Bifidobacterium animalis Causes Extensive Duodenitis and Mild Colonic Inflammation in Monoassociated Interleukin-10-Deficient Mice

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Background: We recently showed that *Bifidobacterium animalis* is more prevalent within the colons of interleukin (IL)-10-deficient (−/−) mice than in wildtype (WT) animals colonized with the same specific pathogen-free (SPF) fecal contents. Here we tested the ability of this organism to cause T-cell-mediated intestinal inflammation by introducing it into germ-free (GF) IL-10−/− mice.

Methods: GF IL-10−/− or WT mice were monoassociated with *Bifidobacterium animalis* subsp. *animalis* ATCC (American Type Culture Collection, Manassas, VA) 25527^{T} or with *B. infantis* ATCC 15697^{T}. Inflammation was measured by blinded histologic scores of the duodenum, cecum, and colon and by spontaneous secretion of IL-12/IL-23 p40 from colonic explants. Bacterial antigen-specific $CD4^{+}$ mesenteric lymph node (MLN) T-cell recall responses were measured in response to antigen-presenting cells (APC) pulsed with bacterial lysates.

Results: *B. animalis* caused marked duodenal inflammation and mild colitis in monoassociated IL-10−/− mice, whereas the intestinal tracts of WT animals remained free of inflammation. *B. infantis* colonization resulted in mild inflammation in the duodena of IL-10−/− mice. $CD4^{+}$ MLN T cells from *B. animalis* monoassociated IL-10−/− mice secreted high levels of IFN-γ and IL-17 in response to *B. animalis* lysate. *B. animalis* equally colonized the different intestinal regions of WT and IL-10−/− mice.

Conclusions: *B. animalis*, a traditional probiotic species that is expanded in experimental colitis in this model, induces marked duodenal and mild colonic inflammation and TH1/TH17 immune responses when introduced alone into GF IL-10−/− mice. This suggests a potential pathogenic role for this commensal bacterial species in a susceptible host.

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Key Words: intestinal inflammation, animal models, IL-10 deficient mice, *Bifidobacterium animalis*

Human inflammatory bowel diseases (IBDs), including ulcerative colitis and Crohn's disease, are believed to be caused by an inappropriate cell-mediated (T cell) immune response to commensal enteric bacteria in genetically susceptible individuals.[1,2] This is supported by clinical observations linking increased concentrations of luminal and adherent bacteria to inflamed regions of the intestinal tract[3–5] and by studies of experimental colitis in genetically susceptible animals, including interleukin (IL)-10-deficient (−/−) mice, where colitis and immune activation fail to develop in the absence of commensal bacteria.[1,6] Colonization of susceptible gnotobiotic rodents has demonstrated that some commensal bacterial species cause inflammation, some have no effect, and others (probiotics) provide protection from the inflammation caused by detrimental species.[3] For example, gnotobiotic IL-10−/− mice develop bacterial species-antigen-specific T-cell-mediated intestinal inflammation when monoassociated with *Enterococcus faecalis* or *Escherichia coli*,[7] but remain healthy when colonized with *Bacteroides vulgatus*, which has been shown to induce colitis in HLA-B27/β2 microglobulin transgenic rats.[8] IL-10−/− mice housed under specific pathogen-free (SPF) conditions and fed *Lactobacillus salivarius* subsp. *salivarius* or *Bifidobacterium infantis* displayed reduced colonic inflammation and attenuated Peyer's patch IFN-γ responses following in vitro stimulation with the enteric pathogen *Salmonella typhimurium*, compared to placebo controls.[9] These observations highlight the importance of understanding how different intestinal inhabitants selectively impact the host's mucosal immune system, and how this immune activation or inhibition influences health in hosts with distinct genetic backgrounds.

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We recently reported significant alterations in the composition of the enteric microbiota in colitic IL-10−/− mice compared to healthy WT animals after germ-free (GF) mice were colonized with fecal microbiota obtained from SPF wildtype (WT) mice.[10] Changes in microbiota composition with progression of colitis were analyzed using polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (PCR/DGGE) and specific DNA:RNA dot blot analysis. Interestingly, *B. animalis* was 1 of 4 bacterial species that increased in abundance as colitis developed. Bifidobacteria are widely considered to be beneficial commensal organisms that are "generally regarded as safe" (GRAS) and have been associated with probiotic activity.[3,11–13] Therefore, the increased prevalence of *B. animalis* in the colons of colitic IL-10−/− mice was unexpected and the inflammatory potential of this species in IL-10−/− mice warranted further investigation.

