



Leukotriene C4 increases the susceptibility of adult mice to Shiga toxin-producing *Escherichia coli* infection



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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) is a food-borne pathogen that causes hemorrhagic colitis. Under some circumstances, Shiga toxin (Stx) produced within the intestinal tract enters the bloodstream, leading to systemic complications that may cause the potentially fatal hemolytic-uremic syndrome (HUS). Despite STEC human infection is characterized by acute inflammation of the colonic mucosa, little is known regarding the role of proinflammatory mediators like cysteine leukotrienes (cysLTs) in this pathology. Thus, the aim of this work was to analyze whether leukotriene C4 (LTC4) influences STEC pathogenesis in mice. We report that exogenous LTC4 pretreatment severely affected the outcome of STEC gastrointestinal infection. LTC4-pretreated (LTC4+) and STEC-infected (STEC+) mice showed an increased intestinal damage by histological studies, and a decreased survival compared to LTC4-non-pretreated (LTC4–) and STEC+ mice. LTC4+/STEC+ mice that died after the infection displayed neutrophilia and high urea levels, indicating that the cause of death was related to Stx2-toxicity. Despite the differences observed in the survival between LTC4+ and LTC4– mice after STEC infection, both groups showed the same survival after Stx2-intravenous inoculation. In addition, LTC4 pretreatment increased the permeability of mucosal intestinal barrier, as assessed by FITC-dextran absorption experiments. Altogether these results suggest that LTC4 detrimental effect on STEC infection is related to the increased passage of pathogenic factors to the bloodstream.

Finally, we showed that STEC infection *per se* increases the endogenous LTC4 levels in the gut, suggesting that this inflammatory mediator plays a role in the pathogenicity of STEC infection in mice, mainly by disrupting the mucosal epithelial barrier.

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1. Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains are important food-borne pathogens, being the major etiological agents of hemorrhagic colitis and typical hemolytic uremic

syndrome (HUS), a life-threatening disease characterized by hemolytic anemia, thrombocytopenia and renal failure (Karmali, 1989).

Although STEC is not invasive and is restricted to the lumen of the gut (Paton and Paton, 1998) in some circumstances, Stx produced within the intestinal tract crosses the epithelial barrier and enters the bloodstream (Acheson et al., 1996; Schuller, 2011). Stx targets its receptor (globotriaosylceramide, Gb3) in the endothelium and other susceptible tissues, resulting in systemic dysfunction, being the kidney and the central nervous system the main organs affected (Ochoa and Cleary, 2003; Palermo et al., 2009). Approximately, 10–15% of STEC infected children evolve to HUS, the most severe systemic complication (Rivas et al., 2006). Several factors from both, the bacteria and the host, contribute to a

Abbreviations: STEC, Shiga toxin-producing *Escherichia coli*; Stx, Shiga toxin; HUS, hemolytic-uremic syndrome; LTs, leukotrienes; LTC4, leukotriene C4.

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poor outcome. Some of them act improving the colonization on the intestine, and others act enhancing Stx toxicity systemically (Ibarra et al., 2013).

The pathogenic mechanism of Stx, as well as the host factors that modulate Stx systemic toxicity have been extensively studied and well delineated (Melton-Celsa et al., 2012). However, the host factors that contribute to or counteract the intestinal dysfunction mediated by STEC infection are much less understood. In part, this is due to the lack of a mouse model of intestinal infection by STEC that mimics the acute colon inflammatory process that occurs in humans. It is a serious handicap to STEC research because it limits basic studies on molecular pathogenesis, and studies aimed at understanding the mechanisms of local innate and adaptive immune responses. Many investigators have focused on identifying agents present in the intestinal environment that might contribute to Stx production by bacterial strains by working in the infant rabbit model of STEC infection or with *in vitro* systems by using intestinal cellular lines or human intestinal primary cultures (Golan et al., 2011; Pacheco and Sperandio, 2012). In this regard, it has been demonstrated that neutrophils and H₂O₂ increase phage induction and Stx production (Los et al., 2010; Wagner et al., 2001) while nitric oxide (NO) inhibits the levels of the phage particle and the synthesis of Stx by STEC (Vareille et al., 2007). In addition, many bacteria, including STEC, share the ability to sense epi/nor-epinephrine via an adrenergic sensor (Sperandio et al., 2003), thus leading to virulence regulation (Clarke et al., 2006). On the other hand, STEC adheres to the apical membrane of colonic epithelial cells and, although it is not invasive for them, several pathogenic bacterial factors trigger an acute inflammation in the intestine (Ibarra and Palermo, 2010). STEC produce several proteins that are candidates for signaling the upregulated production of epithelial chemokines. These include the surface protein intimin (Yu and Kaper, 1992) but also Stx (Thorpe et al., 1999, 2001; Yamasaki et al., 1999) and H7 flagellin (Berin et al., 2002), which stimulates interleukin-8 production in T84 cells (Miyamoto et al., 2006; Zhou et al., 2003). Hemolysin (Hly), the pore-forming cytolysin produced by both eae-positive and eae-negative STEC (May et al., 2000; Taneike et al., 2002) could contribute to disease through lysis of erythrocytes and release of hemoglobin as a potential source of iron for the bacteria. But also, it has been demonstrated that Hly induces a biphasic response in intestinal mast cells (MC), leading to the release of cytokines and inflammatory mediators, including leukotrienes (LTs) (Kramer et al., 2008). Although it remains unknown how this inflammation affects the outcome of STEC infections, it is reasonable to speculate that factors that may affect the mucosal integrity impact negatively on the course of infection, since integrity of the mucosal barrier plays a critical role in avoiding Stx systemic dissemination.

