count agar and incubated overnight at 37 °C. Overnight cultures reached a final concentration of 1×10^{10}–1.8×10^{10} c.f.u. ml^{-1}. The strain was maintained at −70 °C in TSB supplemented with 20 % glycerol.

Mice and oral infection

Immature male and female BALB/c mice were used immediately after weaning (17–20 days of age, ∼8–11 g of body weight). BALB/c mice were bred in the animal facility at the Institute of Experimental Medicine (IMEX), Academia Nacional de Medicina, Buenos Aires. Experiments performed herein were approved by the IMEX Animal Care Committee in accordance with the principles set forth in the Guide for the Care and Use of Laboratory Animals (National Institute of Health, 1985). After 8 h of starvation [22, 23], weaned mice were divided randomly into experimental groups: EHEC-inoculated mice, which were intragastrically inoculated via a stainless steel canulae (model 7.7.1, 0.38 mm × 22G) (Harvard Apparatus, Holliston, MA) with a single oral dose of 0.1 ml of bacterial suspension ($2–6 \times 10^9$ UFC/kg), and PBS-inoculated mice, which were inoculated with PBS. Food and water were provided to mice ad libitum 4 h after inoculation. EHEC-inoculated mice showed systemic symptoms related to Stx toxicity, i.e., intestinal damage, renal disease, neutrophilia, leukopenia and death in 50 % of infected mice as previously demonstrated [21].

Antibodies

The following phycoerythrin (PE), fluorescein isothiocyanate (FITC) or phycoerythrin-cyanine 5 (PE-Cy5)-conjugated monoclonal antibodies (mAbs) were used: anti-CD3 (clone DaA3), anti-CD19 (clone PeCa1), anti-CD69 (clone H1.2F3), anti-CD62L (clone MEL-14) (Immunotools, Friesoythe, Alemania), anti-CD45R (B220) (clone RA3-6B2), anti-IgA (clone M18-254) (BD-Pharmingen, San Diego, CA).

Lymphocyte isolation

Control and infected mice were killed at 12, 24, 48 h and 7 days after inoculation, and their PP and MLN were

dissected. Single-cell suspensions were prepared by homogenization through a stainless steel mesh in RPMI 1640 medium (Thermo Scientific HyClone, UT, EE UU) supplemented with 5 % fetal bovine serum (FBS) (Natocor, Argentina). After washing with RPMI containing 5 % FBS once, 100 µl of each suspension containing 500,000 cells was incubated with 0.5 µg of specific mAbs for 30 min at 4 °C and resuspended in 0.2 ml PBS containing 0.5 % paraformaldehyde. Lymphocytes were identified and gated according to their forward and side scatter properties by using a FACScan cytometer (Becton–Dickinson, Mountain View, CA). The mean fluorescence intensity (MFI) and/or the percentage of positive cells were determined. Percentage of lymphocytes was converted into absolute number according to the lymphocyte count. The analysis was made on 50,000 events on each sample by using WinMDI 2.8 program.

Recombinant Stx2

Recombinant Stx2 was prepared as previously described [5]. Briefly, the culture of recombinant *E. coli* strain JM109 (pStx2) was obtained by overnight incubation in Luria–Bertani (LB) broth (Difco Laboratories) supplemented with 100 µg/ml ampicillin (Sigma-Aldrich) with shaking at 200 rpm at 37 °C. Bacterial cells were centrifuged, and the resultant pellet was resuspended in PBS containing 1 mM phenylmethanesulfonyl fluoride (PMSF) proteases inhibitor (Gybco) (PBS-PMSF) and lysed by sonication. After centrifugation (14,000 rpm, 20 min 4 °C), the supernatant was precipitated with ammonium sulfate solution (75 %). The pellet was resuspended in PBS-PMSF, dialyzed against PBS overnight at 4 °C and stored at −20 °C until its usage.

Intradermal injection of rStx2

Purified rStx2 was diluted to 11 µg/ml and preincubated with 5 ng/ml polymyxin B (Sigma-Aldrich). Fifty microliters of this solution was injected intradermally into the rear foot-pad of adult Balb/c mice. Contralateral foot-pad was injected with 50 µl of PBS containing 5 ng/ml polymyxin B as a control. Draining popliteal lymph nodes (PLNs) were harvested at 24, 48 or 72 h after injection, and leukocytes populations were analyzed by flow cytometry as described above.

