BRIEF COMMUNICATION

TNF-α Sensitizes HT-29 Colonic Epithelial Cells to Intestinal Lactobacilli[1]

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The ability of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) to influence epithelial interleukin (IL)-8 responses to the intestinal bacterium *Lactobacillus plantarum* 299v was analyzed in the human HT-29 colonic epithelial cell line. In the absence of TNF-α, IL-8 mRNA expression was not detectable by Northern blot analysis in HT-29 cells alone or in HT-29 cells co-cultured with *L. plantarum* 299v. However, TNF-α induced IL-8 mRNA expression, and co-culture of TNF-α-treated HT-29 cells with *L. plantarum* 299v significantly increased IL-8 mRNA expression above levels induced by TNF-α alone in an adhesion-dependent manner. The increase in IL-8 mRNA expression was not observed in TNF-α-treated HT-29/*L. plantarum* 299v co-cultures using heat-killed lactobacilli or when *L. plantarum* adhesion was prevented using mannoside or a trans-well membrane. Paradoxically, IL-8 secretion was decreased in TNF-α-treated HT-29 cells with *L. plantarum* 299v relative to cells treated with TNF-α alone. TNF-α-mediated responsiveness to *L. plantarum* 299v was further investigated by analyzing expression of a coreceptor for bacterial cell wall products CD14. HT-29 cells expressed CD14 mRNA and cell-surface CD14; however, TNF-α did not alter CD14 mRNA or cell-surface expression, and blockade of CD14 with monoclonal antibody MY4 did not alter the IL-8 response to *L. plantarum* 299v in TNF-α-treated HT-29 cells. These results indicate that although TNF-α sensitizes HT-29 epithelial cells to intestinal lactobacilli, the bacteria exert a protective effect by downregulating IL-8 secretion.

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Key words: *Lactobacillus*; intestinal epithelial cell; interleukin-8; CD14

Intestinal epithelial cells (IEC) defend the host by producing a diverse array of cytokines (1). Interleukin-8 (IL-8), which produces a chemoattractant gradient that attracts neutrophils to sites of inflammation, is one such cytokine secreted by IEC in response to a variety of pathogenic bacteria as well as proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) (2–4). However, there are limited reports of studies investigating cytokine responses of IECs to nonpathogenic intestinal bacteria.

The potential for proinflammatory cytokines such as TNF-α to sensitize IEC to indigenous bacteria under inflammatory conditions is of interest, particularly for studies of inflammatory bowel disease where intestinal concentrations of TNF-α and epithelial expression of TNF-α receptors are increased (5–7). Furthermore, there is increasing evidence that CD14, a TNF-α-sensitive bacterial pattern recognition receptor (PRR) capable of binding various bacterial cell wall components, is also expressed by IEC, and could increase IEC sensitivity to intestinal bacteria (8–13). To examine the effects of an indigenous, adherent *Lactobacillus* strain on IL-8 expression by human IECs and to investigate the possible role of CD14 in mediating interactions between lactobacilli and IEC, the human colonic epithelial cell line HT-29 was associated with the commensal bacterium *Lactobacillus plantarum* 299v (14) in the presence or absence of the proinflammatory cytokine TNF-α.

First, to evaluate epithelial IL-8 mRNA responses to *L. plantarum* 299v in the presence of TNF-α, near confluent HT-29 cell cultures were washed three times in adhesion

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test medium (serum- and antibiotic-free Dulbeccos' modified Eagles' medium [DMEM], Gibco, Grand Island NY) to remove antibiotics and serum from maintenance medium (15). Adhesion test medium alone or containing 10 ng/ml rhTNF-α was added to each plate after the final wash, and plates were incubated for 3 hr at 37°C in a 95% air/5% CO_2 atmosphere. After 3 hr, *L. plantarum* 299v at a multiplicity of infection (MOI) of 1000 were resuspended in adhesion test medium and were added to epithelial cell cultures and co-cultures incubated for an additional 3 hr. Negative control plates received adhesion test medium alone.

