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Nonpathogenic *Escherichia coli* Strain Nissle 1917 Inhibits Signal Transduction in Intestinal Epithelial Cells▽

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Although the probiotic *Escherichia coli* strain Nissle 1917 has been used for the treatment of inflammatory bowel diseases, the precise mechanisms of action of this strain remain unclear. In the present study, we estimated the anti-inflammatory effect of *E. coli* Nissle 1917 on inflammatory responses in vitro to determine the suppressive mechanism of Nissle 1917 on the inflammatory process. To determine the effect of *E. coli* Nissle 1917, the human colonic epithelial cell line HCT15 was incubated with or without *E. coli* Nissle 1917 or another nonpathogenic *E. coli* strain, K-12, and then tumor necrosis factor alpha (TNF-α)-induced interleukin-8 (IL-8) production from HCT15 cells was assessed. Enzyme-linked immunosorbent assays and real-time quantitative PCR showed that Nissle 1917 treatment suppressed TNF-α-induced IL-8 transcription and production. In addition, results from luciferase assays indicated that Nissle 1917 inhibited IL-8 promoter activity. On the other hand, these anti-inflammatory effects were not seen with *E. coli* K-12. In addition, heat-killed Nissle 1917 or its genomic DNA did not have this anti-inflammatory effect. Surprisingly, Nissle 1917 did not affect IL-8 transactivation pathways, such as NF-κB activation, nuclear translocation, and DNA binding, or even activation of other transcriptional factors. Furthermore, it also became evident that Nissle 1917 induced the anti-inflammatory effect without contact to epithelial cells. In conclusion, these data indicate that the nonpathogenic *E. coli* strain Nissle 1917 expresses a direct anti-inflammatory activity on human epithelial cells via a secreted factor which suppresses TNF-α-induced IL-8 transactivation through mechanisms different from NF-κB inhibition.

Inflammatory bowel disease (IBD) is a disease characterized as chronic intestinal mucosal inflammation (3). In spite of recent clinical and basic research, the etiology of IBD remains unknown (16). Recent basic research has indicated that bacterial flora play an important role in the homeostasis of gut, and a dysregulated interaction between intestinal mucosa and flora may contribute to the development and the perpetuation of intestinal inflammation (22). In some genetically engineered rodent colitis models, for example, in murine lines deficient for the T-cell receptor alpha-chain gene (12), the interleukin-10 (IL-10) gene (10), or the IL-2 gene (20) and in HLA-B27 transgenic rat lines (29), the commensal bacteria are required for the development of chronic colitis. These rodent colitis models do not develop intestinal inflammation under germ-free conditions. Some clinical evidence has also indicated the contribution of intestinal flora to the development of colitis, as antibiotics are often effective in human IBD therapy (28). Furthermore, recent linkage analysis identified a single-nucleotide polymorphism of the CARD15 gene, which is the cytosolic receptor for the bacterial cell wall component muramyl dipeptide, as associated with the risk of Crohn's disease (4, 14). Thus, the regulation of the interaction between intestinal mucosa and gut flora is considered to be a novel therapeutic strategy for IBD. Probiotics are defined as living nonpathogenic organisms that confer health benefits by improving the microbial balance (23). It has been reported that probiotics have demonstrated efficacy in the treatment of human IBD (23). Nonpathogenic *Escherichia coli* strain Nissle 1917 is the active component of the microbial drug Mutaflor (Ardeypharm, Germany). This strain is used in several European countries as a probiotic drug for the treatment of IBD. Recent clinical trials have demonstrated the efficacy of Nissle 1917 for the treatment ulcerative colitis (8, 9, 18). Nissle 1917 had equivalent efficacy to mesalazine, which is commonly used for treating IBD, in the maintenance of remission of ulcerative colitis. Although several mechanisms of action have been suggested to explain the protective and anti-inflammatory effect of probiotics, the precise mechanism remains unclear. In this study, we investigated the anti-inflammatory effect of *E. coli* Nissle 1917 on proinflammatory cytokine production from intestinal epithelial cells (IECs).

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MATERIALS AND METHODS

Cell culture. HCT15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated

fetal bovine serum (Biosource International, CA). The cells were inoculated into six-well plates (1.0×10^6 cells/well) and then incubated overnight at 37°C.

Preparation of bacteria. The *E. coli* strain Nissle 1917 (Mutaflor; DMS 6601, serotype O6:K5:H1) was kindly provided by Ardeypharm GmbH (Herdecke, Germany). Nissle 1917 and the nonpathogenic *E. coli* K-12 DH10B strain were used in this study. Bacteria were incubated in Luria-Bertani medium at 37°C in a shaker to reach mid-log phase with the density determined as 0.5 to 0.7 at A_{600}. Bacteria were collected by centrifugation, washed twice with phosphate-buffered saline (PBS), and resuspended in DMEM at up to 1.0×10^5 CFU/ml. Heat-killed bacteria were prepared by resuspending viable bacteria in PBS, followed by incubation for 30 min at 60°C. Killed bacteria were then washed with PBS and resuspended in PBS. Genomic DNA was isolated from Nissle 1917 using a Genomic DNA isolation kit (Qiagen Inc., Valencia, CA).

