Bifidobacterium lactis Inhibits NF-κB in Intestinal Epithelial Cells and Prevents Acute Colitis and Colitis-associated Colon Cancer in Mice

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Background: The aim of this study was to investigate the anti-inflammatory effects of *Bifidobacterium lactis* on intestinal epithelial cells (IECs) and on experimental acute murine colitis and its tumor prevention effects on colitis-associated cancer (CAC) in mice.

Methods: Human HT-29 cells were stimulated with IL-1β, lipopolysaccharides, or tumor necrosis factor-α with and without *B. lactis*, and the effects of *B. lactis* on nuclear factor kappa B (NF-κB) signaling in IEC were examined. For in vivo study, dextran sulfate sodium (DSS)-treated mice were fed with and without *B. lactis*. Finally, we induced colonic tumors in mice by azoxymethane (AOM) and DSS and evaluated the effects of *B. lactis* on tumor growth.

Results: *B. lactis* significantly suppressed NF-κB activation, including NF-κB-binding activity and NF-κB-dependent reporter gene expression in a dose-dependent manner, and suppressed IκB-α degradation, which correlated with the downregulation of NF-κB-dependent gene products. Moreover, *B. lactis* suppressed the development of acute colitis in mice. Compared with the DSS group, the severity of DSS-induced colitis as assessed by disease activity index, colon length, and histological score was reduced in the *B. lactis*-treated group. In the CAC model, the mean number and size of tumors in the *B. lactis*-treated group were significantly lower than those in the AOM group.

Conclusions: Our data demonstrate that *B. lactis* inhibits NF-κB and NF-κB-regulated genes in IEC and prevents acute colitis and CAC in mice. These results suggest that *B. lactis* could be a potential preventive agent for CAC as well as a therapeutic agent for inflammatory bowel disease.

(Inflamm Bowel Dis 2010;16:1514–1525)

Key Words: Bifidobacterium lactis, colitis, colitis-associated colon cancer, nuclear factor kappa B, inflammatory bowel disease

Inflammatory bowel disease (IBD) is a group of chronic and relapsing intestinal inflammatory disorders of unknown etiology. Currently, no drugs for the treatment of IBD have a nonrelapsing cure rate and few nontoxic therapeutic options are available to modulate intestinal inflammation. Furthermore, epidemiological studies have demonstrated an increased risk of colorectal cancer in patients with ulcerative colitis and Crohn's disease involving the colon.[1,2] In light of these facts, more effective and safe therapeutic and chemopreventive strategies for IBD are urgently needed.

The activation of the proinflammatory gene transcriptional program in intestinal epithelial cells (IECs) in response to challenges by bacterial products such as lipopolysaccharides (LPSs) or inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β), is associated with acute and chronic intestinal inflammation.[3,4] This signal ultimately converges on the nuclear factor kappa B (NF-κB) transcriptional system.[5] Activation of NF-κB then upregulates the expression of various proinflammatory genes involved in intestinal inflammation.[6] Because the NF-κB transcriptional system in IEC plays an essential role in the regulation of inflammation in patients with various intestinal disorders, targeting this signaling pathway may offer a therapeutic avenue for treating these diseases.[7] Moreover, NF-κB is a multifunctional transcription factor that regulates the expression of a number of genes whose products are involved in tumorigenesis[8,9] and in regulating tumor development resulting from chronic

Received for publication January 14, 2010; Accepted January 21, 2010.

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Supported by a faculty research grant of Yonsei University College of Medicine for 2007 (6-20070224).

Presented during UEGW 2008 in Vienna, Austria.

DOI 10.1002/ibd.21262

Published online 22 March 2010 in Wiley Online Library (wileyonlinelibrary.com).

inflammation.[10] Persistent NF-κB activation has been suggested to contribute to cancer development. Furthermore, activation of NF-κB in response to chronic inflammation may be of particular relevance to gastrointestinal carcinogenesis, especially in colitis-associated cancer (CAC).[11]

A growing body of evidence suggests that certain probiotic bacteria can modulate intestinal inflammation, cell proliferation, and apoptosis, and intestinal epithelial homeostasis.[12–14] Such properties may be useful for both immunomodulatory and cancer prevention strategies.[15] Manipulation of intestinal bacterial flora has been used as an alternative health approach for disease prevention and treatment. *Bifidobacterium lactis* is a Gram-positive, anaerobic commensal-derived probiotic species.[16] Interestingly, recent investigations have suggested that *B. lactis* has potent antiinflammatory and antiproliferative effects.[17,18] However, the precise mechanisms by which these probiotic bacteria exert their putative antiinflammatory and antitumorigenic influences are uncertain.

Because of the central role of NF-κB signaling in immune response and cancer development, we speculated that *B. lactis* mediates antiinflammatory and antiproliferative effects by modulating NF-κB signaling pathways in IEC. Therefore, we hypothesized that *B. lactis* could inhibit NF-κB in IEC and suppress in vivo acute and chronic intestinal inflammation and thereby suppress CAC. Thus, we aimed to investigate the effect of *B. lactis* on LPS- or cytokine-induced NF-κB signaling and proinflammatory and protumorigenic gene expression in HT-29 intestinal epithelial cancer cells. Moreover, we evaluated the antiinflammatory effects of *B. lactis* on acute colitis induced by dextran sodium sulfate (DSS) and the cancer prevention effects of *B. lactis* on CAC induced by azoxymethane (AOM) and DSS in a murine model.

MATERIALS AND METHODS

Bacterial Strains and Cell Lines

B. lactis (KCTC 5727, Korean collection for type cultures, Seoul, Korea) was obtained from Cell Biotech (Seoul, Korea). Lyophilized cultures (2×10^{10} colony-forming unit [CFU]/g) of *B. lactis* were dissolved with phosphate-buffered saline (PBS) to feed experimental animals in the acute colitis model. Mice were fed a diet including a 5×10^8 CFU/g diet of *B. lactis* in the CAC model. The *B. lactis* cells were suspended in deMan-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit, MI) and plated in MRS agar plates and cultured at 37°C under microaerobic conditions. Bacteria were counted using a plating technique and the cell counts in the bacterial suspension were estimated by optical density at an absorbance of 600 nm (UV-1601, Shimadzu, Kyoto, Japan). The *B. lactis* cells grown in MRS broth were collected by centrifugation (3000 rpm for 15 minutes), washed, and resuspended in 10 mL RPMI 1640, excluding antibiotics. The bacteria were added to the HT-29 cell culture wells at the appropriate dilution to reach a final concentration of 10^7, 10^8, or 10^9 CFU per mL of the incubation medium without antibiotics. The human colon cancer cell line HT-29 (KCLB 30038, Korean Cell Line Bank, Seoul, Korea) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics.

Reagents

DSS (36,000–50,000 Da; MP Biomedicals, Aurora, OH) was dissolved in distilled water at a concentration of 2% or 3.5% (w/v). A colonic carcinogen, AOM, was purchased from Sigma Chemical (St. Louis, MO).