Lymphocyte trafficking

Peripheral lymph nodes were isolated from adult mice and homogenized by using stainless steel mesh and scissors in RPMI medium supplemented with 3 % bovine seroalbumin (BSA) (Gibco, NY). Cells were counted, diluted up to 1×10^7cells/ml and incubated with 10 µM 5,6-carboxy-fluorescein succinimidyl ester (CFSE) (Molecular probes, Eugene, OR) for 20 min at 37 °C. After washing them thoroughly with PBS containing 10 % FBS, 2×10^7 cells/mouse was injected intravenously 2 h before intragastric bacterial inoculation. Mice were killed 12 h after gavage, and PP, MLN, spleen and popliteal nodes were isolated and processed for flow cytometry analysis as described before.

Fecal extracts

Fifteen days after gavage, the whole intestine from each mouse was recovered and feces were weight and diluted to 1 g/ml with PBS-PMSF. After vigorous homogenization with vortex, feces were incubated for 1 h on ice and centrifuged (4,000 rpm for 30 min at 4 °C). Supernatants were stored in triplicates at −20 °C until IgA determination by ELISA.

Analysis of antibody responses in fecal extracts

Determination of lgA levels on fecal extracts was performed as previously described [24, 25]. Briefly, 96-well polystyrene microtiter plates (GBO, Germany) were coated with a formalin-killed whole-cell suspension diluted to an OD_{600} of 0.05 with 15 mM carbonate, 25 mM bicarbonate (pH 9.6). Intact EHEC cells were prepared as follows. Bacteria were cultured as described above, washed twice with PBS by centrifugation, suspended in PBS containing 0.5 % neutralized formalin, stored at room temperature for 3 days and then washed three times with PBS to remove free Stx. Plates were incubated overnight at 4 °C. After incubation, wells were washed three times with PBS containing 0.01 % Tween 20 (PBS-T). Wells were sequentially incubated with samples (1/4 dilution), 1/3,000 goat anti-mouse lgA coupled to horseradish peroxidase (HRP) (Chemicon International, Inc.) and finally 1 mg/ml O-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, St. Louis, MO) and 30 % H_2O_2 for 20 min at room temperature in darkness. The reaction was terminated by the addition of 50 ml of 4 N sulfuric acid, and the optical density values at 492 nm (OD_{492}) were measured in a microplate ELISA reader. Wells were washed with PBS-T three times between incubations and blocked for non-specific adherence with PBS-T supplemented with 1 % BSA (Sigma-Aldrich) at 37 °C for 1 h before the addition of the samples. Results were expressed as OD_{492} units per gram of feces. In order to determine the cutoff value for the ELISA test, we calculated the mean value + 2SD from PBS-inoculated mice (mean = 0.37, 2SD = 1.69, $n = 13$). Thus, $OD_{492} = 2.06$ was considered as the cutoff for the ELISA test, and absorbance values below this OD were considered as negative.

Western blot assay to detect anti-Stx2 IgG

This assay was performed as previously described [5] with some changes. Briefly, a standard concentration of purified Stx2 (80 µg) was resolved into its A (Stx2A) and B (Stx2B) subunits by SDS-PAGE, by using 6 % stacking and 17.5 % separating gels and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA) for 2 h at 300 mA. After blocking for 1 h in PBS containing 3 % skim milk, each membrane was cut into longitudinal strips. Strips were incubated overnight at 4 °C in plasma specimens diluted 1:100 in PBS containing 3 % skim milk. After washing thoroughly, strips were incubated for 1 h at 37 °C with 1:3,000 goat anti-mouse IgG (H + L) HRP conjugate (BioRad) in PBS containing 3 % skim milk. After washing, strips were developed using a chemiluminescent detection system (ECL; Amersham Pharmacia Biotech, United Kingdom). The strips were exposed to a Kodak AR film (Kodak, Rochester, NY) for 10 min, and the film was then developed. Each plasma sample was tested at least twice. The results of the WB were read in a blinded fashion.

Statistics

Data expressed as the mean ± SEM of individual mice were analyzed for statistical differences by using Student's *t* test and one- or two-way analysis of variance (ANOVA). Comparisons a posteriori between two groups were performed using the Student–Newman–Keuls (SNK) test. Survival curves were compared by using log-rank test. $p \leq 0.05$ was considered significant.

Results

Quantitative changes in lymphocyte subpopulations in the GALT after EHEC infection

GALT plays important functions in controlling intestinal infections. Thus, we studied PP and MLN as inductive tissues at several times post-EHEC infection. Mice were humanely euthanized, and MLN and PP from small intestine were homogenized to quantify T and B lymphocytes by flow cytometry. EHEC-inoculated mice had significantly lower absolute number of B lymphocytes than PBS-inoculated mice (control) in PP, as soon as 12 and 24 h post-EHEC oral delivery (Fig. 1a). Since the number of T lymphocytes did not show significant changes, the relative number of B lymphocytes was also decreased (Fig. 1b). Simultaneously, lymphocytes were measured in MLNs. The absolute and relative number of B lymphocytes were increased in EHEC-inoculated mice compared to control mice at 12 h post-infection (Fig. 2a, b), whereas 7 days after infection the absolute number of B lymphocytes was reduced. On the other hand, the number of T lymphocytes did not show significant changes during the experiment.

