



## Research paper

# The long polar fimbriae of STEC O157:H7 induce expression of pro-inflammatory markers by intestinal epithelial cells

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## ARTICLE INFO

## Keywords:

Enterohemorrhagic *E. coli*  
Shiga toxin *E. coli*  
Long polar fimbriae  
Inflammation  
Interleukin-8

## ABSTRACT

Infection with Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 is characterized by acute inflammation of the colonic mucosa. STEC O157:H7 contains two non-identical loci encoding long polar fimbriae (Lpf), which play a role in the STEC colonization of the intestinal epithelial cells. However, no information is available regarding the involvement of Lpf in the STEC-induced host inflammatory response. Hence, in this study we assess the role of Lpf as an inducer of inflammation on intestinal epithelial cells. Secretion of pro-inflammatory cytokines in response to STEC wild type and *lpf*isogenic mutants was evaluated on intestinal T84 cells. Of the 27 cytokines assayed, IL-6, IL-8, IL-15, FGF, GM-CSF and IP-10 were significantly reduced, when compared to the wild-type strain, in the *lpfA1 lpfA2* double mutant. Further, the host intracellular signaling pathways activated in response to Lpf were determined by using an array containing genes representative of 18 different signal transduction pathways. The analysis indicated that the NF- $\kappa$ B pathway is activated in response to Lpf-expressing STEC. Therefore, our study supports the role of Lpf as a STEC factor mediating intestinal inflammation.

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## 1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 are food-borne human pathogens and the etiological agent associated with acute and bloody diarrhea and in some cases, hemolytic uremic syndrome (HUS) (Pennington, 2010). Ruminants are a major reservoir of *E. coli* O157:H7; however, the mechanisms underlying STEC persistence in this animal host are poorly understood (Menge et al., 2004).

In humans, STEC infections are characterized by acute inflammation of the colonic mucosa (Besser et al., 1999). On intestinal epithelial cells, STEC induces the secretion of several pro-inflammatory markers, such as interleukin-8 (IL-8), as a response to the activation of the MAPK, AP-1 and NF- $\kappa$ B signaling pathways (Dahan et al., 2002; Miyamoto et al., 2006). Several STEC proteins have been studied and correlated with this inflammatory process (as reviewed in Farfan and Torres, 2012). Of these proteins, flagellin and HCP pili, along with LPS, were shown as having a role in the induction of inflammation on intestinal epithelial cells (Berin et al., 2002; Ledesma et al., 2010). Because these inflammatory markers are still produced at a lower level by cells infected with STEC isolates lacking these bacterial inflammatory inducers, the role of other factors cannot be excluded (Berin et al., 2002; Ledesma et al., 2010).

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**Table 1**  
Bacterial strains used in this study.

Strain	Relevant characteristic	Reference
86-24	Prototype <i>E. coli</i> O157:H7	Lab stock
CVD468	86-24 <i>lpfA1::cat</i> Cm <sup>R</sup>	Torres et al. (2004)
AGT201	86-24 <i>lpfA2::tet</i> Tc <sup>R</sup>	Torres et al. (2004)
AGT120	86-24 <i>lpfA1::cat lpfA2::tet</i> Cm <sup>R</sup> Tc <sup>R</sup>	Torres et al. (2004)

Adherence to intestinal cells is a critical step in STEC pathogenesis, and several adhesion factors have been described (as reviewed in Farfan and Torres, 2012). For STEC O157:H7, the intimin protein and the long polar fimbriae (Lpf) are the only two factors that have been demonstrated to play a role in the colonization of the human intestine and the subsequent persistence of the bacteria (Donnenberg et al., 1993; Fitzhenry et al., 2006; Jordan et al., 2004). The intimin protein, together with the bacterial “translocated intimin receptor”, Tir, participate in the formation of A/E lesions, critical for the pathophysiology observed during STEC infections. Interestingly, previous reports showed that the inactivation of the intimin coding gene in wild-type STEC did not result in changes in the production of inflammatory markers on colonic epithelial cells, as observed with the wild-type strain (Berin et al., 2002). This further supports the role of other STEC surface-expressed proteins as inducers of the inflammatory response.

