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Toll-Like Receptor 9 Signaling Mediates the Anti-inflammatory Effects of Probiotics in Murine Experimental Colitis

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Background & Aims: We tested whether the attenuation of experimental colitis by live probiotic bacteria is due to their immunostimulatory DNA, whether toll-like receptor (TLR) signaling is required, and whether nonviable probiotics are effective. Methods: Methylated and unmethylated genomic DNA isolated from probiotics (VSL-3), DNAse-treated probiotics and *Escherichia coli* (DH5α) genomic DNA were administered intragastricly (i.g.) or subcutaneously (sc) to mice prior to the induction of colitis. Viable or γ-irradiated probiotics were administered i.g. to wild-type mice and mice deficient in different TLR or in the adaptor protein MyD88, 10 days prior to administration of dextran sodium sulfate (DSS) to their drinking water and for 7 days thereafter. Results: Intragastric and sc administration of probiotic and *E. coli* DNA ameliorated the severity of DSS-induced colitis, whereas methylated probiotic DNA, calf thymus DNA, and DNase-treated probiotics had no effect. The colitis severity was attenuated to the same extent by i.g. delivery of nonviable γ-irradiated or viable probiotics. Mice deficient in MyD88 did not respond to γ-irradiated probiotics. The severity of DSS-induced colitis in TLR2 and TLR4 deficient mice was significantly decreased by i.g. administration of γ-irradiated probiotics, whereas, in TLR9-deficient mice, γ-irradiated probiotics had no effect. Conclusions: The protective effects of probiotics are mediated by their own DNA rather than by their metabolites or ability to colonize the colon. TLR9 signaling is essential in mediating the anti-inflammatory effect of probiotics, and live microorganisms are not required to attenuate experimental colitis because nonviable probiotics are equally effective.

Probiotics are live commensal microorganisms that confer health benefits to the host by one or more of the following means: production of various antimicrobial metabolites, competitive exclusion of enteric pathogens, neutralization of dietary carcinogens, and modulation of mucosal immune responses.[1–3] Current probiotic therapy, i.e., the daily oral intake of probiotics, is mainly advocated for its immunomodulatory properties and anti-inflammatory activities at mucosal sites.[4]

Inflammatory bowel disease (IBD) includes Crohn's disease and ulcerative colitis, both of which are characterized by flare-up periods with possible lifelong relapses. Clinical and experimental evidence suggest that the etiology of IBD is multifactorial, involving susceptibility genes and environmental factors, such as intestinal microflora or their products, and it is the interaction of these factors with the immune system that leads to dysregulated mucosal immunity and intestinal inflammation.[4]

The rationale for using probiotics in IBD is based on evidence implicating enteric bacteria in the pathogenesis of various models of murine colitis and IBD in humans.[5] Indeed, probiotic therapy has been effective for the attenuation of experimental colitis[6–8]; prevention of pouchitis; and maintenance of remission of pouchitis, Crohn's disease, and ulcerative colitis.[9,10] Despite these beneficial effects, the exact mechanisms and the molecular pathways by which probiotics ameliorate experimental colitis and IBD are largely unknown.

Immunostimulatory DNA sequences (ISS-DNA, also known as CpG-DNA) are unmethylated CpG dinucleotides within consensus sequences, i.e., CpG motifs. CpG motifs are common in certain bacterial and viral genomes and are underrepresented in mammalian genomes. ISS-DNA or its synthetic oligodeoxynucleotide analogs (ISS-ODN or CpG-ODN) activate innate immunity via TLR9.[11] We have recently shown that ISS-ODN

Abbreviations used in this paper: DSS, dextran sodium sulfate; i.g., intragastric; i.r., intrarectal; ISS, immunostimulatory sequences; MPO, myeloperoxidase; ODN, oligonucleotide; TLR, toll-like receptor; TNBS, trinitrobenzenesulfonic acid.

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effectively prevent or ameliorate the severity of colitis in several different animal models.[12]

Because ISS-ODN mimic the immunomodulatory activities of bacterial DNA, we hypothesized a role for probiotic DNA in the inhibition of colonic inflammation mediated by probiotic therapy. If correct, this may imply that some structural components of these microorganisms, recognized by specific receptors of innate immunity (e.g., TLRs),[13] rather than their metabolic activities, are responsible for their beneficial properties.[10] Furthermore, this hypothesis may also suggest that the administration of viable probiotics is not necessary to provide the beneficial effects observed in models of experimental colitis.

To explore the mechanisms by which probiotic therapy ameliorates experimental colitis, we evaluated the impact of viable vs. nonviable (γ-irradiated) probiotics in TLR-deficient mice and in wild-type controls. The results of these experiments, combined with those obtained with isolated probiotic DNA, demonstrate that TLR9 signaling is required to mediate the inhibition of experimental colitis.

Materials and Methods

Animals

Balb/c, C57Bl/6 (B6), 129XB6F_2, congenic mice bearing the C3H-Tlr4$^{Lps\text{-}d}$ mutation (i.e., resistant to LPS) on the Balb/c background, and IL-10-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MyD88 (B6), TLR2 (B6), and TLR9 (129XB6F_2)-deficient mice were used as described[14,15] and are currently bred in the UCSD vivarium.