Stimulation of B and T lymphocytes by purified r Stx2 in vivo

It was previously suggested that Stx2 had a direct toxic effect in B lymphocytes from germinal center [26]. Therefore, purified rStx2 or PBS was inoculated in the rear foot-pad of adult BALB/c mice, and draining PLNs was analyzed at different times [12]. In order to block LPS-contaminating traces in rStx2 preparation, it was preincubated with polimixin B as described in Materials and Methods [27]. As shown in Fig. 3a, b, the absolute number of B and T lymphocytes was significantly increased since

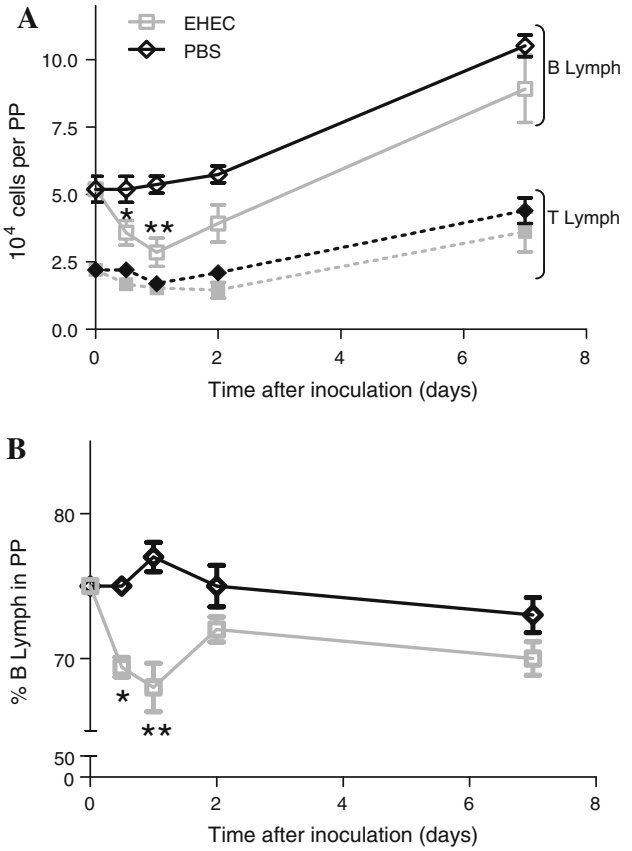


Fig. 1 Absolute and relative number of lymphocytes in PP from experimental mice. BALB/c mice were orally inoculated with EHEC (*grey squares*) or PBS (*black diamonds*) as described in Materials and Methods. PP were isolated at indicated times and labeled with FITC anti-mouse CD3 and PE anti-CD19. The absolute number (**a**) and percentage (**b**) of lymphocytes per PP were determined using a Becton–Dickinson FACScan. Each point represents the mean ± SEM of 5–13 mice. $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with PBS, Student's *t* test

