Host Inflammatory Response Inhibits *Escherichia coli* O157:H7 Adhesion to Gut Epithelium through Augmentation of Mucin Expression

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Escherichia coli O157:H7, a major Shiga toxin-producing pathogen, has a low infectious dose and causes serious illness in humans. The gastrointestinal tract of cattle is the primary reservoir of *E. coli* O157:H7, and thus, it is critical to eliminate or reduce *E. coli* O157:H7 gut colonization. Given that *E. coli* O157:H7 produces effectors that attenuate inflammatory signaling, we hypothesized that the host inflammatory response acts to perturb *E. coli* O157:H7 intestinal colonization. Tumor necrosis factor alpha (TNF-α) treatment of HT-29 cells resulted in increased expression of inflammatory cytokine interleukin 1β (IL-1β), IL-8, and TNF-α genes and increased IL-8 protein and resulted in decreased adhesion of *E. coli* O157:H7. Similarly, *E. coli* O157:H7 adhesion to cattle colonic explants was reduced by TNF-α treatment. Irrespective of the presence of *E. coli* O157:H7, TNF-α enhanced activation of p65, the key mediator of NF-κB inflammatory signaling, whereas *E. coli* O157:H7 infection suppressed this pathway by inhibiting p65 activation in HT-29 cells. To further explore the mechanisms linking the inflammatory response to attenuated *E. coli* O157:H7 adhesion, mucin 2 (MUC2) expression was analyzed, considering that the intestinal mucus layer is the first defense against enteric pathogens and MUC2 is the major secretory mucin in the intestine. MUC2 expression in HT-29 cells was increased by TNF-α treatment and by *E. coli* O157:H7 infection. However, reducing mucin expression by blocking mitogen-activated protein kinase (MAPK) extracellular signal-regulated protein kinases 1/2 (ERK1/2) and/or phosphatidylinositol 3-kinase (PI3K)/Akt signaling increased *E. coli* O157:H7 adherence to HT-29 cells. These data suggest that the inflammatory cytokine response acts to protect host epithelial cells against *E. coli* O157:H7 colonization, at least in part, by promoting mucin production.

Although the food supply in the United States is one of the safest in the world, outbreaks of food-borne illnesses are not uncommon. *Escherichia coli* O157:H7 is a major food-borne pathogen that causes hemorrhagic colitis, hemolytic uremic syndrome, and even death (1). Ruminant animals are primary reservoirs for *E. coli* O157:H7, and fecal shedding of *E. coli* O157:H7 is a major source for *E. coli* O157:H7 contamination in food and water. About 10% to 20% of beef cattle shed *E. coli* O157:H7, and shedding in summer months is higher than in winter months (2). Certain cattle have much higher rates of *E. coli* O157:H7 shedding ($>10^4$ bacteria/g feces) and can maintain a high level of shedding for several weeks or even months (3); such cattle are so-called "supershedders" (4) and are major sources of *E. coli* O157:H7 contamination in meat, typically via contaminated hides (4, 5). In addition, runoff water from livestock farms can contaminate vegetables, as was shown in a 2006 nationwide spinach recall due to *E. coli* O157:H7 contamination (6, 7). Eliminating or reducing *E. coli* O157:H7 gut colonization in cattle could help to solve food safety problems associated with *E. coli* O157:H7 infection. This effort would be helped by understanding the mechanisms leading to *E. coli* O157:H7 supershedders.

E. coli O157:H7 interaction with intestinal epithelial cells is the first step in *E. coli* O157:H7 colonization. Colonization depends on the ability of the bacteria to adhere to the host cells. *E. coli* O157:H7 possesses intimin and several type III secretion effectors that enable it to intimately attach to intestinal epithelial cells, resulting in the formation of attaching and effacing (A/E) lesions (1, 8, 9). In addition, *E. coli* O157:H7 possesses numerous fimbrial and nonfimbrial adhesins, which also play important roles in the initial stages of adhesion (10–13). On the other hand, a healthy gastrointestinal (GI) tract contains multiple defensive barriers. The intestinal mucosa is covered by a thick mucus layer, which serves as a major barrier between the epithelium and the lumen of the host GI tract and plays an essential role in innate immune defense (14, 15). In order to maintain homeostasis of the intestinal mucosa, mucus is continuously secreted from the intestinal epithelium. Major components of mucus are the gel-forming mucins (16), highly glycosylated high-molecular-weight proteins that are secreted mainly by goblet cells. Approximately 20 different mucin (MUC1 to MUC20)-encoding genes have been identified. They are broadly grouped as either membrane associated or secretory (17). The prominent component of secretory mucins in the intestine is mucin 2, which is synthesized by goblet cells and stored as mucin granules that expand dramatically upon secretion (16). The rapid secretion and turnover of mucins generates a virtually sterile layer between the gut luminal contents and the epithelial cells, preventing contact with and colonization of the epithelium by pathogens. It has been reported that inflammatory cytokines,

including interleukin 4 (IL-4), IL-13, and tumor necrosis factor alpha (TNF-α), promote mucin gene expression in intestinal epithelial cells (18–21). The large amount of mucins and antimicrobial compounds secreted at the site of inflammation is expected to wash away pathogens, preventing pathogen infection and colonization. Therefore, we hypothesized that the inflammatory response acts to protect host epithelial cells against *E. coli* O157:H7 colonization, at least in part, by promoting mucin production.

MATERIALS AND METHODS

Cell line, media, and bacterial strains. The human colonic epithelial cell line HT-29 was obtained from the American Type Culture Collection (Manassas, VA). HT-29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Sigma), 100 units/ml penicillin G, and 100 μg/ml streptomycin (Sigma) at 37°C with 5% CO_2. *E. coli* O157:H7 strain EDL933 was obtained from the Shiga toxin-producing *E. coli* (STEC) center at Michigan State University and was routinely grown in 5 ml LB broth in 16- by 150-mm culture tubes at 37°C overnight with shaking at 250 rpm.

Adhesion of *E. coli* O157:H7 to colonic epithelial cells. HT-29 cells were seeded in 24-well plates, cultured until 80 to 90% confluent, and treated with 0 or 10 ng/ml TNF-α (Cell Signaling Technology, Beverly, MA) for 2 or 12 h. The cells in each well were then washed 3 times with phosphate-buffered saline (PBS) (pH 7.4) and challenged with overnight-diluted *E. coli* O157:H7 culture (10^7 CFU/ml) at a multiplicity of infection (MOI) of 10:1. Bacteria and HT-29 cells were cocultured for 3 h at 37°C with 5% CO_2. The cell monolayers were then washed 3 times with PBS, and the cells were lysed with 0.2% Triton X-100. Serial dilutions of the lysates were plated on LB agar, and bacterial colonies were counted after 18 h of incubation at 37°C. The percent adhesion was calculated by dividing the number of colonies (recovered *E. coli* O157:H7) by the total number of *E. coli* O157:H7 bacteria added and multiplying by 100.