Probiotic Preparations

Probiotic bacteria (VSL-3) were purchased from VSL Pharmaceutical Inc. (Gaithersburg, MA). Each packet contains viable lyophilized gram+ bacteria of 4 strains of lactobacilli (*L. casei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii* subsp. *bulgaricus*), 3 strains of bifidobacteria (*B. longum*, *B. breve*, and *B. infantis*), and one strain of *Streptococcus salivarius* subsp. *Thermophilus*. Original packets (450×10^9 CFU per packet) were irradiated with 1.2 megarad using a ^{137}Cs source at a rate of 8 Gy/min overnight. Heat-killed VSL were prepared by resuspending viable probiotics in PBS at 28×10^8 CFU/mL followed by incubation for 30 minutes at 100°C (heat block), centrifuged at 8000 rpm for 5 minutes, washed in PBS, and resuspended in fresh PBS prior to their administration. All VSL preparations were resuspended in PBS at a final concentration of 28×10^8 CFU/mL and then cultured as described.[6] The resulting viability was determined by plating the cells on MRS-agar plates (Difco Laboratories, Detroit, MI) under anaerobic conditions for 16 hours at 37°C. No colonies were detected in the irradiated or heat-killed VSL, whereas $22.1 \times 10^8 \pm 6.1$ CFU/mL were recovered for viable (untreated) VSL (28×10^8 as specified by the manufacturer).

Genomic DNA and Oligodeoxynucleotide Preparations

Genomic DNA was isolated from VSL-3 packets (VSL Pharmaceutical) and from *E. coli* (DH5α, Invitrogen, Carlsbad, CA) using EndoFree DNA Isolation Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purity of DNA was confirmed by measuring the UV 260/280-absorbance ratio (>1.8). LPS levels in the DNA preparations were detected by limulus amebocyte lystate (BioWhittaker Inc., Wakersville, MD) and were <0.2 EU per μg of DNA.

Cytosine methylation of CpG dinucleotides in isolated probiotic DNA was performed by Sss I methylase (CpG methylase) (New England BioLabs, Beverly, MA) according to the manufacturer's instructions. Methylated DNA was extracted with phenol/chloroform for deproteination. Methylation of DNA was confirmed by digestion with restriction endonuclease BstUl followed by agarose gel electrophoresis.

Calf thymus DNA was purchased from Sigma (St. Louis, MO). The ISS-ODN (5′-TGACTGTGAACGTTCGAGATGA-3′) and the control ODN (5′-TGACTGTG AAGGTTAGAGATGA-3′)[12] on a phosphothioate backbone were purchased from Tri-Link (San Diego, CA).

To generate DNA-free probiotics, bacteria (VSL-3) were suspended in saline and disrupted by sonication. Bacterial lysates were incubated with DNase I (Roche, Indianapolis, IN) (10 U/mL) in the presence of 1 mmol/L $MgCl_2$ on ice for 2 hours. Elimination of DNA was confirmed by ethidium bromide staining on a 1% TAE agarose-gel.

Colitis Models

To induce dextran sodium sulfate (DSS) colitis, DSS (Sigma) was given in the drinking water for 7 days. Preliminary studies were performed to identify the concentration of DSS in the drinking water required to elicit a similar disease activity score in different mouse strains. 3.5% Of DSS in Balb/c mice was equivalent to 1.5% DSS in B6 mice and to 1.75% DSS in 129/B6 mice.

Trinitrobenzenesulfonic acid (TNBS) colitis was induced in 8-week-old Balb/c mice by rectal instillation of 0.5 mg/mouse of 2,4,6-trinitrobenzene sulfonic acid (Sigma) dissolved in 0.1 mL of 50% ethanol as described.[12] Mice were killed 7 days after the induction of colitis. All studies were performed in a blind fashion.

Probiotics and Various DNA Treatment Protocols

Probiotics, including live, irradiated, and heat killed, were intragastrically (i.g.) given, starting 10 days prior to the induction of colitis and for 7 days thereafter. In preliminary studies, mice were treated daily by i.g. administration of 0.28×10^8, 2.8×10^8, or 28×10^8 CFU of irradiated probiotics per mouse per day. The administration of 2.8×10^8 CFU/mouse/day was sufficient to inhibit colitis in Balb/c mice, whereas the administration of 28×10^8 CFU/mouse/day was required to inhibit colitis in the other mouse strains. In some

experiments chloroquine (10 mg/kg) (Sigma) was injected subcutanously (sc) daily[16] after the i.g. administration of viable or irradiated probiotics (2.8×10^8 CFU/mouse/day).

Various DNA preparations (ISS-ODN and control-ODN, 30 μg/mouse; probiotic DNA, methylated probiotic DNA, *E. coli* DNA, and calf thymus DNA, 50 μg/mouse) and DNase treated probiotics (i.e., the amount of microorganisms that yielded 50 μg of probiotic DNA) were sc injected 2 hours prior to the administration of DSS or TNBS. In another experiment 50 μg of these DNA preparations were administered i.g. or intrarectally (i.r.) 2 hours prior to DSS administration. In the IL-10-deficient colitis model, 10-week-old mice were treated sc once a week with the various DNA preparations (see above), and this treatment continued for 4 weeks as described.[12] The disease-activity score, histologic score, and MPO activity were determined as described.[12]

Effect of Probiotics on Chronic DSS Induced Colitis

To evaluate whether probiotics are effective not only in the prevention but also in the treatment of colitis, the following experiment was performed: Mice were treated for 7 days with DSS 3.5% added to the drinking water. From the eighth day until death on day 15, the concentration of DSS in the drinking water was reduced to 1.75%. During the 15 days of the experiment, 2 groups of mice were treated daily i.g. with viable or with irradiated probiotics 2.8×10^8 CFU. A third group was treated sc on day 8 with ISS-ODN (10 μg) and a fourth control group was treated i.g. daily with 0.2 mL of saline. Mice were observed for rectal bleeding, weighed, and killed on day 15. The colon was isolated and weighed, sections were taken for histology, and mucosal samples were obtained for myeloperoxidase (MPO) determination.