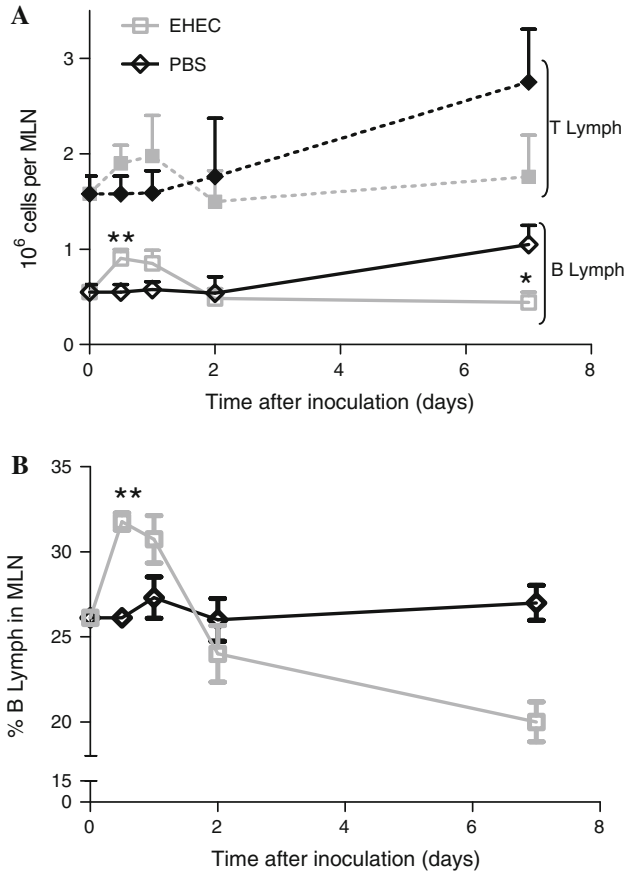


Fig. 2 Absolute and relative number of lymphocytes in MLN from experimental mice. BALB/c mice were orally inoculated with EHEC (*grey squares*) or PBS (*black diamonds*) as described in Materials and Methods. MLN were isolated at indicated times and labeled with FITC anti-mouse CD3 and PE anti-CD19. The total absolute number (**a**) and percentage (**b**) of lymphocytes were determined using a Becton–Dickinson FACScan. Each point represents the mean ± SEM of 5–13 mice. $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with PBS, Student's *t* test

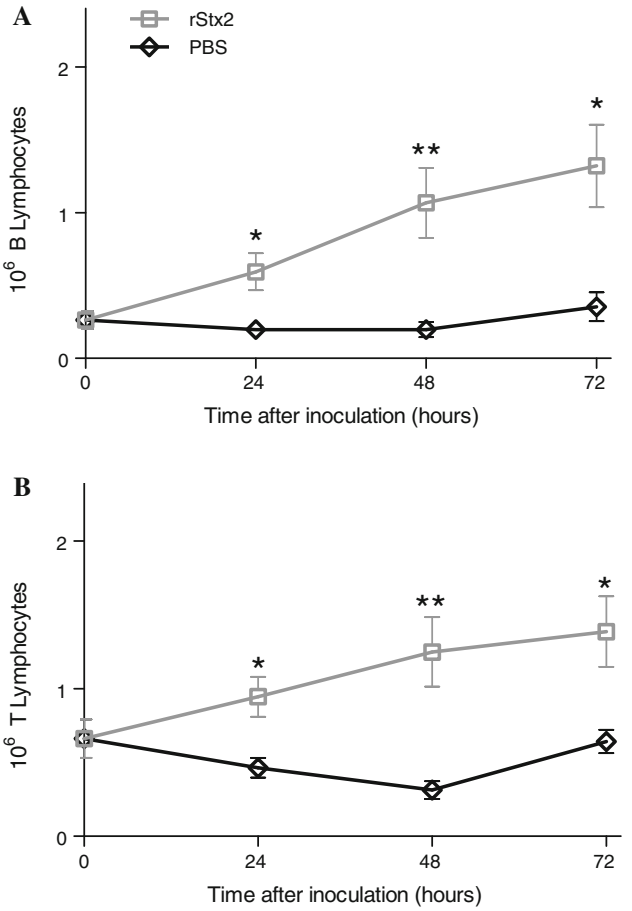


Fig. 3 Assessment of the rStx2 toxic effect on lymphocytes in vivo. BALB/c mice were intradermally inoculated on the rear foot-pad with PBS (*black diamonds*) or rStx2 (*grey squares*) preincubated with 5 ng/ml polymyxin B. Draining PLN was isolated and analyzed by flow cytometry. The absolute number of B (**a**) and T lymphocytes (**b**) was determined using a Becton–Dickinson FACScan. Each point represents the mean ± SEM of 5–10 mice. $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with PBS, Student's *t* test

24 h after rStx2 injection. These results allowed us to rule out a toxic effect of rStx2 on B lymphocytes in vivo.

Activation and trafficking of lymphocytes in PP and MLN after EHEC infection

In order to study lymphocyte activation, we studied the expression of CD69 and CD62L antigens by flow cytometry. Whereas lymphocyte activation increases the expression of CD69 by the transcription factor AP-1 [28], it reduces the expression of CD62L by cleavage [29]. Figure 4a, b shows that EHEC-inoculated mice had an increased percentage of CD69+ B and T lymphocytes in PP at 12 h post-infection compared to control mice. In contrast, no changes in the percentage of CD69+ lymphocytes were observed in MLN at the same time (data not shown).

On the other hand, signs of activation were found in MLN but not in PP from EHEC-inoculated mice at 24 h post-infection. In fact, both T and B lymphocytes presented a reduced CD62L expression compared to controls (Fig. 5a, b). We found similar results when lower bacterium inocula (1–5 × 10^7 UFC/kg) were tested (data not shown).