Electrophoretic Mobility Shift Assay (EMSA)

To assess NF-κB activation, nuclear and cytoplasmic extracts were prepared. Briefly, harvested cells were lysed for 5 minutes in lysis buffer (5 mM KCl, 25 mM HEPES, 0.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 0.2% Triton X-100, complete protease inhibitor cocktail [Roche Diagnostics, Mannheim, Germany]), and then nuclei were pelleted by centrifugation at 6000 rpm for 15 minutes. Nuclei were lysed in a hypotonic buffer (350 mM NaCl, 10 mM HEPES, 25% glycerol, 0.1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, and protease inhibitors as above) for 30 minutes and debris was cleared by centrifugation at 13,000 rpm for 5 minutes. Bradford reagent (Bio-Rad Laboratories, Hercules, CA) was used to measure protein content. EMSA was performed using Lightshift chemiluminescent EMSA kit (Pierce, Rockford, IL) by following the manufacturer's protocol. Complementary NF-κB oligonucleotides 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; 3′-GCC TGG GAA AGT CCC CTC AAC T-5′ were biotin-labeled separately using the Biotin end labeling kit (Pierce) and then annealed before use. Each binding reaction contained 1 × binding buffer (100 mM Tris, 500 mM KCl, 10 mM dithiothreitol, pH 7.5), and 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/μL poly (dIdC), 0.05% NP-40, 10 μg of nuclear extract, and 40 nM of biotin end-labeled target DNA. The contents were incubated at room temperature for 20 minutes. Complexes were separated on 4% nondenaturing polyacrylamide gel and were transferred to a nylon membrane. When the transfer was complete, DNA was crosslinked to the membrane at 120 mJ/cm^2 using a UV crosslinker equipped with 254 nm bulbs. The biotin end-labeled DNA was detected using streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate. The membrane was exposed to X-ray film and developed using a Kodak film processor (Eastman Kodak, Rochester, NY).

Luciferase Reporter Gene Expression Assay

To examine reporter gene expression, cells were transiently transfected with the pNF-κB Luc plasmid and control pRenilla Luc plasmid (Invitrogen, Carlsbad, CA) for 24 hours. Transfected cells were treated with *B. lactis* at 10^8 to 10^9 CFU per mL of the incubation medium without antibiotics for 16 hours and stimulated with TNF-α (10 μM). They were analyzed for NF-κB reporter gene and control vector activity using lysis buffer and reagents from Promega (Madison, WI).

Western Blot Analysis

Cells were harvested on ice by washing twice with cold PBS, scraping, and resuspending. Protein concentration was determined using the Bradford assay with bovine serum albumin as a reference. Samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared by mixing aliquots of the protein with NuPAGE sample buffer (Invitrogen) and heated at 70°C for 10 minutes. Protein samples were run on NuPAGE 4%–12% gradient Bis-Tris gels at 150V for 1 hour with MES SDS running buffer (Invitrogen). For Western blot analysis, gels were electrotransferred to a polyvinylidene difluoride membrane (Invitrogen) using the Xcell Surelock electrophoresis and transfer apparatus (Invitrogen). Blots were blocked with 5% (w/v) skim milk in Tris-buffered saline solution containing 0.1% Tween 20 (Pierce) and incubated overnight at 4°C with antibodies against p65 NF-κB, active NF-κB, IκB-α, phospho-IκB-α (p-IκB-α), cyclooxygenase-2 (COX-2), Histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma). Blots were additionally incubated with secondary antibodies conjugated with horseradish peroxidase for 1 hour at room temperature and, finally, revealed with the Enhanced Chemiluminescence Western blot detection reagent (Amersham Biosciences, Freiburg, Germany).

Quantitative Real-time Reverse-transcription Polymerase Chain Reaction (RT-PCR)

B. lactis-pretreated cells were stimulated with TNF-α for 4–8 hours. Total RNA was extracted using TRIzol Reagent (Invitrogen) and 1 μg of RNA was reverse-transcribed using SuperScript First-Strand Synthesis kit (Invitrogen) according to the manufacturer-recommended protocol. The cDNAs were mixed in triplicate using SYBR Green master mix (Applied Biosystems, Foster City, CA) and pairs of primers (4 pmol of each primer). PCR was done using primers for COX-2 (5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′ and 5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′), matrix metallopeptidase 9 (MMP-9; 5′-TGA CAG CGA CAA GAA GTG-3′ and 5′-CAG TGA AGC GGT ACA TAG G-3′), vascular endothelial growth factor-A (VEGF-A; 5′-GCT GCT CTA CCT CCA CCA TGC-3′ and 5′-GTT AAC TTC CGC GTT TGC TC-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 5′-TGA TGA CAT CAA GAA GGT GG-3′ and 5′-TTT CTT ACT CCT TGG AGG CC-3′). Samples were amplified in a 7500 real-time PCR System (Applied Biosystems) for 40–45 cycles using the following PCR variables: 95°C for 30 seconds, 60–62°C for 1 minute, and 72°C for 1 minute. Finally, quantitative analysis was performed using the relative standard curve method and the results were reported as the relative expression or fold change as compared to the calibrator after normalization of the transcript level to the endogenous control, GAPDH.

Animal Model and Assessment

Six-week-old male C57BL/6 mice (Orient, Seongnam, Korea) were maintained on a 12:12-hour light:dark cycle under specific pathogen-free conditions. The mice had access to a standard diet and water until they reached the desired age (9 weeks). All experiments using animals were reviewed and approved by the Institutional Animal Care and Use Committee of Yonsei University Severance Hospital, Seoul, Korea.

In the acute colitis model, mice were given 3.5% DSS in drinking water for 7 days. *B. lactis* was administered daily by oral gavage until Day 6 in the low-dose group (2×10^9 CFU/day) and the high-dose group (2×10^{10} CFU/day). DSS-treated groups received 0.9% normal saline in a comparable volume by the same route. Normal control mice received filtered water alone. Mice were sacrificed on Day 7 and clinical parameters and pathology were evaluated.

To test the physiological relevance of *B. lactis*-mediated blockade of CAC in vivo, we used an AOM/DSS-induced colon cancer model. The mice were divided into three groups: a control group, an AOM group in which vehicle were fed, and an AOM + BL (*B. lactis*) group in which mice were fed a 5×10^8 CFU/g diet of *B. lactis* ($n = 5$ each group). Mice in the AOM and AOM + BL groups were injected intraperitoneally with AOM (10 mg/kg). After 1 week they were fed 2% DSS in the drinking water over 5 days, followed by 16 days of regular water. This cycle was repeated three times. On the ninth experimental week all the mice were sacrificed and their colons were resected.