Effect of Chloroquine on Normal Flora and on Probiotic Bacterial Strains

To test whether chloroquine has antimicrobial activity on probiotics and on the commensal flora, mice were treated sc daily for 7 days with 10 mg/kg of chloroquine (Sigma). Control group was treated sc daily with 0.2 mL of saline. After 7 days, stool samples were collected; homogenized; and cultured on blood, Maconkey, phenylethanol, chocolate, M.R.S., and anaerobic agars. In another experiment, all strains of fecal flora and all probiotic strains were tested for susceptibility to chloroquine by the agar dilution method. Concentrations tested ranged from 0.3 to 250 μg/mL of choloroquine. No significant differences were observed as to the identity or quantity of bacterial strains grown from the stool of chloroquine and saline-treated mice. The flora that grew included the following: *Bacillus* spp., *Enterococcus* sp., *Escherichia coli*, *Diphtheroid* sp., *Lactobacillus* sp., and *Bacteroides* sp. All strains, inclusive of the probiotic strains, grew on all plates including those containing 250 μg/mL. The MIC of all bacterial strains is therefore >250 μg/mL.

Activation of Bone Marrow-Derived Macrophages by DNA

Bone marrow-derived macrophages (BMDM) were prepared from Balb/c mice as described.[12] BMDM (1×10^6) were incubated for 48 hours with 0.1–10 μg/mL of the various DNA preparations. The levels of IL-6 and IL-12 in the supernatants were determined by ELISA (BD-Pharmingen, San Diego, CA) 24 hours poststimulation.

Detection of Absorbed DNA in Mice

For the detection of plasmid DNA (pDNA), 1 mg of pBudCE4 (Invitrogen, Carlsbad, CA) was administered i.g. or i.r. to Balb/c mice. Mice were killed at various time points after pDNA administration, and DNA was extracted from liver and spleen using DNeasy Tissue Kit (Qiagen). For the detection of probiotic DNA, 28×10^8 CFU of irradiated probiotics was delivered i.g. for 10 days before DSS administration and for 7 days thereafter as described above. Ten micrograms each of isolated DNA were run on 1% TAE-agarose gel, transferred onto Hybond-N$^+$ membrane (Amersham, Piscataway, NJ), and hybridized to ^{32}P-labeled pDNA or VSL DNA using hybridization solution (Clontech, Palo Alto, CA). The hybridized membrane was exposed to X-ray film (Kodak, Rochester, NY) at −80°C overnight.

Signaling Assays

Translocation of nuclear factor-B (NF-κB) was detected by EMSA as described.[17] For JNK and IKK kinase assays, lysates of cells or tissues were prepared, and JNK1 or IKK were immunoprecipitated using anti-JNK1 or anti-IKK antibodies (Santa Cruz Biotech, Santa Cruz, CA). The kinase activities were determined by an in vitro kinase assay using GST-cJun for JNK or GST-IκBα for IKK as a substrate, respectively.[17]

Statistical Analysis

Data are expressed as ± SEM. Statistical analyses for significant differences were performed according to parametric, Student *t* test (MPO activity) and nonparametric, Mann–Whitney test (disease activity score and histologic score). In some assays, the χ^2 test was applied.

Results

Probiotic and *E. coli* DNA Have Immunostimulatory Activities

To evaluate the immunostimulatory properties of probiotic DNA, we assessed the ability of probiotic DNA to activate NF-κB and JNK, 2 major signaling pathways involved in TLR activation.[14] Probiotic DNA, but not methylated probiotic DNA or calf thymus DNA, activated NF-κB (EMSA), as did ISS-ODN but not control-ODN (Figure 1*A*). Similar results were obtained for JNK activation (Figure 1*B*). The activation of these

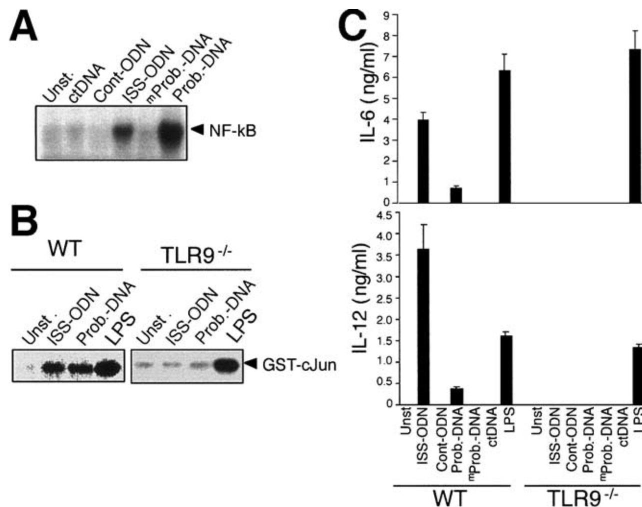

Figure 1. Probiotic DNA has immunostimulatory activities that depend on TLR9. BMDM were unstimulated (Unst) or stimulated with ISS-ODN, control (Cont)-ODN (5 μg/mL), probiotic (Prob) DNA, methylated (m) probiotic DNA, or calf thymus (ct) DNA (20 μg/mL) for 2 hours. (*A*) The activation of NF-κB was determined by EMSA. (*B*) JNK1 activation (kinase assay). (*C*) Cytokine levels in the supernatants were measured 24 hours poststimulation (ELISA). Results are mean ± SEM.

signaling pathways resulted in the induction of IL-12 (p40) and IL-6, which was mediated via TLR9 because both probiotic DNA and ISS-ODN did not induce the secretion of p40 or IL-6 in TLR9 null macrophages (Figure 1*C*). A similar immunostimulatory profile was observed with *E. coli* genomic DNA (data not shown).

