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Escherichia coli LF82 Differentially Regulates ROS Production and Mucin Expression in Intestinal Epithelial T84 Cells: Implication of NOX1

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Background: Increased reactive oxygen species (ROS) production is associated with inflamed ileal lesions in Crohn's disease colonized by pathogenic adherent-invasive *Escherichia coli* LF82. We investigated whether such ileal bacteria can modulate ROS production by epithelial cells, thus impacting on inflammation and mucin expression.

Methods: Ileal bacteria from patients with Crohn's disease were incubated with cultured epithelial T84 cells, and ROS production was assayed using the luminol-amplified chemiluminescence method. The gentamicin protection assay was used for bacterial invasion of T84 cell. The expression of NADPH oxidase (NOX) subunits, mucin, and IL-8 was analyzed by quantitative real-time PCR and Western blots. Involvement of NOX and ROS was analyzed using diphenyleneiodonium (DPI) and N-acetylcysteine (NAC).

Results: Among different bacteria tested, only LF82 induced an increase of ROS production by T84 cells in a dose-dependent manner. This response was inhibited by DPI and NAC. Heat- or ethanol-attenuated LF82 bacteria and the mutant LF82Δ*FimA*, which does not express pili type 1 and poorly adheres to epithelial cells, did not induce the oxidative response. The LF82-induced oxidative response coincides with its invasion in T84 cells, and both processes were inhibited by DPI. Also, we observed an increased expression of NOX1 and NOXO1 in response to LF82 bacteria versus the mutant LF82Δ*FimA*. Furthermore, LF82 inhibited mucin gene expression (MUC2 and MUC5AC) in T84 cells while increasing the chemotactic IL-8 expression, both in a DPI-sensitive manner.

Conclusions: Adherent-invasive *E. coli* LF82 induced ROS production by intestinal NADPH oxidase and altered mucin and IL-8 expression, leading to perpetuation of inflammatory lesions in Crohn's disease.

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Key Words: Crohn's disease, NADPH oxidase, *Escherichia coli* AIEC LF82, intestinal microbiota, reactive oxygen species

Crohn's disease (CD) and ulcerative colitis are chronic inflammatory bowel diseases (IBDs) with a growing incidence in Western countries. They are complex diseases in which prolonged and abnormal intestinal immune response occurs because of environmental and microbial factors in genetically predisposed individuals.¹ There is mounting clinical and experimental

evidence that intestinal bacteria play an active role in the onset and perpetuation of IBD.^{2,3} The diversity of gut microbiota is low in patients with IBD with a decrease in beneficial bacterial groups and an increase in potentially pathogenic bacteria.^{4,5} For example, *Faecalibacterium prausnitzii* is decreased, while *Escherichia coli* is increased in the mucosa of the ileum of patients with CD.⁶ A protective effect of *F. prausnitzii* has been shown in experimental colitis.⁷ In contrast, some strains of *E. coli*, the major gram-negative species in healthy intestine, have acquired virulence factors, are highly adherent and invasive, and are termed adherent-invasive *E. coli* (AIEC).^{8,9} The role of AIEC in the process of chronic inflammation has been well studied in the gut for the prototype AIEC strain LF82.¹⁰ Adhesion depends on type 1 pili of the bacteria that binds to cell adhesion molecule 6 related to carcinoembryonic antigen (CEACAM6) present on the surface of epithelial cells.¹¹ LF82 invades cultured intestinal epithelial cells and survives extensively in macrophages.¹² It also promotes proinflammatory cytokines and enhances chemically induced colitis in mice.¹³

The AIEC strain LF82 has been shown to be relatively resistant to reactive oxygen species (ROS),¹⁴ which represent a common antimicrobial mediator produced by NADPH oxidase

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of macrophages (NOX2) in innate immunity.¹⁵ ROS have been implicated in intestinal oxidative damage in IBD,¹⁶ but they can also act as a second key message in multiple signaling pathways, in response to cytokines, growth factors, or bacteria.¹⁷ For example, ROS production can induce the renewal of the intestinal epithelium in *Drosophila* during bacterial infection of the intestine.¹⁸ Intestinal epithelial cells express high level of NADPH oxidase isoform, NOX1, and to a lesser extent DUOX2, and these isoforms may locally produce ROS.^{19–21} The cytokine IL-10, by reducing ROS generated by NOX1 in response to interferon gamma and tumor necrosis factor alpha, could protect against spontaneous colitis in mice.²² The role of NOX1/DUOX2 in IBD has been strengthened by recent data showing that NOX1 is responsible for ileocolitis in mice²³ and that an abnormal increase of DUOX2 expression was detected in association with an expansion of Proteobacteria in both ulcerative colitis and CD.²⁴

Changes in intestinal mucosal barrier or inflammatory lesions in CD can allow bacteria to modulate epithelial ROS production. In this context, we investigated whether AIEC strain LF82 and other bacteria isolated from inflamed ileal lesions of patients with CD were able to modulate ROS production of cultured intestinal epithelial T84 cells. We showed that LF82 induced an increase of ROS production by T84 cells during its process of invasion. This was also accompanied by an increase of NOX1 expression and its regulatory subunit, NOXO1. In parallel, expression of MUC2 and MUC5AC decreased, whereas the expression of IL-8 increased in T84 cells. The data and the observed sensitivity of these events to DPI, a flavoprotein inhibitor, suggest that the host epithelial ROS/NOX1 contribute to LF82 pathogenicity.