In both the acute colitis and the CAC model the mice were examined daily for behavior, water/food consumption, body weight, stool consistency, and the presence of gross blood in the stool or at the anus. A previously validated clinical disease activity index (DAI) that ranged from 0 to 4 was calculated based on the following parameters: stool consistency (0, normal; 2, loose; 4, diarrhea), gross bleeding (0, absence; 2, blood tinged; 4, presence), and weight loss (0, none; 1, 1%–5%; 2, 5%–10%; 3, 10%–20%; 4, >20%).[19] The calculated DAI = (weight loss + stool

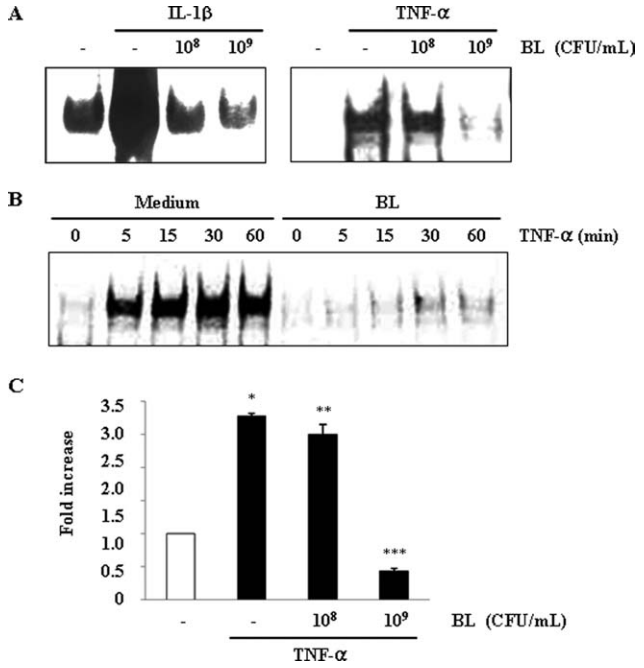

FIGURE 1. *B. lactis* inhibits NF-κB activation in HT-29 cells. (A) Cells were preincubated with *B. lactis* for 16 hours, followed by an incubation with 10 μM of IL-1β or TNF-α for 1 hour and the nuclear extracts were analyzed for NF-κB activation by EMSA, as described in Materials and Methods. (B) Cells were preincubated with *B. lactis*, treated with TNF-α (10 μM) for the indicated times, and then subjected to EMSA. (C) To examine reporter gene expression, cells were transiently cotransfected with the NF-κB and control renilla Luc reporter plasmid for 24 hours. Transfected cells were treated with *B. lactis* for 16 hours and stimulated with TNF-α (10 μM). The cell culture medium was harvested after 16 hours of TNF-α treatment and analyzed for NF-κB reporter gene and control vector activity. The firefly luciferase (NF-κB) activity was normalized against renilla luciferase activity. Data are expressed as mean ± SEM ($n \geq 4$). Error bars indicate standard deviations. *$P < 0.001$ compared with untreated; **$P < 0.05$ compared with TNF-α only treated; ***$P < 0.001$ compared with TNF-α only treated. BL, *Bifidobacterium lactis*.

consistency + gross bleeding)/3. The severity of colitis was evaluated by an independent observer who was blinded to the treatment.

Histology and Immunohistochemistry

Postmortem, the entire colon was removed from the cecum to the anus and opened longitudinally. Subsequently, samples of colonic tissue were either fixed in 10% buffered formalin, embedded in paraffin, and stained hematoxylin-eosin. Histological examination was performed on three samples of the distal colon for each animal. All histological quantification was performed in a blinded fashion using a scoring system described previously.[20] Briefly, three parameters were measured: severity of inflammation (0, none; 1, slight; 2, moderate; 3, severe), extent of injury (0, none; 1, mucosal; 2, mucosal and submucosal; 3, transmural), and crypt damage (0, none; 1, basal one-third damaged; 2, basal two-thirds damaged; 3, only surface epithelium intact; 4, entire crypt and epithelium lost). The score of each parameter was multiplied by a factor that reflected the percentage of tissue involvement (31, 0%–25%; 32, 26%–50%; 33, 51%–75%; 34, 76%–100%), and all numbers were summed. The combined histopathological scores ranged from 0 to 40. Colon length as an indirect marker of inflammation was also measured. For the precise evaluation of CAC in colon tissue the presence and tumor load was determined in the whole colon by a blinded pathologist. Tumor counts were performed and tumor sizes were calculated. Paraffin blocks were sectioned and stained for COX-2, IL-6, and IκB-α (Santa Cruz Biotechnology) using the Dako REALTM Envision Kit (DakoCytomation, Carpinteria, CA) and the slides were counterstained with hematoxylin–eosin. Images were obtained using a microscope (Olympus BX41; Olympus Optical, Tokyo, Japan).

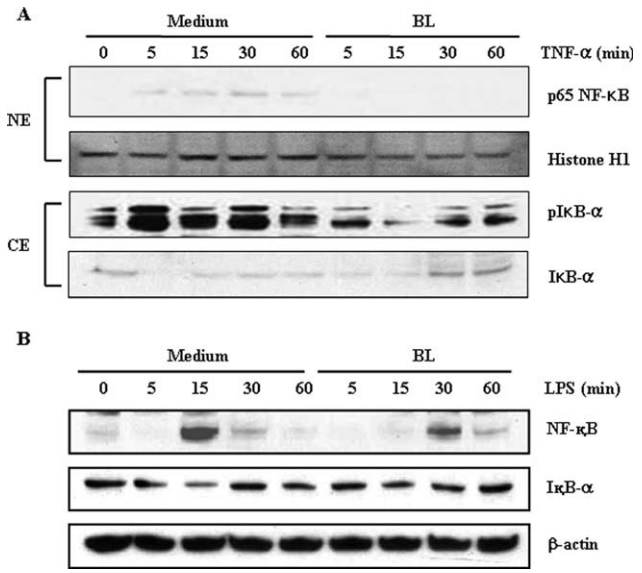

FIGURE 2. *B. lactis* inhibits TNF-α-and LPS-induced p65 NF-κB translocation and IκBα degradation in HT-29 cells. (A) Cells were preincubated with *B. lactis* for 16 hours, followed by an incubation with TNF-α (10 μM) for 1 hour. Nuclear (NE) and cytoplasmic (CE) extracts were analyzed for p65 localization and IκB-α phosphorylation/degradation by Western blot. Histone H1 was used as control. (B) Cells were preincubated with BL for 16 hours, followed by an incubation with LPS (10 μg/ml) for 1 hour. This experiment was performed in triplicate. BL, *Bifidobacterium lactis*.