To study whether B lymphocytes activated in PP during EHEC infection traffic to MLN, we systemically transferred allogeneic CFSE-stained lymphocytes into PBS- or EHEC-inoculated mice as described in Materials and Methods [30]. The percentage of CFSE-B lymphocytes in MLN was slightly but significantly higher in EHEC-inoculated mice than in control mice at 12 h after infection (EHEC: 15.0 ± 1.0 %, $n = 4$; control: 12.2 ± 0.5 %, $n = 4$, $p < 0.05$, Student's *t* test), whereas the percentage of CFSE-T lymphocytes was similar in both experimental

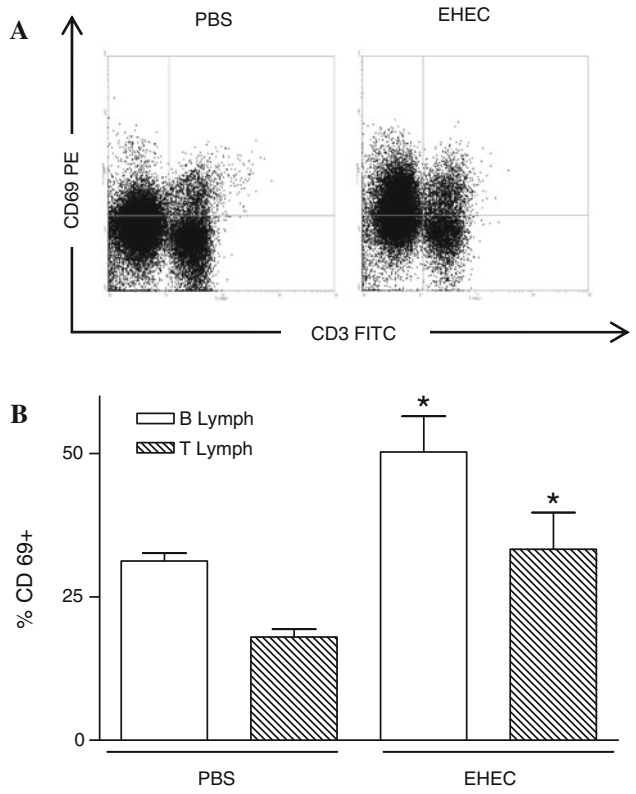


Fig. 4 CD69 expression in lymphocytes from PP at 12 h after EHEC infection. BALB/c mice were orally inoculated with EHEC or PBS as described in Materials and Methods, and PP were isolated and analyzed by flow cytometry. **a** Representative dot plot of CD69 and CD3 surface markers on PP lymphocytes from each experimental group. **b** Mean ± SEM percentage of CD3+ CD69+ (*shaded bars*) and CD3-CD69+ (*open bars*) lymphocytes ($n = 3$). Percentages were calculated within the respective CD3+ or CD3- subpopulation of lymphocytes. Similar results were obtained in two independent experiments. *$p < 0.05$ compared with PBS, Student's *t* test

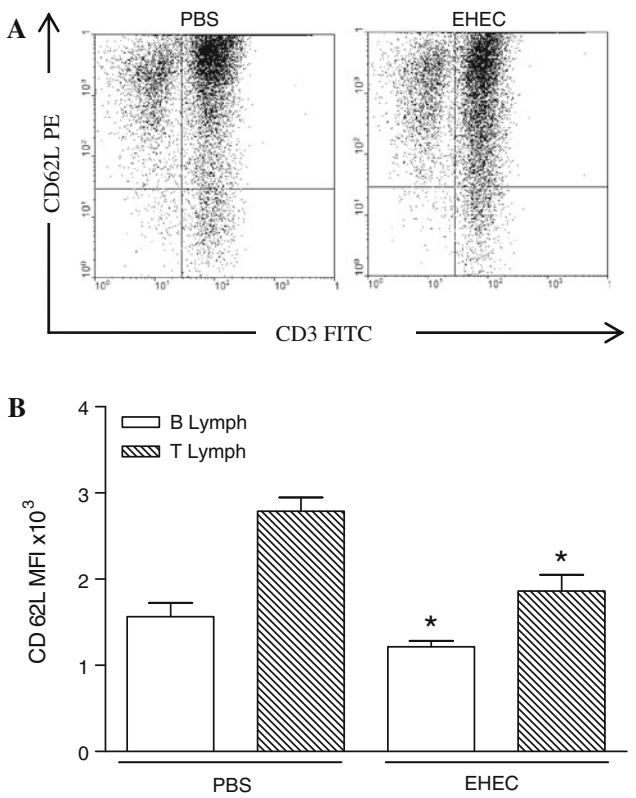


Fig. 5 CD62L expression in lymphocytes from MLN at 24 h after EHEC infection. BALB/c mice were orally inoculated with EHEC or PBS as described in Materials and Methods, and MLN were isolated and analyzed by flow cytometry. **a** Representative dot plot of CD62L and CD3 surface markers on MLN lymphocytes from each experimental group. **b** Mean ± SEM MFI of CD3+ CD62L+ (*shaded bars*) and CD3-CD62L+ (*open bars*) lymphocytes ($n = 3$). Similar results were obtained in two independent experiments. *$p < 0.05$ compared with PBS, Student's *t* test

groups (data not shown). In contrast, there were not differences in the percentage of B or T lymphocytes adoptively transferred in PP, inguinal lymph nodes or spleen from all mice (data not shown).