***E. coli* O157:H7 adherence to cattle colonic explants.** Distal colonic tissues (5 to 10 cm from the anus) were aseptically collected from cattle slaughtered in the University of Wyoming Meat Laboratory and transferred to the microbiology laboratory on ice within 5 min. The time lapse between the death of the cattle and processing of explant tissues was less than 30 min. Specimens were washed 5 times with 0.9% (wt/vol) NaCl, and the mucosa was dissected from the underlying tissue with sterile dissecting scissors. The trimmed specimens were reduced to 8-mm round pieces using Miltex disposable biopsy punches (Cardinal Health, OH) and were transferred to individual wells of 24-well plates (without a cover to enhance air circulation). The specimens were cultured in DMEM with or without 10 ng/ml TNF-α for 2 h. *E. coli* O157:H7 was then added at 10^7 CFU/well, and culture was continued at 37°C with 5% CO_2. Three hours postinfection, *E. coli* O157:H7-attached explants were washed, serially diluted, plated, and enumerated. The percent adhesion was calculated by dividing the number of colonies (recovered *E. coli* O157:H7) by the total number of *E. coli* O157:H7 bacteria added and multiplying by 100. In the inhibitor study, LY294002 (phosphatidylinositol 3-kinase [PI3K]/Akt inhibitors; Cell Signaling Technology) and/or U0126 (mitogen-activated protein kinase [MAPK]/extracellular signal-regulated protein kinases 1/2 [ERK1/2] inhibitor; Cell Signaling Technology) was added to the culture medium (20 μM each) 60 min prior to TNF-α treatment.

Immunofluorescent staining of *E. coli* O157:H7. *E. coli* O157:H7 adhesion was conducted as described above. After interaction, the cells were washed with PBS three times and incubated in DMEM at 37°C for an additional 3 h. The HT-29 monolayer or explants were then fixed in 4% paraformaldehyde. Explant tissues were paraffin embedded, sectioned (5 μm), and rehydrated for immunofluorescent staining. The cells or tissue sections were washed with PBS and then permeabilized with 0.1% Triton X-100 for 10 min. After blocking with 1.5% goat serum for 30 min, the cells or tissue sections were stained with anti-*E. coli* O157:H7-fluorescein isothiocyanate (FITC) antibody (KPL, Gaithersburg, MD), followed by

incubation with 5 μg/ml phalloidin-tetramethyl rhodamine isothiocyanate (TRITC) (Sigma) for 40 min at room temperature, and mounted with Vectashield mounting medium with DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratory). Attached *E. coli* O157:H7 was visualized on a Leica DMI6000 B fluorescence microscope equipped with a DFC340 FX digital camera (Leica, Buffalo Grove, IL).

Alcian blue staining of HT-29 cells and cattle gut explants. HT-29 cell monolayers were seeded and treated or not with 10 ng/ml TNF-α for 12 h as described above and then fixed in 4% paraformaldehyde, and mucins were stained with alcian blue (22). Quantification of mucins produced by HT-29 cells was performed using Image J software (split color channels; NIH, Bethesda, MD). Six randomly selected fields were examined. The results were quantified by dividing the positive (blue) area by the number of cells in each selected area. Cattle gut explants were treated or not with 10 ng/ml TNF-α for 2 h, fixed in 4% (wt/vol) paraformaldehyde, embedded in paraffin, and sectioned (5 μm) as described above. For each colonic explant, 12 5-μm sections evenly spaced over a 450-μm area were obtained. The sections were rehydrated and stained with alcian blue (22). The quantification of mucins in alcian blue-stained explant sections was performed using Image J software as described above. The results were quantified by dividing the positive (blue) area by the total tissue area.

qRT-PCR analyses. Total RNA was extracted from HT-29 cells using an RNeasy minikit (Qiagen, Valencia, CA) and reverse transcribed using a QuantiTect reverse transcription kit (Qiagen). The resulting cDNA was used as a template for quantitative reverse transcription-PCR (qRT-PCR) analysis of selected genes using a CFX96 Real-Time PCR Detection System (Bio-Rad, CA). SYBR green Master Mix (Bio-Rad, CA) was used for all qRT-PCRs. The primers for qRT-PCR are listed in Table 1. The relative expression of selected genes was normalized to that of the gene encoding glyceraldehyde 3-phosphate dehydrogenase (*gapdh*). The amplification efficiency was 0.90 to 0.99 (23).

Immunoblotting. Immunoblotting analysis was conducted according to procedures previously described (23). Antibodies against protein kinase B (Akt) (catalog number 9272), phospho-Akt (catalog number 9271), ERK1/2 (catalog number 9102), phospho-ERK1/2 (catalog number 9101), NF-κB p65 (catalog number 4764), and phospho-p65 (Ser 536) (catalog number 3033) were purchased from Cell Signaling Technology (Beverly, MA). Anti-GAPDH antibody (catalog number MA116757) was purchased from Affinity BioReagents (Golden, CO). Binding of antibodies was detected using horseradish peroxidase (HRP)-coupled anti-rabbit or anti-mouse immunoglobulin and visualized by chemiluminescence. The density of bands was quantified using an Imager Scanner II (Amersham Bioscience) and ImageQuant TL software (Amersham Bioscience) and normalized to the GAPDH content.

IL-8 and IL-18 analysis by ELISA. After *E. coli* O157:H7 infection, spent HT-29 cell medium was collected and analyzed for IL-8 using a human IL-8 enzyme-linked immunosorbent assay (ELISA) kit (Thermo Scientific, IL) according to the manufacturer's directions. The intra-assay coefficient of variation (CV) and the interassay CV were <10%. IL-18 levels were analyzed using a human IL-18 ELISA kit (eBioscience, San Diego, CA) according to the manufacturer's directions. The intra-assay CV and the interassay CV were 6.5% and 8.1%, respectively.

Statistical analyses. Statistical analyses were conducted as previously described (23). Data were analyzed as a complete randomized design using GLM (General Linear Model of Statistical Analysis System; SAS, 2000). Means ± standard errors of the mean (SEM) are reported. A P value of <0.05 was considered significant.