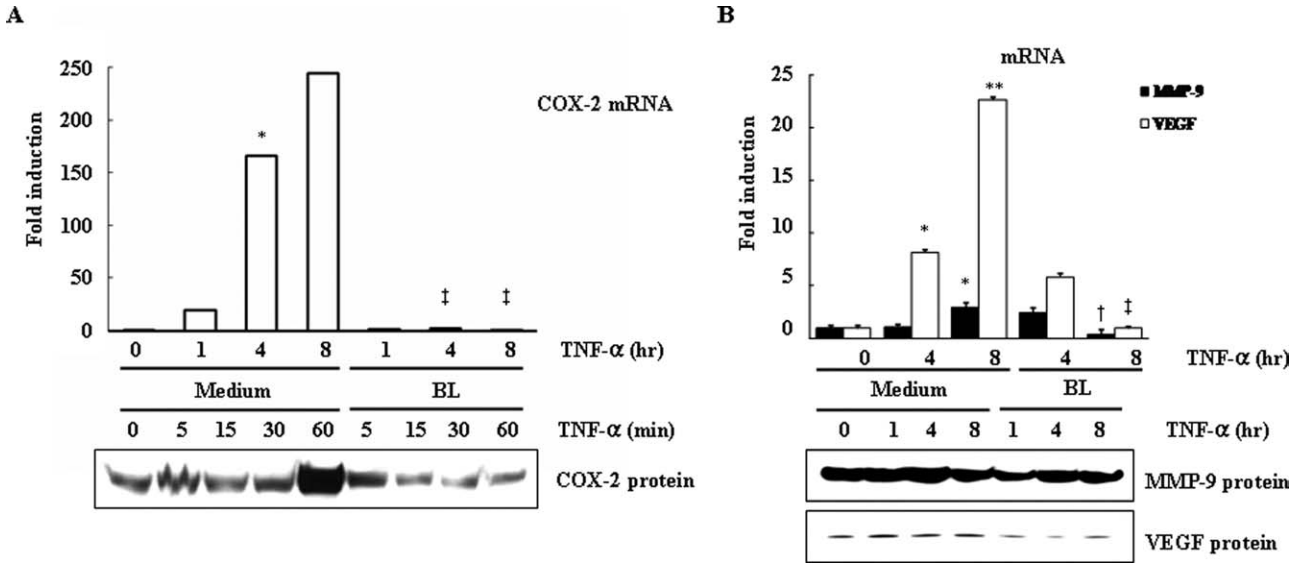

FIGURE 3. *B. lactis* suppresses TNF-α-induced NF-κB-dependent expression of tumorigenic genes. (A) Cells were preincubated with *B. lactis* for 16 hours, followed by an incubation with TNF-α (10 μM) for the indicated times. Total RNA and whole-cell extracts were prepared. COX-2 mRNA was determined by quantitative real-time RT-PCR and the protein level was analyzed by Western blot. (B) VEGF and MMP-9 mRNA were measured by quantitative real-time RT-PCR and the protein level analyzed by Western blot, respectively. Each mRNA expression level is depicted as the relative amount of each gene divided by the amount of GAPDH gene. Data are expressed as mean ± SEM ($n \geq 3$). Error bars indicate standard deviations. *$P < 0.05$ compared with control; **$P < 0.001$ compared with control; †$P < 0.05$ compared with *B. lactis*-untreated cells; ‡$P < 0.01$ compared with *B. lactis*-untreated cells. BL, *Bifidobacterium lactis*.

Isolation of Primary Mouse IEC

Colons were cut longitudinally, washed three times in calcium/magnesium-free HBSS (Invitrogen), cut into pieces 0.5 cm long, and incubated at room temperature in 40 mL of calcium/magnesium-free HBSS containing 10 mM DTT for 30 minutes, then incubated for 60 minutes in 1 mM EDTA in CMF-HBBS at 4°C. Epithelial cells were detached as intact crypts by 10 vigorous shakes of the vessel. The supernatant was filtered on cell strainer (BD Biosciences, San Jose, CA), centrifuged for 5 minutes at 400 *g*, and the IEC pellets were lysed for subsequent Western blot analysis.

Statistical Analysis

Experimental results are expressed as mean values ± standard error of the mean (SEM). Statistical Package for the Social Sciences (SPSS/PC+ 11.0, Chicago, IL) was used for all analyses. Significance was determined using the Mann–Whitney *U*-test or Student's *t*-test and were accepted when *P*-values were less than 0.05.

RESULTS

B. lactis Inhibits IL-1β or TNF-α-mediated NF-κB Activation in IEC

First, we evaluated the molecular effects of *B. lactis* in a colorectal cancer cell line, HT-29, which possess a range of endogenous NF-κB activity. We performed EMSA to determine whether *B. lactis* decreases the DNA binding activity of NF-κB. *B. lactis* inhibited IL-1β or TNF-α-dependent NF-κB activation in a dose-dependent manner (Fig. 1A; Supporting Fig. 1A). Furthermore, TNF-α activated NF-κB in a time-dependent manner in *B. lactis*-untreated cells, which was significantly inhibited in *B. lactis*-treated cells (Fig. 1B; Supporting Fig. 1B).

To evaluate the effects of *B. lactis* on NF-κB gene expression, an NF-κB-dependent luciferase (Luc) reporter gene assay was performed because DNA binding may not correlate with effects on gene expression. The results of luciferase reporter assay showed that *B. lactis* suppressed TNF-α-mediated NF-κB activation in a dose-dependent manner (Fig. 1C).

B. lactis Inhibits p65 NF-κB Translocation by Blocking IκBα Degradation

Since the degradation of IκB-α results in nuclear translocation of p65 NF-κB, the effects on TNF-α- or LPS-induced nuclear translocation and the state of IκB-α were examined using Western blot of nuclear and cytosolic extracts. Translocation of NF-κB was abolished by *B. lactis* in a time-dependent manner (Fig. 2A; Supporting Fig. 2). Because nuclear translocation of NF-κB is preceded by proteolytic degradation of IκB-α by phosphorylation level, phosphorylation of IκB-α in the cytoplasm was analyzed.

TNF-α-or LPS-induced IκB-α degradation occurred in control cells not treated with *B. lactis*, but *B. lactis* pretreatment stabilized IκB-α (Fig. 2; Supporting Fig. 2).

B. lactis Suppresses NF-κB-dependent Tumorigenic Genes

COX-2 contributes to carcinogenesis by promoting cell proliferation. TNF-α can induce the expression of genes in IEC involved in tumor metastasis, including MMP, VEGF, and COXs that are regulated by NF-κB. We investigated whether *B. lactis* can modulate TNF-α-induced expression of COX-2, VEGF, and MMP-9 in vitro by real-time RT-PCR and Western blot, respectively. *B. lactis* strongly inhibited transcriptional activation of these genes in HT-29 cells challenged with TNF-α (Fig. 3). Similarly, protein levels of COX-2, MMP-9, and VEGF decreased in a time-dependent fashion as determined by Western blot analysis (Fig. 3; Supporting Fig. 3). These overall patterns of *B. lactis* responsiveness appear to correlate best with the level of inactivated NF-κB and demonstrate that *B. lactis* suppresses NF-κB activation and the expression of the related tumorigenic genes.

B. lactis Prevents the Development of Acute Colitis in Mice

To test the physiological relevance of *B. lactis*-mediated blockade of NF-κB activation in vivo we used a DSS-induced acute murine colitis model. The mice were divided into four groups: Control, DSS, DSS + LBL (mice were supplemented with *B. lactis* in a low dose, 2×10^9 CFU/day), and DSS + HBL (a high dose of *B. lactis*, 2×10^{10} CFU/day). There were no significant differences in the starting body weights between control and experimental groups in the acute preventive model. Administration of a high dose of *B. lactis* produced a significant recovery of body weight induced by DSS-induced colitis (Fig. 4A).

On gross examination, *B. lactis*-treated groups (DSS + LBL and DSS + HBL) had lower levels of clinical DAI than DSS groups (Fig. 4B). Blinded histological injury scoring was quantified in the distal colon. In the DSS group the histological severity of colitis assessed by the overall score was significantly higher than that in controls (Fig. 4C). DSS induced complete destruction of epithelial architecture with a loss of crypts and epithelial integrity, submucosal edema, and intense infiltration of inflammatory cells including neutrophils and lymphocytes in all layers (Fig. 5; Supporting Fig. 4A). Treatment of DSS-fed mice with *B. lactis* led to a significant attenuation of experimental colitis, with a reduced total score compared with the DSS group (Fig. 4D). These observations correlate well with clinical and macroscopic findings in a dose-dependent manner.