TLR Signaling Is Required for Antiinflammatory Effects of Irradiated Probiotics

The administration of nonviable irradiated or viable probiotics attenuated the severity of DSS-induced colitis as reflected in the disease-activity score, histologic score, and colonic MPO activity. In contrast, the administration of heat-killed probiotics had no effect on the severity of DSS induced colitis (Table 1). Recently, chloroquine has been shown to inhibit the activation of TLR9 induced by its natural ligand, bacterial DNA.[18] Indeed, when mice were treated with chloroquine, it completely abolished the protective effect of both viable and irradiated probiotics on experimental colitis (Table 1). Histologically, the extensive superficial ulceration with mucosal inflammatory reaction induced by DSS (Figure 2*E*) was totally abolished in mice treated with irradiated probiotics (Figure 2*D*), whereas, in mice cotreated with viable probiotics, only minimal superficial ulceration with minimal inflammatory reaction was observed (Figure 2*C*).

Irradiated and viable probiotics as well as ISS-ODN were also found to equally attenuate the severity of a chronic model of DSS-induced colitis (Table 2). In this model, the probiotic preparations and the ISS-ODN were administered with or after induction of colitis, respectively, indicating their therapeutic capacity.

To confirm further the requirement of TLR signaling in the antiinflammatory effect observed above, irradiated probiotics were delivered to mice deficient in TLR2, TLR4, and TLR9 in which colitis was induced by DSS. Both TLR2- and TLR4-deficient mice responded favorably to irradiated probiotics, as did their wild-type littermates. In contrast, the administration of irradiated probiotics to TLR9-deficient mice had no effect on the severity of DSS-induced colitis (Table 3 and Figure 2*B*). TLR9 signaling is fully dependent on the adaptor protein MyD88.[14] As presented in Table 3, MyD88-deficient mice did not respond to irradiated probiotics, further indicating the essential role of the TLR9-signaling pathway in mediating the antiinflammatory effects of probiotics in this model.

Probiotic and *E. coli* DNA Inhibit DSS-Induced Colitis

To evaluate the antiinflammatory role of probiotic DNA in experimental colitis, we delivered it i.g., i.r. (Table 4), or sc (Table 5) once, 2 hours prior to DSS administration. Intragastric and sc administration of probiotic DNA or ISS-ODN inhibited the severity of DSS-induced colitis, whereas i.r. administration of these com-

Table 1. Effect of Probiotics and Chloroquine on DSS-Induced Colitis

Treatment	N	Disease activity score	MPO (*U/g*)	Histologic score
None	8	8.0 ± 0.9	1.90 ± 0.09	7.5 ± 0.9
Viable probiotics	8	2.7 ± 1.1[a]	0.78 ± 0.20[a]	2.8 ± 0.8[a]
Irradiated probiotics	8	0.1 ± 0.1[a]	1.10 ± 0.10[a]	2.5 ± 0.3[a]
Heat-killed probiotics	9	7.0 ± 0.8	1.60 ± 0.10	5.4 ± 1.0
Chloroquine	5	6.2 ± 0.4	1.54 ± 0.16	8.0 ± 0.8
Irradiated probiotics + chloroquine	5	6.6 ± 0.8	1.60 ± 0.10	7.7 ± 0.6
Viable probiotics + chloroquine	5	6.8 ± 0.1	2.30 ± 0.48	6.8 ± 0.7

NOTE. Balb/c mice were intragastrically treated daily with 2.8×10^8 CFU of viable, irradiated, or heat-killed probiotics 10 days prior to the addition of DSS (3.5%) to the drinking water and for 7 days thereafter. Three groups were also subcutaneously treated with chloroquine (10 mg/kg) dissolved in 0.1 mL of saline once daily (see Materials and Methods section). Disease activity score, colonic MPO activity, and histologic score were determined after 7 days of DSS administration as described. Results are mean ± SEM and represent 1 of 3 experiments. The following statistical analyses were used: for MPO activity, Student *t* test; for disease activity score as well as for histologic score, Mann–Whitney test.
[a]Significantly different from no treatment or chloroquine treatment ($P < 0.05$).