Total RNA was isolated from cells using a single-step guanidinium isothiocyanate protocol (16), was size-separated on a formaldehyde agarose gel (10 μg/lane), transferred to a nylon membrane using standard Northern blotting techniques, and screened for IL-8 mRNA expression using an α^{32}P-labeled partial cDNA probe. The IL-8 probe was generated using an reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Perkin Elmer, Branchburg, NJ) following the manufacturer's protocol and using primers specific for IL-8 (GenBank accession number Z11686): 5′-ATGACTTCCAAGCTGGCCGTGGCT (sense), 5′-TCTCAGCCCTCTTCAAAAACTTCTC (antisense). Hybridization was also performed with a probe specific for 28S rRNA to ensure equal loading of RNA (17). After hybridization, bands were visualized by phosphorimager analysis (Molecular Devices, Menlo Park, CA). To determine relative expression of target and 28S rRNA, densitometry was performed on a photograph of the gel using Diversity Database™ version 2.1 software (Bio-Rad, Hercules, CA).

In the absence of TNF-α, IL-8 mRNA expression was not detectable by Northern analysis in untreated HT-29 cells and was not altered in cells co-cultured with *L. plantarum* 299v (Fig. 1). Treatment with TNF-α induced IL-8 expression in HT-29 cells (Fig. 1). Co-culture of *L. plantarum* 299v with TNF-α-treated HT-29 cells increased IL-8 mRNA expression 6.6-fold over cells treated with TNF-α alone (Fig. 1). A lactate dehydrogenase cytotoxicity assay (data not shown) demonstrated that the relative increase of IL-8 mRNA expression in TNF-α-treated HT-29 cells associated with *L. plantarum* 299v was not a result of *Lactobacillus*-induced killing of HT-29 cells.

Supernatant IL-8 concentrations were detected using antibody pairs and standards provided in the OPT-EIA Human IL-8 ELISA set (BD PharMingen, San Diego, CA). IL-8 concentrations in medium of HT-29 cells co-cultured with *L. plantarum* 299v for 3 hr (130 ± 12 pg/ml) were not significantly different from baseline concentrations (90 ± 13 pg/ml; Fig. 2). Similar to previous reports (2, 4), treatment with TNF-α increased ($P < 0.05$) IL-8 concentrations to 75,851 ± 3123 pg/ml. However, IL-8 concentrations were reduced ($P < 0.05$) to 30,877 ± 2127 pg/ml in TNF-α-treated HT-29 cells co-cultured with *L. plantarum* 299v.

Although TNF-α-stimulated IL-8 mRNA and protein secretion by IEC are typically closely linked (2, 18), Northern blot and ELISA results did not agree in our study. Specifically, although IL-8 mRNA expression and protein secretion were sharply increased in response to TNF-α, the decreased concentrations of IL-8 in TNF-α-stimulated HT-29 cells co-cultured with *L. plantarum* 299v, relative to cells treated with TNF-α alone, differ from the consistent increase in IL-8 mRNA expression. We are not aware of other examples of dissociation between RNA expression and secretion of a cytokine in response to a microbial stimulus. The potential for lactobacilli to absorb or degrade the secreted IL-8 protein, thus resulting in decreased apparent concentrations of IL-8, was evaluated by incubating adhesion test medium containing IL-8 for 3 hr in the presence or absence of *L. plantarum* 299v; however, ELISA performed on these supernatants demonstrated that the presence of the bacteria did not alter IL-8 concentrations (data not shown). The mechanisms whereby lactobacilli increase IL-8 transcription, yet partially inhibit secretion, remain unknown.