The role of fimbrial proteins in the inflammatory phenotype is plausible because, for example, the aggregative adherence fimbriae (AAF), the main adherence factor of enteroaggregative *E. coli* (EAEC), contributes to the inflammatory response of intestinal epithelial cells infected with EAEC (Harrington et al., 2005). Considering that there are no experimental data available about the role of the Lpf fimbriae in STEC-induced inflammation on the intestinal epithelia, we sought to characterize the involvement of these fimbriae on the induction of IL-8 secretion by host cells. Here, we provide evidence that Lpf contributes to the IL-8 secretion, as well as the production of other inflammatory markers, by intestinal cells infected with STEC O157:H7. Also, gene expression analysis revealed that Lpf participate in the induction of the NF- $\kappa$ B signaling pathway.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. All STEC strains were grown in static conditions overnight in Dulbecco's modified Eagle's medium (DMEM)/0.5% glucose (DMEM-HG) or Luria–Bertani (LB) broth with the addition of streptomycin (100  $\mu$ g/ml), chloramphenicol (30  $\mu$ g/ml), tetracycline (12.5  $\mu$ g/ml) and kanamycin (50  $\mu$ g/ml) when appropriate.

### 2.2. STEC binding to fibronectin

STEC binding to fibronectin was carried out according to a previously described protocol (Farfan et al., 2011).

Briefly, wells of 24-well plates were coated with a solution of 10  $\mu$ g/ml of fibronectin (from human plasma; Sigma). Unbound protein was removed, and wells were blocked with 1% BSA for 1 h at room temperature. One milliliter of DMEM-HG containing  $\sim 1 \times 10^8$  STEC bacteria was added to each well and the plate was incubated at 37 °C for 4 h. Bacterial cells that adhered to wells were collected by scraping them into distilled water. Serial dilutions were plated onto LB agar plates, and the number of adherent bacteria was determined by counting the resulting colonies.

### 2.3. T84 cell culture conditions

Human colonic T84 intestinal epithelial cells (ATCC CCL-248) were routinely maintained in DMEM-F12 media, supplemented with 10% fetal bovine serum (FBS), penicillin (10 U/ml) and streptomycin (10  $\mu$ g/ml), at 37 °C under 5% CO<sub>2</sub>.

### 2.4. T84 adherence assays

Adherence assays were performed with non-polarized T84 cells as previously described (Farfan et al., 2011). Briefly, 24-well tissue culture plates were seeded at a density of  $1 \times 10^5$  cells/well and incubated at 37 °C under 5% CO<sub>2</sub> until a  $\sim 90\%$  confluence was achieved. Prior to bacteria inoculation, cell monolayers were incubated with DMEM-F12 medium for 30 min. Then, the medium was aspirated and 200  $\mu$ l containing  $\sim 1 \times 10^8$  CFU/ml bacteria added to the monolayer (in triplicate), with a multiplicity of infection (MOI) of 100. The plates were centrifuged at 500  $\times$  g for 10 min and incubated at 37 °C in 5% CO<sub>2</sub> for 4 h. Cells were lysed with a solution containing 0.1% (v/v) Triton X-100/PBS, and serial dilutions of the lysates were plated on LB agar. The number of adherent bacteria was determined by counting the resulting colonies in duplicate.

### 2.5. T84 infection assays

Infection assays were performed in triplicate with non-polarized T84 cells as described above. After infection, cells were washed and incubated with DMEM-F12 containing gentamicin (100  $\mu$ g/ml) for 18 h. After this time point, the culture medium was obtained and stored at –20 °C.