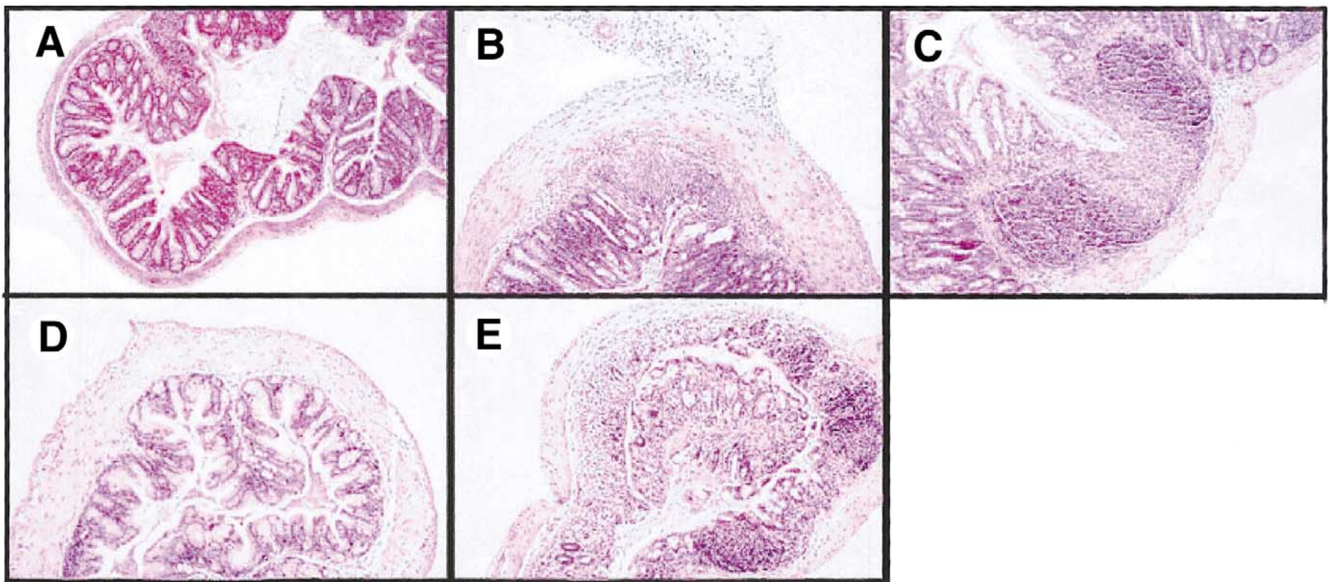

Figure 2. Histologic evaluation. (*A*) Colonic segment of naïve Balb/c mice showing normal colonic mucosa, submucosa, and muscularis propria. (*B*) Colonic segment of TLR9 null mice treated with DSS (1.75%) and irradiated probiotic bacteria, showing superficial ulceration with severe acute inflammation involving mucosa, submucosa, muscularis propria, and mesenteric fat tissue. (*C*) Colonic segment of Balb/c mice treated with DSS (3.5%) and viable probiotic bacteria, showing minimal superficial ulceration over a lymphoid nodule along with minimal inflammatory reaction involving the mucosa only. (*D*) Colonic segment of Balb/c mice treated with DSS (3.5%) and irradiated probiotic bacteria, showing normal colonic mucosa, submucosa, and muscularis propria. (*E*) Colonic segment of Balb/c mice following 7 days of DSS (3.5%) administration, showing extensive superficial ulceration with mucosal inflammatory reaction. (H&E staining; original magnification ×10.)

pounds had no effect on the outcome of colitis. The i.g. administration of methylated probiotic DNA (i.e., with CpG methylase), calf thymus DNA, or DNase treated probiotics (i.e., the amount of microorganisms that yielded 50 μg of probiotic DNA) also did not affect the course or the severity of colitis (Table 4). Intragastric or sc administration of *E. coli* DNA also inhibited the severity of DSS-induced colitis (Table 6). Taken together, these data outline the antiinflammatory role of certain microbial DNA and the required i.g. or sc route of administration for the attenuation of experimental colitis.

Table 2. Effect of Probiotics on Chronic DSS-Induced Colitis

Treatment	N	Disease activity score	MPO (*U/g*)	Histologic score
None	9	5.4 ± 0.6	1.60 ± 0.10	8.0 ± 0.5
Viable probiotics	7	0.9 ± 0.5[a]	0.97 ± 0.10[a]	6.5 ± 0.7
Irradiated probiotics	8	1.6 ± 0.5[a]	1.18 ± 0.10[a]	5.9 ± 0.1[a]
ISS-ODN	8	1.1 ± 0.4[a]	1.25 ± 0.19[a]	6.9 ± 0.8

NOTE. Balb/c mice were treated for 7 days with DSS (3.5%) added to the drinking water and for an additional 7 days with DSS (1.75%). One group was treated sc on day 8 with ISS-ODN (10 μg) and 2 other groups were treated daily i.g. with viable or irradiated probiotics 2.8×10^8 CFU. Mice were killed on day 15. Results are mean ± SEM and represent 1 of 3 experiments. For MPO activity, Student *t* test was employed. For disease activity score and for histologic score, Mann-Whitney test was employed.

[a]Significantly different from no treatment ($P < 0.05$).

Probiotic DNA Attenuates Different Models of Experimental Colitis

We further evaluated whether the protective effect of probiotic DNA can be reproduced by its SC injection in TNBS-induced colitis as well as in spontaneous colitis observed in IL-10-deficient mice.[19] A single sc injection of probiotic DNA, but not of methylated probiotic DNA or of calf thymus DNA, decreased the disease-activity score, histologic score, and colonic MPO activity of TNBS-induced colitis (Table 7) and attenuated the course and the severity of the colitis that had developed in IL-10-deficient mice over time (Table 8).

Absorption of Bacterial DNA From the Gastrointestinal Tract

Because i.g. or sc but not i.r. administration of probiotic DNA ameliorates experimental colitis, we reasoned that the probiotic DNA might be absorbed from the upper gastrointestinal tract as was described for phage DNA[20] and act in systemic sites. To explore this possibility, a purified form of bacterial DNA was delivered, i.e., plasmid DNA (pDNA), i.g. once to wild-type mice, and the presence of this bacterial DNA in their liver and spleen was evaluated. Indeed, we identified the

Table 3. Effect of Irradiated Probiotics on DSS-Induced Colitis

Background	N	Treatment	Disease activity score	Histologic score
B6 (wt)	8	None	4.5 ± 0.5	7.7 ± 1.3
	4	Irradiated probiotics	1.8 ± 0.6[a]	1.0 ± 0.6[a]
MyD88 KO	6	None	4.8 ± 0.3	9.2 ± 0.5
	6	Irradiated probiotics	5.0 ± 0.2	9.0 ± 0
TLR2 KO	7	None	4.9 ± 0.2	9.7 ± 0.5
	7	Irradiated probiotics	2.1 ± 0.5[a]	6.6 ± 0.7[a]
Balb/c (wt)	14	None	7.3 ± 1.1	5.3 ± 1.2
	8	Irradiated probiotics	1.7 ± 0.5[a]	1.7 ± 0.5[a]
TLR4 KO	5	None	4.9 ± 0.5	8.0 ± 0.9
	6	Irradiated probiotics	1.4 ± 0.3[a]	1.8 ± 0.9[a]
B6/129F_2 (wt)	11	None	6.1 ± 0.4	8.3 ± 0.3
	4	Irradiated probiotics	2.5 ± 0.3[a]	1.8 ± 0.9[a]
TLR9 KO	6	None	5.7 ± 0.6	7.5 ± 1.7
	6	Irradiated probiotics	5.7 ± 0.6	9.2 ± 0.9