In the current study we evaluated *B. animalis* for its ability to induce intestinal inflammation in gnotobiotic WT and IL-10−/− mice using the type strain *B. animalis* subsp. *animalis* ATCC (American Type Culture Collection, Manassas, VA) 25527^T for colonization. For comparison, we also colonized gnotobiotic WT and IL-10−/− mice with *B. infantis* ATCC 15697^T. We further investigated the antigen specificity of the T-cell-mediated immune response of *B. animalis* colonized mice and assessed possible crossreactivity of the T cell response to other bacteria. Here we report that IL-10−/− mice monoassociated with *B. animalis* developed extensive duodenal and mild colitic inflammation with $CD4^+$ T cell responses directed against both unique and shared bacterial antigens expressed by *B. animalis*.

MATERIALS AND METHODS

Bacterial Strains and Media

Bifidobacterium animalis subsp. *animalis* ATCC 25527^T, *B. infantis* ATCC 15697^T and *B. bifidum* ATCC 11863^T were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and propagated anaerobically at 37°C in Lactobacilli MRS medium (BD Biosciences, Sparks, MD). A murine *Escherichia coli* strain designated NC101[7] and a human oral isolate of *Enterococcus faecalis* (strain OG1RF provided by Mark Huycke, MD)[14] have been previously described. Bacterial lysates were prepared as previously described.[7]

Mice

GF IL-10−/− mice on the 129S6/SvEv background and WT control (inbred 129S6/SvEv) mice were from breeding colonies maintained at the National Gnotobiotic Rodent Resource Center (NGRRC, University of North Carolina, Chapel Hill) or the Center for Gastrointestinal Biology and Disease (CGIBD) Gnotobiotic Animal Core (North Carolina State University, Raleigh). These mouse colonies were originally derived by hysterectomy at the Gnotobiotic Laboratory (University of Wisconsin, Madison).[6] Mice were monoassociated at 10–26 weeks of age with *B. animalis* or *B. infantis* by gavage feeding and rectal swabbing with viable cultured bacteria. Monoassociated mice were maintained in either the NGRRC or the CGIBD gnotobiotic facilities. Mice colonized with *B. animalis* or *B. infantis* were housed in separate isolators. Bacterial monoassociation and absence of contamination by other bacterial species were confirmed by periodic aerobic and anaerobic culture of stool samples. Mice were killed 11, 17, or 20–24 weeks after colonization with *B. animalis* and 22–23 weeks after colonization with *B. infantis*. WT 129S6/SvEv mice (Taconic Laboratories, Germantown, NY) maintained under SPF conditions free of *Helicobacter* species were used to prepare antigen-presenting cells (APC). Animal use protocols were approved by the Institutional Animal Care and Use Committees of North Carolina State University and the University of North Carolina at Chapel Hill.

Investigation of Bacterial Colonization of Mice

At necropsy after 24 weeks of colonization with *B. animalis*, a portion of the cecal contents from 2 129 WT and 2 IL-10−/− mice was used to inoculate sterile Lactobacilli MRS liquid media and cultures were incubated in sealed glass culture tubes overnight at 37°C. Fecal samples were collected from 2 129 WT and 2 IL-10−/− *B. infantis* monoassociated mice after 8 weeks of colonization and were similarly inoculated into sterile Lactobacilli MRS liquid media. An aliquot from each culture was streaked onto Lactobacilli MRS agar supplemented with 0.05% L-cysteine and plates were incubated for 72 hours under anaerobic conditions. Cell and colony morphology of these isolates were identical to the *B. animalis* and *B. infantis* strains initially used to colonize the animals (data not shown). Genomic DNA was isolated from randomly selected individual colonies of *B. animalis* and *B. infantis* stock cultures and from each ex vivo isolate using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). 16S ribosomal RNA gene sequence was amplified by PCR using the universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1491R (GGTTACCTTGTTACGACTT). The following reagents were included in each PCR tube: 5 μL 10 × buffer, 1.5 μL 50 mM $MgCl_2$ (Invitrogen, Carlsbad, CA), 0.5 μL 5 mM dNTP (GE Healthcare, Piscataway, NJ), 1 μL each of primers 27F and 1491R (5 pmol/μL), 30–40 ng DNA template, 1.25 U Taq DNA Polymerase (Invitrogen), and deionized water to a final volume of 50 μL. The PCR cycling was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) under the following conditions: 95°C for 2 minutes 30 seconds, then 35 cycles consisting of 95°C for 60 seconds, 51°C for 60 seconds, and 72°C for 120 seconds. The PCR products were incubated at 4°C until used.