### 2.6. IL-8 secretion

The culture medium from triplicate wells of non-polarized T84 cell monolayers infected with STEC obtained at 18 h after infection was evaluated in duplicate by ELISA for IL-8 as described (Harrington et al., 2005). Briefly, microtitre plates were coated with 1  $\mu$ g/ml of anti-human

IL-8 in carbonate buffer, pH 9.6 at 4 °C. After washing, plates were blocked with 5% skim milk in PBS for 1 h at 37 °C. Plates were washed again and serial dilutions of a recombinant human IL-8 standard were added. Plates were then incubated at room temperature for 2 h and then 0.25 µg/ml biotinylated mouse α-human IL-8 was added, and plates incubated at room temperature for 1 h. Avidin–horseradish peroxidase conjugate was added and plates were incubated at room temperature for 30 min. IL-8 was detected with TMB substrate solution using optical densities at 450 nm with a 96-well plate reader. IL-8 concentrations were determined from a best-fit standard curve.

### 2.7. Cytokine measurement

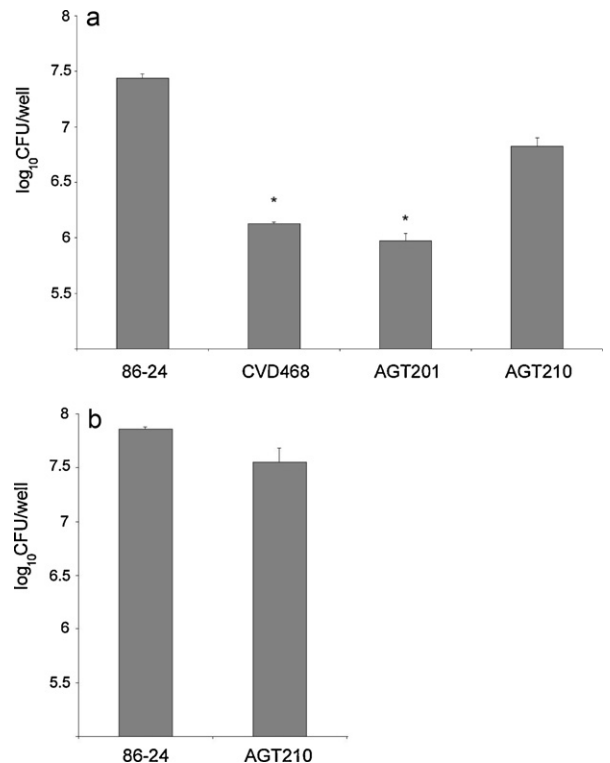
Cytokine levels were measured by using a Bio-Plex human cytokine 27-plex assay (Bio-Rad). This multiplex assay detects IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic FGF, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF. Frozen samples of culture medium from triplicate wells of T84 cell monolayers infected with STEC were assayed according to the manufacturer's instructions.

### 2.8. PCR arrays

Whole-cell RNA was isolated from triplicate wells of T84 cell monolayers infected with a STEC wild-type strain 86-24 or its isogenic *lpf1 lpf2* double mutant strain AGT210, by using the Trizol method according to the manufacturer's instructions (Invitrogen). RNA was treated with RNase-free DNase I to eliminate contaminating DNA by using the RNeasy kit (Qiagen). One microgram of RNA was used for cDNA synthesis with the RT<sup>2</sup> First Strand kit (SuperArray Bioscience) following the manufacturer's instructions. The cDNA synthesized was used to perform a Human Signal Transduction PathwayFinder RT<sup>2</sup> Profiler™ PCR Array (Qiagen). Two arrays were performed with preparations from T84 cells infected separately with STEC strains 86-24 and AGT210 or uninfected cells. Changes in cycle threshold (ΔCt) values for each gene were obtained by subtracting the mean threshold cycle (Ct) of the reference genes (Gusb, HPRT, Hsp90ab1, Gapdh, and Actβ) from the threshold cycle value of the gene. Normalized transcription was calculated as  $2^{\hat{C}-\Delta Ct}$ . The fold up- or down-regulation was calculated relative to the uninfected cells or cell infected with the strain AGT210. The genes that showed more than 2-fold differences in expression were identified as described in the RT<sup>2</sup> Profiler™ PCR Array Data Analysis (<http://www.sabiosciences.com/pcrarraydataanalysis.php>).