MATERIALS AND METHODS

Culture of T84

T84 cells, derived from human colorectal carcinoma, were cultured in an atmosphere containing 5% CO₂ at 37°C in DMEM/HAM'S F-12 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin). Cells were maintained in 25-cm² flasks (T_{PP}). The intestinal epithelial cells were seeded at a density of 500,000 cells per well in 24-well plates and cultured for 48 hours before use.

Bacterial Strains

We studied bacteria *E. coli* strains (AIEC LF82, noninvasive Y54-1, nonenteropathogenic) and *Klebsiella oxytoca*, which were isolated from a chronic ileal lesion of a patient with CD as previously described.¹⁰ The LF82Δ*FimA* mutant harboring Tn5*phoA* transposon in *fimA* that does not synthesize type 1 pili was a kind gift of A. Darfeuille-Michaud (Université d'Auvergne, Clermont-Ferrand, France). Bacteria were cultured in LB media at 37°C for 18 hours in a shaking incubator (180 RPM). The multiplicity of infection used for most experiments was 14:1.

Coculturing of T84 Cells with Bacteria and Measurement of ROS Production

T84 cells were seeded at a density of 500,000 cells per well in 24-well plates for 48 hours. Overnight-cultured bacteria were resuspended in Hank's medium at 10⁷ CFU/mL and cocultured with T84 cells at 37°C under microaerobic conditions (5% O₂, 5% CO₂). ROS production was measured by the luminol-enhanced chemiluminescence method in 1 mL of Hank's assay buffer containing 10 μM luminol and 2.5 U/mL horseradish peroxidase. In some assays, diphenyleneiodonium (DPI), a flavoprotein inhibitor, or N-acetylcysteine (NAC), a ROS scavenger, was added as indicated in figure legends. Cells were only preincubated for 15 minutes at 37°C with DPI (2.5 μM) and washed, while NAC (1 mM) was maintained throughout the incubation. Also, ROS generation was measured as superoxide dismutase-inhibitable reduction of cytochrome *c* at 550 nm in a dual-beam recording Uvikon 860 spectrophotometer. To this end, T84 cells were similarly seeded and incubated as above with cytochrome *c* (100 μM final concentration) in the presence or absence of superoxide dismutase (75 U) and LF82 *E. coli* strain (10⁷ CFU/mL). After an incubation of 3 hours, cells were eliminated by centrifugation at 600g, the supernatant was recovered, and total superoxide production was then measured as an endpoint reduction of cytochrome *c* at 550 nm.

Bacterial Invasion in T84 Cells

Bacterial invasion in T84 cells was performed using the gentamicin protection assay as described by Darfeuille-Michaud et al.⁹ Cells were seeded in 24-well plates at a density of 500,000 cells per well and incubated for 48 hours. T84 cells were incubated with bacteria (10⁷ CFU) in 1 mL of Hank's buffer for 3 hours. In each condition studied, absence or presence of DPI (2.5 μM) and NAC (1 mM), we checked the bacterial number outside the cells and the viability of T84 cells (Lactic Dehydrogenase Assay; Sigma Aldrich, Lyon, France) and found no significant differences (data not shown). After this infection period of 3 hours at 37°C, T84 cells were washed 3 times in phosphate-buffered saline (pH 7.2). Then, 100 μg/mL gentamicin (Sigma) was added over 1 hour to remove extracellular bacteria. To determine the number of intracellular bacteria, the epithelial cells were then lysed with 1% Triton X-100 (Sigma) in deionized water. The samples were diluted, plated on agar plates, and incubated overnight at 37°C. The number of colonies in each agar plate corresponds to the number of intracellular bacteria.

Messenger RNA Quantification

Total RNA was extracted from T84 cells using the NucleoSpin RNA kit (Macherey-Nagel, Hoerd, France) according to the manufacturer's protocol and stored at -70°C until use. A quantitative real-time PCR (qPCR) was used to determine relative messenger RNA (mRNA) levels of the gene of interest. Complementary DNA was synthesized from 50 ng of total mRNA using OligodT and M-MLV reverse transcriptase (Invitrogen, Cergy-Pontoise, France). The following sequence of DNA oligos—primers for qPCR were used: NOX1 forward TAGGCGCCCTAAGTTTGAAG,