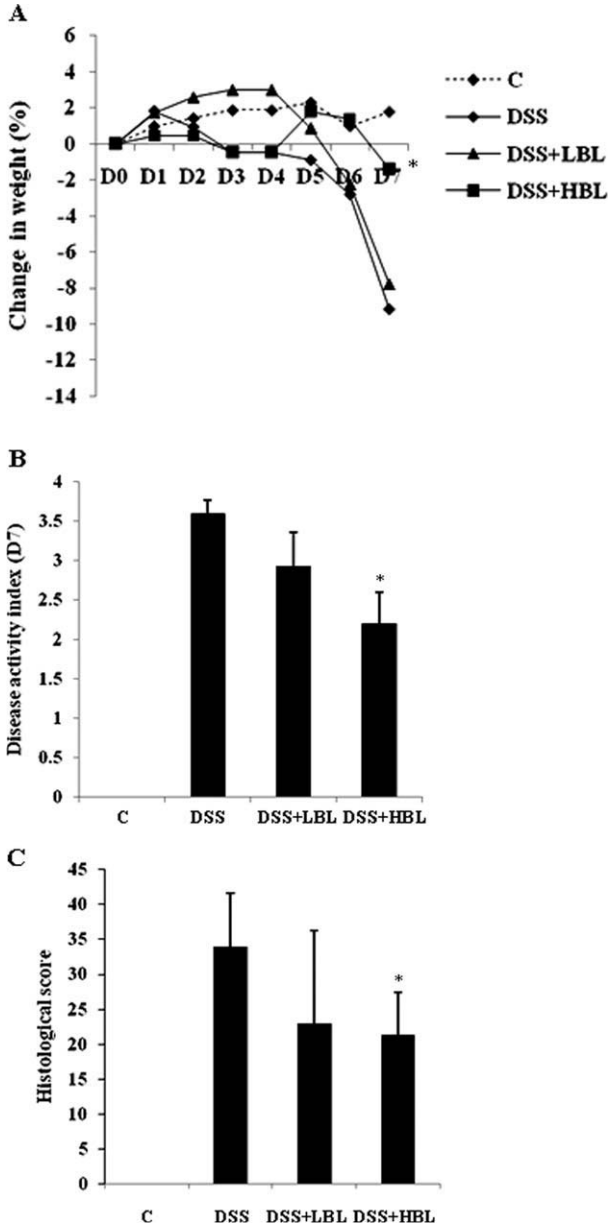

FIGURE 4. *B. lactis* prevents DSS-induced colitis. (A) Changes in body weight. (B) Changes in colon length. (C) Changes in the disease activity index. (D) Histological score. Data are expressed as mean ± SEM ($n = 5$). *$P < 0.05$ compared with DSS group. LBL, low-dose *B. lactis*-treated group (2×10^9 CFU/day); HBL, high-dose *B. lactis*-treated group (2×10^{10} CFU/day); BL, *Bifidobacterium lactis*.

Because our data from the in vitro study suggested that *B. lactis* could exert its antiinflammatory effects by blocking NF-κB in IEC, we investigated this signaling in the DSS-colitis model to reconfirm it in vivo. The expression of IκB, the active form of NF-κB, and IL-6 in colonic