Despite LTs play an important role in mucosal integrity during colitis from different etiologies (Groschwitz et al., 2009; Wallace and Ma, 2001), very little is known about the involvement of these mediators in STEC infection and HUS pathogenesis.

The rate-limiting step in LT synthesis from arachidonic acid is the enzyme 5-lipoxygenase (5-LOX) in association with membrane-bound 5-lipoxygenase-activating protein. LTA₄, an unstable precursor of all LTs, is quickly metabolized to one of the two different classes of LTs, LTB₄ (by LTA₄ hydrolase) or LTC₄ (by LTC₄ synthase) and its metabolites (LTD₄ and LTE₄) (Peters-Golden and Henderson, 2007; Vannella et al., 2007). Collectively, LTC₄, LTD₄, and LTE₄ were previously known as the slow-reacting substance of anaphylaxis and are currently termed the cysteinyl LTs (cysLTs) (Peters-Golden and Henderson, 2007).

The receptors for LTB₄ (BTL1 and BTL2) and cysLTs (CysLT1 and CysLT2) (Matuk et al., 2004) are cell surface G protein-coupled receptors (Bensinger and Tontonoz, 2008). The expression of these

receptors differs between cell types, but they are mainly expressed in immune cells (Neves et al., 2010; Rogerio and Anibal, 2012). Endothelial expression and activation of these receptors is linked to vasoactive responses and to the promotion of vascular permeability (Barajas-Espinosa et al., 2011).

LTB₄ is involved in leukocyte chemotaxis, lysosomal enzyme secretion, neutrophil degranulation, induction of the expression of adhesion molecules, defensins and production of nitric oxide (NO), phagocytosis, and other functions (Peters-Golden et al., 2005). It has been reported that mucosal LTB₄ levels increase during enterohemorrhagic *Escherichia coli* (EHEC) infection in a model of ten-day-old rabbits. However, a correlation between LTB₄ and colonic dysfunction could not be established, and a complex role of eicosanoids during EHEC infection has been suggested (Bell et al., 2000).

On the other hand, LTs can increase the permeability of the vascular endothelium (Groschwitz et al., 2009), play important roles in innate and adaptive immune responses, and are involved in several inflammatory and infectious diseases (Peters-Golden and Henderson, 2007). CysLTs induce edema and chemotaxis of eosinophils and neutrophils to the bronchial mucosa in asthma (Laitinen et al., 1993) and patients with prolonged inflammatory diseases that affect intestinal tissues, such as inflammatory bowel diseases, exhibit increased levels of cysLTs (Stenson, 1990). In addition, LTs are produced during the interaction of phagocytes and microorganisms *in vitro*, but also during experimental infections *in vivo* (Peters-Golden et al., 2005). Of interest, mast cells and eosinophils are a major source of cysLTs in the intestinal mucosa and, the most important, they affect mucosal permeability (Wallace and Ma, 2001).

Since there are no reports concerning the role of cysLTs in STEC infections, the aim of this work was to analyze whether LTC₄ influences STEC pathogenesis and HUS development in mice.

2. Materials and methods

2.1. Mice

BALB/c mice were maintained in the animal facility of the IMEX-CONICET, Academia Nacional de Medicina, Buenos Aires, Argentina. Mice were housed in standard polypropylene transparent cages under environmentally controlled conditions (Temperature, 24 ± 2 °C; Humidity, 50 ± 10%) with a 12 h light:12 h dark cycle.

Experiments performed herein were approved by the local Experimental Care Committee at IMEX-CONICET in accordance with the principles set forth in the Guide for the Care and Use of Laboratory Animals (National Research Council, National Research Council (U.S.), Committee for the Update of the Guide for the Care and Use of Laboratory Animals, et al., 2011) (protocol number 1016). Health and behavior of mice were assessed three times a day. Any unnecessary pain, discomfort or injury to animals was avoided. Any mice becoming moribund were humanely euthanized by CO₂ inhalation in a closed chamber to minimize animal suffering. Institutional Animal Care & Use Committee (IACUC) guidelines were used to define humane endpoints.

2.2. LTC₄ administration

Mice were orally inoculated with two doses of 200 μL of exogenous LTC₄ (10⁻⁸ M) (Calbiochem, Argentina) directly into the stomach via a stainless steel canula (model 7.7.1, 0.38 mm outside diameter, 22 gauge; Harvard Apparatus) at seven and three days before the inoculation of bacteria or PBS.

2.3. Bacterial strain

The bacterial strain STEC O157:H7 (125/99) used in this study was isolated from fecal specimens of a patient with HUS. The strain belonged to seropathotype and clade 8 (Amigo et al., 2015). This STEC strain harbored the *eae* and *ehxA* genes (Brando et al., 2008). The strain 125/99 has the Stx2 phage inserted in *yehV* site (Amigo et al., 2015), a high Stx2 activity measured on Vero cells *in vitro* ($17.0 \pm 6.2 \times 10^{11}$ CD50/mL) and a high toxicity *in vivo* (Brando et al., 2008).

2.4. STEC infection

Bacterial suspensions of STEC O157:H7 were diluted to an appropriate concentration and delivered directly into the stomach of mice after 3 h of food starvation, by using the stainless steel cannula described above. Animals received a sub-lethal inoculum of 5×10^{11} to 7×10^{11} CFU of STEC/kg body weight. Control animals received the same volume of sterile PBS. After 4 h of bacterial ingestion, both food and water were provided *ad libitum*.