NOX1 reverse AAACCGGAGGATCCTTTTCAC; NOX1 forward AGATCAAGAGGCTCCAAACG, NOXO1 reverse GGAA GGTCTCCTTGAGGGTCT; HPRT forward GTTATGGCGA CCCGACG, HPRT reverse ACCCTTCCAAATCCTCAGC; MUC2 forward ACTGGGAGTGC GACTGCT, MUC2 reverse CCTCCACCAGCACGTAGG; MUC5AC forward GTCCCTTCAATATTCCACCTC, MUC5AC reverse GCCCAGTCTCTCACCTTTCTT; IL-8 forward TGGCTCTCTTGGCAGCCTTC, IL-8 reverse GTTCTTTAGCACTCCTTGGC. All real-time PCR reactions were performed using the Roche LightCycler 480 device and the CliniSciences Kapa Sybr Fast qPCR kit according to the manufacturer's protocol. Samples were run in duplicate, and the melting curve and melting peak were controlled for each primer pair. Relative expression levels for each gene were calculated using the $2^{-\Delta\Delta Ct}$ method, with normalization to HPRT. Results are representative of 3 to 4 independent experiments.

Western Blot Analysis

T84 cells were incubated in Hank's buffer for 3 hours at 37°C, with or without bacteria as described above. Then, cells were washed 3 times in phosphate-buffered saline (pH 7.4) on ice, and lysis was performed as described previously.²⁵ The lysis buffer contained 20 mM Tris-HCl, pH 7.4; 0.5% Triton X-100; 150 mM NaCl; 2.5 mM EGTA; 2.5 mM EDTA; 10 µg/mL leupeptin; 10 µg/mL pepstatin; 10 µg/mL aprotinin; 2 mM PMSF; 1 mg/mL NaF; 1 mg/mL Na₃VO₄ 0.5 mg/mL glycerophosphate; 0.5 mg/mL p-nitrophenyl phosphate (pNPP); 0.2 mg/mL levamisole; and 8% sucrose in the presence of 1 mg/mL DNase I and 2.5 mM DFP. The suspension was sonicated on ice 3 times for 15 seconds each. The lysate was centrifuged at 100,000g for 20 minutes at 4°C in a TL-100 Ultracentrifuge (Beckman Coulter, Villepinte, France), and the recovered supernatant was complemented with 5x Laemmli buffer, boiled, and subjected to Western blot analysis with the laboratory's anti-NOXO1 antibody (1:2000) and HRP-labeled goat anti-rabbit antibodies (1:30,000). The blots were revealed using enhanced chemiluminescence.

Statistical Analysis

The Prism graphing and analysis program was used for calculation of statistical measures including mean values, 1-way analysis of variance with the Tukey–Kramer's post hoc test for multiple comparisons.

RESULTS

Effect of Bacteria on T84 ROS Production

To study the effect of the *E. coli* strain AIEC LF82 on ROS production by the intestinal epithelial cell line T84, either cells or bacteria were incubated alone or in combination in the presence of luminol and peroxidase at 37°C, and chemiluminescence was measured over time in a thermostated chemiluminometer. The results in Figure 1A show that T84 cells alone or the bacteria alone produced a minimal amount of ROS as measured by luminol-amplified chemiluminescence. Interestingly, a dramatic

increase of ROS production occurred only when LF82 was incubated with T84 cells. This was confirmed by further experiments and quantification of the chemiluminescence signals from several experiments as represented in Figure 1B. In similar conditions, different *E. coli* strains and *K. oxytoca* isolated from patients with CD were tested for ROS production, and clearly, the strain *E. coli* AIEC LF82 was the most effective, Figure 1C. Also, the increase in ROS production by LF82 was dose-dependent as shown in Figure 1D. Furthermore, the use of the superoxide dismutase–inhibitable cytochrome *c* reduction assay showed that LF82 (10⁷ CFU/mL) induced extracellular superoxide production when incubated with T84 cells (Fig. 1E).

Intact Pili of LF82 Is Required for Inducing ROS Response

To investigate whether viable LF82 was important for its interaction with T84 intestinal epithelial cells, we used heat- or ethanol-attenuated bacteria. The results in Figure 2A indicate that heat- or ethanol-attenuated bacteria were ineffective and that only living LF82 were able to induce ROS production by T84 cells, suggesting that an intact extrabacterial component is required to fulfill this effect. Thus, we questioned whether type 1 pili, which is essential for LF82 adhesion, might be involved in the ROS response.²⁶ The mutant LF82Δ*FimA*, without pili, known to have impaired ability to adhere to and invade epithelial cells, showed no significant effect on T84 intestinal cell ROS production as compared with wild-type (WT) LF82, Figure 2B. Taken together, the data suggest that there is a close interrelationship between host ROS production and bacterial adhesion/invasion.

N-acetylcysteine and Diphenylethylideneiodonium Inhibit LF82-induced ROS Response

First, to validate ROS response in T84 intestinal epithelial cells exposed to LF82, we used NAC (1 mM), a scavenger of ROS. Second, to investigate the source of ROS, we used DPI (2.5 µM), an inhibitor of flavoenzymes such as NADPH oxidases (NOX). The results in Figure 3 show that NAC and DPI prevented ROS production by T84 cells in the presence of LF82. This suggests the likely involvement of NADPH oxidase as the ROS-generating system.