Sequencing of PCR products was carried out using 27F and 1491R as sequencing primers. DNA was sequenced at the UNC-CH Genome Analysis Facility on a 3730 DNA Analyzer (Applied Biosystems). Sequence analysis using the BLASTN algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) confirmed that all ex vivo isolates from mice monoassociated with *B. animalis* or *B. infantis* were *B. animalis* and *B. infantis*, respectively.

Bacterial numbers of *B. animalis* in the gut of 129S6/SvEv WT and IL-10−/− mice (6 mice each) were measured in the duodenum, cecum, proximal colon, and distal colon by quantitative plating of serial dilutions of homogenized intestinal segments on Lactobacilli MRS agar supplemented with 0.05% L-cysteine (Sigma, St. Louis, MO). Plates were incubated for 72 hours under anaerobic conditions at 37°C and colonies were enumerated.

Histological Scoring

Sections of colon (proximal and distal), cecum, and duodenum were fixed in 10% neutral buffered formalin. The fixed tissue was embedded in paraffin and stained with hematoxylin and eosin (H&E). Sections were scored blindly for severity of inflammation by a single individual using a well-validated scale.[6,7,15,16] Histological scores (0 to 4) were based on the degree of lamina propria and submucosal mononuclear cellular infiltration, crypt hyperplasia, goblet cell depletion, and architectural distortion. These scoring criteria, which were developed previously to evaluate colitis were modified for use with the duodenal tissue sections. In the duodenum a score of 0 represents no inflammation and normal villus architecture. A score of 1 represents mild focal cellular infiltration and normal villous architecture. A score of 2 represents mild lamina propria cellular infiltration and early crypt epithelial hyperplasia with normal villus architecture. A score of 3 represents more pronounced cellular infiltration, thickened mucosa, marked epithelial hyperplasia, and moderate distortion of villus architecture. A score of 4 represents extensive cellular infiltration throughout the section and severe architectural distortion.

Colonic Tissue Fragment Cultures

Colonic tissue fragment cultures were prepared from the large intestine as previously described.[6,7] Colonic tissue was thoroughly irrigated with phosphate-buffered saline (PBS), shaken at room temperature in RPMI containing 50 μg/mL gentamicin for 30 minutes at 280 rpm, cut into 1-cm fragments, blotted to remove excess media, and weighed. Colonic tissue fragments were distributed (0.05 g per well) into 24-well plates (Costar, Cambridge, MA; 3524) and incubated in 1 mL of RPMI 1640 medium supplemented with 5% fetal bovine serum, 50 μg/mL gentamicin, and 1% antibiotic/antimycotic (penicillin/streptomycin/amphotericin B; Invitrogen) for 20 hours at 37°C. Supernatants were collected and stored at −20°C prior to use for IL-12/IL-23 p40 quantification by enzyme-linked immunosorbent assay (ELISA).

$CD4^+$ T-cell Isolation

$CD4^+$ T cells were enriched by negative selection from mesenteric lymph node (MLN) cells harvested from IL-10−/− or WT mice using magnetic beads coated with B-cell-specific (anti-CD45R) antibody plus beads coated with CD8-specific (anti-CD8a) antibody according to the manufacturer's instructions (Miltenyi Biotech, Auburn, CA) as previously described.[7,16] Over 95% of the enriched MLN cell populations expressed CD4 as determined by flow cytometry.