NOTE. Mice were intragastrically treated daily with irradiated probiotics 10 days prior to the addition of DSS to the drinking water and for 7 days thereafter. Mice were killed 7 days after DSS administration (see Materials and Methods section). Results are mean ± SEM and represent 1 of 2 experiments. For MPO activity, Student *t* test was employed. For disease activity score and for histologic score, Mann-Whitney test was employed.
wt, wild type.
[a]Significantly different from no treatment ($P < 0.05$).

pDNA and its fragments in these organs (Southern blot) within 2–6 hours post-i.g. but not post-i.r. administration (Figure 3*A*). Interestingly, the efficacy of bacterial DNA absorption was by far lower when the pDNA was delivered i.r. rather than i.g. (Figure 3*A*). The localization of this bacterial DNA in these organs coincided with its immunostimulatory activities, i.e., the activation of JNK and NF-κB (Figure 3*B*), the major signaling pathways initiated by the engagement of TLR9 with its ligand, bacterial DNA.[11,14] We also identified the probiotic DNA in the liver and spleen after daily i.g. administration of irradiated probiotics, which was initiated 10 days prior to induction of colitis with DSS, and for 7 days thereafter (Figure 3*C*). Taken together, these data indicate that most of the probiotic DNA is absorbed from the upper gastrointestinal tract and most probably acts systemically as occurs with sc injection of other types of immunostimulatory DNA (e.g., ISS-ODN).

Table 4. Effect of Intragastric or Intrarectal Administration of Various Probiotic DNA on DSS-Induced Colitis

Treatment	N	Disease activity score	MPO (*U/g*)	Histologic score
None	19	7.3 ± 0.5	1.90 ± 0.09	7.5 ± 0.9
Probiotic DNA (i.g.)	8	3.2 ± 0.7[a,b]	1.30 ± 0.10[a,b]	3.0 ± 0.4[a]
Probiotic DNA (i.r.)	10	5.9 ± 1.2	1.90 ± 0.10	6.5 ± 1.1
Methylated probiotic DNA (i.g.)	4	7.8 ± 0.5	1.93 ± 0.35	5.7 ± 0.7
DNase-treated probiotics (i.g.)	6	7.8 ± 1.2	1.60 ± 0.20	7.4 ± 1.4
Calf thymus DNA (i.g.)	8	5.7 ± 1.0	1.98 ± 0.20	6.3 ± 1.4
ISS-ODN (i.g.)	8	3.6 ± 0.7[a]	1.09 ± 0.10[a]	2.8 ± 0.6[a]
ISS-ODN (i.r.)	10	6.3 ± 0.7	1.90 ± 0.30	5.9 ± 0.7
Control-ODN (i.g.)	10	6.7 ± 0.8	1.90 ± 0.20	5.7 ± 0.3

NOTE. Balb/c mice were intragastrically (i.g.) or intrarectally (i.r.) treated with various DNA preparations 2 hours before induction of colitis with DSS. Results are mean ± SEM and represent 1 of 3 experiments. For MPO activity, Student *t* test was employed. For disease activity score and for histologic score, Mann-Whitney test was employed.
[a]Significantly different from no treatment or treatment with calf thymus DNA ($P < 0.05$).
[b]Significantly different from DNase treated probiotics ($P < 0.05$).

Table 5. Effect of Subcutaneous Administration of Various DNA on DSS-Induced Colitis

Treatment	N	Disease activity score	MPO (*U/g*)	Histologic score
None	10	5.7 ± 0.5	3.6 ± 0.3	10.7 ± 0.8
Probiotic DNA	8	1.1 ± 0.3[a]	0.8 ± 0.1[a]	0.3 ± 0.3[a]
Methylated probiotic DNA	8	3.4 ± 0.4[b]	1.7 ± 0.2[b]	4.3 ± 1.3[b]
DNase-treated probiotics	4	5.0 ± 1.1[b]	1.5 ± 0.2[b]	4.3 ± 1.2[b]
Calf thymus DNA	4	5.5 ± 1.5	3.3 ± 0.5	6.0 ± 1.3
ISS-ODN	4	0.4 ± 0.4[a]	0.9 ± 0.1[a]	0 ± 0[a]
Control-ODN	4	3.8 ± 0.7	4.2 ± 0.8	8.0 ± 0.9

NOTE. Balb/c mice were subcutaneously injected with various DNA preparations 2 hours before induction of colitis (see Materials and Methods). Results are mean ± SEM and represent 1 of 3 experiments. For MPO activity, Student *t* test was employed. For disease activity score and for histologic score, Mann-Whitney test was employed.
[a]Significantly different from no treatment ($P < 0.05$).
[b]Significantly different from treatment with probiotic DNA ($P < 0.05$).

Table 6. Effects of Administration of *E. coli* DNA on DSS-Induced Colitis

Treatment	N	Disease activity score	MPO (*U/g*)	Histologic score
None	7	3.0 ± 0.8	1.40 ± 0.20	6.70 ± 0.7
E. coli DNA (sc)	7	0.3 ± 0.2[a]	0.55 ± 0.10[a]	3.10 ± 0.6[b]
E. coli DNA (i.g.)	7	0.6 ± 0.3[a]	0.70 ± 0.19[b]	6.40 ± 0.9

NOTE. Balb/c mice were subcutaneously or intragastrically treated with *E. coli* DNA 2 hours before induction of colitis DSS (see Materials and Methods section). Results are mean ± SEM and represent 1 experiment. For MPO activity, Student *t* test was employed. For disease activity score and for histologic score, Mann-Whitney test was employed.
[a]Significantly different from no treatment ($P < 0.01$).
[b]Significantly different from no treatment ($P < 0.03$).