Treatment for *E. coli* on human IECs. The *E. coli* prepared at 1.0×10^5 CFU/ml were applied to HCT15 cells and coincubated for various lengths of time. After *E. coli* preincubation, HCT15 cells were stimulated with 20 ng/ml of tumor necrosis factor alpha (TNF-α) for 30 min without removing the bacteria. Cells were washed once with cold PBS and scraped with a cell scraper. Cells were collected and washed twice with ice-cold PBS.

ELISA) for IL-8. Collected cells were suspended in lysis buffer (0.5% NP-40, 10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and left to stand on ice for 25 min. The cell lysate was centrifuged at 15,000 rpm for 5 min, and then insoluble debris was removed. The interleukin-8 (IL-8) concentration of the HCT15 cell extraction was measured by an enzyme-linked immunosorbent assay (ELISA) kit (Biosource).

Analysis of IL-8 expression by RT-PCR. HCT15 cells were incubated with live Nissle 1917 (1.0×10^5 CFU/ml), heat-killed Nissle 1917 (1.0×10^8 CFU/ml), or its genomic DNA (10 μg/ml) for 4 h and then stimulated by TNF-α. Cells were harvested after a 30-min stimulation, and total RNA was isolated using an RNeasy mini kit (Qiagen). In this process, RNA was treated with RNase-free DNase I (Qiagen) to prevent carryover of genomic DNA. The cDNA was synthesized from 2 μg of total RNA with Omniscript reverse transcriptase (Qiagen). For quantitative reverse transcription-PCR (RT-PCR), equivalent amounts of cDNAs (2 μl) and 0.5 μM concentrations of the forward and the reverse primers were used with a DyNAmo Sybr Green quantitative PCR (qPCR) kit (MJ Research, Waltham, MA). The PCRs were carried out in a thermocycler DNA Engine, Opticon2 (MJ Research). Cycling conditions for PCR amplification were 95°C for 10 min and 40 cycles of 95°C for 10 s and 58°C for 50 s. Human specific primers for IL-8 were 5′-TCTGCAGCTCTGTGTGAAGGTGCAGTT-3′ (forward) and 5′-AACCCTCTGCACCCAGTTTTCCT-3′ (reverse). β-Actin primers were 5′-CTACGTCGCCCTGGACTTCGAGC-3′ (forward) and 5′-GATGGAGCCGCCGATCCACACG-3′ (reverse).

Construction of IL-8 luciferase reporter constructs. The 5′ deletion constructs of the human IL-8 promoter corresponding to sequences from −1481 (pNAF), −130 (pN130), −112 (pN112), and −78 (pN78) to +44 bp and a promoterless plasmid (pLuc0) were kindly provided by S. Abe (Yamagata University, Japan). Site-directed mutagenesis of the IL-8 promoter was performed using a QuickChange site-directed mutagenesis kit (Stratagene). The construct containing the −130-bp sequence upstream from the transcription start site of the IL-8 gene (pN130) was used as a template plasmid. The primers used for point mutations for the activator protein 1 (AP-1) site (at −126 to −120, TGACTCA to TATC TCA; mutation is underlined) were 5′-GTGTGATATCTCAGGTTTGCCCTG AGGG-3′ (forward) and 5′-CCCTCAGGGCAAACCTGAGATATCACAC-3′ (reverse). For C/EBPβ (point mutations at −94 to −81, CAGTTGCAAATCGT to AGCTTGCAAATCGT), the primers were 5′-GGATGGGCCATAGCTTG CAAATCGTGG-3′ (forward) and 5′-CCACGATTTGCAAGCTATGGCCCA TCC-3′ (reverse). For NF-κB (point mutations at −80 to −71, GGAATTTCCT to TAACTTTCCT), the primers were 5′-GTTGCAAATCGTTAACTTTCCTC TGACATAATG3′ (forward) and 5′-CATTATGTCAGAGGAAAGTTAACG ATTTGCAAC-3′ (reverse). These plasmid constructs were confirmed by sequencing.

DNA transfection and luciferase reporter assay. A total of 1×10^6 HCT15 cells/well were seeded on six-well plates. After 24 h, the cells were transiently transfected with the indicated reporter plasmid (1 μg/well) with the *Renilla* luciferase expression plasmid pRL-TK (0.1 μg/ml) (Promega), using FuGENE 6 transfection reagent (Roche). After incubation of 24 h, cells were washed twice with PBS and cultured for 4 h in serum and antibiotic-free Hank's balanced buffered salt containing calcium chloride with or without Nissle 1917 (1.0×10^7 CFU/ml). Cells were then stimulated by TNF-α (20 ng/ml) for 30 min. The luciferase activity of total cell lysates was measured using a dual luciferase reporter assay system (Promega). The *Renilla* luciferase reporter gene pRL-TK was used as an internal control.