2.5. Stx2 intravenous inoculation

Stx2 was prepared as described previously (Bentancor et al., 2013). In order to analyze Stx2-dependent specific effects, different doses of Stx2 were injected *i.v.* into the retro-orbital plexus of isoflurane-anesthetized control mice. We selected a 50% lethal dose (LD50) of 80 ng/kg that induced 50% mortality in control mice between 72 and 96 h after injection. This dose was selected on the basis of the necessity to highlight the modulatory effect of LTC4 pretreatment on mortality rate. The same batch and dose of Stx2 was used for all experiments.

2.6. FITC-dextran assay

To assess the intestinal barrier function, the FITC-dextran (FITC-Dx) assay was performed as previously described with modifications (Gibson et al., 2008). Briefly, 0.15 mL of PBS containing 80 g/L of FITC-Dx (FD4; Sigma–Aldrich, Oakville, ON, Argentina) was administered by oral gavage after 18 h of one LTC4 dose (10^{-8} M). The fluorescence of FITC-dextran was measured both in the intestine and in serum after 4 h of treatment. Blood was collected from the retroorbital plexus under isoflurane anesthesia. The intestines were excised and cell lysates were prepared in a final volume of 0.5 mL of RPMI (0.25% sodium dodecyl sulfate), adjusted at pH 9.6 with 0.5 M of carbonate buffer. Cell lysates were centrifuged at 400 g and fluorescein fluorescence was measured in the supernatant (and serum) with a spectrofluorometer (SPF-500C; SLM/AMINCO, Urbana, IL) using an excitation wavelength of 495 nm and a detection wavelength of 514 nm.

2.7. Histology

Small and large intestines from euthanized mice of all experimental groups were excised and fixed in a 10% formal–PBS solution at 96 h post-infection. At least three mice per experimental group were studied. Sections were stained with hematoxylin and eosin (H&E), or Giemsa and examined by light microscopy. Histological examination was performed in a blinded fashion by the pathologist to analyze epithelial damage and mucosal or submucosal inflammatory cell infiltration, as previously reported (Brando et al., 2008; Cabrera et al., 2014).

Apoptosis was evaluated on H&E-stained sections. Fifteen high power fields (hpf) were counted of each section by using a 1000 \times magnification, and were expressed as the mean \pm standard

deviation of the apoptotic cell number per hpf. Morphological identification of apoptosis was performed according to criteria previously reported (Vanzulli et al., 2005). Selected samples were also reacted using the deoxynucleotidyl transferase-mediated dUTP–biotin nick end labeling (TUNEL) method according to the manufacturer's instructions (Boehringer Mannheim, UK) (Vanzulli et al., 2005).

2.8. Urea and polymorphonuclear neutrophils (PMNs)

Blood samples were obtained by puncture of the retro-orbital plexus under isoflurane anesthesia at 96 h post STEC or PBS inoculation. PMNs were counted in Neubauer chambers. To determine urea concentrations, the blood samples were centrifuged at $2000 \times g$ for 10 min, and the plasma was separated and stored at -20°C . Urea concentrations were measured using the Urea Colour Kit, according to the manufacturer's instructions (Wiener Lab, Rosario, Argentina).

2.9. Evaluation of LTC4 and LTB4 production in intestinal samples by ELISA

The whole intestines from mice were excised 96 h after STEC or PBS inoculation. The intestines were washed extensively with PBS and incubated at 37°C in PBS/ β -mercaptoethanol (0.1%). Then, the intestines were cut into small pieces and treated with a solution of type I collagenase (250 U/mL) for 30 min at 37°C . After the incubation time, the reaction was stopped using a solution of PBS supplemented with 5% fetal bovine serum (FBS). Subsequently, the intestine fragments were incubated with DNase I (50 U/mL) for 40 min at 37°C . Finally, intestinal suspensions were collected, washed in PBS and resuspended in a buffer solution containing 1×10^6 cells/mL. Cells were incubated overnight and supernatants were collected. The LTC4 and LTB4 concentrations in the supernatants were determined using the LTC4 or LTB4 ELISA Kit from R&D (limit of detection 10 pg/mL) according to the manufacturer's instructions (Cayman Chemical, Migliore-La Clastra SRL, Buenos Aires, Argentina).

2.10. Statistical analysis

Levels of significance were determined using ANOVA, two-tailed Student's *t* test or log-rank test, and a confidence level greater than 95% ($p < 0.05$) was used to establish significance.

3. Results

3.1. Intestinal changes by LTC4 pretreatment

Since LTC4 is considered an inflammatory mediator in the gut (Groschwitz et al., 2009; Wallace and Ma, 2001), we analyzed intestinal alterations induced by exogenous administration of LTC4, in noninfectious conditions and during STEC experimental infection. The small and large intestines from mice receiving LTC4 or PBS were histologically analyzed at 96 h post STEC infection. The small intestines from LTC4-pretreated mice showed Peyer's Patches (PP) with activated germinal centers even before STEC infection (Fig. 1A). After infection, PP from PBS or LTC4-pretreated STEC+ mice showed hyperplasia and also activation of germinal centers (Fig. 1A). Interestingly, there was a marked increase in the number of eosinophils in the small intestine of LTC4-pretreated mice, similar in both STEC– and STEC+ mice, as compared to PBS-pretreated mice (Fig. 1B and C). On the other hand, although the colon of PBS/STEC+ infected mice showed increased mucosal mononuclear leukocyte infiltration as compared to noninfected mice (PBS/STEC–), the colon