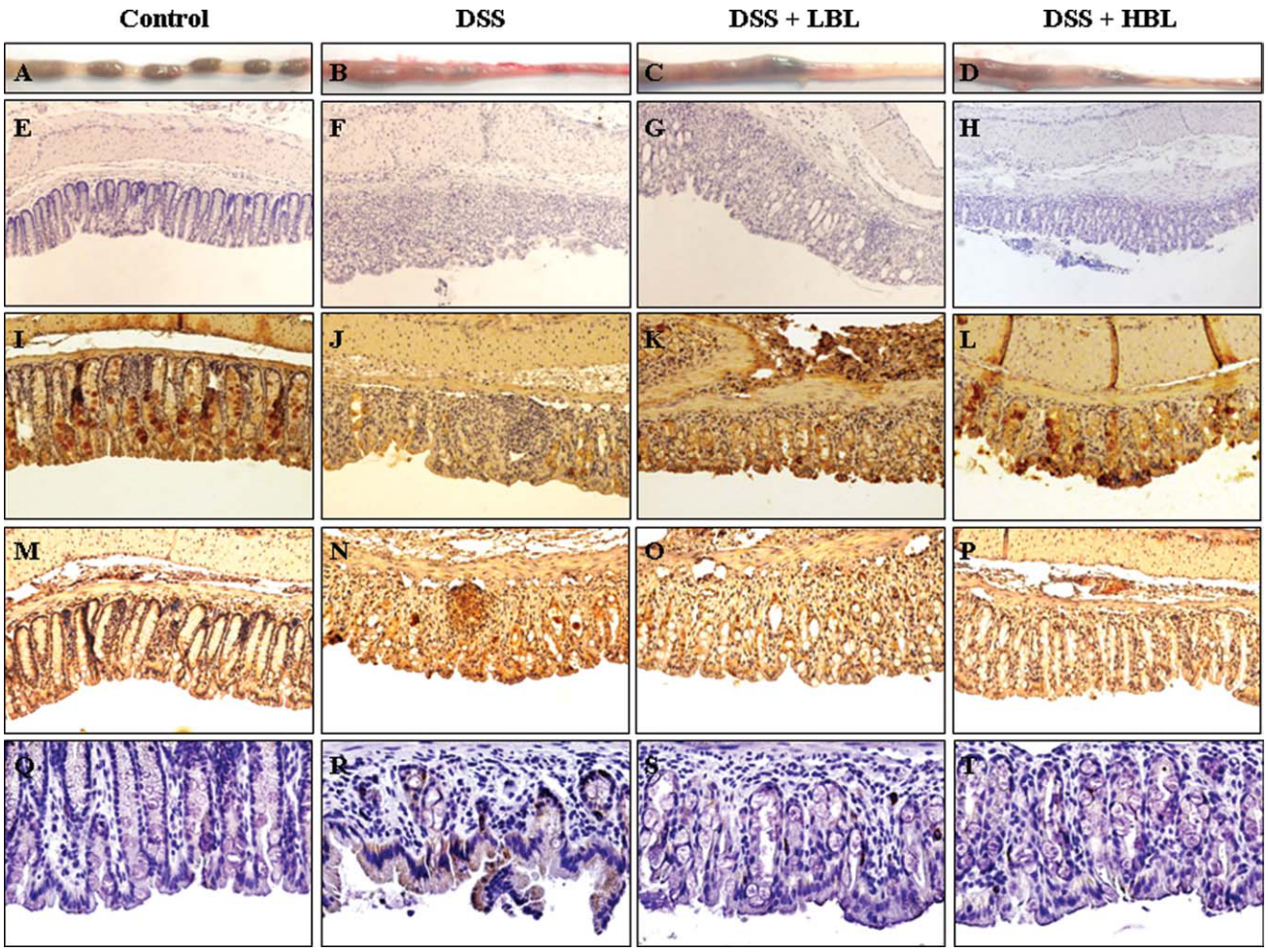

FIGURE 5. Histopathology. (A–D) Representative colon specimens taken from mice at day 7 of DSS-treatment. (E–H) Hematoxylin and eosin stain (magnification: ×100). Immunohistochemistry for IκB-α (I–L), active NF-κB (M–P), and IL-6 (Q–T) of colonic samples taken from mice that received water, 3.5% DSS, 3.5% DSS + LBL, or 3.5% DSS + HBL in the acute colitis model (magnification: × 200). LBL, low-dose *B. lactis*-treated group (2×10^9 CFU/day); HBL, high-dose *B. lactis*-treated group (2×10^{10} CFU/day); BL, *Bifidobacterium lactis*. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mucosa was examined by immunohistochemistry staining. In mice with DSS-induced colitis, IκB-α was strongly negative in both epithelial cells and submucosal inflammatory cells, but treatment with *B. lactis* markedly increased the degree of IκB-α (Fig. 5I–L; Supporting Fig. 4A,B) staining and decreased the active form NF-κB (Fig. 5M–P; Supporting Fig. 4C) and IL-6 (Fig. 5Q–T; Supporting Fig. 4D) in the colonic tissue. Moreover, the expression of IκB, phosphorylated IκB, and IL-6 in colonic tissue extracts and isolated IEC from colonic tissues were examined by Western blot analysis (Supporting Fig. 4E). Administration of *B. lactis* significantly increased the expression of IκB but decreased the phosphorylated IκB and IL-6 expression in both colonic tissues (Fig. 6A–C) and isolated IEC (Fig. 6D).

B. lactis Suppresses the Development of Colitis-associated Colon Cancer in Mice

Next we examined the tumor prevention effects of *B. lactis* in a murine CAC model. Body weights were measured daily during the experimental period. Weight gain in DSS-treated mice was much lower than in control mice. Rapid weight reduction started 19 days after the second cycle of 2% DSS, and this reduction was sustained in both the AOM and the AOM + BL groups. However, *B. lactis* attenuated the DSS-induced weight loss (Fig. 7A). The mice that received AOM had markedly shorter colons compared to control mice, whereas the colons of mice that received AOM + BL were markedly longer than the colons of mice in the AOM group (Fig. 7B). Although partial destruction of epithelial architecture, submucosal edema,

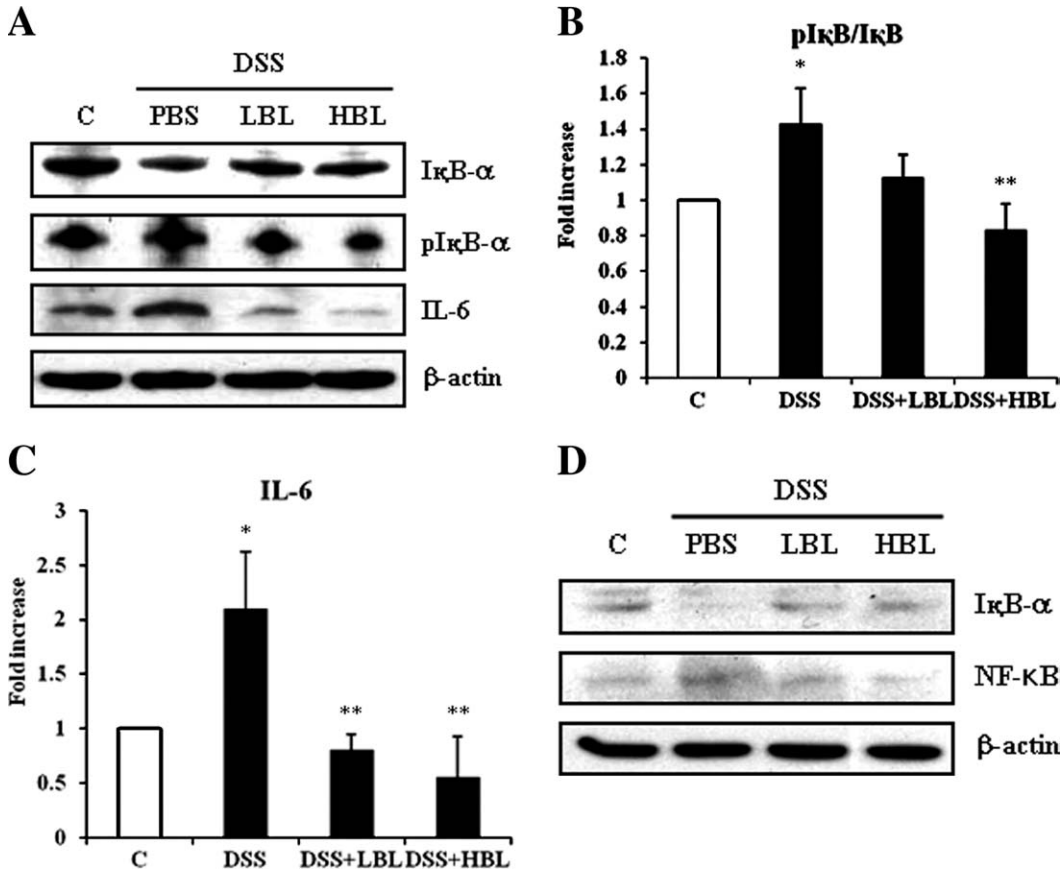

FIGURE 6. (A–C) Western blot analysis for IκB-α, pIκB-α, and IL-6 in murine colonic samples and (D) isolated intestinal epithelial cells taken from mice that received water, 3.5% DSS, 3.5% DSS + LBL, or 3.5% DSS + HBL in the acute colitis model. (B,C) Graphs show the ratio of band intensity using densitometry. Data are expressed as mean ± SEM. *$P < 0.05$ compared with control; **$P < 0.05$ compared with DSS group. LBL, low-dose *B. lactis* group (2×10^9 CFU/day); HBL, high-dose *B. lactis* group (2×10^{10} CFU/day); BL, *Bifidobacterium lactis*.

predominant lymphocytic infiltration, and several lymphoid follicles were observed in the AOM group, the maintenance of crypt architecture and minimal infiltration of inflammatory cells were observed in the AOM + BL group (Fig. 8A–C).

On magnified gross examination, no tumors were found in mice treated with saline. All mice treated with AOM plus DSS developed tumors. These tumors were located in the middle to distal colon and were primarily broad-based adenomas with high-grade dysplasia and varying degrees of inflammatory cell infiltration. We observed a dramatic decrease in tumor incidence in *B. lactis*-treated mice (Fig. 7C). The mean number of tumors was 4.5 in the *B. lactis* group, which was markedly lower than that of the AOM group (10.9). When tumors were classified into three groups on the basis of size (below 1 mm, more than 1 mm, and not more than 3 mm, or above 3 mm), none of the tumors observed in the *B. lactis* group were found to be more than 3 mm in diameter and most tumors were less than 1 mm. In contrast, the number of tumors between 1 mm and 3 mm was significantly higher in the AOM ($n = 7.6$) group compared with the *B. lactis* ($n = 1.3$) group ($P = 0.014$).