Western blot analysis. HCT15 cells were grown in six-well plates. The cells were pretreated with 1.0×10^5 CFU/ml Nissle 1917 for 4 h and then stimulated with 20 ng/ml of TNF-α. Total cell lysates and nuclear protein from HCT15 cells were obtained using mammalian protein extraction reagent and a nuclear and cytosol protein extraction kit (Pierce). Each protein was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 5% milk and incubated overnight with anti-p65 polyclonal antibody (Santa Cruz), anti-IκBα polyclonal antibody (Santa Cruz), or anti-β-actin polyclonal antibody (Sigma) at 4°C, followed by incubation at room temperature for 1 h with horseradish peroxidase-linked anti-rabbit antibody (Cell Signaling). Staining was detected using ECL Western blotting detection reagent (Amersham Biosciences).

Nuclear protein extraction and electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from HCT15 cells using a nuclear and cytosol protein extraction kit (Pierce). Both double-stranded oligonucleotide probes corresponding to the wild-type NF-κB binding site (5′-AGTTGAGGGGACTT TCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′ [Promega]) and the mutated NF-κB binding site (5′-AGTTGAGGCGACTTTCCCAGG C-3′ and 3′-TCAACTCCGCTGAAAGGGTCCG-5′; mutations are underlined [Santa Cruz]) were used as probes or cold competitors to analyze the interaction between NF-κB and DNA. The probes were labeled with [γ-^{32}P]ATP (3,000 Ci/mmol at 10 mCi/ml) (Amersham Biosciences) using T4 polynucleotide kinase (Promega) and purified by MicroSpin G-25 columns (Amersham Biosciences). The binding reaction was performed using a gel shift assay system (Promega) and 20 μg of nuclear extract. For the competition assay, a 100-fold excess of unlabeled oligonucleotide was added to the reaction mixture. Samples were electrophoresed by a 4% nondenaturing acrylamide gel in 0.5× Tris-borate-EDTA buffer at 150 V for 1.5 h. The gel was placed on a filter paper and dried with a Gel Dryer (Bio-Rad) for 1 h. Then, the gel was exposed to a phosphorimaging plate overnight and analyzed by BAS 2000 systems.

ChIP assay. The HCT15 cells were cross-linked by 1% formaldehyde for 15 min at 37°C. Cross-linking was stopped by the addition of 0.125 M glycine, and cells were incubated for 10 min at 4°C and then washed twice with ice-cold PBS. Following cross-linking, the cells were collected and resuspended in sodium dodecyl sulfate lysis buffer (Upstate) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin), and the chromatin was cleaved into 500- to 1,000-bp fragments by sonication. Samples were then diluted with chromatin immunoprecipitation (ChIP) dilute buffer and incubated overnight with 3 μg of specific antibodies for p65 (Upstate) or RNA polymerase II (Santa Cruz), followed by incubation with protein A-agarose saturated with bovine serum albumin and salmon sperm DNA. Agarose-bound immune complexes were collected, washed, and eluted. The protein-DNA cross-links were incubated overnight at 65°C to reverse cross-links. After proteinase K digestion, DNA was purified using a PCR purification kit (Qiagen). Purified immunoprecipitated chromatin and input samples were analyzed by real-time qPCR using a DyNAmo Sybr Green qPCR kit and the thermocycler DNA Engine Opticon2 (MJ Research). Primer pairs for monitoring the IL-8 promoter region were 5′GGGCCATCAGTTGCAAATC-3′ (forward) and 5′-TTCCTTCCGGTGGTTTCTTC-3′ (reverse). Cycling conditions for PCR amplification were 95°C for 10 min and 40 cycles of 95°C for 10 s and 58°C for 50 s.

Analysis of the suppressive effect of Nissle 1917 secreted factor. HCT15 cells were cultured in the lower well of the Transwell filter membrane system (0.4-μm pore size; Costar). *E. coli* Nissle 1917 or K-12 DH10B prepared at 1.0×10^5 CFU/ml was applied to the upper well and coincubated for 4 h. After preincubation, cells were stimulated with 20 ng/ml of TNF-α for 30 min. In a separate test, Nissle 1917 or K-12 DH10B (1.0×10^5 CFU/ml) alone was incubated in DMEM for 4 h, and then supernatants were separated by passage through 0.22-μm-pore-size filters. Filtered culture supernatants were then applied to the HCT15 cells and incubated for 30 min. After incubation, cells were stimulated with 20 ng/ml of TNF-α for 30 min. For both culture methods, after stimulation with TNF-α, cells were washed once with cold PBS and scraped with a cell scraper. Cells were then collected and washed twice with ice-cold PBS. Cellular proteins were extracted by the protein extraction method, described above, and IL-8 concentrations were measured by ELISA.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software, version 4.0 (San Diego, CA). Differences at a P value of <0.05 were considered to be significant. All data are expressed as means ± standard error of the mean (SEM).