### 2.9. Statistical analysis

Statistical significance between the individual groups was analyzed by using the unpaired Student's *t* test with a threshold of  $P < 0.01$ . Values are expressed as the means with error bars indicating one standard deviation.



**Fig. 1.** Lpf involvement in the adherence of STEC to T84 cells. (A) Numbers of adherent STEC strain 86-24 and its isogenic mutant in *lpfA1* (strain CVD468), *lpfA2* (strain AGT201) and *lpfA1 lpfA2* (strain AGT210) infecting T84 cells and (B) Binding of STEC strains 86-24 and its isogenic mutant in *lpfA1 lpfA2* (strain AGT210) to fibronectin-coated wells. \* $P < 0.01$ , as compared to the wild-type strain.

## 3. Results

### 3.1. *Lpf* fimbriae play a role in STEC adherence to intestinal cells

We first confirmed the role of the STEC Lpf fimbriae on binding to intestinal T84 epithelial cells. STEC wild-type strain 86-24 and its isogenic single and double mutants in the *lpfA1* and *lpfA2* genes (the A subunits are the first genes in both *lpf1* and *lpf2* loci) were separately added to T84 monolayers, and the number of adherent bacteria was determined after 4 h of infection. A significant reduction in the number of bacteria was observed in cells infected with the *lpfA1* (strain CVD468) and *lpfA2* (strain AGT201) single mutants as compared to that found in the wild-type strain. Surprisingly, the number of adherent bacteria from cells infected by the double mutant (strain AGT210) was comparable to that in cells infected with the wild-type strain (Fig. 1a;  $P = 0.02$ ), and when we assessed the ability of these strains to bind to fibronectin (Fig. 1b;  $P = 0.4$ ), an extracellular matrix protein that binds Lpf fimbriae (Farfan et al., 2011), the findings were similar to those above.

Considering that the adherence of the *lpfA1 lpfA2* double mutant (strain AGT210) was similar to that of the wild-type strain, we compared the ability of these strains to induce the secretion and expression of inflammatory markers by T84 cells.

**Table 2**Pro-inflammatory markers secreted by T84 cells infected with wild-type STEC and its *lpfA1 lpfA2* double mutant.

Cytokine	86-24 <sup>a</sup> (pg/ml)	AGT210 <sup>a</sup> (pg/ml)	Uninfected <sup>a</sup> (pg/ml)	86-24 vs. uninfected fold increase (P value)	AGT210 vs. uninfected fold increase (P value)
IL-4	1.085 (0.4)	0.34 (0)	0.34 (0)	3.2 (P=0.18)	1.0 (P=1)
IL-8	246.37 (51.5)	93.13 (13.7)	43.4 (8.6)	5.7 (P=0.002)	2.1 (P=0.006)
IP-10	313.9 (73.6)	230.4 (10.9)	208 (25.4)	1.6 (P=0.08)	1.1 (P=0.25)

Abbreviations: IL-4, interleukin 4; IL-8, interleukin 8; and IP-10, interferon gamma-induced protein 10.

<sup>a</sup> Secreted cytokines by T84 cells infected with STEC strains or uninfected cells determined by Bio-Plex. Results are expressed as the mean (standard deviation) of three wells per group from a single representative experiment.

### 3.2. *Lpf* fimbriae contribute to the release of pro-inflammatory markers by T84 cells

Previous studies demonstrated that STEC is able to induce IL-8 secretion by T84 cells (Dahan et al., 2002); therefore, we evaluated the role of *Lpf* fimbriae in the secretion of pro-inflammatory markers. We assayed the ability of the *lpfA1 lpfA2* double mutant (strain AGT210) to induce IL-8 secretion and found that the amount of this cytokine in the supernatant of T84 cells infected with the *lpfA1 lpfA2* double mutant was significantly lower when compared to that in cells infected with the wild-type strain (Fig. 2).