RESULTS

TNF-α treatment reduced *E. coli* O157:H7 adhesion. To test the effect of the inflammatory response on *E. coli* O157:H7 adhesion to epithelial cells, we treated HT-29 cells with TNF-α, which is known to induce inflammatory cytokine expression (24). As indicated in Fig. 1, TNF-α pretreatment resulted in increased mRNA expression of the proinflammatory cytokines IL-1β, IL-8, and

TABLE 1 Primer sets used for qRT-PCR for HT-29 cells

Gene name	Accession no.	Product size (bp)	Direction	Sequence (5′→3′)
IL-1β	NM_000576	126	Forward	GTGGCAATGAGGATGACTTGT
			Reverse	TGTAGTGGTGGTCGGAGATTC
IL-8	NM_000584	88	Forward	GAGGGTTGTGGAGAAGTTTTTG
			Reverse	CTGGCATCTTCACTGATTCTTG
IL-18	NM_001562	100	Forward	TGAAGATGATGAAAACCTGGAAT
			Reverse	TTGGTCAATGAAGAGAACTTGGT
MUC1	NM_001018017.2	108	Forward	CAGCCAGCGCCTGCCTGAAT
			Reverse	GCACTGTGAGGAGCAGCAGCA
MUC2	M94132.1	138	Forward	CCTGCCGACACCTGCTGCAA
			Reverse	ACACCAGTAGAAGGGACAGCACCT
MUC3	AB038784.1	130	Forward	CTCGTGTTGCCATTGCCTCTCTCG
			Reverse	CTGCAGGTTGCCTCCAGGTTCAGA
TNF-α	AF043342	100	Forward	AACCTCCTCTCTGCCATCAA
			Reverse	GGAAGACCCCTCCCAGATAG
gapdh	NM_002046	94	Forward	ACAGTCAGCCGCATCTTCTT
			Reverse	ACGACCAAATCCGTTGACTC

TNF-α in HT-29 cells (Fig. 1A). Furthermore, TNF-α treatment resulted in increased production of IL-8 protein (Fig. 1B), which was associated with increased phosphorylation of NF-κB p65 (Fig. 1C). The NF-κB signaling pathway is a major regulator of inflammation, and its activation is associated with phosphorylation of p65 (25). Longer (12-h) TNF-α exposure resulted in enhanced p65 activation in HT-29 cells compared with shorter (2-h) exposure (Fig. 1C). Given that a longer duration of TNF-α exposure is more similar to chronic inflammation in the gut *in vivo*, we chose 12-h TNF-α pretreatment for further experiments using HT-29 cells.

Accompanying the increased inflammatory cytokine response, the adhesion of *E. coli* O157:H7 to HT-29 cells treated with TNF-α was reduced compared with that of untreated controls (Fig. 2A). We evaluated the significance of this observation using colonic explant tissues separated from the rectoanal region of beef cattle, considering that the terminal rectoanal junction (RAJ) is the principal colonization site of *E. coli* O157:H7 (26, 27). We note that this claim has recently been disputed, and it has been proposed that *E. coli* O157:H7 colonizes other sites in the GI tract, including the small intestine, in addition to the RAJ (28). In agreement with our findings using HT-29 cells, TNF-α treatment also reduced adhesion of *E. coli* O157:H7 in colonic explant tissues (Fig. 2B). Since bacteria can also attach to the exposed basal lamina and other tissues and cells of explant tissues, in addition to epithelial cells, the bacterial counts might be artificially increased, which might partially mask the treatment effect. Immunofluorescent staining indicated that fewer *E. coli* O157:H7 bacteria (green fluorescent signal) were associated with HT-29 cells (Fig. 2C) or colonic explant tissues treated with TNF-α (Fig. 2D), showing once again that TNF-α treatment reduced *E. coli* O157:H7 adherence to epithelial cells. Examination of paraffin-embedded post-*E. coli* O157:H7-infected cattle explant sections imaged under a bright-field microscope (Fig. 2E) demonstrated that the overall integrity of epithelial cells after *E. coli* O157:H7 infection was preserved.

In line with its proinflammatory function, TNF-α increased the phosphorylation of p65, the key mediator of NF-κB inflammatory signaling, while *E. coli* O157:H7 infection resulted in suppression of phosphorylated p65 and therefore NF-κB activation (Fig. 3A). The ability of *E. coli* O157:H7 to reduce NF-κB activation, thereby diminishing the host inflammatory response, correlated with increased *E. coli* O157:H7 colonization. As indicated in Fig. 3B, the presence of *E. coli* O157:H7 resulted in enhanced expression of IL-1β and TNF-α mRNAs in HT-29 cells, and TNF-α incubation further enhanced TNF-α mRNA (Fig. 3B). IL-8, a chemotactic cytokine for T lymphocytes and neutrophils, is induced under inflammatory conditions (29). *E. coli* O157:H7 infection resulted in exaggerated IL-8 mRNA expression and protein production, which was further increased by TNF-α treatment (Fig. 3C). IL-18 is another important proinflammatory cytokine. Consistent with its effects on NF-κB p65 signaling pathways, TNF-α treatment stimulated the activation of IL-18, as indicated by increased mRNA expression and secretion, which were blocked after *E. coli* O157:H7 infection (Fig. 3C).