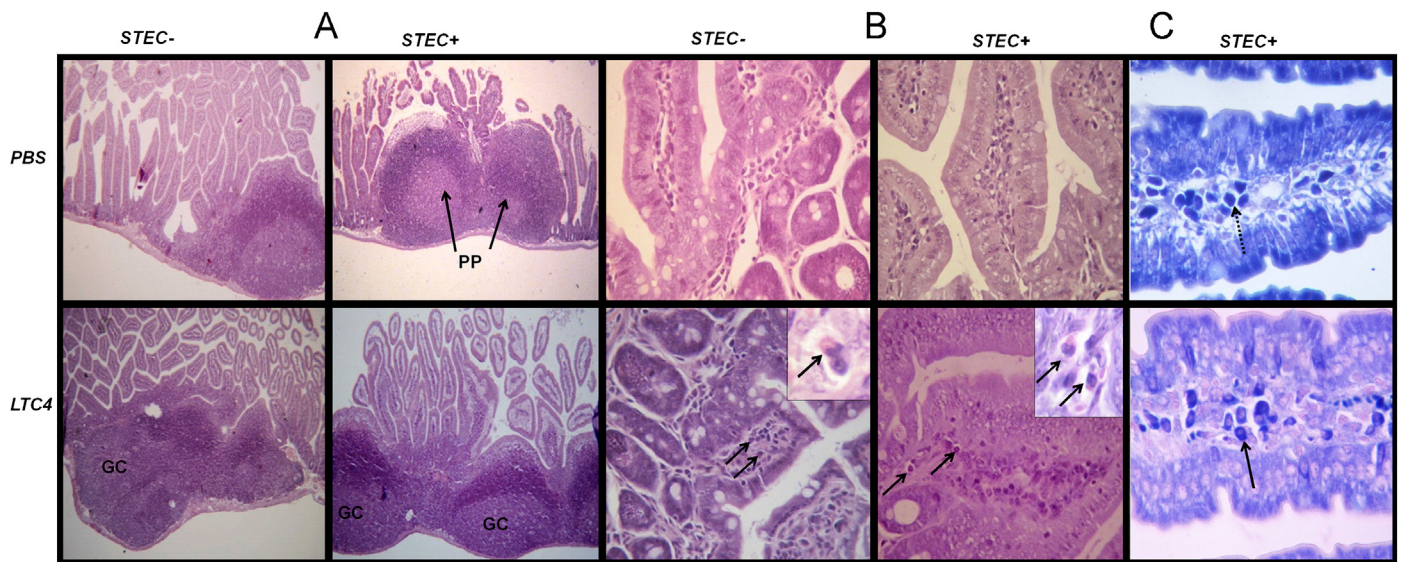


Fig. 1. LTC4 effect on small intestines. Representative H&E-stained sections of small intestines from non-pretreated (PBS) or pretreated with LTC4 (10^{-8} M) (LTC4), uninfected (STEC⁻) or infected mice (STEC⁺) are shown. At least three mice per each experimental group were studied. (A) Representative images from Peyer's Patches (PP) of PBS/STEC⁻ mice showing normal appearance; LTC4/STEC⁻ mice showing high activation of germinal centers (GC); and PBS/STEC⁺ and LTC4/STEC⁺ mice showing hyperplasia of PP and activated germinal centers (original magnification, 100 \times). (B) Representative images from PBS/STEC⁻ and PBS/STEC⁺ mice showing only mild lymphocyte infiltration of lamina propria. LTC4/STEC⁻ and LTC4/STEC⁺ showing lymphocyte infiltration, and also a marked eosinophilic interstitial infiltration (arrows) (original magnification, 200 \times , insert 800 \times). (C) Representative images of Giemsa-stained sections of small intestines from PBS/STEC⁻ and LTC4/STEC⁺ mice, showing infiltration of lymphocytes (small cells with round and blue dark nuclei and scanty cytoplasm, dashed arrow) or eosinophils (cells with lobulated and marginal nuclei and central red purple cytoplasm, arrow) (original magnification, 400 \times).

of STEC⁺ mice pretreated with LTC4 (LTC4/STEC⁺) showed the highest inflammatory status, as evidenced by vasocongestion and the presence of both mucosal and submucosal cell infiltration (Fig. 2A–C). In addition, the colon from LTC4/STEC⁺ mice showed more severe damage than the colon from PBS/STEC⁺ mice, as indicated by focal areas of epithelial microerosion (Fig. 2C). Furthermore, the colon from LTC4/STEC⁺ mice showed an increased number of apoptotic epithelial cells (number of apoptotic cells by field PBS/STEC⁺ = 1.79 ± 0.66 ; LTC4/STEC⁺ = 3.9 ± 0.70) (Fig. 2D and E).

Taking together, these results suggest that LTC4 pretreatment induces an inflammatory status in the intestine that increases the damage caused by STEC infection.

3.2. LTC4 pretreatment alters the permeability of mucosal barrier

Since disruption of mucosal barrier makes it permissive to the passage of pathogenic factors (LPS, Stx) to bloodstream, the mucosal permeability was analyzed using FITC-Dx as described in Section 2.