To evaluate other cytokines secreted by T84 cells infected with STEC, we measured the levels of 27 cytokines by using the Bio-Plex human cytokine 27-plex assay. The cytokines IL-4, IL-8 and IP-10 were secreted at high levels (>2-fold) into the supernatant of T84 cells infected with wild-type strain 86-24, when compared to the levels found in uninfected cells (Table 2). Of these, only IL-8 was found at high levels in the supernatant of T84 cells infected with the *lpfA1 lpfA2* double mutant strain AGT210 as compared to IL-8 levels in uninfected cells, but was secreted at a lower level when compared with the levels found in the wild type

strain 86-24 (6-fold compared to 2-fold). Altogether, the data obtained led us to suggest that *Lpf* fimbriae stimulate the secretion of pro-inflammatory cytokines from intestinal epithelial cells.

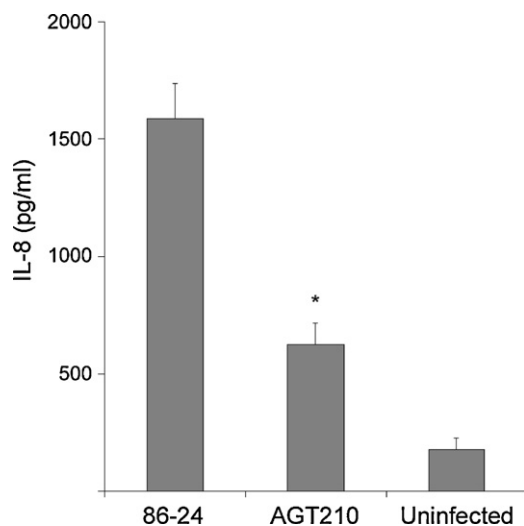
### 3.3. Activation of pro-inflammatory genes in cells infected with STEC

STEC induced up- and down-regulation of several genes in epithelial cells (Dahan et al., 2002; Gobert et al., 2008). To evaluate the signaling pathways that might be activated by *Lpf*-expressing STEC, the expression of 84 key markers found in 18 different signal transduction pathways was evaluated upon infection of T84 cells by STEC strains (Table 3). When compared with their levels in uninfected cells, *CCL20*, *IL8*, *ICAM1*, *IL1A*, *TANK*, *TNF*, *SELE*, *CSF2*, *BIRC3*, *HSPB1* and *MMP7* genes were up-regulated in T84 cells infected with wild-type STEC strain 86-24. In contrast, only *CCL20*, *IL8*, *ICAM1*, *TANK* and *HSPB1* were up-regulated in T84 cells infected with the *lpfA1 lpfA2* double mutant strain AGT210 when compared to the levels of those gene in uninfected cells, but the expression levels were lower than those observed in cells infected with the wild-type strain.

## 4. Discussion

Inflammation is a prominent feature of intestinal lesions after infection with STEC O157:H7. The enterocytes produce chemokines and cytokines in response to STEC to attract neutrophils to the infection site (Gobert et al., 2008). Among the pro-inflammatory factors secreted by infected cells, IL-8 plays a key role in the host innate immune response against STEC as well other enteric pathogens (Dahan et al., 2002). Of all STEC-virulence factors, H7 flagellin and LPS appear to be the major contributors to cytokine stimulation (Berin et al., 2002; Miyamoto et al., 2006; Stahl et al., 2006). Considering that pathogen-induced inflammation on epithelial cells is frequently a process initiated by multiple virulence factors, we evaluated the role of *Lpf* in the induction and subsequent secretion of pro-inflammatory cytokines by intestinal cells infected with STEC.