Induction of a local and systemic specific antibody response

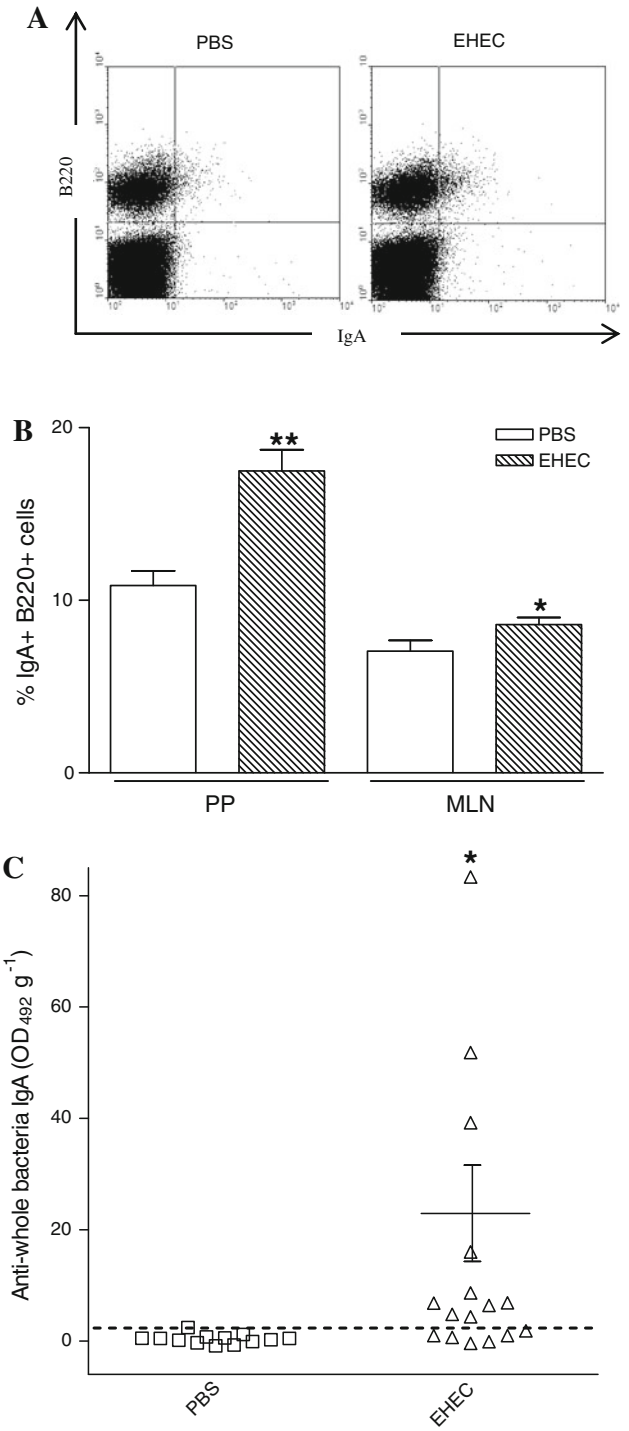
Because class switching to IgA typically occurs in PP, we analyzed by FACS whether oral infection induces changes in the IgA+ B cell subset in PP and MLN at 15 days [31]. We found that the relative number of IgA+ B220+ cells was increased in PP and MLN of EHEC-infected mice (Fig. 6b).

To determine the specificity of the secretory IgA, fecal extracts were assayed by an ELISA against the whole EHEC. Figure 6c shows that EHEC-infected mice secreted IgA antibodies that specifically recognize antigens expressed on the membrane of the bacteria used in the oral infection.

Severe complications of EHEC infections, such as HUS, derive from systemic effects of Stx2. Therefore, the raise of a systemic anti-Stx2 antibody response is very important in terms of protection. In order to evaluate systemic protection against Stx2, mice were challenged with an i.v. lethal dose of purified rStx2 (2.5 ng per mice) at 21 days after infection. EHEC-infected mice were partially protected against Stx2, thus suggesting the systemic induction of anti-Stx2 neutralizing antibodies (Fig. 7a). Besides, we found serum anti-Stx2B IgG antibodies in protected mice but not in controls by Western blot (Fig. 7b).