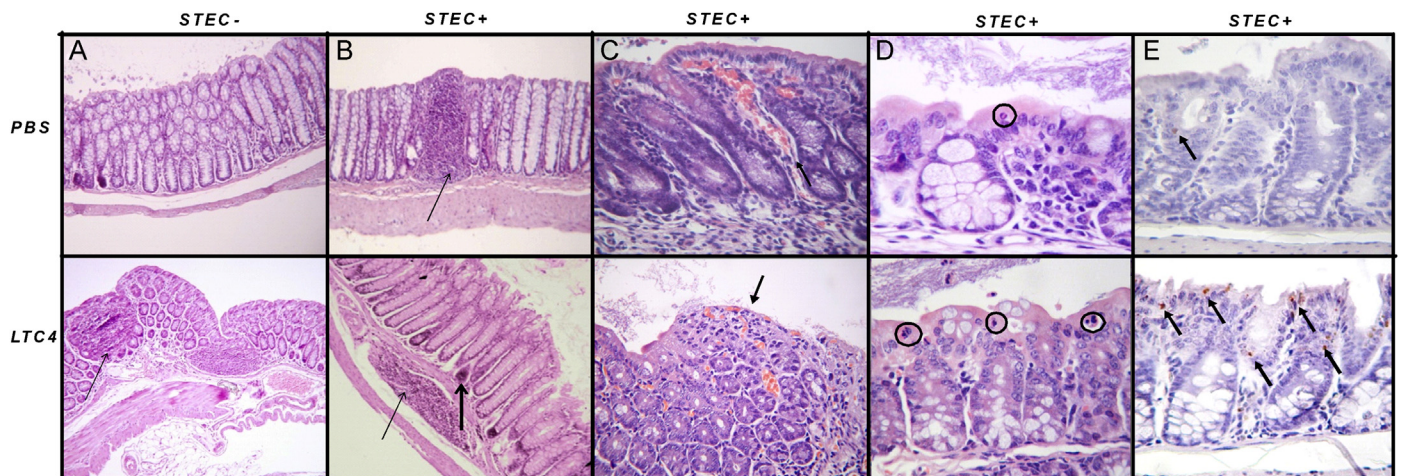


Fig. 2. LTC4 effect on colonic damage induced by STEC infection. Representative H&E-stained sections of colon from non-pretreated (PBS) or pretreated with LTC4 (10^{-8} M) (LTC4), non-infected (STEC⁻) or infected mice (STEC⁺) are shown. At least three mice per each experimental group were studied. (A) Representative image from colonic mucosa of PBS/STEC⁻ control mice showing normal thickness, preserved architecture and abundant goblet cells, and from LTC4/STEC⁻ mice showing lymphocyte inflammatory infiltrate in lamina propria (arrow); (B) PBS/STEC⁺ mice showing presence of inflammatory infiltrate in the mucosa (arrow) and LTC4/STEC⁺ mice showing presence of inflammatory infiltrate, both in mucosa (thick arrow) and submucosa (thin arrow) (original magnification, 100 \times). (C) The colon from PBS/STEC⁺ mice shows vasocongestion in lamina propria, edema and microhaemorrhagic foci (arrow). The colon from LTC4/STEC⁺ mice shows areas of epithelial erosion (thick arrow) and vasocongestion (original magnification, 200 \times). (D) PBS/STEC⁺ infected mice show isolated apoptotic cells (circle), while LTC4/STEC⁺ infected mice show an increased number of apoptotic cells (circles) (original magnification, 400 \times); (E) TUNEL technique was used to detect apoptosis *in situ*. The experimental procedure used is described in Section 2. The image representative of PBS/STEC⁺ infected mice shows only one stained cell (arrow), while LTC4/STEC⁺ infected mice shows high number of stained cells (arrow) (original magnification, 400 \times , 3,3'-diaminobenzidine).

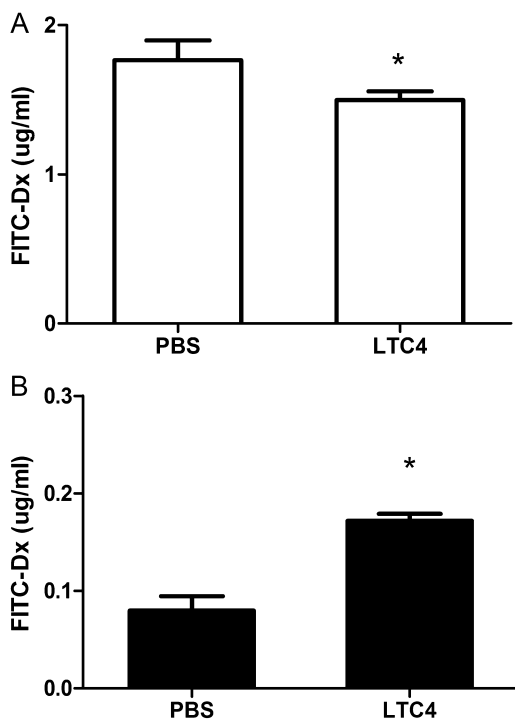


Fig. 3. LTC4 effect on intestinal barrier function. Study of the functional integrity of intestinal barrier by oral administration of FITC-Dx to PBS-pretreated mice ($n=4$) or LTC4-pretreated mice ($n=4$). Bars represent the mean \pm SEM of FITC-Dx levels in supernatants of intestinal tissue (A) and serum (B) from individual mice. Data are representative of two independent experiments. * $p \leq 0.05$ by t test.

BALB/c mice were pretreated with or without LTC4 and 18 h later 0.15 mL of FITC-Dx was orally administered. Mice pretreated with LTC4 showed a decreased level of FITC-Dx in the intestine (Fig. 3A), and an increased level of FITC-Dx in serum (Fig. 3B), as compared to LTC4 nonpretreated mice. These results suggest that LTC4 affects the functional integrity of epithelial barrier.