In the AOM group, IκB-α was weakly positive in both destroyed epithelial cells and submucosal inflammatory cells. Treatment with *B. lactis* markedly attenuated the degree of IκB-α degradation in the colonic tissue. There was a significant increase of IκB-α in the AOM + BL group compared with the AOM group (Fig. 8D–F; Supporting Fig. 5A). As inhibition of COX-2 is an accepted chemopreventive strategy, we tested the effect of COX-2 inhibition in our model. Although strong COX-2 expression was seen in both epithelial cells and submucosal inflammatory cells in the AOM group, reduced immunoreactivity for COX-2 was observed in epithelial cells and in the basal layer in *B. lactis*-treated mice (Fig. 8G–I; Supporting Fig. 5B).

DISCUSSION

The present study was undertaken to investigate the potential antiinflammatory and cancer preventive effects of

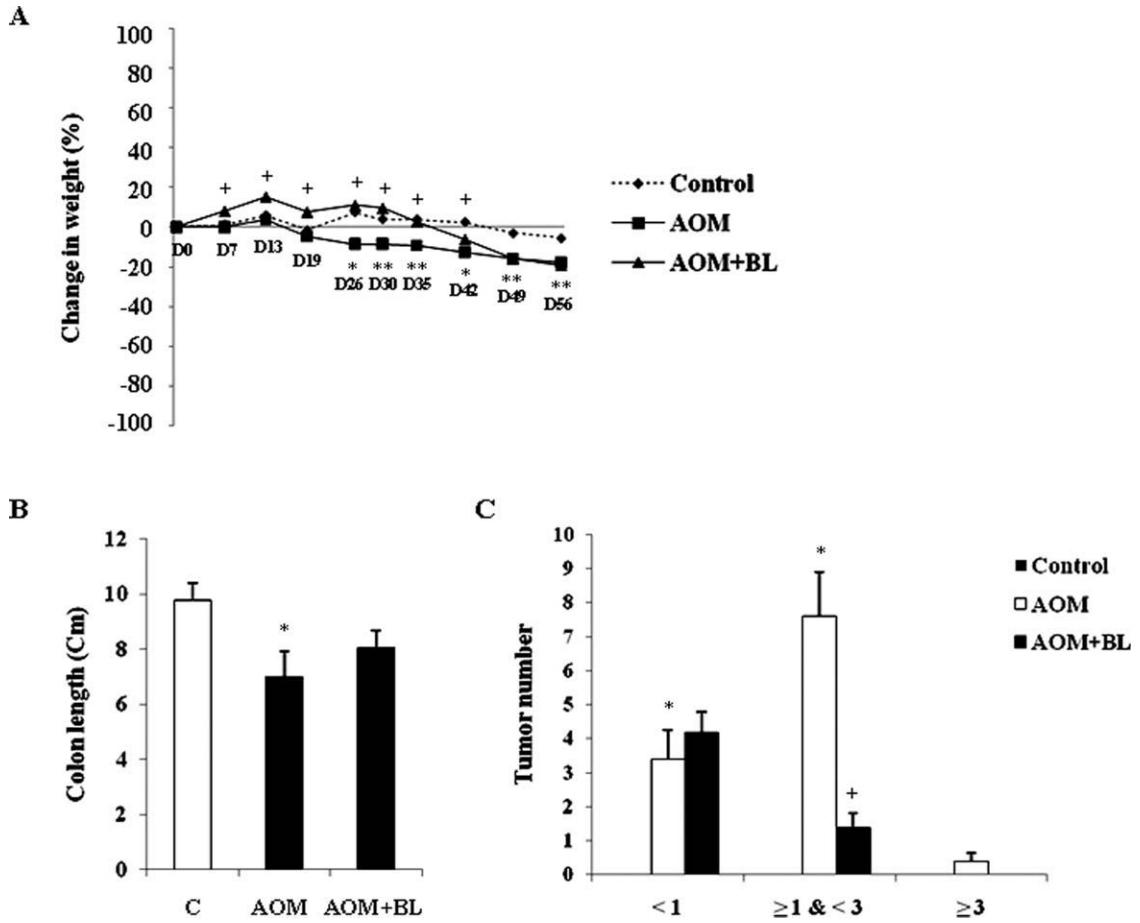

FIGURE 7. *B. lactis* prevents AOM-induced colitis-associated cancer. (A) Changes in body weight expressed as percentages. (B) Differences in colon length. (C) Tumor number and size distribution. Data are expressed as mean ± SEM. *$P < 0.01$ compared with control; **$P < 0.05$ compared with control; $^{+}P < 0.01$ compared with AOM group ($n = 5$ per each group). AOM, azoxymethane; BL, *Bifidobacterium lactis*.

B. lactis on intestinal inflammation and cancer and to understand the mechanisms involved. Recently, a few studies have demonstrated the antiproliferative and proapoptotic effects of *Bifidobacterium* on various cancer cell lines.[12,16,21] However, the precise mechanism of their anti-inflammatory and antitumorigenic influences remains unclear, although a speculative mechanism implicates the immunomodulating properties of probiotic bacteria.[22]

Because NF-κB is considered a key player in inflammatory processes and cell proliferation, it provides a mechanistic link between inflammation and cancer.[23] We hypothesized that *B. lactis* might modulate NF-κB activation in IEC, through which it could also inhibit acute colitis and CAC in mice. To test these hypotheses, we first investigated the impact of *B. lactis* on cytokine signaling and elucidated its mode of action using IEC. Our results demonstrate that *B. lactis* strongly suppresses TNF-α- and IL-1β-induced NF-κB signaling in IEC, which highlights a possible mechanism for its antiinflammatory action in the intestine. We used EMSA and a nuclear assay for the NF-κB p65 in HT-29 cells to demonstrate that *B. lactis* inhibits NF-κB DNA binding activity and translocation of NF-κB into the nucleus by suppression of IκB-α degradation. Moreover, NF-κB-dependent reporter gene activation by TNF-α could be inhibited by preincubation with *B. lactis* in a dose-dependent manner. Next, to clarify whether *B. lactis* would modulate the inflammation in the intestines of animals as well as IEC, we investigated the effects of *B. lactis* in a murine DSS-induced colitis model that is a commonly used model for the inflammatory component of IBD. In our study, *B. lactis* appeared to prevent DSS-induced colitis. Furthermore, it was clearly demonstrated by immunohistochemistry and Western blot that *B. lactis* is associated with the inhibitory modulation of IκB-α degradation. This is in line with the results of the in vitro study that demonstrated a *B. lactis*-mediated NF-κB inhibition in IEC. These