LF82 Increases NOX1 and NOXO1 Expression in T84 Cells

The ROS-generating NOX1 complex is highly expressed in intestinal epithelial cells as compared with DUOX2.²⁶ NOX1 is associated with NOXO1 and NOXA1, and both subunits are crucial for the ROS production by NOX1. We evaluated the mRNA expression of DUOX2, NOX1, NOXO1, and NOXA1 by qPCR in T84 cells treated with WT LF82 bacteria or mutant LF82Δ*FimA*. The expression of DUOX2 and NOXA1 was inconsistently variable in the presence or absence of bacteria (data not shown), whereas NOX1 and NOXO1 expression was significantly upregulated by WT LF82 as shown in Figure 4A and 4B, respectively. LF82 increased NOX1 expression significantly at 2 hours as

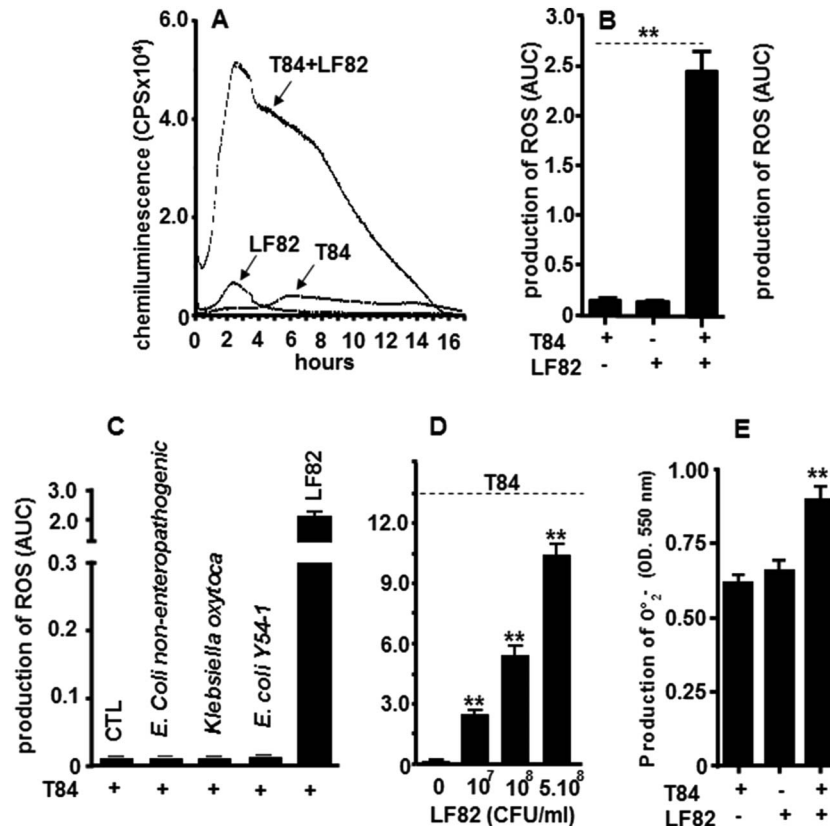


FIGURE 1. Effect of bacteria on ROS production by T84. Cultured T84 cells were incubated at 37°C in the absence or the presence of different bacterial strains, and ROS production was measured by chemiluminescence method as described in Methods section. A, Kinetics of chemiluminescence detected in the presence of LF82 *E. coli* strain (10⁷ CFU/mL) alone or T84 alone and T84 in combination with LF82 are shown. B, The areas under the corresponding curves are represented. C, Effect of other bacteria strains (10⁷ CFU/mL) isolated from ileal lesions of patients with CD on ROS production by T84. D, ROS production of T84 cells in response to different doses of *E. coli* LF82 was further studied. E, T84 cells were incubated with LF82 *E. coli* strain (10⁷ CFU/mL), and superoxide anion production was assayed by spectrophotometric analysis of reduced cytochrome *c* at 550 nm as described in Methods section. Results are means \pm SEM of 3 separate experiments (***P* < 0.01 versus control [CTL] or T84 samples without bacteria).

compared with the control without bacteria, and mutant LF82 Δ *FimA* was ineffective. Also, the expression of NOXO1 was significantly increased within 2 and 3 hours of incubation with the WT LF82 bacteria versus the control in Figure 4B. No significant effect was observed with the mutant LF82 Δ *FimA*. The antibody directed against NOXO1 developed in our laboratory²⁵ allowed us to perform Western blot analysis of T84 cell extracts. A clear increase in NOXO1 expression was detected when T84 cells were incubated with LF82 versus the mutant LF82 Δ *FimA* (Fig. 4C). The ability of WT AIEC LF82 to increase NOX1 and NOXO1 expression suggests that NOX1 is involved in AIEC LF82–induced ROS production by T84 cells.