3.3. LTC4 pretreatment decreases the survival of STEC infected mice

Taking into account the alterations induced by LTC4 on intestinal tissue we decided to evaluate whether LTC4 affected the outcome of STEC-infected mice. For this purpose, survival studies were performed. As shown in Fig. 4A, LTC4 pretreatment caused an increase in the mortality rate of STEC-infected mice (LTC4/STEC+) as compared to the control group (PBS/STEC+). As expected, the survival of noninfected mice was 100% in LTC4-pretreated (LTC4/STEC-) or nonpretreated mice (PBS/STEC-) (data not shown).

3.4. LTC4 pretreated mice that died after STEC infection show clinical parameters related to Stx2 toxicity

We have previously shown that STEC infection of BALB/c mice can be lethal, as a consequence of systemic damage related to Stx2 toxicity (Brando et al., 2008; Cabrera et al., 2014). In addition, we have also demonstrated that neutrophilia and renal dysfunction secondary to STEC infection is blocked when anti-Stx2 polyclonal serum is simultaneously inoculated, confirming that Stx2 is responsible for renal damage (Mejias et al., 2013).

In order to confirm that the death of LTC4+/STEC+ mice was related to Stx2 toxicity, urea concentration and the percentage of PMNs in blood were measured at 96 h postinoculation and data were analyzed retrospectively according to the final outcome of the inoculated mice. Fig. 5A and B shows that only

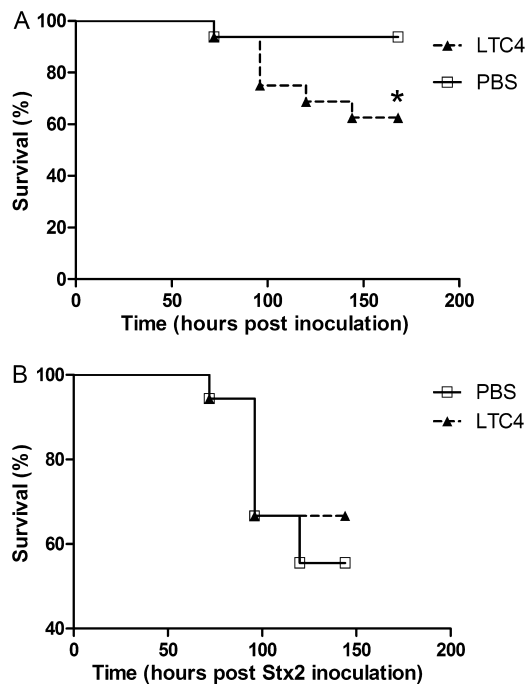


Fig. 4. LTC4 effect on STEC susceptibility. (A) Doses of $5-7 \times 10^{11}$ CFU of STEC/kg of body weight were intragastrically administered to three-month-old mice. Survival rates of PBS/STEC+ mice (control; white squares) or LTC4/STEC+ (black triangles) are shown. Sixteen mice were used per each experimental group. * $p < 0.05$, log-rank test. (B) PBS or LTC4-pretreated mice were intravenously inoculated with 1LD₅₀ of Stx2 and the survival was evaluated. Eighteen mice were used per each experimental group. No differences were found between LTC4/Stx2+ (black triangles) and PBS/Stx2+ mice (control, white squares), by log-rank test.

LTC4-pretreated mice that died after STEC infection showed neutrophilia and increased urea levels in blood. These results demonstrate that LTC4 pretreatment increases Stx2-associated systemic complications after STEC infection in mice.

3.5. LTC4 pretreatment does not affect the mortality rate to Stx2 intravenous injection

Next, one lethal dose 50 (1LD50) of Stx2 was intravenously (i.v.) inoculated to evaluate whether LTC4 pretreatment influenced mice susceptibility to Stx2 toxic effects. PBS and LTC4-pretreated mice showed similar survival rates (Fig. 4B), strongly suggesting that LTC4 worsened the outcome of STEC infection by affecting the intestinal phase of the infection and not Stx2 systemic toxicity.

3.6. STEC infection increases LTC4 levels in the gut

Having demonstrated the impact that LTC4 has on the pathogenesis of STEC infection, we finally analyzed whether STEC ingestion affected LTC4 gut levels. Our results showed that STEC-infected mice had higher LTC4 intestinal levels as compared to noninfected control mice at 96 h postinfection (Fig. 6A). In contrast, LTB4 gut levels were not modified after STEC infection (Fig. 6B). This result supports that LTC4 plays a pathogenic role in STEC infections, mainly by disrupting the mucosal epithelial barrier.

4. Discussion

LTs have been implicated in the pathophysiology of both acute and chronic inflammatory diseases including asthma, arthritis, psoriasis, and inflammatory bowel disease (IBD) (Wang and DuBois, 2007). The role of LTs in the pathogenesis of inflammatory diseases other than bronchial asthma has always been neglected. One