Discussion

Data presented herein demonstrate that oral administration of EHEC into weaned mice is an accessible and feasible animal model in which to study the early local immune response of the host upon intestinal colonization. Our results also highlight the impact of EHEC strains on local



◀**Fig. 6** Induction of local IgA after oral EHEC infection, BALB/c mice were orally inoculated with EHEC (*shaded bars*) or PBS (*open bars*) as described in Materials and Methods. Fifteen days later, PP and MLN were isolated and labeled with PE-Cy5 anti-B220 and FITC anti-IgA. Feces from the whole intestine were removed and further processed to analyze the IgA specificity as described in Materials and Methods. **a** Representative dot plot from MLN lymphocytes gated according to their forward and light scattering properties. **b** Mean ± SEM percentage of IgA+ B220+ lymphocytes ($n = 10$–16). Percentages were calculated within B220+ lymphocytes. $^{**}p < 0.001$ and $^{*}p < 0.05$ compared with PBS, Student's *t* test. **c** Detection of anti-EHEC IgA by ELISA in fecal extracts from PBS-inoculated (*square*) and EHEC-inoculated (*triangle*) mice. The OD_{492} per gram of feces is shown. *Symbols* represent individual mice ($n = 13$–16). *Broken line* represents the cutoff level, and *solid line* represents the mean ± SEM of positive values. $^{*}p < 0.05$ compared with PBS, Student's *t* test

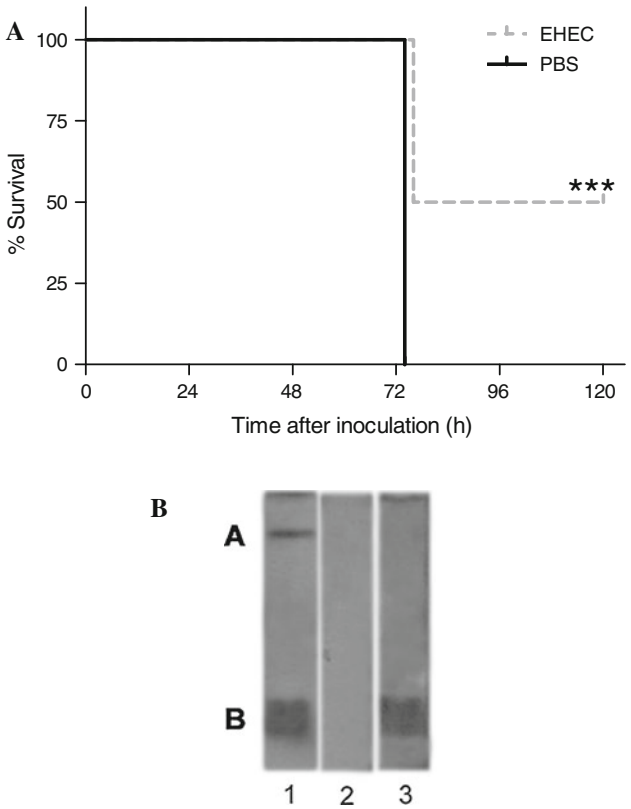


Fig. 7 Induction of systemic neutralizing antibodies in EHEC-infected mice at 21 days post-infection. **a** EHEC-inoculated (*dashed gray line*) or PBS-inoculated (*solid black line*) mice ($n = 6$–7) were challenged with a lethal dose of rStx2, and survival was monitored daily as indicated in Materials and Methods. $^{***}p < 0.001$, log-rank test compared with PBS. **b** Representative immunoblot strips from PBS- and EHEC-inoculated mice. Plasma samples were obtained by puncture of the retroorbital plexus at 21 days post-infection, before challenge with rStx2. *Lane 1* shows a mouse polyclonal plasma reactive against Stx2 A and B subunits. EHEC-inoculated mouse (*lane 3*) shows anti-Stx2B subunit antibodies, whereas PBS-inoculated mouse shows a complete absence of binding signal (*lane 2*)

and systemic adaptive immune response, which would be relevant in case of subsequent infections by or exposition to those strains.

It is well established that absorption of mucosally administered antigens, especially particulate ones, occurs via M cells that overly PP [32]. Our results showed that T and B lymphocytes of PP from EHEC-infected mice have

an increased expression of CD69+, thus indicating lymphocyte activation since 12 h after infection. Simultaneously, these mice showed an absolute and relative decrease in B lymphocytes in PP, which was maximal at 24 h after infection. In this regard, it has been previously reported that Stx2 has toxic effects on B lymphocytes, such as Burkitt's lymphoma cell lines [33, 34] and centroblasts, which could prevent an efficient antibody immune response against Stx [26]. However, we did not find direct toxicity of Stx2 on B lymphocytes in vivo. In agreement with these results, Imai et al. [35] showed that B lymphocytes from mouse germinal center do not express binding sites for Stx. In addition, the impairment of the specific antibody response was questioned by epidemiological evidence on the presence of anti-Stx antibodies, not only in convalescent HUS patients but also in their close relatives and healthy children of endemic areas [5–7].