TNF-α treatment enhanced mucin production, which possibly contributed to the reduced *E. coli* O157:H7 epithelial adhesion. The mucus layer functions as a critical protective barrier against bacterial adhesion to and invasion of the epithelial layer; therefore, we analyzed mucin mRNA expression in HT-29 cells in the presence of *E. coli* O157:H7 and/or TNF-α. In the presence or absence of *E. coli* O157:H7 infection, for both 2 h (Fig. 4A) and 12 h (Fig. 4B), TNF-α treatments resulted in enhanced MUC1, MUC2, and MUC3 mRNA expression. Because mucin proteins are highly glycosylated and have high and variable molecular weights, we were not able to analyze the mucin protein content by Western blotting. To determine whether there was a change in mucin protein levels in response to TNF-α treatment, we used alcian blue staining on HT-29 cells (Fig. 4C) and on cattle colonic explants (Fig. 4D), each of which indicated increased mucin excretion/production in response to TNF-α treatment. Of note, the
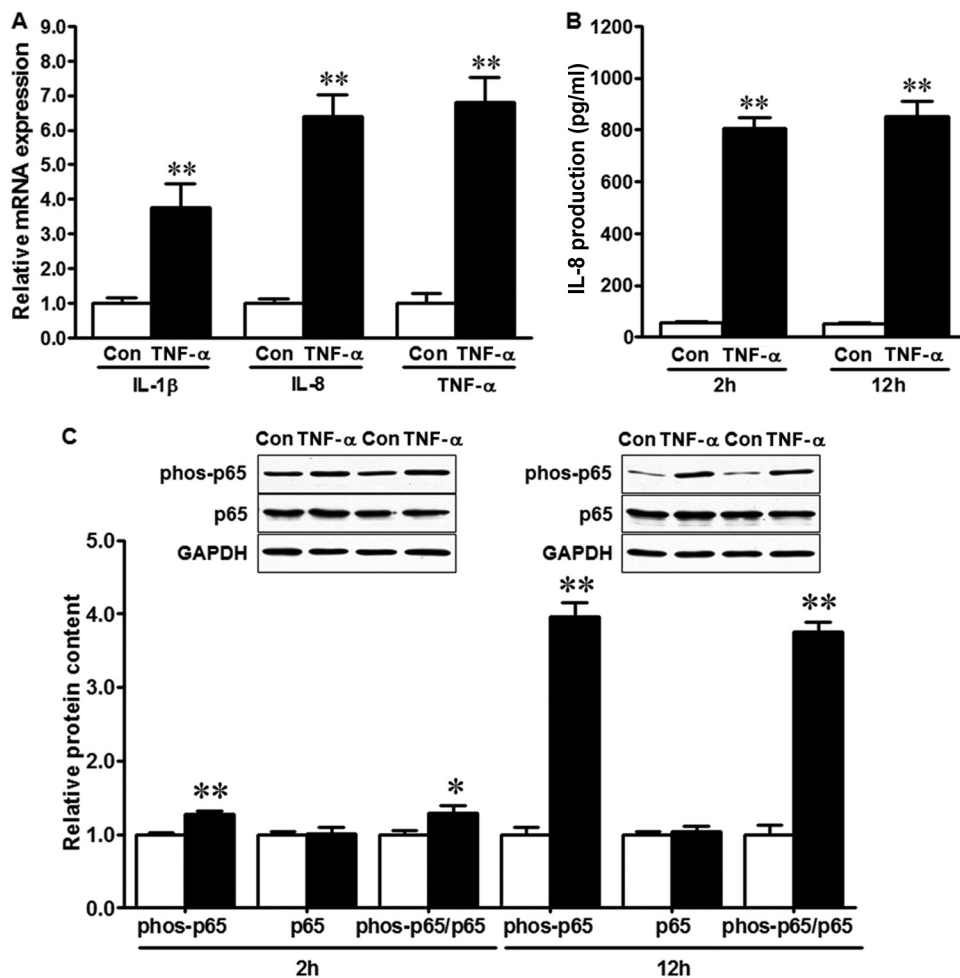
FIG 1 TNF-α treatment induces inflammatory response in HT-29 cells. (A) Relative mRNA expression of proinflammatory cytokines in HT-29 cells untreated (Con) or treated with 10 ng/ml TNF-α (TNF-α) for 12 h. (B) IL-8 production in HT-29 cells 2 h or 12 h after TNF-α treatment. (C) NF-κB p65 signaling in HT-29 cells at 2 h or 12 h after TNF-α treatment. (Top) Representative Western blotting bands. (Bottom) Statistical data. Shown are means and SEM; $n = 4$. **, $P < 0.01$; *, $P < 0.05$.

detected mucin level in explant tissues might be an underestimate caused by possible loss of crypt epithelium due to postmortem autolysis, processing, and culturing. These results are consistent with our hypothesis that TNF-α reduces *E. coli* O157:H7 attachment by enhancing mucin secretion and synthesis.

Blocking mucin production by inhibition of PI3K/Akt and MAPK/ERK1/2 signaling. To explore possible mechanisms by which TNF-α enhances mucin production, we analyzed PI3K/Akt and MAPK/ERK1/2 signaling pathways. Both *E. coli* O157:H7 infection and TNF-α treatment enhanced Akt signaling (Fig. 5A), a pathway-regulating protein synthesis, including the synthesis of mucins, as indicated by increased phosphorylation of Akt and an increased ratio of phospho-Akt to total Akt. Similarly, TNF-α treatment activated ERK1/2 signaling in HT-29 cells; however, upon *E. coli* O157:H7 infection, ERK1/2 signaling was downregulated (Fig. 5B).

To elucidate the role of PI3K/Akt and/or MAPK/ERK1/2 signaling in mucin production and *E. coli* O157:H7 epithelial cell adherence, LY294002, a specific inhibitor of the PI3K/Akt pathway, and U0126, a specific inhibitor of MAPK/ERK1/2 signaling, were used to block PI3K/Akt and/or MAPK/ERK1/2 signaling.

Expression of MUC2 mRNA was inhibited in HT-29 cells treated or not with TNF-α following addition of LY294002 and/or U0126. The effect of U0126 was far more dramatic than that of LY294002 (Fig. 6A). The ability of TNF-α to reduce adhesion of *E. coli* O157:H7 to epithelial cells was negated by treatment with LY294002 and/or U0126. These data indicate that mucin expression and adhesion of *E. coli* O157:H7 to gut epithelial cells following TNF-α treatment are at least in part regulated by the PI3K/Akt and MAPK/ERK1/2 pathways. These data also provide further correlative evidence that blocking mucin expression enhances *E. coli* O157:H7 adhesion to epithelial cells.