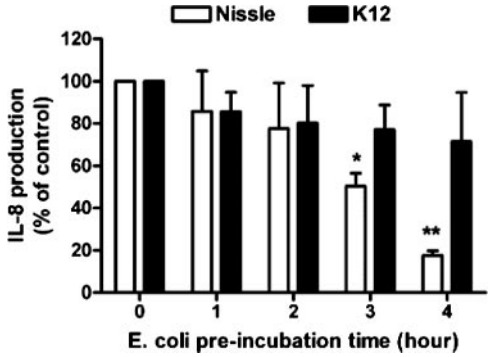

FIG. 1. *E. coli* Nissle 1917 suppresses TNF-α-induced IL-8 production. HCT15 cells were pretreated for various lengths of time with 1×10^5 CFU/ml nonpathogenic *E. coli* strain Nissle 1917 or K-12 DH10B and were then stimulated with 20 ng/ml TNF-α for 30 min. After stimulation, intracellular proteins were extracted, and IL-8 protein amounts were determined by ELISA. Data are expressed as the percentage of IL-8 production in HCT15 cells stimulated with TNF-α without Nissle 1917 treatment (defined as 100%) and are given as means ± SEM of three to four experiments. Significance was determined by comparison with the values of TNF-α-induced IL-8 in the *E. coli* untreated group (Dunnett's test). *, $P < 0.05$; **, $P < 0.01$.

RESULTS

***E. coli* Nissle 1917 inhibited TNF-α-induced IL-8 production by IECs.** To determine the effect of Nissle 1917, human IEC line HCT15 cells were untreated or pretreated with *E. coli* Nissle 1917 and another nonpathogenic *E. coli* strain, K-12 DH10B (1×10^5 CFU/ml), for 1 to 4 h, followed by stimulation with TNF-α (20 ng/ml) for 30 min. After stimulation, the intracellular protein levels of the proinflammatory chemokine IL-8 were assayed by ELISA. The basal production of IL-8 was not affected by *E. coli* Nissle 1917 and K-12; however, TNF-α-induced IL-8 production was significantly inhibited by a 3- to 4-h pretreatment with Nissle 1917 but not by K-12 (Fig. 1). Another enteric *E. coli* strain, ATCC 25922, did not show the inhibitory effect on TNF-α-induced IL-8 production, similar to K-12 (data not shown). These results suggest that only the probiotic strain Nissle 1917 has an anti-inflammatory effect among the nonpathogenic *E. coli* strains. Next, we examined the effect of Nissle 1917 on IL-8 mRNA expression after TNF-α stimulation using real-time qPCR. Consistent with the result of protein production, TNF-α-induced IL-8 mRNA transcription was also suppressed by pretreatment with *E. coli* Nissle 1917 (Fig. 2A) but not by *E. coli* K-12 (data not shown). Moreover, the proinflammatory chemokine methyl-accepting chemotaxis protein 1, which was strongly induced by TNF-α, was also suppressed by Nissle 1917 (data not shown). Because recent studies have reported that genomic DNA itself has anti-inflammatory effects in vitro and in vivo (5, 7, 17), we investigated the effect of genomic DNA from Nissle 1917 and heat-killed Nissle 1917 on IL-8 production from TNF-α-treated HCT15 cells. As shown in Fig. 2B, heat-killed Nissle 1917 and its genomic DNA did not show such an anti-inflammatory effect. These results indicated that only the live *E. coli* Nissle 1917, and not heat-killed and genomic DNA alone, can inhibit TNF-α-induced IL-8 production, even in the transcriptional process.