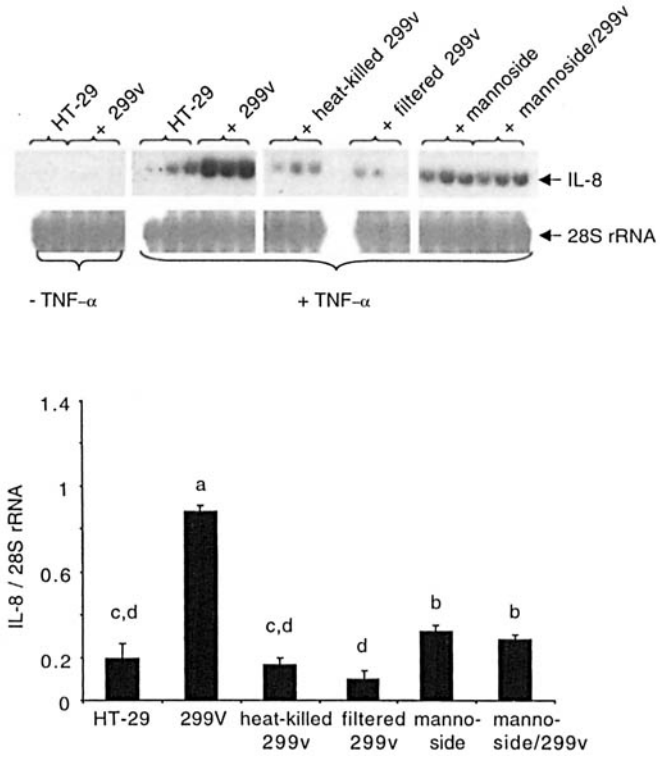

Figure 1. TNF-α-dependent alterations of *L. plantarum*-induced IL-8 expression in HT-29 cells. (Top) HT-29 cells (3×10^7/plate) were incubated for 3 hr in adhesion test medium ± TNF-α (10 ng/ml), and then co-cultured for 3 hr with *L. plantarum* 299v (3×10^{10} CFU/plate). Indicated samples contained lactobacilli in the presence of 60 m*M* mannoside, or heat-killed lactobacilli (30 min of boiling), or contained transwell filters that prevented contact between bacteria and HT-29 cells. Total RNA was extracted and Northern blot analysis of IL-8 mRNA expression was performed. Expression of 28S ribosomal RNA was assayed as a constitutive control. Treatments: HT-29 cells alone; + *L. plantarum* 299v; + TNF-α; + TNF-α + *L. plantarum* 299v; + TNF-α + heat-killed *L. plantarum* 299v; + TNF-α + filtered *L. plantarum* 299v; + TNF-α + mannoside; and + TNF-α + mannoside + *L. plantarum* 299v. (Bottom) Ratios of IL-8/28S rRNA expression from HT-29 cells associated with bacteria after treatment with TNF-α (bands from samples not treated with TNF-α were too faint for densitometry). Treatments with different superscripts are statistically different ($P < 0.05$).

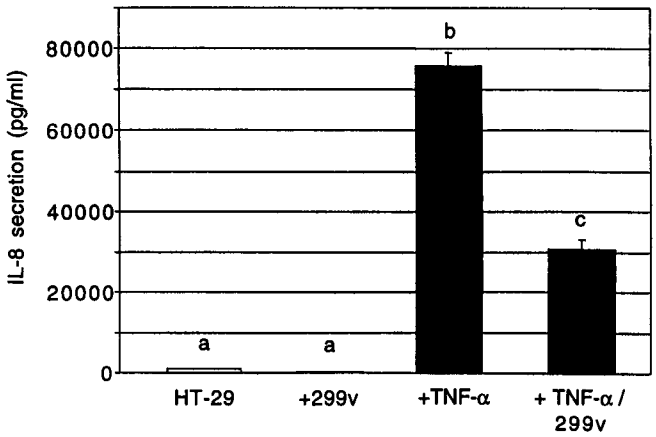

Figure 2. Effects of TNF-α and co-culture with *L. plantarum* 299v on HT-29 cell IL-8 secretion. HT-29 cells were incubated for 3 hr in adhesion test medium in the absence or presence of 10 ng/ml TNF-α. Subsequently, cells were cultured for 3 hr in the absence or presence of *L. plantarum* 299v, and cell-culture supernatants were collected for ELISA analysis. Values represent the means from three wells for each treatment in a representative experiment. Each experiment was repeated at least three times with comparable results. Treatments with different superscripts are statistically different as measured by Student's *t* tests ($P < 0.05$).