Besides, our results show parameters of activation in MLN lymphocytes from EHEC-inoculated mice, together with an absolute and relative increase in B lymphocytes in this tissue at 24 h post-infection, thus suggesting trafficking and/or clonal expansion. In this regard, the adoptive transfer of CFSE-labeled lymphocytes yielded an increased percentage of CFSE-labeled B lymphocytes only in MLN from EHEC-infected mice. Since it has been reported that CFSE-labeled B lymphocytes are not only less capable of entering lymph nodes but may be even more prone to cell death upon transfer [30], this slight but significant increase suggests that this migratory population gives rise during the anti-EHEC immune response in MLN. The selective intestinal migration of B lymphocytes induced in secondary lymphoid organs during a primary immune response has been well documented [36]. Changes in adhesion molecules or chemokine receptors in activated B lymphocytes, in addition to changes in the profile of chemokine secretion by MLN, would explain that migration [37, 38]. However, we cannot rule out that the clonal expansion of reactive B lymphocytes could have contributed to the observed increase. Additional studies are needed to investigate the role of these factors in our model.

In agreement with the temporal evolution of immune responses, EHEC-inoculated mice showed a significant increase in the percentage of IgA-bearing B lymphocytes in PP and MLN 15 days after EHEC infection. PP have three important features in IgA switching. First, PP include germinal centers that promote the interaction between antigen-specific T and B lymphocytes as well as the expression of activation-induced cytidine deaminase, a B cell-specific enzyme required for the diversification of Ig genes through class-switch DNA recombination and somatic hypermutation [39]. Second, they contain a higher proportion of B versus T lymphocytes (four to six more times) compared to peripheral lymph nodes [40]. Third, PP are rich in cytokines with IgA-inducing functions, including transforming growth factor β (TGF-β) [11, 32], interleukin-4 (IL-4), IL-6 and IL-10, which facilitate the expansion of IgA-expressing B lymphocytes and their differentiation to IgA-secreting plasma cells [41]. Besides, DCs from PP may utilize retinoic acid, IL-6 and inducible nitric oxide synthase (iNOS) to enhance intestinal IgA responses [42].

Appropriate lymphocyte activation leads to the terminal differentiation of B lymphocytes to plasmatic cells. Due to the presence of fecal anti-EHEC IgA antibodies, it is possible to conclude that oral administration of EHEC leads to the effective differentiation of IgA class-switched B lymphocytes into IgA-secreting plasma cells. In this regard, Itoh et al. [43] have reported that EHEC-infected children exhibit specific anti-bacterium IgA in feces. It is known that adaptive immune responses, which include the emergence of specific antibodies, arise to eliminate pathogens and prevent or reduce the severity of reinfections. Indeed, it has been suggested that an increase in total IgA in intestinal secretion could prevent adherence and colonization of pathogen such as EHEC [31]. On the other hand, there is some evidence that the immune response to EHEC colonization factors, acquired after a first infection, may protect against diarrhea in a subsequent infection [44]. In agreement with this, it was previously demonstrated that the shedding of *Escherichia coli* O157:H7 in calves is reduced by prior colonization with the homologous strain [45]. Since adult BALB/c mice are naturally resistant to EHEC infection, we could not evaluate if this mucosal response is able to counteract a subsequent intestinal infection. However, we demonstrated that EHEC-infected mice were partially protected against an intravenous challenge with Stx2, thus suggesting serum anti-Stx antibodies with neutralizing activity. In agreement with this result, epidemiological evidence supports the importance of a specific systemic immune response in HUS patients in case of reinfection. Besides, an increased frequency of anti-Stx antibodies has been reported in higher age population which is in general refractory to HUS [7]. In addition, anti-Stx2 seroreactivity has been correlated with the absence of symptoms in family outbreaks of STEC infection [46, 47], thus suggesting that a specific and protective immune response is also triggered by nonpathogenic doses of STEC. This epidemiological evidence is in line with our results showing that EHEC-inoculated mice have signs of GALT activation even when non-lethal doses were administered.

Data presented herein are the first experimental approach demonstrating that only one episode of EHEC infection, which emulates natural infection, is enough to induce GALT activation, especially the B cell compartment, and lead to the production of specific IgA in mucosal

tissue and the generation of a systemic protection against Stx2. These data also support the epidemiologic observation that a second episode of typical HUS is very rare.

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