Table 7. Effect of Various DNA on TNBS-Induced Colitis

Treatment	N	Disease activity score	MPO (*U/g*)	Histologic score
None	10	1.8 ± 0.7	1.50 ± 0.01	9.0 ± 1.8
Probiotic DNA	8	0[a]	0.88 ± 0.08[a]	1.7 ± 0.9[a]
Methylated probiotic DNA	8	1.5 ± 0.3[b]	1.50 ± 0.09[b]	5.0 ± 0.9[b]
DNase-treated probiotics	8	1.0 ± 0[b]	1.60 ± 0.06[b]	5.5 ± 0.9[b]
Calf thymus DNA	8	3.4 ± 0.5	1.20 ± 0.07	7.0 ± 2.0
ISS-ODN	8	0.4 ± 0.3[a]	0.8 ± 0.10[a]	0 ± 0[a]
Control-ODN	4	1.3 ± 0.2	2.00 ± 0.30	4.8 ± 1.8

NOTE. Balb/c mice were subcutaneously injected with various DNA preparations 2 hours before induction of colitis (see Materials and Methods). Results are mean ± SEM and represent 1 of 3 experiments. For MPO activity, Student *t* test was employed. For disease activity score and for histologic score, Mann–Whitney test was employed.
[a]Significantly different from no treatment or treatment with calf thymus DNA ($P < 0.05$).
[b]Significantly different from treatment with probiotic DNA ($P < 0.05$).

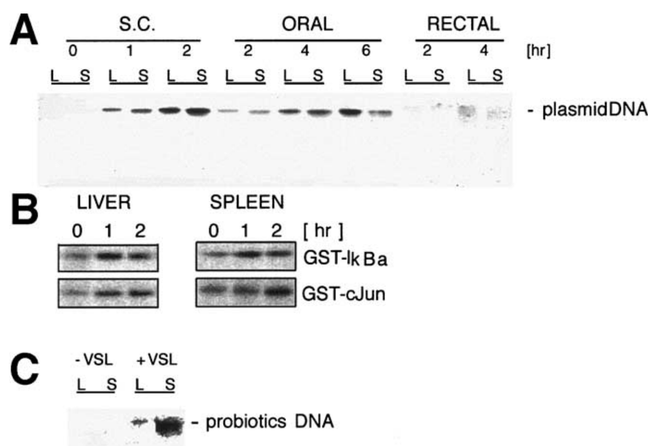

Figure 3. Detection of bacterial DNA at systemic sites. (*A*) Plasmid DNA is detected after its oral administration (1 mg/mouse) in the liver (L) and spleen (S) but is not detected after rectal administration (Southern blot). The uptake of pDNA in these organs after SC injection (100 μg/mouse) is shown as control. (*B*) The localization of orally administered pDNA coincides with the activation of IKK and JNK1 in these organs. (*C*) VSL DNA is detected in the liver (L) and the spleen (S) after 17 days of oral administration of irradiated probiotic bacteria (Southern blot).

Discussion

Persuasive evidence indicates that intestinal microflora play an important role in the initiation and the perpetuation of murine experimental colitis[19] and human IBD.[5] This fact initially provided the rationale for the use of probiotics to manipulate the intestinal microenvironment. In fact, recent studies have demonstrated the therapeutic effects of probiotics in various models of experimental colitis and in several clinical trials in patients with IBD or related disorders.[10] However, the molecular mechanisms by which probiotics exert their therapeutic effects have not been identified. The impact of probiotics on mucosal barrier function,[6] their diverse metabolic activities, their competitive exclusion of intestinal indigenous microflora, and their interaction with the mucosal immune system have all been implicated in mediating their therapeutic effects.[1–4]

Table 8. Effect of Various DNA on Spontaneous Colitis in IL-10 KO Mice

Treatment	N	Rectal prolapse (N)	MPO (*U/g*)	Histologic score
None	13	11	1.0 ± 0.10	8.1 ± 0.9
Probiotic DNA	10	2[a]	0.2 ± 0.04[a]	3.0 ± 0.4[a]
Calf thymus DNA	8	5	0.7 ± 0.10	6.3 ± 1.4
ISS-ODN	6	1[a]	0.8 ± 0.10	1.8 ± 1.2[a]

NOTE. IL-10 KO mice (B6) were subcutaneously injected once a week with various DNA (see Materials and Methods section). The following statistical analyses were used. For rectal prolapse, χ^2; for MPO activity, Student *t* test; and for histologic score, Mann–Whitney test. Results are mean ± SEM.
[a]Significantly different from no treatment ($P < 0.05$).