DPI or NAC Inhibits the Invasion of LF82 in T84 Epithelial Cells

The observed peak of ROS production occurred at 3 hours, which corresponds to the time of invasion in T84 cells. Thus, we investigated whether DPI, an inhibitor of NOX, could affect the invasion of T84 cells by LF82. To this end, we used the adapted

technique described by Darfeuille-Michaud et al⁹ to study invasion of WT LF82 bacteria. T84 cells or the bacteria were preincubated in the presence or the absence of DPI (2.5 μ M) before performing the invasion assay as described in Materials and Methods section. The effect of NAC (1 mM) was also tested. The data in Figure 5A show that LF82 was invasive as compared with the LF82 Δ *FimA* mutant, which was noninvasive in accordance with a previous study.²⁷ When T84 cells were treated with DPI or NAC, LF82 invasion was inhibited, suggesting a NOX-mediated process. Furthermore, DPI or NAC was effective only when T84 cells rather than LF82 were incubated with these compounds, Figure 5B.

LF82 Inhibits MUC2 and MUC5AC and Upregulates IL-8 Expression in T84 Cells in a ROS-dependent Manner

The interaction between bacteria of the intestinal flora and intestinal epithelial cells is important as this may modulate cytokine secretion and the mucous layer, which represent the

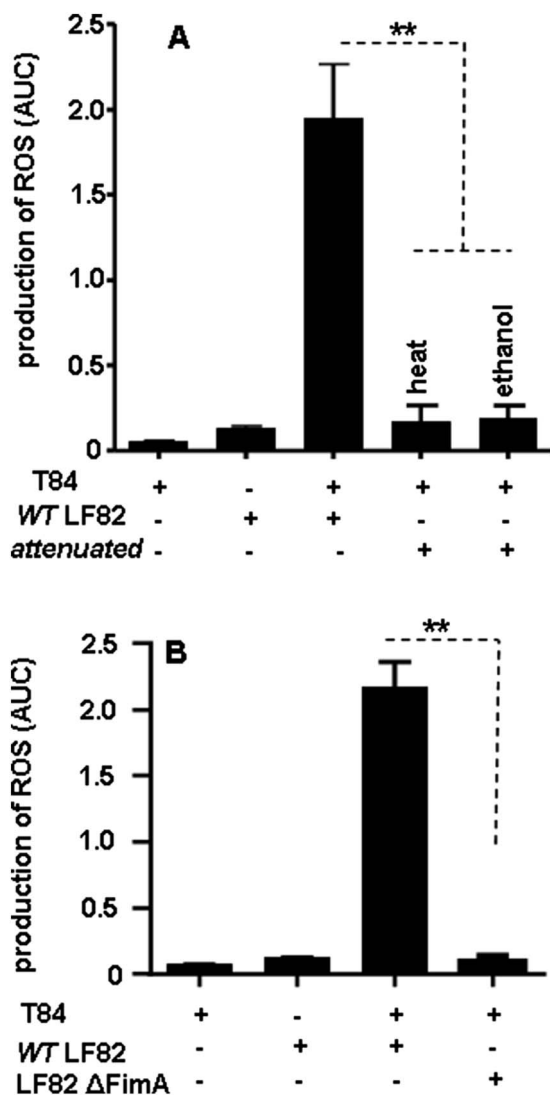


FIGURE 2. Effect of modified LF82 on T84 cell ROS production. A, ROS production by T84 cells was measured when incubated at 37°C alone and in the presence of untreated WT LF82 (10⁷ CFU) or WT LF82 treated with heat or ethanol. B, ROS production by T84 cells incubated with WT LF82 or LF82 Δ FimA, a deletion mutant that has an impaired ability to adhere to and invade epithelial cells, was compared. The results are expressed as corresponding area under the curve and are means \pm SEM of 3 separate experiments (***P* < 0.01).

first line of host defense.²⁸ We evaluated whether the mRNA expression of MUC2 and MUC5AC in T84 cells was modulated by LF82. The results in Figure 6 show that after 3 hours of incubation, LF82 significantly inhibited MUC5AC mRNA expression (Fig. 6B) as compared with untreated cells and showed a tendency (*P* = 0.06) toward diminishing MUC2 expression (Fig. 6A). Interestingly, these effects were abrogated in the presence of DPI. Similarly, we studied whether the T84 cell expression of IL-8, a powerful chemokine, could be modulated by LF82. Data in Figure 6C indicate that LF82 induced a 10-fold increase in