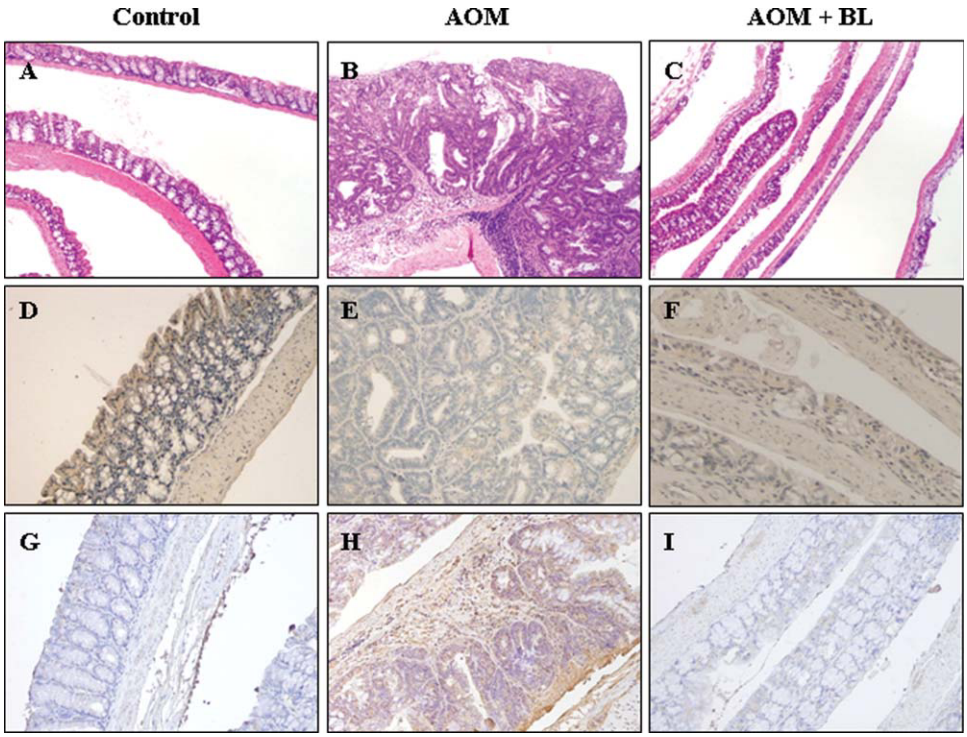

FIGURE 8. Histopathology. (A–C) Hematoxylin-eosin stain (magnification: ×100). (D–I) Immunohistochemistry for IκB-α (D–F), and COX-2 (G–I) of colonic samples taken from mice that received water (A,D,G), AOM (B,E,H), or AOM + BL (C,F,I) in the CAC model (magnification: × 200). AOM, azoxymethane; BL, *Bifidobacterium lactis*. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

findings indicate the novel protective effects of *B. lactis* against inflammation in the colon, suggesting its potential clinical value in the treatment of IBD.

Our results are consistent with the results of several other studies. It was recently reported that some strains of bifidobacteria are effective in inhibiting LPS-induced activation of IL-8 production and the NF-κB activation pathway.[16,24] However, only NF-κB promoter transcriptional activity was measured in this study. For in vivo study it was reported in one study that *B. lactis* showed intestinal antiinflammatory activity in a TNBS model of rat colitis without the demonstration of its action mechanisms.[25] Our finding is further supported by the recent observation that *Bifidobacterium* inhibits DSS-induced colitis in mice with a similar dose of bacteria to ours.[26] On the contrary, commensal bacteria, including *Lactobacillus* spp., have been shown to influence the regulatory pathways of the mammalian intestinal epithelium by directly modulating the ubiquitin–proteasome system.[12] Moreover, a previous report suggested that *B. lactis* can transiently activate NF-κB and proinflammatory gene expression both in vitro and in vivo.[27] *Bifidobacterium animalis* was also shown to cause duodenitis and colitis in a susceptible host.[28] Taken together, these findings suggest that all *Bifidobacterium* strains are not protective in the intestine and different probiotics may mediate different signaling pathways. Further studies are necessary to elucidate the exact roles of each strain of *Bifidobacterium* in NF-κB signaling. Moreover, recent evidence indicated that IEC-derived NF-κB signaling might be essential to protect these cells against acute injury such as DSS or radiation.[29–31] Further research is necessary to draw a concrete conclusion concerning this controversial impact of NF-κB blockage on acute intestinal damage.

In addition to its key role in inflammation, NF-κB activates the transcription of numerous genes capable of suppressing apoptosis, suggesting a pivotal role in inflammation-related carcinogenesis.[9] In particular, a recent investigation demonstrated that IKKβ is related to inflammation and tumorigenesis in murine models of colitis-associated cancer.[10] The anti-NF-κB action of sulfasalazine, which is known to prevent colon cancer in patients with IBD, is mediated by the direct inhibition of IKKα and IKKβ.[32] Based on these findings, *B. lactis* may have similar effects on CAC and IBD. Encouraged by the antiinflammatory effects of *B. lactis* on intestines in mice, we further sought to investigate the effect of *B. lactis* on CAC in a murine model. *B. lactis* significantly reduced the number of colon tumors in an inflammation-related colon cancer model. Although the antiinflammatory activity of probiotics

has been reported previously, to our knowledge, this is the first report on a specific inhibitory effect of bifidobacteria on colitis-induced carcinogenesis in epithelial cells through NF-κB modulation, suggesting a role for bifidobacteria in down-modulation of inflammation and tumorigenesis. This effect of *B. lactis* which suppressed IκB-α degradation in both acute colitis and colonic cancer suggests a mechanistic effect comparable to sulfasalazine. Our findings correspond to those of some previous reports. It was reported that *B. lactis* treatment reduced colonic TNF-α production and COX-2 expression.[33] Moreover, it has been reported that dietary intake of *Bifidobacterium* culture significantly inhibited the development of AOM-induced aberrant crypt foci and blocked the induction of colon and liver tumors by 2-amino-3-methyl-imidazo [4, 5-f] quinolone, a food mutagen.[17]

In this study, *B. lactis* inhibited the expression of COX-2, an NF-κB-dependent mediator, in colitis-related cancer. COX-2 is an enzyme that catalyzes the production of prostaglandin E_2 (PGE_2) from arachidonic acid, and has been linked to proliferation and metastasis of tumor cells.[34] VEGF, a key molecule involved in angiogenesis, and MMP, a class of enzymes involved in tissue remodeling, are known as positive regulators of intestinal tumorigenesis,[35] which is also regulated by NF-κB and COX-2. COX-2, VEGF, and MMP-9, all of which have NF-κB binding sites in their promoters, are also induced by TNF-α. mRNA or protein induction of these genes was inhibited by *B. lactis* in our study. Similar to our findings, a COX-2 inhibitor inhibited both colon carcinogenesis and colitis in a murine colitis model.[36,37] The COX-2 level has been shown to be increased in IBD[38] and in colon cancer.[39] However, in other studies *B. lactis* upregulated COX-1 but downregulated COX-2 in Caco-2 cells[40] and one such probiotic, *Lactobacillus rhamnosus* GG, prevented cytokine-induced apoptosis in two different IEC models[41] and induced COX-2 expression in T84 colon epithelial cells.[42] Moreover, COX-1 and -2 has been shown to have a crucial role in the defense of the intestinal mucosa.[43] Therefore, the complex, interactive mechanisms of COX-2 in intestinal inflammation or cancer remain to be further determined.

In conclusion, the results of this study indicate that *B. lactis* blocks cytokine-induced NF-κB signaling and proneoplastic gene expression in IEC. Moreover, *B. lactis* prevents acute colitis and decreases AOM-induced carcinogenesis in a murine model of CAC by the inhibition of IκB-α degradation. These results provide clues to understanding the molecular mechanisms by which *B. lactis* mediates its antiinflammatory and anticarcinogenic effects both in vitro and in vivo. *B. lactis* has great potential as a therapeutic agent for disorders of chronic intestinal inflammation and as an agent for cancer prevention.

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