To test for the role of bacterial adhesion in induction of IL-8 mRNA, methyl-α-D-mannoside (60 m*M*; United States Biochemical, Cleveland, OH), which prevents adhesion of *L. plantarum* 299v to a mannose-containing receptor on HT-29 cells (19), was also added to cells 3 hr prior to treatment with lactobacilli (MOI = 1000). Treatment of HT-29 cells with mannoside significantly decreased adhesion of *L. plantarum* 299v (data not shown) and the *Lactobacillus*-mediated increase in IL-8 mRNA expression in TNF-α-treated HT-29 cells (Fig. 1). Mannose-sensitive binding to epithelial cells occurs in many gram-negative organisms, including *Enterobacter, Klebsiella, Shigella,* and *Vibro* species (20). The present finding that prevention of *L. plantarum* 299v adhesion to HT-29 cells by mannoside inhibited the IL-8 response in TNF-α-treated HT-29 cells further reveals the importance of the mannose receptor for lactobacilli adhesion to IEC (19).

As an additional test for the requirement of direct contact between live bacteria and epithelial cells, bacteria were prevented from contacting epithelial cells by a 0.22-μm Anopore membrane filter (Nalge Nunc International, Naperville, IL) or were killed by boiling for 30 min. IL-8 mRNA expression was similar to TNF-α-treated control cells when bacterial adhesion was prevented using 0.22-μm transwell filters (Fig. 1). Similarly, heat-killed lactobacilli did not increase IL-8 expression above levels expressed by HT-29 cells treated with TNF-α alone (Fig. 1). Thus, enhancement of IL-8 mRNA expression in TNF-α-treated HT-29 cells required contact of viable lactobacilli with the cell surface.

Recent studies have reported that IEC express CD14 (13), a PRR capable of binding a variety of bacterial cell wall components (8–12), and whose expression can be activated by TNF-α (9). Therefore, we evaluated the potential for CD14 to mediate TNF-α sensitization of HT-29 cells to *L. plantarum* 299v. RT-PCR was performed with a kit following the manufacturer's protocol (Perkin Elmer). Samples were also prepared without RT to confirm absence of contaminating genomic DNA using primers for CD14 (GenBank accession number M86511): 5'-GCTCAGAGG-TTCGGAAGA (sense), 5'-GTCCTCGAGCGTCAGTT (antisense) and GAPDH (GenBank accession number X01677) 5'-TCATCTCTGCCCCCTCTGCT (sense), 5'-CGACGCCT-GCTTCACCACCT (antisense) as a constitutive control. RT-PCR products were electrophoresed in 2.0% agarose gels stained with ethidium bromide for visualization.

As reported previously (13), CD14 mRNA was expressed in HT-29 cells; however, CD14 mRNA was neither increased by TNF-α alone nor in combination with *L. plantarum* 299v (Fig. 3). Identity of CD14 RT-PCR products was confirmed by purifying products on TE-100 columns (Clontech, Palo Alto, CA) and sequencing using an automated sequencing system (Applied Biosystems, Foster City, CA). Sequence data were analyzed using Sequencer 3.0 (Gene Codes Corp., Ann Arbor, MI), and a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) demonstrated that the RT-PCR sequence was identical to published sequences for human CD14 (GenBank accession number M86511).