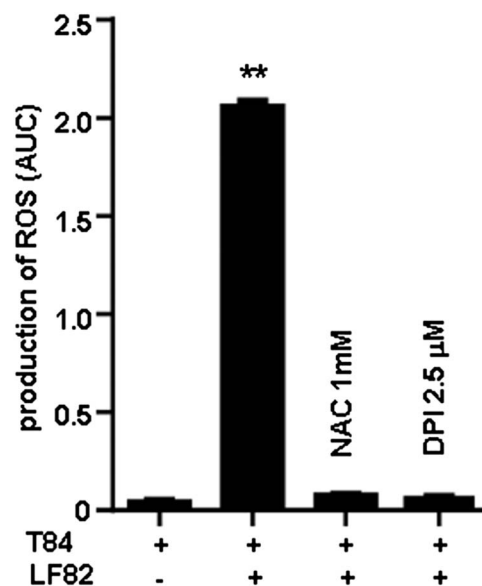


FIGURE 3. Effect of NAC or DPI on T84 ROS response induced by LF82. T84 cells were incubated with a ROS scavenger N-acetylcysteine or NAC (1 mM), and the ROS production was measured as described in Methods section. Also, T84 cells were incubated for 15 minutes at 37°C with the diphenyleioidonium or DPI (2.5 μ M), a flavoenzyme inhibitor before assaying ROS production. The response is expressed as corresponding area under the curve and represents means \pm SEM of 3 separate experiments (***P* < 0.01 versus T84 alone).

the expression of IL-8 as compared with control values, and this positive response was inhibited by DPI. As with other studies above performed with DPI, the number of bacteria and the T84 cell viability were not different from control values (absence of DPI, data not shown). These results suggest that LF82 inhibits MUC2 and MUC5AC expression while increasing IL-8 expression in T84 cells, in a ROS-dependent manner.

DISCUSSION

Mucosal flora has been shown to play a key role in the development and the perpetuation of inflammatory process in IBD in adult and pediatric patients.^{2,24} The initial step in the pathogenicity of many bacteria is their adhesion to epithelial cells, followed by colonization of the gut. AIEC, which abundantly colonizes ileal mucosa of patients with CD, induces persistent inflammation that can breach mucosal barrier.^{29,30} Such tissue lesions can be promoted by a local ROS production by NADPH oxidase (NOX1/DUOX) in intestinal mucosa of patients with IBD.^{17,31} In this context, we investigated the effect of different bacteria isolated from ileal lesions of patients with CD on ROS production by cultured epithelial T84 cells.

Our results showed that among strains issued from biopsies of patients with CD, only bacteria *E. coli* AIEC LF82 induced an increase in ROS production by the T84 cells. This *E. coli* adheres and invades intestinal cells and is able to survive and multiply in macrophages without inducing cell death.¹³ In our model, the

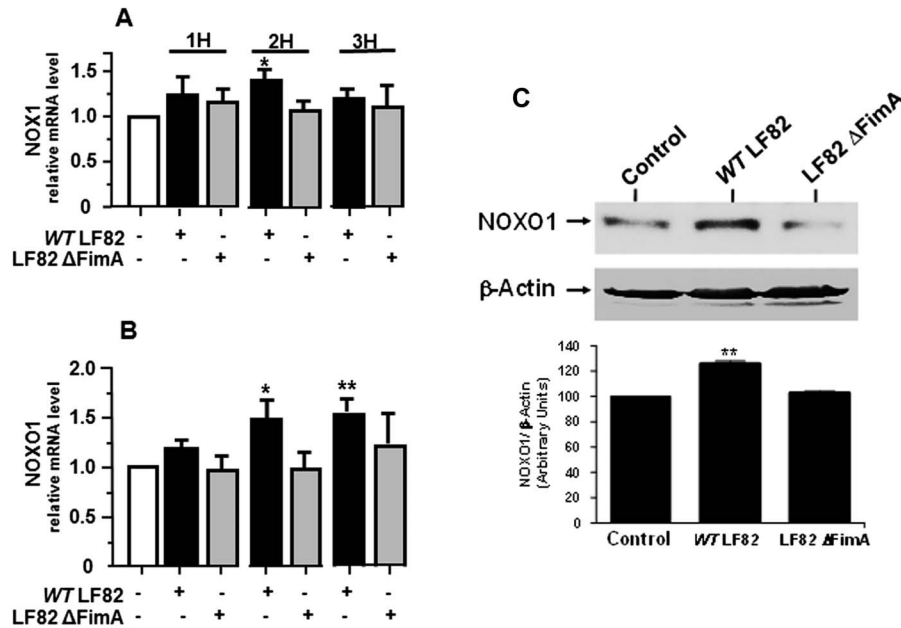


FIGURE 4. Effect of LF82 on NOX1 and NOXO1 expression. T84 cells were incubated at 37°C alone (CTL) and in the presence of WT LF82 (black columns) or LF82Δ*FimA* deletion mutant (gray columns) (10⁷ CFU/mL). After 1, 2, and 3 hours, mRNA was extracted using a NucleoSpin RNA kit, and qPCR was performed as described in Methods section. The gene expression of NOX1 in A and NOXO1 in B is relative to HRPT (internal control) and is represented in arbitrary units. LF82 was effective in regulating the studied gene expression in contrast to LF82Δ*FimA* (**P* < 0.05, ***P* < 0.01 versus CTL [white], n = 3, ± SEM values). C, A representative Western blot analysis of proteins extracted from T84 cells incubated alone or with LF82 or LF82Δ*FimA* during 3 hours is shown. Samples were analyzed with a NOXO1-specific antibody developed in our laboratory.²⁵ The corresponding mean densitometric quantification of 3 Western blot autoradiographs is shown below. A significant ***P* < 0.01 versus CTL (white) increase of NOXO1 expression is observed with WT LF82. All the above experiments were performed at least 3 times.