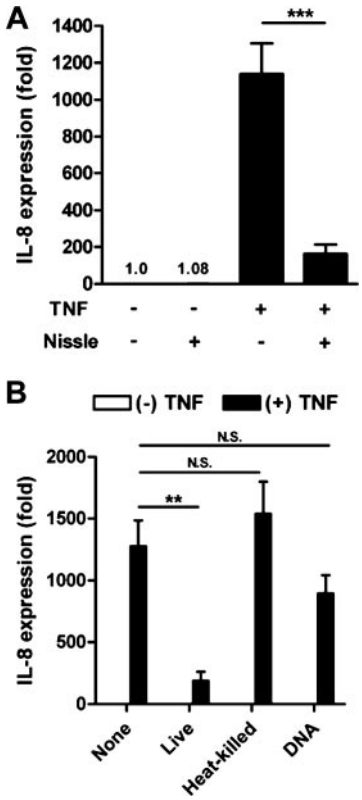

FIG. 2. *E. coli* Nissle 1917 suppresses TNF-α-triggered transcription of IL-8 via its viable form. (A) HCT15 cells were pretreated with Nissle 1917 (1×10^5 CFU/ml) for 4 h and then stimulated with 20 ng/ml TNF-α. After stimulation for 30 min, IL-8 mRNA expression was analyzed by real-time RT-PCR. (B) HCT15 cells were pretreated with living Nissle 1917 bacteria (1×10^5 CFU/ml), heat-killed Nissle 1917 (1×10^8 CFU/ml), or its genomic DNA (10 μg/ml) for 4 h and then stimulated by 20 ng/ml TNF-α for 30 min. IL-8 mRNA expression was analyzed by real-time RT-PCR. Data are shown as relative expression against β-actin mRNA and given as means ± SEM of five independent experiments. Significance was determined by comparison with the values of TNF-α-induced IL-8 in the *E. coli* untreated group (Sheffe's test). **, $P < 0.01$; ***, $P < 0.001$.

***E. coli* Nissle 1917 suppresses the promoter activity of human IL-8.** To identify the transcriptional regulation of IL-8 by Nissle 1917, a series of luciferase reporter plasmids of the IL-8 promoter were used. As shown in Fig. 3A, luciferase activity in HCT15 cells transfected with a vector containing the entire 1,481-bp upstream DNA fragment (pNAF1481) was higher than that obtained with the empty pLuc0 vector by TNF-α stimulation. Promoter activity was significantly decreased in pN78 compared with pN112. This result indicated that the sequence upstream of −78 is required for TNF-α-induced IL-8 promoter activity. Consistent with the results of real-time PCR and ELISA, Nissle 1917 pretreatment suppressed the TNF-α-induced transactivation of IL-8 promoter activity in both pN130 and the deletion mutant of the AP-1 binding sequence, pN112. This result suggests that AP-1 is not the crucial target for the suppressive effect of Nissle 1917 on TNF-α-induced IL-8 transactivation. In addition, the nonpathogenic *E. coli* strain K-12 did not inhibit IL-8 promoter activity in either ELISA or RT-PCR analysis (data not shown). To identify the

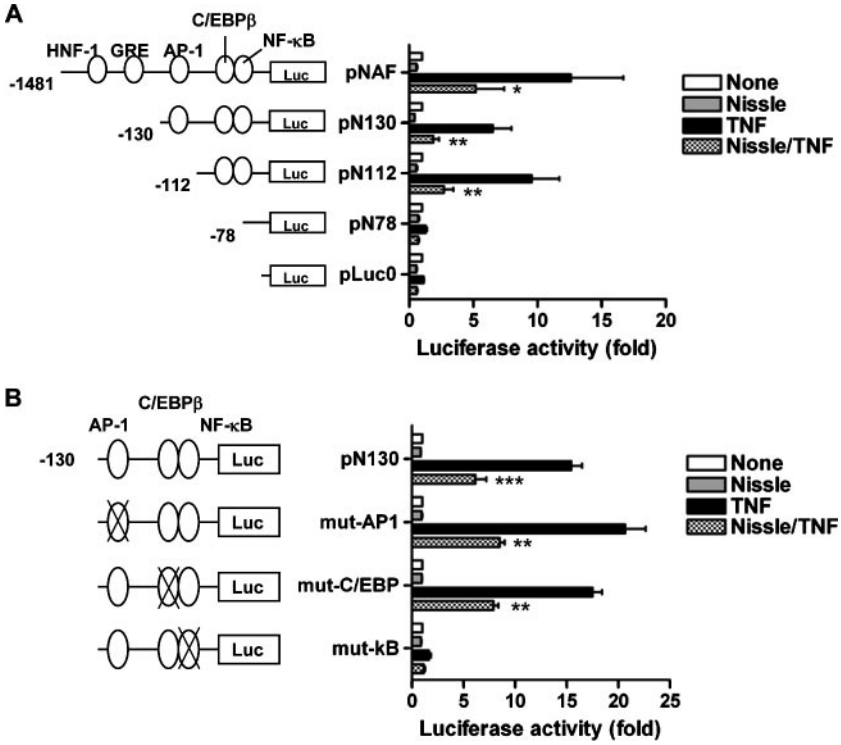

FIG. 3. Nissle 1917 inhibits IL-8 promoter activity. (A) HCT15 cells were transfected with 5′ deletions of the IL-8 promoter-driven luciferase. Each sample contained *Renilla* luciferase vector DNA (pRL-TK) for normalization of transfection efficiency. Twenty-four hours after transfection, cells were treated with Nissle 1917 bacteria (1.0×10^7 CFU/ml) for 4 h in serum-free Hank's balanced salt solution and then stimulated with TNF-α for 30 min. Cells were lysed and assayed for luciferase activity. (B) The proximal human IL-8 promoter region (position −130) was used for mutation assays. Mutant (mut) vectors of NF-κB, C/EBPβ, or AP-1 binding sites were transfected into HCT15 cells and then treated with Nissle 1917 and TNF-α, as described above. Cells were lysed and assayed for luciferase activity. The results are presented as the relative increase in activation (*n*-fold) of control cells and are given as means ± SEM of four to six independent experiments. Significance was determined by comparison with the values of TNF-α-induced IL-8 in the *E. coli* untreated group (paired Student's *t* test). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. GRE, glucocorticoid response element.