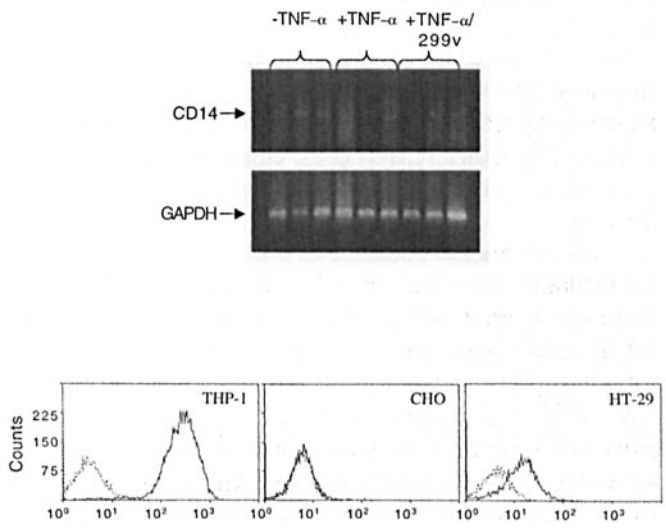

Figure 3. HT-29 expression of CD14. (Top) HT-29 mRNA expression was demonstrated by incubating HT-29 cells for 3 hr in adhesion test medium ± TNF-α (10 ng/ml), then for 3 hr with *L. plantarum* 299v or with adhesion test medium alone. Relative expression of CD14 mRNA in HT-29 cells was determined by performing RT-PCR for 25 amplification cycles. Identity of PCR amplicons was verified by sequence analysis. Results are representative of triplicate samples from three separate adhesion experiments. GAPDH was used as a constitutive control. (Bottom) Cells were treated with the anti-CD14 monoclonal antibody (mAb) MY4 (solid line) for 30 min, followed by 30 min incubation with FITC-labeled goat anti-mouse IgG. A hapten-specific mouse IgG2b (MsIgG2b; hatched line) was used as an isotype control. Vitamin D_3-treated THP-1 cells, which express cell-surface CD14, were also stained with MY4 as a positive control for CD14 expression. CHO cells served as a negative control for CD14 expression. The MsIgG2b and MY4 lines overlap for the CHO-1 sample.

The apparent lack of TNF-α regulation of CD14 expression by HT-29 cells is comparable with findings for other lipopolysaccharide (LPS)-hyporesponsive epithelial cells, such as murine bronchiolar epithelium (21).

To investigate the possibility that the TNF-α-mediated upregulation of IL-8 mRNA expression results from potential increased interactions between *L. plantarum* 299v and CD14, HT-29 cells were treated with a blocking antibody to CD14. First, expression of CD14 on HT-29 cells was confirmed by flow cytometric analysis using the anti-CD14 mAb MY4 (10). HT-29 cells were grown for 3 hr in adhesion test medium ± 10 ng/ml rhTNF-α to evaluate the potential for TNF-α to regulate CD14 expression. As a positive control for CD14 expression, THP-1 cells were cultured for 48 hr in RPMI medium (Gibco) containing 10% fetal calf serum (Summit Biotechnology, Ft. Collins, CO), 40 U penicillin/ml, and 40 μg streptomycin/ml and were treated with $1,25(OH)_2D_3$ (vitamin D3; Sigma) (22). For flow cytometric analysis, 5×10^5 cells of each population were incubated on ice in 50 μl of phosphate-buffered saline with saturation concentrations of the anti-CD14 mAb MY4 (Coulter, Miami FL) or the isotype control (MsIgG2b; Coulter) and counterstained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (BD PharMingen) as per manufacturer's instructions. Fluorescence intensity was measured using an XL flow cytometer (Coulter, Hialeah, FL). Cell-surface CD14 expression was observed for HT-29 cells as well as for vitamin D_3-treated THP-1 cells (Fig. 3). Cell-surface CD14 expression was not altered significantly by treatment with TNF-α (data not shown). Chinese hamster ovary (CHO) cells, cultured in the same medium used for HT-29 cells, were negative for CD14 staining (Fig. 3).

Effects of mAb blockade of CD14 on the *Lactobacillus*-mediated increases in IL-8 mRNA expression were evaluated by performing adhesion tests on TFN-α-treated HT-29 cells as described above, except that 10 μg/ml of the anti-CD14 MY4 mAb or MsIgG2b were added 30 min before addition of bacteria. Nonadherent antibody was removed by washing cells three times with adhesion test medium immediately before adding bacteria. Blockade of CD14 did not prevent the *L. plantarum* 299v-induced increase of IL-8 mRNA expression in TNF-α-treated HT-29 cells (Fig. 4).