Antigen-presenting Cell Preparation and $CD4^+$ T-cell Stimulation

Splenic APCs were prepared from SPF 129S6/SvEv WT mice and pulsed overnight with 10 μg/mL of *B. animalis, B. bifidum, B. infantis, Enterococcus faecalis*, or *E. coli* lysate or keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL) as an unrelated antigen control, as described previously.[7,16] $CD4^+$ T cells (2×10^5 $CD4^+$ T cells/well) were cocultured for 72 hours with antigen-pulsed APC (3×10^5 APC cell/well) in flat-bottom 96-well cell culture plates (Costar 3595), 0.2 mL per culture. Supernatants were collected after 3 days and stored at –20°C.

Dendritic Cell Stimulation with Bacterial Cell Lysates

Bone marrow-derived dendritic cells (BMDC) were isolated and cultured from femora and tibiae of SPF 129S6/SvEv mice, as previously described.[17] BMDC (2×10^4) were seeded in triplicate wells of flat-bottom 96-well cell culture plates (Costar 3595) in the presence of media alone, LPS (1 μg/mL), or various bacterial lysates (10 μg/mL) at 0.2 mL per culture. Supernatants were collected after 3 days and stored at –20°C.

Cytokine Measurements

We used commercially available monoclonal anti-mouse IFN-γ, IL-12/IL-23 p40 (BD Biosciences Pharmingen, San Diego, CA), and IL-17 (e-Bioscience, San Diego, CA) specific capture and detection reagents to measure amounts of secreted cytokines by ELISA. For detection of IL-12/IL-23 p40, plates were coated with anti-mouse IL-12 p40/p70 (clone C15.6) and bound ligand was detected with biotin anti-mouse IL-12 p40/p70 (clone C17.8). To quantify IFN-γ, plates were coated with anti-mouse IFN-γ (clone R4-6A2) and bound ligand was detected with biotin anti-mouse IFN-γ (clone XMG1.2). For detection of IL-17, plates were coated with anti-mouse IL-17A, clone eBioTC11-18H10.1, and IL-17 was detected using biotin anti-mouse IL-17A, clone eBioTC11-8H4. Cytokine levels were measured in triplicate

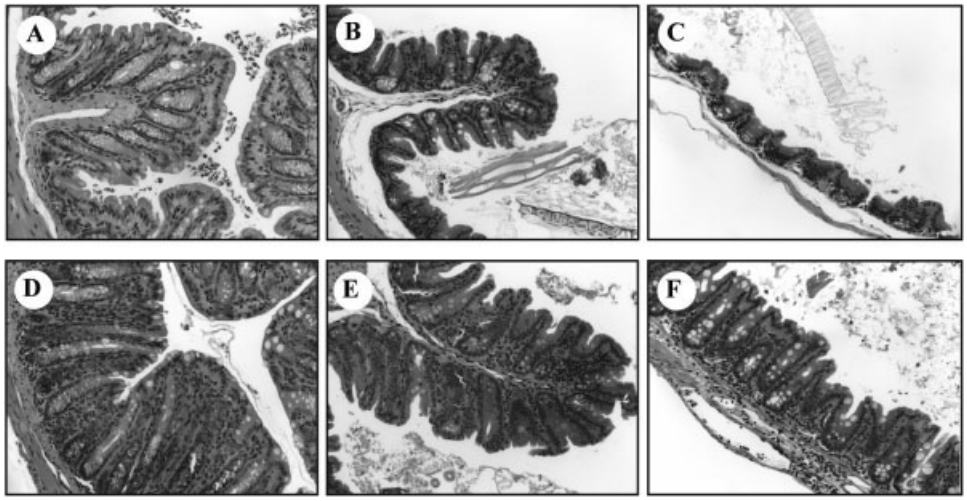

FIGURE 1. Histological evidence of inflammation in the colons of *B. animalis* monoassociated mice. WT (A–C) and IL-10−/− (D–F) mice were monoassociated with *B. animalis* for 23 weeks. At necropsy portions of distal colon (A,D), proximal colon (B,E), and cecum (C,F) were removed. H&E-stained paraffin sections reveal mild inflammation in the colons of IL-10−/− mice, characterized by crypt hyperplasia, goblet cell depletion, and lamina propria cellular infiltration. All images are at 20× magnification.

supernatants and compared to standard curves generated using recombinant murine cytokines.