Several studies have demonstrated the importance of *Lpf* in the adherence process of STEC O157:H7 to intestinal cells (Torres et al., 2002, 2004), and now we showed that the inactivation of both *lpf1* and *lpf2* loci results in a significant reduction in the secretion of IL-8 (Fig. 2), IL-4 and IP-10 (Table 2) by T84 cells. One explanation for this finding may be that there is a reduced binding of the double



**Fig. 2.** *Lpf* double mutant induces a reduced amount of IL-8 secretion by infected T84 cells. Intestinal T84 monolayers were infected with the wild-type STEC strain 86-24 and its isogenic *lpfA1 lpfA2* double mutant (strain AGT210). After 18 h of incubation, the media from the basolateral side of the cells was collected and levels of secreted IL-8 measured by ELISA. Bars represent the means for three experiments, with error bars indicating one standard deviation. \*Significantly different from IL-8 secretion induced by wild-type strain ( $P < 0.01$ ).

**Table 3**Up-regulation of genes in T84 cells infected with wild type STEC and its *lpfA1 lpfA2* double mutant.

86-24 vs. uninfected (fold increase)	AGT210 vs. uninfected (fold increase)	86-24 vs. AGT210 (fold increase)
<i>CCL20</i> (35.2)	<i>CCL20</i> (3.6)	<i>CCL20</i> (9.7)
<i>IL8</i> (10.1)	<i>IL8</i> (2.4)	<i>IL8</i> (4.1)
<i>ICAM1</i> (14.1)	<i>ICAM1</i> (2.9)	<i>ICAM1</i> (2.3)
<i>IL1A</i> (8.7)	<i>HSPB1</i> (2.6)	<i>IL1A</i> (5.1)
<i>TANK</i> (7.0)	<i>TANK</i> (2.1)	<i>TANK</i> (3.4)
<i>TNF</i> (6.3)		<i>TNF</i> (3.4)
<i>SELE</i> (4.9)		<i>SELE</i> (6.6)
<i>CSF2</i> (3.3)		<i>CSF2</i> (3.3)
<i>BIRC3</i> (3.4)		<i>BIRC3</i> (3.1)
<i>HSPB1</i> (2.4)		
<i>MMP7</i> (2.4)		

Abbreviations: *CCL20*, Chemokine (C-C motif) ligand 20; *IL8*, interleukin 8; *ICAM1*, intercellular adhesion molecule 1; *IL1A*, interleukin-1 alpha; *TANK*, TRAF family member-associated NF- $\kappa$ B activator; *TNF*, tumor necrosis factor; *SELE*, selectin E; *CSF2*, colony stimulating factor 2 (granulocyte-macrophage); *BIRC3*, baculoviral IAP repeat containing 3; *HSPB1*, heat shock 27 kDa protein 1; and *MMP7*, matrix metalloproteinase 7 (matrilysin, uterine).

mutant to intestinal cells. However, we found that the double *lpf* mutant adhered similarly to the wild-type strain, in contrast to the reduced adherence observed with the *lpfA1* and *lpfA2* single mutants (Fig. 1a). We obtained similar findings when we evaluated the ability of these strains to bind to fibronectin. However, no significant reduction was observed in the binding to fibronectin with the *lpfA1 lpfA2* double mutant when we compared these findings with those in the wild-type strain (Fig. 1b). An explanation of the lack of reduction may be that other STEC adhesins could be up-regulated in the *lpfA1 lpfA2* double mutant (Lloyd et al., 2012). Recently, transmission electronic microscopy analyses on the surface of the *lpfA1 lpfA2* double mutant revealed the presence of curli-like structures. Adherence analyses to T84 cells by using an *lpfA1 lpfA2 csgA* (*CsgA*-coding gene; major constituent of curli) triple mutant revealed that this strain showed a reduced, but not significant binding to T84 cells compared to the wild-type strain (Lloyd et al., 2012). Considering that curli have been implicated in the induction of pro-inflammatory cytokines (Bian et al., 2000), the role of these organelles in the inflammation response induced by Lpf deserves future investigation.