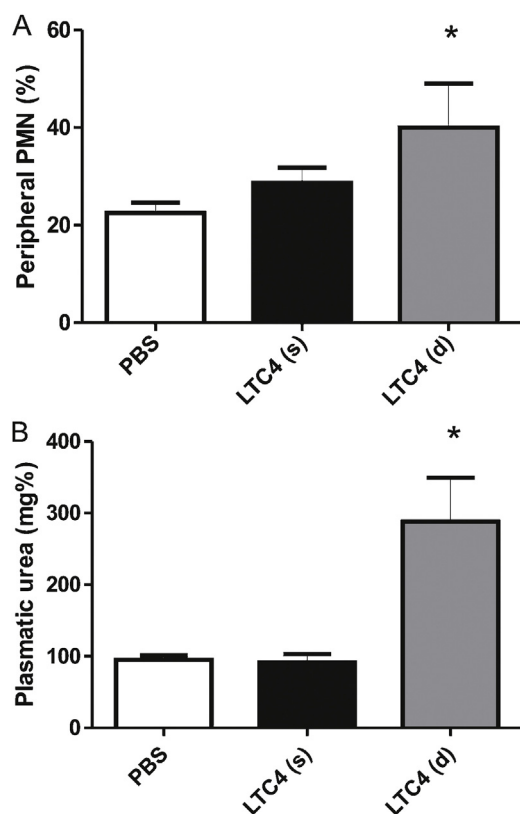


Fig. 5. Clinical parameters associated to Stx2 toxicity. Analysis of PMN percentage in the blood and plasma urea levels were assessed 96 h post STEC infection of PBS or LTC4-pretreated mice. LTC4/STEC+ mice were classified retrospectively according to the outcome into survivors (s) (black bars) ($n = 10$), or dead (d) (gray bars) ($n = 6$). PBS-pretreated mice has only the survivor bar (white bar) ($n = 15$), because only one mouse died at 72 h post STEC infection, and bleeding was not possible. (A) PMN percentage is shown as the mean \pm SEM. (B) Plasma levels of urea are expressed as the mean urea concentration (mg%) \pm SEM. All data were analyzed by ANOVA and *a posteriori* Tukey test. * $p < 0.05$.

possible reason is that 5-LOX is expressed in a very limited number of cells, mostly leukocytes. Furthermore the distribution of secondary enzymes within cells possessing 5-LOX activity exhibits remarkable cellular specificity: neutrophils possess LTA4 hydrolase and generate LTB4, a compound with very potent chemoattractant activities; on the other hand, eosinophils (Weller et al., 1983) and mast cells possess LTC4 synthase and preferentially generate LTC4, a potent bronchocontracting and vasoactive compound. The specific profile of LTs formed by different cells well explains the involvement of cys-LT in asthma and gastric inflammation, where mast cells and eosinophils are believed to play a significant role in the pathogenesis of different diseases (Sala and Folco, 2001).

In this report we showed that LTC4 concentration increased in the intestinal mucosa during STEC infection in mice, while LTB4 levels remained unchanged. To reach this conclusion, we measured, after overnight incubation, the LTs secreted by a standardized number of intestinal cells coming from STEC-infected mice in comparison to the same number of cells from control non-infected mice. Considering that LTC4 could be metabolized to LTE4 or LTD4, the amount of LTC4 could be under-estimated in these experimental conditions. On the other hand, the amount of LTB4 is not metabolized, remaining stable for longer times. Thus, under the present experimental protocol we can ensure that the differences between LTC4 and LTB4 are at least not over-estimated, reinforcing the conclusion that LTC4 secretion is specifically increased in the gut upon STEC-infection. The levels of LTs detected in our work are similar to the levels reported in other pathophysiological models, such as

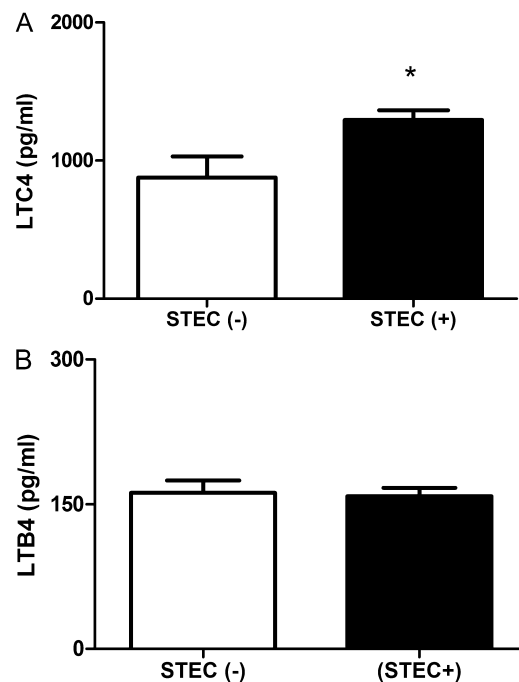


Fig. 6. STEC infection increases intestinal LTC4 levels. Doses of $5\text{--}7 \times 10^{11}$ CFU of STEC/kg of body weight were intragastrically administered to three-month-old mice (STEC-infected). These animals (without LTC4 pretreatment) and the corresponding uninfected control group were sacrificed at 96 h post-infection, the intestines were excised, and LTC4 (A) or LTB4 (B) were measured by ELISA as described in Section 2. One representative experiment out of two is shown. Data are expressed as the mean \pm SEM of uninfected (white bars) or STEC-infected (black bars) ($n = 4$ mice per group). * $p \leq 0.05$ by *t*-test.

a gastric inflammatory model that uses ethanol in rats (Hattori et al., 2008). On the other hand, our values of LTC4 are lower as compared to the levels described in some reports that induce peritonitis using Zymosan, one of the strongest stimuli available to trigger LTs secretion (Kanaoka et al., 2001). This indicates that the levels of LTs are deeply dependent on the stimuli that trigger their secretion.

In apparent contradiction, it has been previously reported that STEC infection increases LTB4 concentration in the intestine of rabbits (Bell et al., 2000). Differences can be ascribed to the animal model, since STEC infection in rabbits courses with an important neutrophil intestinal infiltration, while the cellular infiltration in mice is predominantly mononuclear, as has been reported by others (Wadolowski et al., 1990) and it is confirmed in the present manuscript. On the other hand, LTC4 release has been observed in fecal samples from patients suffering *Shigella dysenteriae* (Cruz et al., 1995). Since both LTC4 and LTB4 are potent inflammatory mediators, these data support that LTs may play a role in the establishment of a proinflammatory milieu at the intestinal mucosa during STEC infections.