Flow Cytometry

MLN cell subpopulations (before and after negative selection) were evaluated as previously described[7] on the FACScan (BD Biosciences, Mountain View, CA) using FITC anti-CD4, PE anti-CD8, or FITC anti-B220 (all from Invitrogen).

Statistical Analyses

Nonparametric histologic scores were analyzed with the Mann–Whitney test (SAS, Cary, NC) by statisticians in the Biostatistics Core of the Center for Gastrointestinal Biology and Disease. The paired Student's *t*-test was used to compare all other data. Statistical significance was defined as $P < 0.05$ for comparisons indicated. Data are reported as mean values ± standard error of the mean (SEM) unless otherwise indicated.

RESULTS

B. animalis Monoassociated IL-10−/− Mice Develop Mild Colitis and Marked Inflammation in the Duodenum

To test whether *B. animalis* could elicit intestinal inflammation in IL-10−/− mice, we colonized inbred GF IL-10−/− (129S6/SvEv background) and GF 129S6/SvEv WT mice with *B. animalis* ATCC 25527^T for 20–24 weeks. We confirmed monoassociation by analyzing the 16S ribosomal RNA sequence of several ex vivo bacterial isolates from cecal contents at necropsy (data not shown). Two of 19 monoassociated IL-10−/− mice studied developed rectal prolapse; however, most of the mice did not display outward signs of distress beyond lethargy and soft stools. Histological evidence of mild inflammation was apparent throughout the colons of IL-10−/− mice, characterized by moderate crypt hyperplasia, infiltration of predominantly mononuclear cells into the lamina propria, but not the submucosa, and moderate goblet cell depletion (Fig. 1D–F). WT 129S6/SvEv mice, similarly colonized for 20-24 weeks, had no histological evidence of colitis (Fig. 1A–C). Importantly, there was considerable grossly evident thickening of the duodena in IL-10−/− mice with evidence of partial obstruction, demonstrated by gastric and proximal duodenal distention with fluid and luminal content retention (Fig. 2B). However, there were no significant differences in the weights of age-matched WT and IL-10−/− mice at 20–24 weeks after colonization, suggesting the absence of high-grade duodenal obstruction (data not shown). Histological examination of duodenal tissue revealed massive cellular infiltration of mononuclear cells into the lamina propria, including the villi and significant crypt hyperplasia, compared to healthy duodenal tissue from monoassociated WT animals (compare IL-10−/−, Fig. 2D,E with WT Fig. 2C). The typical architecture of the mucosa was altered by the formation of abnormal crypt and villus structures consisting of branched and fused villi, but no mucosal ulceration (Fig. 2D,E). This inflammation was localized to the duodenum and did not extend into the jejunum or ileum (data not shown). Histological scores for the colons of IL-10−/− mice reflected a mild pancolitis in these animals (distal colon: 1.9 ± 0.9; proximal colon: 1.6 ± 0.8; cecum: 2.3 ± 0.6) and were significantly higher than the scores for WT colons (distal colon: 0.7 ± 0.2; proximal colon: 1.0 ± 0.5; cecum: 1.2 ± 0.4) (Fig. 3A). This histologic evidence of colitis in *B. animalis*-monoassociated IL-10−/− mice was further supported by the spontaneous release of higher amounts of IL-12/IL-23 p40 from colonic explants from IL-10−/− mice compared to WT mice (Fig. 3B). Scores for histologic detection of inflammation in the duodenum reflected the extensive abnormalities within this intestinal seg-