DISCUSSION

Mucins produced by goblet cells form a mucus layer that provides a defensive barrier between the epithelial cells and invading pathogens, including *E. coli* O157:H7. In order to colonize epithelial cells, *E. coli* O157:H7 must penetrate the mucus layer. The precise role that mucins play in *E. coli* O157:H7 epithelial colonization has not been fully elucidated. Mucin2 is the major secreted mucin, whereas mucin2 and mucin3 are membrane-bound mucins in the intestine. HT-29 cells express MUC2
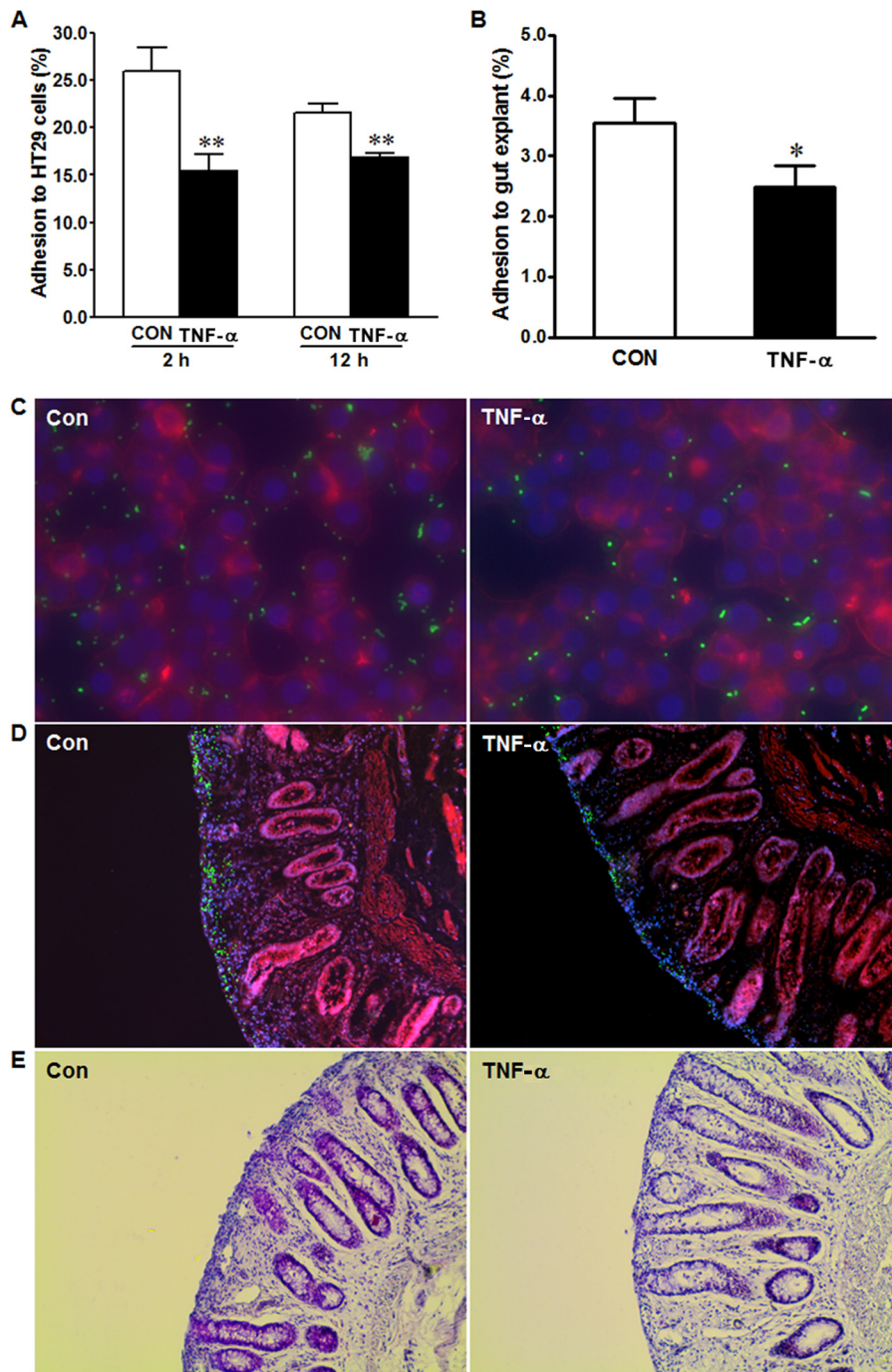
FIG 2 TNF-α treatment reduces adhesion of *E. coli* O157:H7 to HT-29 cells and cattle colonic explant tissues. (A) Adhesion of *E. coli* O157:H7 to HT-29 cells untreated (Con) or treated with 10 ng/ml TNF-α (TNF-α) for 2 h or 12 h. **, $P < 0.01$. (B) Adhesion of *E. coli* O157:H7 to cattle colonic epithelial cells untreated or treated with 10 ng/ml TNF-α for 2 h. *, $P < 0.05$. Shown are means and SEM; $n = 8$ for each treatment of HT-29 cells and explant. (C and D) Representative images of immunofluorescent staining of *E. coli* O157:H7 adherence to HT-29 cells (C) and cattle colonic explant tissues (D) treated or not with 10 ng/ml TNF-α. Blue, DAPI-stained nucleus; green, FITC-labeled *E. coli* O157:H7; red, phalloidin-TRITC to indicate cell skeletal elements. (E) H&E staining of postinfection cattle colonic explant tissues untreated (Con) or treated with 10 ng/ml TNF-α (TNF-α) for 2 h.

(18, 30). Mucin expression is upregulated by inflammatory cytokines (18–21), which are induced by NF-κB signaling (31), as well as STAT signaling (32). Consistent with these reports, we observed that TNF-α treatment enhanced mucin mRNA expression in HT-29 cells and enhanced mucin staining in both HT-29 cells and cattle colonic explant tissues. These data are consistent with previous reports showing that upon *E. coli* O157:H7 infection, MUC2 mRNA expression increased, which is in line with an innate defense role. Consistent with our observations, it has been reported that mucin secretion is increased in response to
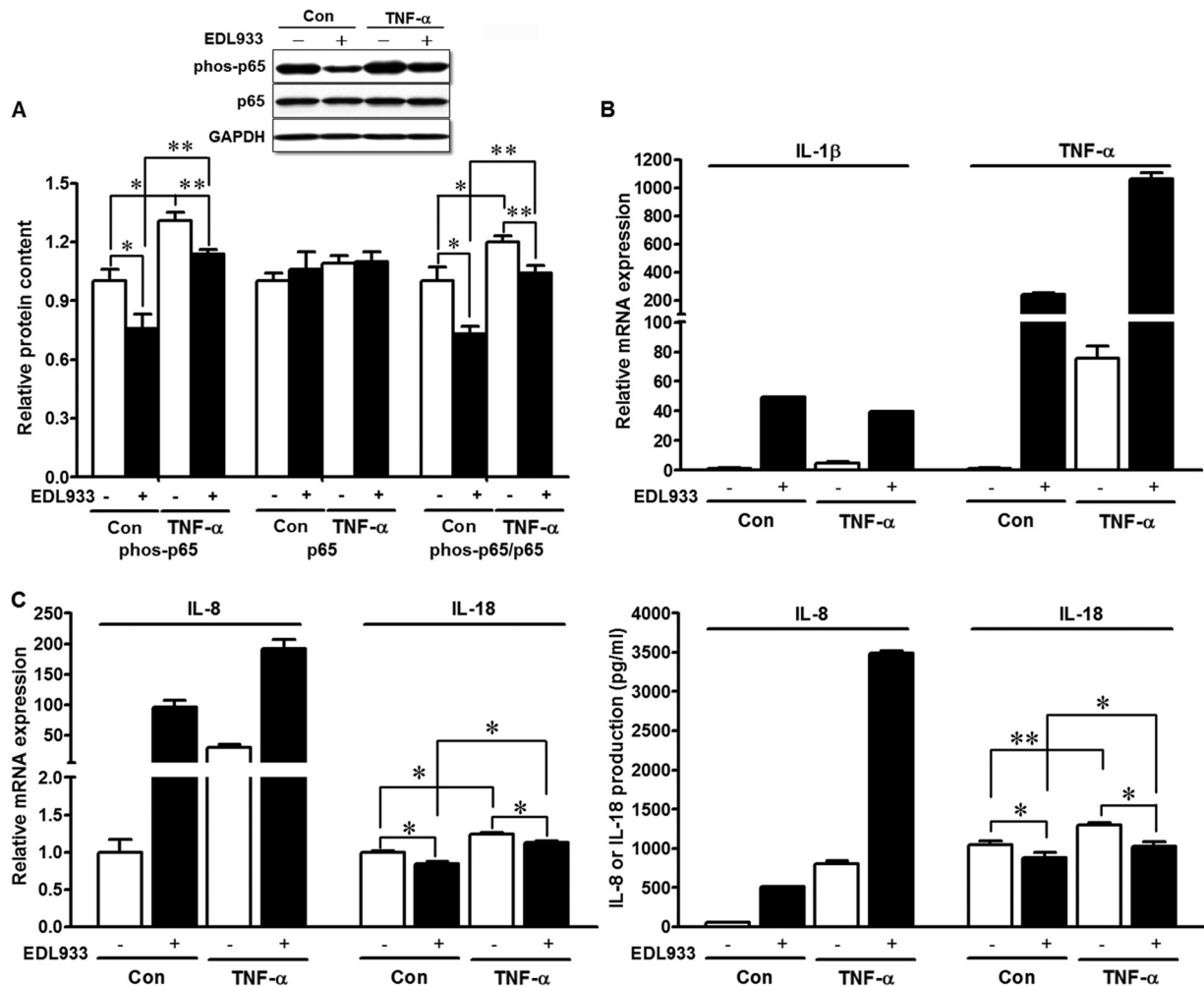
FIG 3 Inflammatory responses in HT-29 cells untreated (Con) or treated with 10 ng/ml TNF-α for 12 h (TNF-α) and infected (+) or not (−) with *E. coli* O157:H7. (A) NF-κB p65 signaling analyzed by Western blotting. (Top) Representative bands. (Bottom) Statistical data. (B) IL-1β and TNF-α mRNA expression. (C) IL-8 and IL-18 mRNA expression and protein production. **, $P < 0.01$; *, $P < 0.05$. Shown are means and SEM; $n = 4$.