responsible element of the IL-8 promoter sequence in the Nissle 1917-mediated inhibition, mutated constructs of each transcription factor binding sequence were generated (Fig. 3B). As a result of the deletion study, a mutated construct in the AP-1 binding site showed increased luciferase activity as a result of TNF-α stimulation and the inhibitory effects of Nissle 1917. Moreover, the construct with a mutation of the C/EBPβ (CCAAT/enhancer-binding protein β) binding site also had a normal response to TNF-α stimulation and decreased luciferase activity with Nissle 1917 pretreatment. These results suggest that AP-1 and C/EBPβ were not responsible for the suppressive effect of Nissle 1917. However, up-regulation of luciferase activity by TNF-α stimulation was completely suppressed by mutation of the NF-κB binding site, as indicated by the results of the deletion study.

To investigate whether Nissle 1917 can inhibit NF-κB activation, which is considered the element responsible for the augmentation of IL-8 transcription by TNF-α, we next analyzed the activation of NF-κB by Western blot analysis. In the normal state, NF-κB exists in the cytoplasm as a nonactivated complex consisting of three subunits: a transactivating subunit p65 (also called RelA), p50, and an inhibitory subunit, IκBα. With exposure to an inflammatory stimulus such as TNF-α, the inhibitory subunit IκBα is phosphorylated and degraded; then the transactivating subunit p65-p50 heterodimer is translocated into the nucleus. As shown in Fig. 4A and B, IκBα degradation and nuclear translocation of the p65 subunit were observed after TNF-α stimulation. However, treatment with Nissle 1917 did not suppress these processes. These data indicate that Nissle 1917 does not affect the nuclear translocation of p65. To determine whether Nissle 1917 prevented the DNA binding process of transcriptional factors, we carried out EMSAs. As shown in Fig. 4C, TNF-α stimulation increased DNA binding of NF-κB, but this was not inhibited by Nissle 1917 treatment (lanes 3 and 4). Another transcriptional factor, C/EBPβ, did not prevent DNA binding with Nissle 1917 treatment (data not shown). Since EMSAs assessed the binding of NF-κB consensus motifs in vitro, p65 recruitment to the endogenous IL-8 promoter in intact cells was tested by ChIP assay. Stimulation of TNF-α caused extensive recruitment of the p65 NF-κB subunit and transcriptional cofactor CBP to the IL-8 promoter, and this was not blocked by Nissle 1917 treatment (Fig. 4D). Thus, the suppressive effect of Nissle 1917 on the transcription of TNF-α-induced IL-8 is independent of the NF-κB activation process.

Nissle 1917 secreted factor suppressed TNF-a induced IL-8 production. To determine the anti-inflammatory effect of Nissle 1917 secreted factors, we used the Transwell system