Also, the direct addition of bacterial cell wall components (*Escherichia coli* LPS and *Staphylococcus aureus* lipoteichoic acid [LTA]), capable of binding to CD14 (23, 24), to TNF-α-treated HT-29 cells did not affect IL-8 mRNA levels compared with cells treated with TNF-α alone (Fig. 5). Together, these results indicate that CD14 is not directly involved in HT-29 responsiveness to *L. plantarum* 299v.

The present data agree with previous reports of IEC responsiveness to indigenous bacteria (25, 26). For example, the presence of leukocytes was reported to sensitize human Caco-2 cells to nonpathogenic *E. coli* and *Lactobacillus sakei*, increasing epithelial mRNA expression of IL-1β, IL-8, monocyte chemoattractant protein 1 (MCP-1), and TNF-β (26). TNF-α produced by the underlying leukocytes was identified as a principal mediator responsible for increased responsiveness to the nonpathogenic bacteria. Similarly, increased expression of IL-8 mRNA in response to *L. plantarum* 299v only in TNF-α-stimulated HT-29 cells indicates that the local cytokine environment can sensitize IEC to normal gut bacteria. Additional studies are required to elucidate the molecular basis of HT-29 responsiveness to lactobacilli, and to determine why IL-8 RNA and protein expression are discordant in TNF-α-stimulated HT-29 cells exposed to *L. plantarum* 299v.