intestinal infection with *Citrobacter rodentium* (33) and *Shigella dysenteriae* (34).

The inflammatory response is an innate defense mechanism. However, some pathogens can disrupt host inflammatory responses to avoid recognition by the innate immune system and may even exploit it to enhance their colonization. It has been previously reported that enterohemorrhagic *E. coli* (EHEC) infection suppresses TNF-α-induced activation of the NF-κB signal transduction cascade in host cells (35–37). Our results indicated that *E. coli* O157:H7 attenuated both basal and TNF-α-induced activation of NF-κB p65 signaling in HT-29 cells 3 h postinfection. In contrast to our results, a previous study indicated that EHEC infection enhanced NF-κB signaling in T84 cells (24). IL-18 is an important inflammatory cytokine involved in both innate and adaptive immunity. IL-18 mRNA expression in cardiomyocytes was upregulated by TNF-α through activation of NF-κB (38). Consistently, we found that TNF-α enhanced IL-18 expression and secretion in HT-29 cells, while *E. coli* O157:H7 infection suppressed IL-18 production, which is consistent with data indicating that *E. coli* O157:H7 infection downregulates NF-κB signaling (35–37).

Despite the suppression of NF-κB signaling and IL-18 secretion, *E. coli* O157:H7 upregulated IL-8 production in control and TNF-α-treated HT-29 cells upon infection, indicating that *E. coli* O157:H7 infection might stimulate IL-8 expression/production independently of the NF-κB signaling pathway. This is in agreement with previous studies showing that *E. coli* O157:H7 infection elevated IL-8 mRNA expression in T84 cells (24, 39) and Caco-2 cells (40). In addition, Bellmeyer et al. (24) reported that EHEC infection reduced IL-8 production in HT-29 cells in response to TNF-α challenge. MAPK/ERK1/2 signaling was reported to be involved in upregulation of IL-8 mRNA expression by epithelial cells in response to *E. coli* O157:H7 infection (39, 40). Phosphorylation of ERK 1/2 was increased in Caco-2 cells 1 to 2 h post-EHEC infection; however, the extent of phosphorylation of ERK was dramatically decreased by 3 h postinfection (40). We found that *E. coli* O157:H7 inhibited ERK 1/2 activation in HT-29 cells 3 h postinfection. Similar results were found in Caco-2 cells (40), but not in T84 cells (39). A possible reason for these discrepant results could be differences among these cell lines in expression of globotriaosylceramide-3 (Gb3), a receptor for Shiga toxins. Only about 5% of T84 cells are Gb3 positive, while 50% of Caco-2 cells
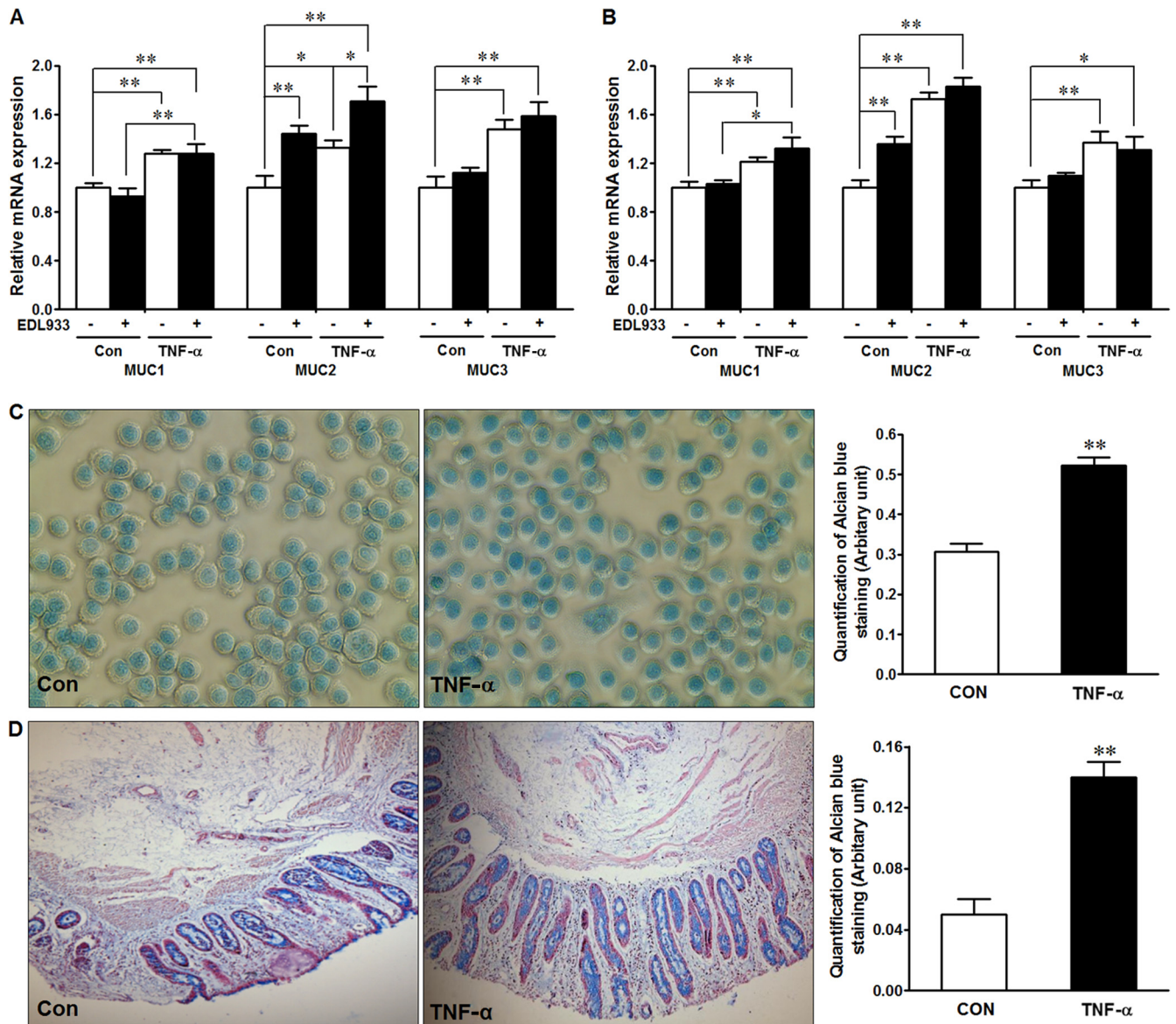
FIG 4 Mucin expression and production in response to TNF-α treatment and *E. coli* O157:H7 infection. (A and B) mRNA expression of selected mucin genes in HT-29 cells untreated (Con) or treated with 10 ng/ml TNF-α (TNF-α) for 2 h (A) and 12 h (B) and infected (+) or not (−) with *E. coli* O157:H7. (C) Alcian blue staining of HT-29 cells not pretreated (Con) or pretreated with 10 ng/ml TNF-α (TNF-α) for 12 h. (Left) Representative images. (Right) Statistical data. (D) Alcian blue mucin staining in cattle colonic explant tissues not pretreated or pretreated with 10 ng/ml TNF-α for 2 h. (Left) Representative images. (Right) Statistical data. **, $P < 0.01$; *, $P < 0.05$. Shown are means and SEM; $n = 4$ for mRNA expression; $n = 8$ for alcian blue staining.