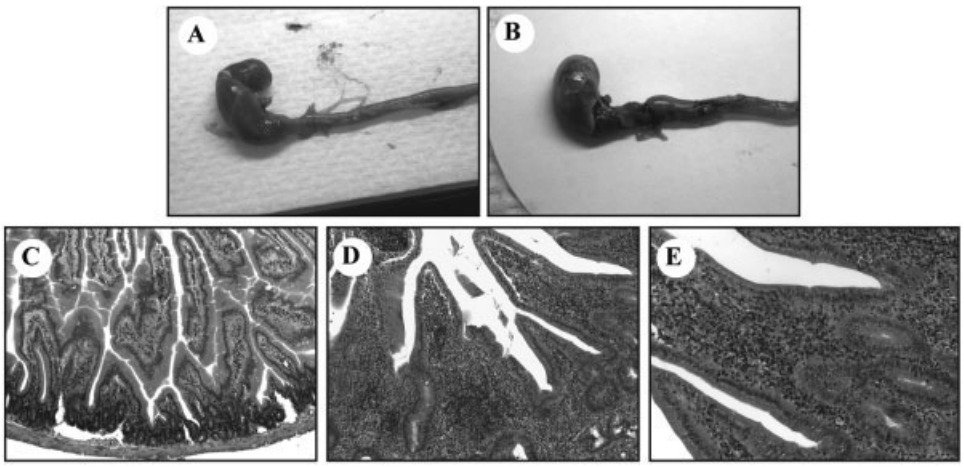

FIGURE 2. Extensive inflammation in the duodena of *B. animalis* monoassociated IL-10−/− mice. Stomach through proximal small intestine was excised from WT (A) and IL-10−/− (B) mice monoassociated with *B. animalis* for 23 weeks. Duodenal thickening and partial obstruction was noted in IL-10−/− animals. H&E-stained paraffin sections revealed healthy WT duodenal tissue (C) and severe inflammation in the duodenum of an IL-10−/− mouse (D,E), characterized by massive cellular infiltration and loss of villus architecture. C,D at 10× magnification and E at 20× magnification.

ment in IL-10−/− animals (3.1 ± 0.3) compared to WT duodenal tissue (0.6 ± 0.2) (Fig. 3A). In preliminary experiments we evaluated WT and IL-10−/− mice monoassociated with *B. animalis* for 11 and 17 weeks. Low levels of inflammation were detected in the distal colons and ceca of IL-10−/− animals by histological analysis and by spontaneous IL-12/IL-23 p40 release from colonic explants (data not shown). Duodena were not evaluated at these earlier timepoints. Together, these results show that IL-10−/− mice monoassociated with *B. animalis* developed a mild pancolitis and extensive duodenal inflammation with proximal duodenal and gastric dilation, whereas similarly colonized WT mice remained healthy.

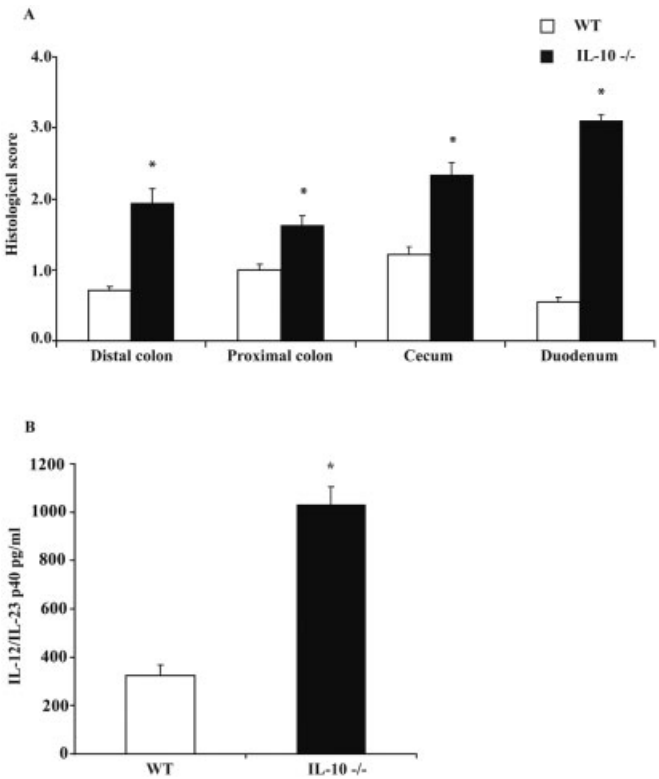

FIGURE 3. Quantitative evidence of inflammation in the intestinal tracts of *B. animalis* monoassociated IL-10−/− mice. (A) Blinded histological scores (mean score ± SEM) of distal colon, proximal colon, cecum ($n = 19$), and duodenum ($n = 10$ for WT and 16 for IL-10−/−) from WT and IL-10−/− mice after 23 weeks of monoassociation with *B. animalis*. *$P < 0.01$ versus WT. (B) Spontaneous IL-12/IL-23 p40 secretion was quantified from colonic explant culture supernatants incubated for 20–24 hours. WT mice, $n = 18$, IL-10−/− mice, $n = 19$. *$P < 0.0001$ versus WT.