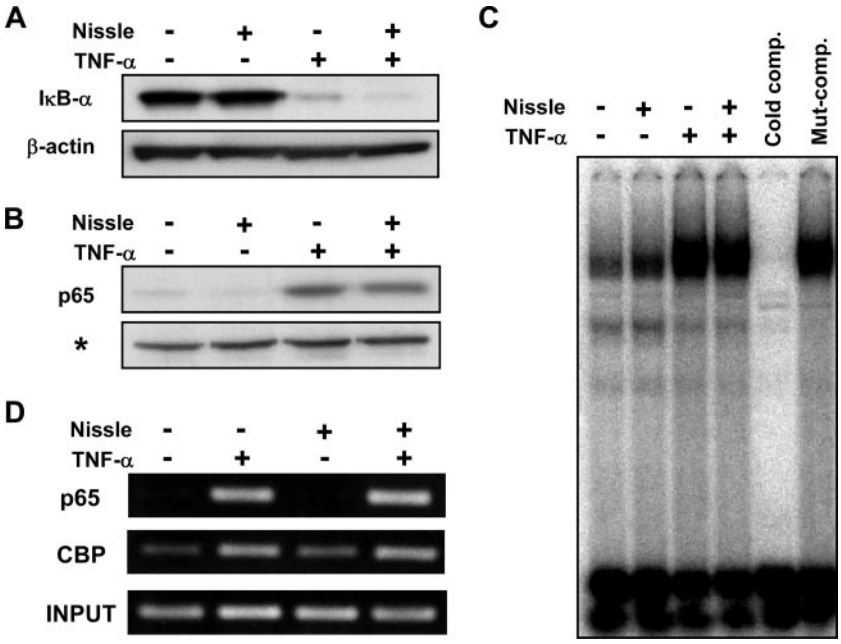

FIG. 4. Nissle 1917 does not affect NF-κB transactivation pathways. (A) HCT15 cells were pretreated with Nissle 1917 bacteria (1×10^5 CFU/ml) for 4 h and then stimulated with 20 ng/ml TNF-α. After stimulation for 30 min, whole cellular proteins were extracted and used for Western blotting of IκBα and β-actin. (B) HCT15 cells were treated with Nissle 1917 and TNF-α as before, and then nuclear proteins were extracted. Extracted nuclear proteins were used for NF-κB p65 Western blotting. A nonspecific protein recognized by the antibody was used as loading control (asterisk). Data are representative of one of three independent experiments. (C) HCT15 cells were pretreated with Nissle 1917 bacteria (1×10^5 CFU/ml) for 4 h and then stimulated with 20 ng/ml TNF-α. After stimulation for 30 min, nuclear proteins were extracted. EMSAs were performed with a ^{32}P-labeled probe containing the NF-κB binding site and nuclear extracts from TNF-α-stimulated HCT15 cells with or without Nissle 1917 pretreatment. Data are representative of one of three independent experiments. (D) HCT15 cells were pretreated with Nissle 1917 bacteria (1×10^5 CFU/ml) for 4 h and then stimulated with 20 ng/ml TNF-α for 30 min. ChIP assays were performed with the indicated antibodies. The detection of the immunoprecipitated human IL-8 promoter was analyzed by PCR with promoter-specific primers. Data are representative of one of three independent experiments. Mut, mutant; comp, competitor.

(Fig. 5A) and *E. coli* culture supernatant (Fig. 5B) in tandem with control strain K-12 for the IL-8 prevention assay. Interestingly, Nissle 1917 inhibited TNF-α-induced IL-8 production but not through contact with the cells. These results suggest that a currently unknown Nissle 1917 secreted factor in the culture supernatant suppressed the TNF-α-triggered inflammatory processes.

DISCUSSION

IECs play a role as a barrier both functionally and structurally. IECs separate the host's internal milieu from the external environment. In addition to the barrier function, it has become evident recently that IECs play an important role in maintaining homeostasis. IECs produce antimicrobial peptides, such as defensins, and protect the host from attachment of luminal bacteria (21, 30). Moreover, in addition to a direct bactericidal role, IEC-derived factors can promote an anti-inflammatory type of dendritic cells and macrophages differentiation to induce mucosal tolerances against luminal bacteria (19, 26). Furthermore, IECs can produce several chemokines and proinflammatory cytokines to induce the migration of granulocytes, lymphocytes, and dendritic cells, resulting in the induction of host immunity (1). Previous studies have demonstrated that IECs produce IL-8 in response to several pathogenic bacteria (6). Thus, IECs function as a defensive frontline of host mucosal immunity. Although the therapeutic mechanism of action

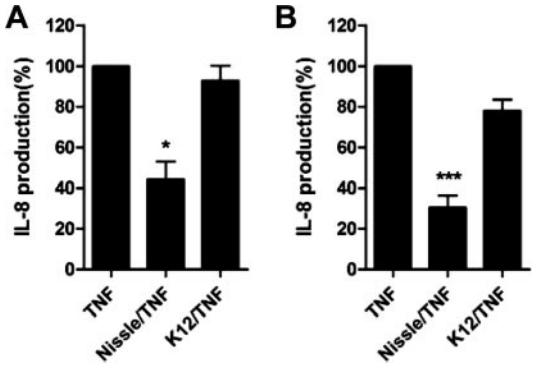

FIG. 5. Nissle 1917 inhibited TNF-α-induced IL-8 production without adherence of bacteria to the epithelial cells. (A) HCT15 cells were cultured in the lower well of a Transwell filter membrane system (0.4-μm pore size). *E. coli* Nissle 1917 or K-12 DH10B prepared at 1.0×10^5 CFU/ml was applied to the upper well and coincubated for 4 h. After preincubation, cells were stimulated with 20 ng/ml of TNF-α for 30 min. (B) *E. coli* Nissle 1917 or K-12 DH10B (1.0×10^5 CFU/ml) alone was incubated in DMEM for 4 h, and then supernatants were separated by passage through a 0.22-μm-pore-size filter. Filtered culture supernatants were then applied to the HCT15 cells and incubated for 30 min. After incubation, cells were stimulated with 20 ng/ml of TNF-α for 30 min. After stimulation, intracellular proteins were extracted, and IL-8 protein amounts were determined by ELISA. Data are expressed as the percentage of IL-8 production in *E. coli* untreated controls (defined as 100%). Data are given as means ± SEM of the at least three experiments. *, $P < 0.05$; ***, $P < 0.001$ (Dunnett's test).