and 90% of HT-29 cells are Gb3 positive (41). Thus, in Caco-2 and HT-29 cells, Shiga toxins might be involved in inhibiting the host immune response via a largely Gb3-mediated mechanism, which would have minor influence in T84 cells (42, 43). Inhibition of both NF-κB and ERK 1/2 signaling may be strategies by which *E. coli* O157:H7 evades the host response, facilitating pathogen survival and further colonization of the GI tract.

As a key inflammatory cytokine, the role of TNF-α in inflammatory signaling has been well defined; however, its role in pathogen colonization of host cells is less defined. TNF-α exerts its biological effects mainly through TNF-α receptor 1 (TNFR1) and TNFR2 (44). Very recently, it was demonstrated that these two receptors have different functions in mucin expression and secretion. TNFR1 is needed for goblet cells to secrete mucus, while TNFR2 is needed for upregulation of mucin expression and synthesis (21). Both TNF-α receptors activate inflammatory NF-κB signaling. In addition, these receptors also activate PI3K/Akt and ERK1/2 signaling (45, 46). In line with previous observations (19), we observed that TNF-α treatment augmented both NF-κB and PI3K/Akt signaling in HT-29 cells. In addition, HT-29 cells exhibited enhanced Akt activation in response to *E. coli* O157:H7 infection. To further assess the roles of PI3K/Akt and MAPK/ERK1/2 signaling in mucin production and in *E. coli* O157:H7 epithelial adherence in response to TNF-α treatment, we used the specific inhibitors LY294002 and U0126 to inhibit PI3K/Akt and MAPK/ERK1/2 signaling, respectively (47, 48). Inhibition of PI3K/Akt
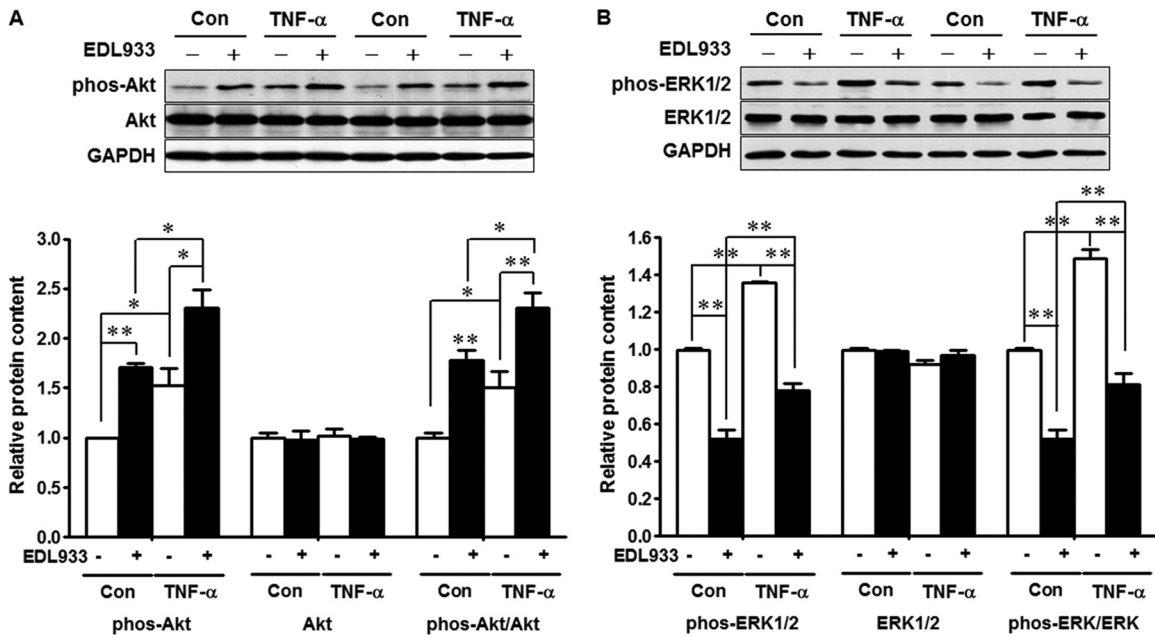
FIG 5 Akt and mitogen-activated protein kinase/ERK1/2 signaling in HT-29 cells untreated (Con) or treated with 10 ng/ml TNF-α (TNF-α) and uninfected (−) or infected with *E. coli* O157:H7 (+). (A) Akt signaling. (Top) Representative Western blotting bands. (Bottom) Statistical data. (B) ERK1/2 signaling. (Top) Representative Western blotting bands. (Bottom) Statistical data. HT-29 cells at 80 to 90% confluence were incubated without or with 10 ng/ml TNF-α for 12 h, and then the HT-29 cells were subjected to *E. coli* O157:H7 infection for 3 h and washed, lysed, and collected. The cell lysates were subjected to 10% SDS-PAGE, and total and phosphorylated Akt (A) and ERK1/2 (B) were detected by probing with their respective antibodies. The ratios of phosphorylated Akt to total Akt and phosphorylated ERK1/2 to total ERK1/2 were used to indicate activation/inhibition of Akt and ERK1/2 signaling, respectively. **, $P < 0.01$; *, $P < 0.05$. Shown are means and SEM; $n = 4$.