To gain insight into the influence of cysLTs, we pretreated mice with exogenous LTC4 before STEC infection, and histological and functional studies were performed. Our results showed that the administration of a standard 10^{-8} M dose of LTC4, previously reported to be active *in vivo* (Hui et al., 2004; Badr et al., 1984) increased mucosal barrier permeability and induced an overall greater intestinal inflammation, resulting in a more severe damage upon STEC infection, mainly at the colon. This is not surprising, since colon has been described as the main target of STEC in humans and mice (Cabrera et al., 2014; Brando et al., 2008; Golan et al., 2011). It should be noted that the LTC4 concentration used in our study is comprised within the range described at mucosal tissues during the course of inflammatory reactions (Leite et al., 2007; Muz et al., 2006) and was previously used for stimulation of goblet cells

in vitro (Dartt et al., 2011), thus indicating that a small increase in the concentration of LTC4 may have a relevant biological effect.

The gastrointestinal epithelium is a selective barrier that allows the absorption of nutrients, electrolytes and water, but restricts the passage of larger potentially toxic compounds into the circulation, thereby preventing bacterial translocation and systemic infection.

The structural integrity of the epithelium is guaranteed by three adhesion systems: tight junctions (TJs), adherent junctions and desmosomes. Of these, TJs form a selective barrier that restricts paracellular diffusion, being the rate-limiting step for paracellular permeability (Shin et al., 2006). Many lines of evidence indicate that the disruption of TJs and loss of epithelial barrier function play a crucial role in the pathogenesis of gastrointestinal disorders, such as IBD, alcoholic endotoxemia, infectious enterocolitis, celiac disease, and necrotizing enterocolitis (Laukoetter et al., 2006; Rao, 2008; Turner, 2006). All of which are associated with the increase of LTC4 or their receptors (Reims et al., 2005).

Although several mechanisms have been proposed, it remains to be completely defined the mechanism by which Stx-produced by STEC in the intestine reaches systemic circulation (Ibarra and Palermo, 2010). It has been demonstrated that STEC interaction with a cellular line of intestinal epithelium (T84) induces the basolateral-to-apical transmigration of PMN, which in turn, significantly increases the movement of Stx1 and Stx2 across polarized T84 cells in the opposite direction, by increasing the paracellular permeability (Hurley et al., 2001).

In this regard, STEC resistance of adult BALB/c mice contrasts with the susceptibility of weaned mice during a short time window of 2–3 days, during which pups develop a renal injury resembling HUS following STEC intragastric inoculation, probably due to immaturity of their epithelial barrier (Brando et al., 2008; Pai et al., 1986). Conversely, in a system characterized by a tight barrier such as the colonic epithelium of adult mice, resistance to STEC in adults is likely to be linked to the inability of this pathogen to unlock this barrier, and to reverse the state of bowel tolerance to the intestinal microbiota that is particularly dense in the colon.

Our results showed that the intestinal mucosa of LTC4-pretreated mice was severely altered by STEC, thus indicating that the refractory state does not reside in an inability of bacteria to disrupt epithelial cells. Indeed, the same number of bacteria elicited a significant degree of cytotoxicity to the colonic epithelium (*i.e.* areas of epithelial erosion and increase of apoptosis) and a stronger inflammatory response at the intestine of LTC4-pretreated mice. The striking feature of this infectious pattern in mice is the lack of PMN infiltrating the tissue (Wadolowski et al., 1990), in sharp contrast to the neutrophilic infiltration observed in the rabbit ileal loops (Pai et al., 1986), which have been associated to the experimental lesions, and the clinical lesions observed in humans (Griffin et al., 1990). Of interest, after LTC4-pretreatment, a strong eosinophilic infiltration was observed in the small intestine, which probably contributes to inflammation and tissue damage.

We have previously shown that Stx2 systemic toxicity is the cause of death after STEC infection in mice. In addition, Stx toxicity strongly correlates with neutrophilia and increased urea levels in blood (Brando et al., 2008). Herein we observed the same correspondence between mortality and alterations in the parameters associated to Stx2 systemic toxicity. In fact, only LTC4-pretreated mice that died after the infection showed neutrophilia and increased urea levels in blood. Thus, the increased mortality secondary to STEC in LTC4-pretreated mice was dependent on Stx2 toxicity. The increased Stx2-associated toxicity could be consequence of: (1) an increased passage of pathogenic factors from the gut to the bloodstream; (2) an increased systemic susceptibility of mice to them, particularly to Stx; (3) a sum of both previous mechanisms. An unusual increased passage of FITC-Dx in LTC4-pretreated mice directly demonstrated the impairment of the intestinal barrier

function, and strongly suggests that more Stx2 could be reaching the bloodstream in those mice.

On the other hand, no differences in the mortality rate were found between LTC4-pretreated and nonpretreated mice after the same dose of Stx2 *i.v.* inoculation, thus indicating that LTC4 did not alter systemic susceptibility to Stx, and further supporting that LTC4 only affected the intestinal phase of the disease.

We have demonstrated for the first time that LTC4 increases the pathogenicity of STEC in mice. We have shown that LTC4 pretreatment affects intestinal integrity by histological and functional studies, thus suggesting that the disruption of the mucosa may facilitate the passage of pathogenic factors, mainly Stx, to bloodstream. This report sheds light on one mechanism by which the local inflammatory response may influence the evolution of STEC infections.

Disclosures

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