In the case of signaling mechanisms, the MAPK, AP-1 and NF- $\kappa$ B pathways are activated in intestinal cells after STEC infection (Dahan et al., 2002; Miyamoto et al., 2006). Analysis of key genes from 18 different signal transduction pathways showed that several genes of the NF- $\kappa$ B signaling pathway, such as *CCL20*, *IL-8*, *ICAM1*, *IL1A*, *TANK*, *TNF* and *BIRC3*, are up-regulated in cells infected with the wild-type strain when compared to results in cells that are uninfected or infected with the *lpfA1 lpfA2* double mutant (Table 3). All of the pro-inflammatory markers that are up-regulated in cells infected with the wild-type strain play key roles in host responses to pathogens. IL-1 $\alpha$  (a cell-associated pro-inflammatory cytokine), TNF- $\alpha$  (a pro-inflammatory cytokine playing a crucial role in the inflammatory response induced by pathogens), IL-8 (a pro-inflammatory chemokine), BIRC3 (regulator of NF- $\kappa$ B and TNF- $\alpha$  with anti-apoptotic activity), TANK (activator of the NF- $\kappa$ B pathway), SELE (a soluble leukocyte adhesion molecule), HSPB1 (a protein involved in stress resistance and actin organization), and MMP7 (a metalloproteinase that participates in the host cell responses to infection).

Among these up-regulated genes, high expression levels (>10 fold) were observed for *IL8*, *ICAM1* and *CCL20* genes, all of which were dramatically reduced in cells infected with the *lpfA1 lpfA2* double mutant or uninfected cells. We have previously reported complementation studies of the mutant strains (Torres et al., 2002, 2004), and decided not to include them in this study because we have noticed that expression of the complemented Lpf causes an aggregation of bacterial cells, which might interfere with the interpretation of the results in the current study.

Intercellular adhesion molecule-1 (ICAM-1 or CD54) is a surface molecule that mediates leukocyte adhesion to epithelial cells. Expression of ICAM-1 on epithelial cells is up-regulated by several stimuli, including inflammatory cytokines and bacterial infection, and it is a reliable marker of epithelial inflammation and an inducer of neutrophil recruitment (Coburn et al., 2005). On the other hand, the chemokine ligand 20 (*CCL20*) is responsible for the chemo-attraction of immature dendritic cells and also recruitment of memory T lymphocytes (Rodriguez-Bores et al., 2007). *CCL20* expression in intestinal epithelial cells is increased by a variety of inflammatory stimuli, as well as enteropathogenic bacteria (Sierro et al., 2001). Considering these previously published data, it is plausible to propose that Lpf fimbriae are playing a role in the inflammatory activity by inducing neutrophil recruitment to the site of infection in response to IL-8, ICAM-1 and *CCL20*. In contrast, it has been shown that *E. coli* O157:H7 cytotoxins, particularly Stx1, inhibit the proliferation of bovine lymphocytes in vitro and influence STEC persistence in calves, which may mean that bacterial suppression of mucosal inflammation is important in vivo during colonization of their animal reservoir (Menge et al., 2004).

Mucosal inflammation in human intestinal epithelial cells induced by STEC is a multifactorial process, in which several pro-inflammatory and anti-inflammatory factors are involved. STEC induce the activation of pro-inflammatory cascades at an early stage during infection; however, some studies postulate that the inflammatory response may be repressed at later stages to prevent a host immune response against STEC, by interfering with the NF- $\kappa$ B pathway (Hauf and Chakraborty, 2003; Yen et al., 2010). Considering that STEC possesses multiple factors

that activate the host pro-inflammatory effect by modulating the NF- $\kappa$ B pathway, it would be interesting to determine whether activation of this pathway is affected by STEC anti-inflammatory effectors.

In this report, we described a STEC virulence factor, the Lpf fimbriae, as one more player in the induction of host pro-inflammatory cytokines. Taken together, the data relative to the role of Lpf fimbriae in STEC adherence to intestinal cells and as an inflammatory-inducing factor indicate that these fimbriae participate in STEC pathogenesis in more than one way.

### Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

### Acknowledgements

This work was supported by grant FONDECYT 11080180 and 1120809 to M.J.F, FONDECYT 1110260 to R.V., and NIH/NIAID 5R01AI079154 to A.G.T. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIAID or NIH.

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