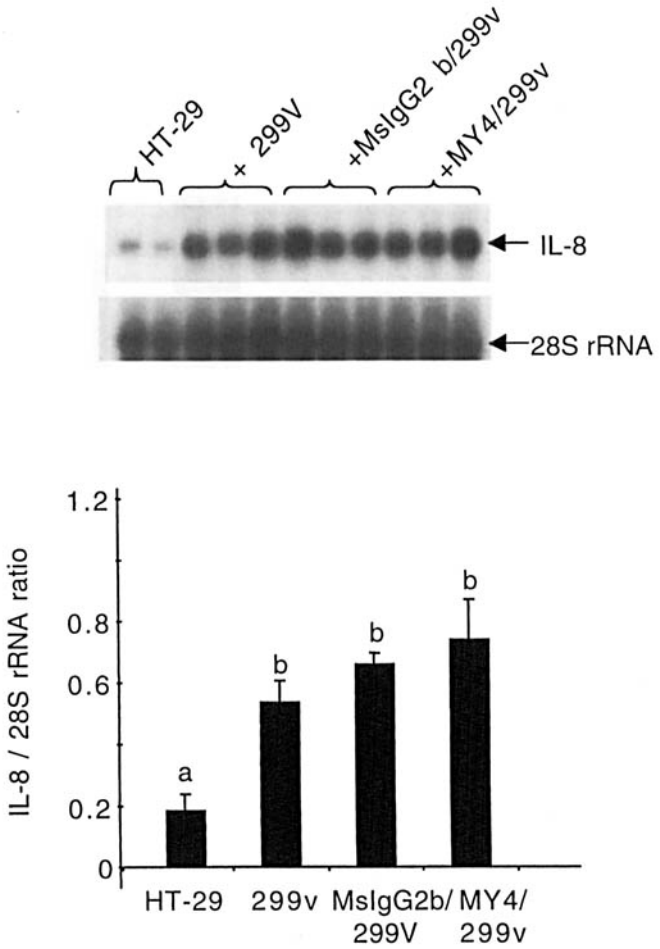

Figure 4. mAb blockade of CD14 does not inhibit TNF-α-dependent alterations of *L. plantarum*-induced IL-8 expression in HT-29 cells. (Top) HT-29 cells were incubated for 3 hr in adhesion test medium containing TNF-α (10 ng/ml), then co-cultured for 3 hr with *L. plantarum* 299v. Indicated samples were also treated with anti-CD14 antibody MY4 or the isotype control MsIgG2b. Total RNA was extracted and Northern blot analysis of IL-8 mRNA expression was performed. Expression of 28S ribosomal RNA was assayed as a constitutive control. Treatments: HT-29 cells; + *L. plantarum* 299v; + MsIgG2b + *L. plantarum* 299v; and MY4 + *L. plantarum* 299v. (Bottom) Ratios of IL-8/28S rRNA expression from HT-29 cells associated with bacteria after treatment with TNF-α. Following densitometric analysis, the ratio of IL-8 to 28S rRNA expression was calculated for each lane. Results represent the means and SEM for three samples from each treatment group. Treatments with different superscripts are statistically different ($P < 0.05$).

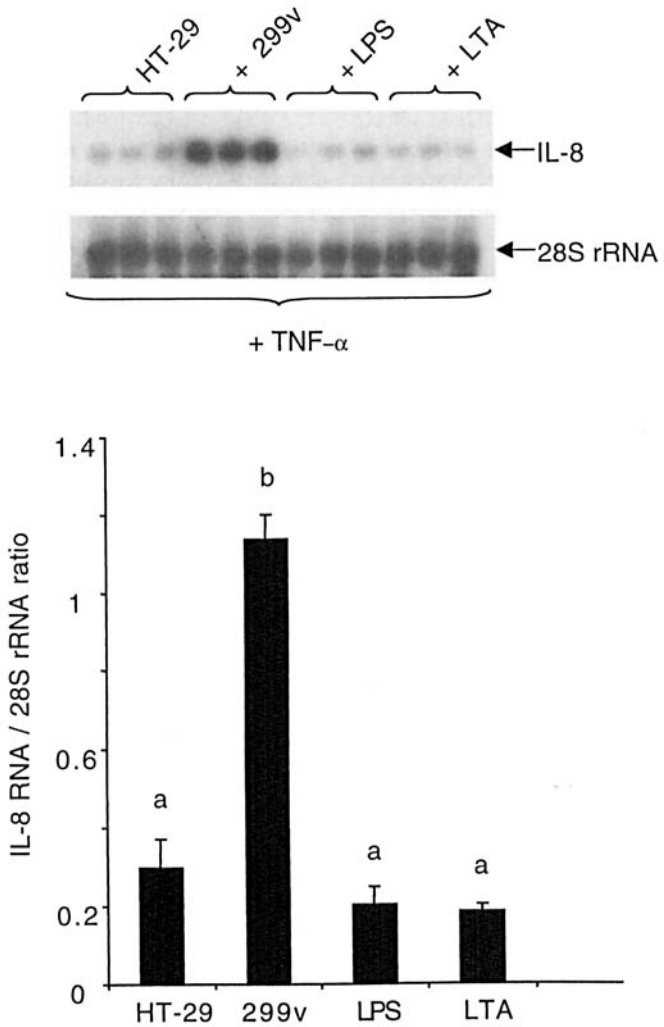

Figure 5. Effects of bacterial cell wall components on IL-8 mRNA expression by TNF-α-treated HT-29 cells. (Top) The effect of bacterial cell wall components on IL-8 mRNA expression was evaluated by administration of 10 ng/ml LPS (*E. coli*) and 1 μg/ml LTA (*S. aureus*) to TNF-α-treated HT-29 cells (3×10^7 cells/plate) for 3 hr. Treatments: HT-29 cells; + *L. planarum* 299v (3×10^{10} CFU); + LPS; and + LTA. (Bottom) Ratios of IL-8/28S rRNA expression from HT-29 cells associated with bacteria after treatment with TNF-α. Following densitometric analysis, the ratio of IL-8 RNA to 28S rRNA expression was calculated for each lane. Results represent the means and SEM for three samples (two controls for group A in Fig. 1A) from each treatment group. Treatments with different superscripts are statistically different ($P < 0.05$).

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