of probiotics in IBD remains unclear, it was previously reported that some nonpathogenic resident bacteria and probiotic bacteria strains suppress IL-8 production from IECs. For example, nonpathogenic *Salmonella enterica* serovar Typhimurium (13), *Lactobacillus reuteri* (11), or the probiotic VSL#3 (5) prevented TNF-α-induced IL-8 production. These suppressive functions of nonpathogenic resident bacteria and probiotic strains may contribute to maintaining intestinal homeostasis or show therapeutic effects as a probiotics. Consistent with studies of other probiotic bacteria, we found a suppressive effect of nonpathogenic *E. coli* Nissle 1917 on TNF-α-induced IL-8 production. This function is one of the therapeutic mechanisms of Nissle 1917 on IBD. There have been some reports on the inhibitory mechanisms of the signal transduction pathway by probiotic bacteria: a nonpathogenic *Salmonella* species was found to inhibit IκB ubiquitination and the subsequent NF-κB activation (13); *L. reuteri* inhibited IκB degradation (11); VSL#3 suppressed the NF-κB activation pathway through proteosome inhibition (15); and genomic DNA from VSL#3 inhibited the phosphorylation of p38 mitogen-activated protein kinase (5). In our present study, Nissle 1917 did not inhibit any of the signal transduction pathways described above, although mRNA transcription of IL-8 was suppressed. In fact, IκB degradation, NF-κB p65 nuclear translocation, and DNA binding were not dramatically altered even after Nissle 1917 treatment. These results indicated that Nissle 1917 does not suppress the NF-κB activation pathway, in contrast to *L. reuteri*. Moreover, our study demonstrated that Nissle 1917 does not affect another signal transduction pathway for IL-8 expression, namely, activation of C/EBPβ and phosphorylation of p38 mitogen-activated protein kinase (data not shown). Therefore, how does Nissle 1917 prevent IL-8 transcription if not through the inhibition of NF-κB or activation of other transcriptional factors? There is a possibility that Nissle 1917 may suppress the post-DNA binding process of transcriptional factors, prevent activation of basic transcriptional factors or RNA polymerase, or suppress chromatin remodeling processes, such as phosphorylation or acetylation of histones.

What is the active component of probiotic bacteria that is responsible for their anti-inflammatory function? Ma et al. demonstrated that *L. reuteri* inhibited TNF-α-induced IL-8 expression only through the viable form (11), and Petrof et al. reported that the VSL#3 secreted factor prevented NF-κB activation (15). In contrast, it was also demonstrated by Jijon et al. and Rachmilewitz et al. that VSL#3 genomic DNA had an anti-inflammatory function in vitro and in vivo (5, 17). We have already demonstrated that Nissle 1917 heat-killed antigen and its genomic DNA can prevent murine colitis (7). However, in our present data, heat-killed Nissle 1917 or its DNA could not suppress the TNF-α-induced IL-8 expression from IECs. This suppressive effect on the production of IL-8 from IECs was displayed only by live Nissle 1917 bacteria. Recently, the therapeutic mechanisms of Nissle 1917 have been proposed based on results from in vitro experiments. For example, Nissle 1917 increased the expression of antibacterial peptides, human β-defensins, by epithelial cell lines through the NF-κB and AP-1 pathways (24, 31); Nissle 1917 modulated T-cell cycling and expansion via Toll-like receptor 2 (TLR2) signaling (27). In addition, an anti-inflammatory effect of Nissle 1917 was reported by some investigators using in vivo experiments in several models of murine experimental colitis. We along with other groups have reported that Nissle 1917 treatment prevented both acute and chronic colitis via suppression of the production of proinflammatory cytokines by mesenteric lymph nodes or lamina propria mononuclear cells (7, 25). Moreover, Grabig et al. reported that Nissle 1917 ameliorated acute dextran sodium sulfate-induced colitis via a TLR2- and TLR4-dependent pathway (2). In the present study, we have demonstrated the first direct evidence of the anti-inflammatory effect of Nissle 1917. The findings presented here could support the anti-inflammatory effect of Nissle 1917 in murine and human intestinal inflammation.

In conclusion, we have provided evidence that the probiotic *E. coli* strain Nissle 1917 as well as other probiotic strains suppresses TNF-α-induced IL-8 secretion from IECs. Moreover, the mechanism by which Nissle 1917 suppresses IL-8 might be independent of the inhibition of the NF-κB signaling pathway. Thus, our findings may provide new insight into the mechanisms of action of the probiotic strain Nissle 1917 in IBD.

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