strong response of ROS reaches its maximum after 3 hours, which coincides with the invasion of the bacteria in T84 cells.⁹ We showed that increased ROS production was dependent on the amount of LF82 bacteria. Also, another ROS indicator method based on the specific reduction of cytochrome *c* was used. It showed that T84 cells produced an increased amount of superoxide anion in the milieu in response to LF82. Furthermore, the action of LF82 was inhibited by heat or ethanol treatment, suggesting a direct contact between an intact bacterial component with the host rather than diffusible factors for ROS production. In agreement, the mutant LF82Δ*FimA*, which does not express type 1 pili and has consequently lost the ability to adhere and invade epithelial,²⁷ did not induce ROS response by T84 cells. Taken together, these results suggest that intestinal ROS response to WT bacteria AIEC could be involved in the process of its adherence and invasion.

The ROS produced by T84 cell in response to LF82 were scavenged by N-acetylcysteine, a potent antioxidant. Furthermore, the pretreatment of T84 cells with flavoenzyme inhibitor diphenyleneiodonium also blunted the ROS production and invasion of AIEC LF82 in T84 cells. This suggests the involvement of NADPH oxidases, especially NOX1 and DUOX2, which are ROS-generating multicomponent isoforms expressed in intestinal epithelial cells.^{21,32} Among these isoforms, we found that LF82 consistently upregulated the mRNA expression of NOX1 and its

regulatory component NADPH oxidase organizer 1 (NOXO1) in T84 cells. The use of our specific NOXO1 antibody in Western blot analysis revealed the upregulation of NOXO1 protein in response to LF82 versus LF82Δ*FimA*. Increased transcription of NOXO1 in T84 epithelial cells has been implicated with increased ROS production.³³ This result may partly explain the observed increase of ROS production. Indeed, other mechanisms such as the phosphorylation of the regulatory proteins NOXO1 and NOXA1 and the activation of Rac1 GTPase may participate in the ROS increase.^{25,34} Taken together, the data are consistent with a role of NOX1 in the ROS response by T84 cells during its invasion by LF82.

Similarly, another gram-negative bacterium, *Campylobacter jejuni* has been shown to induce ROS production through upregulation of NOX1 and NOXO1 expression upon invasion of intestinal epithelial HCT-8 cells.³⁵ The resulting epithelium-derived H₂O₂ was beneficial to the host as it impaired the bacterial capsule formation, thus its virulence. Unlike *C. jejuni* and LF82, which require direct contact with the host cell, *Helicobacter pylori* (gram-negative) increases the production of O₂⁻ in gastric mucosa of rodents through diffusible lipopolysaccharide (LPS) and LPS/TLR4 interaction. This ROS production coincided with increased mRNA expression of NOX1, NOXO1, and Rac1 protein. The amount of O₂⁻ produced by gastric epithelial colonic cells is not sufficient enough to kill *H. pylori* in vitro. However,

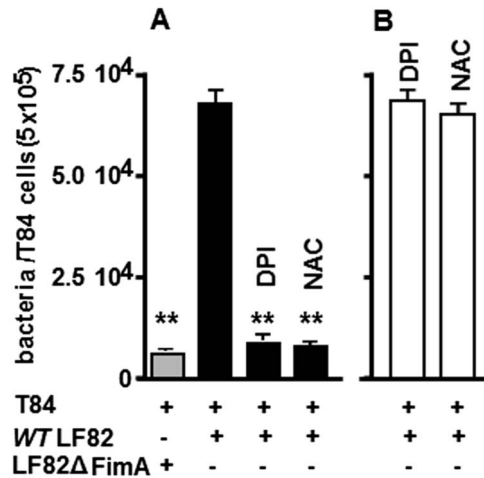


FIGURE 5. Effect of DPI or NAC on the invasion of T84 cells by LF82. The bacterial invasion study was performed as described previously⁹ in Methods section. Briefly, cultured T84 cells were incubated with either WT LF82 or LF82ΔFimA deletion mutant at a multiplicity of infection of 14 bacteria per epithelial cell for 3 hours at 37°C. After eliminating extracellular bacteria with gentamicin, the T84 cells were lysed and samples were plated on to Mueller-Hinton agar plates to determine the number of CFU per wells. The data are expressed as CFU/5 × 10⁵ T84 cells. A, The results indicate effective invasion by LF82 (black) as opposed to LF82ΔFimA (gray). Furthermore, when T84 cells were pretreated with DPI (2.5 μM) or NAC (1 mM), the invasion by LF82 was blunted. B, In contrast, incubation of LF82 with DPI or NAC did not affect the invasion. Results are means ± SEM of 3 separate experiments (**P < 0.01).