In this study, we provide biochemical, immunologic, and genetic evidence that implicates TLR signaling, especially TLR9, in mediating the protective effect of probiotics on experimental colitis. VSL-3 was used because of its well-proven efficacy in clinical trials and in animal models of colitis.[6,9] The administration of irradiated probiotics effectively ameliorated experimental colitis, as did the administration of viable probiotics (Tables 1 and 2). Because the irradiated probiotics were unable to grow in culture, it is unlikely that either their metabolites or their competitive inhibition with indigenous microflora were responsible for the protective effects on the colonic mucosa. Therefore, we reasoned that the antiinflammatory activities could be the product of the activation of innate immunity (e.g., via TLR) by structural microbial components such as peptidoglycans (a cell wall component of gram+ bacteria such as those in VSL-3) or their genomic DNA. Because chloroquine was recently demonstrated to inhibit specifically TLR9 signaling in in vitro and in vivo systems,[11,18] we explored whether its administration could counterbalance the protective effects of probiotics. Chloroquine administration abolished the beneficial effects of i.g. delivery of probiotics, and, consequently, these mice developed severe colitis. In the present study, chloroquine was found to have neither effect on the normal fecal flora nor on the probiotic strains, and, therefore, its observed effect cannot be ascribed to antibacterial activity. These studies further support a previous observation that identified bacterial resistance to chloroquine.[21] To further verify

the role of TLR signaling in the probiotics-induced amelioration of experimental colitis, mice deficient in TLR2, TLR4, TLR9, and MyD88 were treated with DSS and irradiated probiotics. The administration of irradiated probiotics ameliorated the clinical, biochemical, and histologic parameters of colitis in TLR2-deficient mice, indicating that bacterial peptidoglycan or lipopeptides, which are cell wall components of gram+ bacteria and the ligands of TLR2,[14] are not involved in this process. Similar conclusion can be drawn for LPS, the ligand of TLR4. In contrast, the administration of irradiated probiotics to TLR9 and MyD88 null mice did not affect the course and the severity of colitis (Table 3), indicating the involvement of the TLR9-signaling pathway in the observed amelioration of colonic inflammation.

To validate further the pivotal role of TLR9 signaling and to exclude the involvement of other TLR, MyD88-dependent pathways in the inhibition of colonic inflammation, DNase-treated probiotics or different DNA preparations were delivered to the mice prior to the induction of colitis by DSS. The administration of DNase-treated probiotics did not inhibit colonic inflammation (Table 4), whereas the administration of probiotic DNA ameliorated DSS-induced colitis, as did ISS-ODN.[12] In contrast, the administration of methylated probiotic DNA, calf thymus DNA, or control ODN did not suppress colonic inflammation (Table 4). Taken together, these data indicate the specific ameliorating properties of probiotic DNA on experimental colitis and potentially exclude a similar role for other microbial components or microbial compounds.[6]

Interestingly, the sc injection of probiotic DNA also resulted in marked inhibition of various parameters of DSS-induced colitis (Tables 4 and 5). This finding was reproduced in TNBS-induced colitis (Table 7) as well as in spontaneous colitis in IL-10-deficient mice (Table 8). The similar beneficial effect on experimental colitis mediated by i.g. or SC but not by i.r. administration of immunostimulatory DNA led us to speculate that the probiotic DNA is absorbed through the upper gastrointestinal tract and acts systemically as does its SC injection. Indeed, plasmid (p) DNA could be detected within 2–8 hours after i.g. administration in the liver and spleen of pDNA-fed animals. This kinetics coincided with activation of the major signaling events of the TLR pathway, JNK, and NF-κB (Figure 3), suggesting that the absorbed DNA was biologically active. In contrast to i.g. administration, an insignificant quantity of pDNA was detected in these organs following i.r. administration, suggesting that most of the probiotic DNA is absorbed in the small intestine rather than in the colon. Similarly, probiotic DNA was detected in the liver and spleen of DSS-treated animals that had been fed irradiated probiotics (Figure 3). Because most bacterial genomic DNA is immunostimulatory, our data suggest that the ability of other commensal or nonpathogenic bacteria to alleviate experimental colitis relies on the availability of their DNA to be absorbed in the upper gastrointestinal tract. Their natural colonization of the lower gastrointestinal tract prevents commensal bacteria from systemically delivering their potential health benefit properties. In this respect, irradiated probiotics function as a modified natural encapsulated delivery system that releases its cargo DNA in the right portion of the intestinal tract.

The data presented here indicate that the antiinflammatory effect of probiotics on experimental colitis is preserved in irradiated bacteria. This effect is induced by TLR signaling mediated by TLR9-probiotic DNA interaction, rather than mediated by a variety of other metabolic activities attributed to probiotics.[1–4] Our data also indicate that the i.g. and sc administration of certain other microbial DNA (i.e., *E. coli*) also ameliorated experimental colitis (Table 6). Thus, evaluation of the immunostimulatory activities of microbial genomic DNA in vitro and in models of experimental colitis in vivo may predict its ability to attenuate human colitis. It also provides an easy screening system for selection of commensal or other nonpathogenic bacteria with potential therapeutic effects for IBD.

As mentioned above, probiotic DNA most probably acts systemically to ameliorate experimental colitis (Table 5, Figure 3), which may be the result of antiapoptotic effects on colonocytes and/or inhibition of a variety of proinflammatory mediators produced in the colonic mucosa.[12] This concept is further substantiated by a recent study that identified mucosal TLR9+, CD8a+ plasmacytoid dendritic cells, which, upon ISS-ODN stimulation, secrete IFN-α and support the differentiation of naïve CD4+ T lymphocyes to T regulatory cells (Treg).[22] This important finding suggests a physiologic role for bacterial (ISS) DNA in the maintenance of mucosal homeostasis and potentially provides a cellular basis (i.e., Treg) for the antiinflammatory properties of probiotic DNA (and most probably for probiotics) in models of experimental colitis.

The role of probiotic DNA in mediating health benefits in other models of human diseases has yet to be confirmed. Thus, probiotic DNA provides a unique set of immunostimulatory and immunoregulatory effects. This combination of bioactivities most likely limits bacterial invasion into the colonic tissue and reduces the resulted

colonic inflammation. Finally, irradiated probiotics are safer than the viable probiotic preparations, and their use in infants or immunocompromised hosts is expected to provide therapeutic benefits without risk of bacteremia.[23]

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