and/or MAPK/ERK1/2 signaling reduced MUC2 expression and enhanced *E. coli* O157:H7 adherence, consistent with previous studies showing that Akt signaling (19) and ERK1/2 signaling (18) were involved in TNF-α-augmented MUC2 mRNA expression. Of note, in our study, U0126 was more effective than LY294002 in inhibiting MUC2 expression and promoting *E. coli* O157:H7 adhesion. This effect may be due to the dose and efficacy of the

inhibitors and may not necessarily indicate that ERK1/2 is more critical for MUC2 expression than Akt.

Our data indicate that the inflammatory response induced by TNF-α reduces *E. coli* O157:H7 adhesion to gut epithelial cells and explants. The decreased adhesion was partially mediated by activating Akt and ERK1/2 signaling, which also enhanced the production and secretion of mucins (Fig. 7). These data suggest that
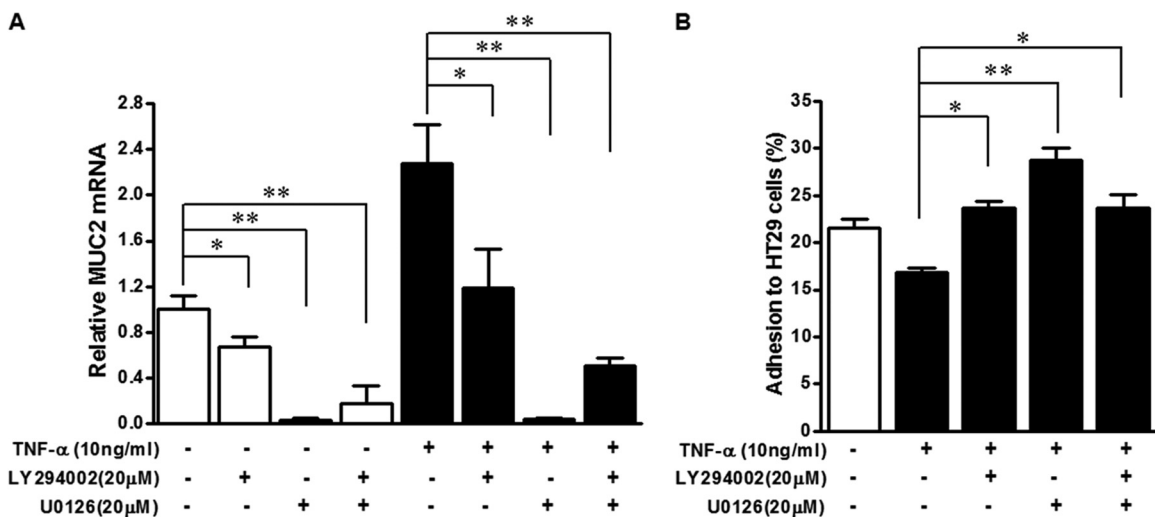

FIG 6 Akt and ERK1/2 inhibition blunt MUC2 expression and increase *E. coli* O157:H7 adhesion to HT-29 cells. (A) MUC2 mRNA expression in HT-29 cells untreated (−) or treated with 10 ng/ml TNF-α (+) and untreated (−) or treated with PI3K/Akt inhibitor (LY294002) or MAPK/ERK1/2 inhibitor (U0126) (+). (B) Adhesion of *E. coli* O157:H7 to HT-29 cells untreated (−) or treated with 10 ng/ml TNF-α (+) and untreated (−) or treated with LY294002 or U0126 (+). **, $P < 0.01$; *, $P < 0.05$. Shown are means and SEM; $n = 4$ for mRNA expression and $n = 8$ for adhesion.
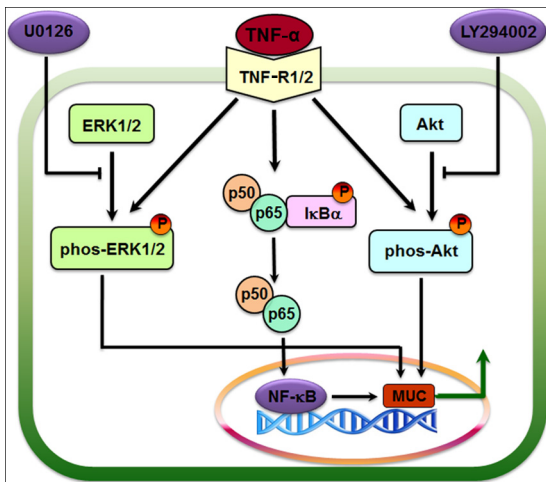
FIG 7 Proposed signaling pathways regulating MUC synthesis and secretion. TNF-α induces an inflammatory response through the NF-κB signaling pathway mediated by TNFR, with concomitant activation of MAPK/ERK1/2 and PI3K/Akt pathways. These pathways synergistically regulate MUC expression and synthesis. LY294002 and U0126 are specific inhibitors of the PI3K and ERK1/2 signaling pathways, respectively. The arrows indicate demonstrated effects. Steps blocked by inhibitors are indicated.

the inflammatory cytokine response has a critical role in preventing *E. coli* O157:H7 epithelial colonization. Mucins, previously underappreciated factors in *E. coli* O157:H7 colonization, may have key roles in mediating *E. coli* O157:H7 gut colonization; thus, stimulation of gut epithelium inflammatory responses and associated mucin secretion might be a useful strategy to reduce *E. coli* O157:H7 gut colonization in beef cattle.

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