NOX1 represents a key molecule for initiating the innate and inflammatory responses against microbial pathogens.³⁶ LF82 also induces innate immune response by stimulating a strong secretion of IL-8 and tumor necrosis factor alpha by cultured intestinal epithelial Caco-2 and T84 cells.^{30,37} In coculturing experiment, LF82-treated epithelial cells induced migration of neutrophils and dendritic cells.³⁰ In agreement with these studies, we showed that LF82 induced a robust upregulation of IL-8 mRNA expression. This response was blunted by DPI treatment, suggesting an involvement of NOX1 in maintaining low-grade chronic inflammation observed in CD.

To further characterize additional factors that contribute to LF82 colonization of ileal lesion of patients with CD, we investigated whether LF82 could regulate mucin gene (MUC2, MUC5AC) in cultured T84 cells. The mucus layers acts as a physical barrier to exclude bacteria from the epithelium, and mucus defect will allow bacteria to gain entry in deeper intestinal tissues and trigger inflammation.³⁸ MUC2 is the main mucin component, and MUC2-deficient mice spontaneously develop colitis.³⁹ Low MUC2 mRNA levels have been observed in patients with IBD.⁴⁰ In our study, we observed that LF82 tended to inhibit the mRNA expression of MUC2 and MUC5AC in cultured T84 epithelial cells. This is in agreement with recent data showing that transgenic mice (CEBAC10) fed on high fat and high sugar

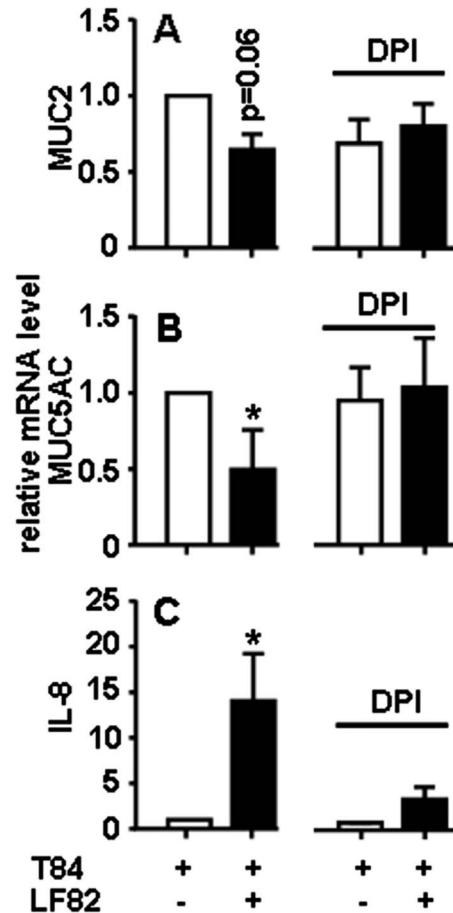


FIGURE 6. Effect of LF82 on mucin and IL-8 expression. T84 cells pretreated or not with DPI (2.5 μM) were incubated at 37°C alone and in the presence of WT LF82 (10⁷ CFU/mL). After 3 hours, RNA was extracted using a NucleoSpin RNA kit, and qPCR was performed as described in Methods section. The gene expression of MUC2 (A), MUC5AC (B), and IL-8 (C) is relative to HRPT (internal control), and values are normalized to CTL values or T84 samples without bacteria and are represented in arbitrary units. Data indicate that LF82 lowers the expression of mucin while increasing IL-8 as compared with CTL (white bar). Results are means ± SEM of 4 separate experiments (*P < 0.05 versus CTL, the P value for MUC2 versus CTL was 0.06). The above negative and positives responses induced by LF82 were diminished when T84 cells were pretreated with DPI. However, the use of NAC was omitted as it was found to significantly diminish the mucin gene expression by itself.

diet allowed AIEC bacteria to better colonize gut mucosa with an associated decreased of MUC2 expression.⁴¹ Other gram-negative bacteria such as *H. pylori* can also lower MUC2 and MUC5AC gene expression, while involving different cellular mechanisms.⁴² In our in vitro study, DPI was able to lower the effect of LF82 to inhibit mucin gene expression of T84 cells, suggesting the role of ROS and NOX1. This is in contrast with ROS upregulating MUC5AC mucin expression in human airway epithelial cells by *Pseudomonas aeruginosa* lipopolysaccharide.⁴³ Differences may be due to airway or intestinal cell-specific activity, and those

dynamic changes in ROS can have varied activities on the involved regulatory virulence factors or secondary signals including NF- κ B.⁴⁴ However, our results are in line with *in vivo* data suggesting a negative control of mucin genes in intestinal epithelium by AIEC colonization.⁴¹

In conclusion, our results indicate that *E. coli* AIEC, strain LF82, can disrupt the redox state of the intestinal mucosa by an increase in ROS. This may be generated by NOX1, leading to a less effective mucus barrier and a local increased amount of IL-8 for recruiting phagocytes. These data further extend the characteristics of AIEC LF82 strain that may perpetuate chronic inflammation observed in CD.

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