B. infantis Monoassociated IL-10−/− Mice Develop Low-grade Colonic and Duodenal Inflammation

We wondered whether *B. animalis* was unique among Bifidobacteria in its ability to induce intestinal inflammation in IL-10−/− mice, or whether other *Bifidobacterium* species shared this capacity. Therefore, we colonized GF WT and IL-10−/− mice with *B. infantis* for 22–23 weeks. All WT and IL-10−/− mice appeared healthy throughout the colonization period and lacked macroscopic evidence of intestinal inflammation or gastric or duodenal dilation. Histological scores for different regions of the intestinal tract and spontaneous IL-12/IL-23 p40 secretion from colonic explants reflected a low level inflammatory response in IL-10−/− animals (Table 1). Only the duodenal histologic scores showed significant differences between IL-10−/− and WT mice, with no differences in the colon. Importantly, these measurements of inflammation in the intestines of *B. infantis* monoassociated IL-10−/− animals were lower than those obtained from *B. animalis* monoassociated IL-10−/− animals shown in Figure 3.

Distribution of Inflammation Among Intestinal Compartments in *B. animalis* Monoassociated IL-10−/− Mice Is Not Explained by Bacterial Colonization Patterns

We hypothesized that the severe inflammation seen within the duodena of IL-10−/− mice might be due to

TABLE 1. *B. infantis* Induces Low Levels of Inflammation in IL-10-/- Mice

	WT	IL-10-/-
Histology score (0-4)[a]		
distal colon	0.6 ± 0.0	0.7 ± 0.1
cecum	0.9 ± 0.1	1.3 ± 0.2
proximal colon	0.8 ± 0.1	1.1 ± 0.2
duodenum	0.6 ± 0.1	1.5 ± 0.2[c]
IL-12/IL-23 p40 (pg/ml)[b]		
colonic explant	158.2 ± 27.0	377.6 ± 35.7[c]

[a]Blinded histological scores (mean score ± SEM) of distal colon, proximal colon and duodenum; WT (n = 10) and IL-10-/- (n = 9) mice after 22-23 weeks of monoassociation with *B. infantis*.
[b]Spontaneous IL-12/IL-23 p40 secretion (pg/mL) from colonic explants.
[c]Significantly different (*$P < 0.001$ vs. WT).

selective colonization of this compartment by *B. animalis*. To test this we performed quantitative bacterial cultures on homogenized intestinal segments from the distal colon, proximal colon, cecum, and duodenum of WT and IL-10−/− mice. This technique measures both luminal and adherent bacteria colonizing each compartment.[18] Comparing WT and IL-10−/− mice, there were no statistically significant differences in the numbers of viable bacteria colonizing any of these compartments (Table 2). Also, bacterial concentrations were 2–3 orders of magnitude higher in the colons than in the duodena of these animals. These data suggest that the severe inflammation observed within the duodena of IL-10−/− mice compared to healthy WT mice was not due to differential colonization of this compartment by *B. animalis*.

$CD4^+$ MLN T-cell Response to *B. animalis* Displays Bacterial Species-Antigen Specificity

We have previously shown that IL-10−/− mice monoassociated or dual-associated with bacterial species that cause intestinal inflammation display $CD4^+$ MLN T-cell responses to lysates of the species that induces inflammation with bacterial species-antigen specificity.[7,19] Here we asked whether *B. animalis* monoassociated IL-10−/− mice developed similar T-cell responses directed against *B. animalis* bacterial antigens and whether T cells would crossreact with antigens expressed by other bacterial species. MLN-derived $CD4^+$ T cells (isolated from IL-10−/− and WT monoassociated mice) were cultured with WT APC that had been pulsed in vitro with either KLH (as an unrelated antigen control) or with *B. animalis, B. infantis, B. bifidum, E. coli,* or *E. faecalis* lysates. $CD4^+$ MLN T-cells from IL-10−/− mice, but not from WT mice, produced high levels of IFN-γ (Fig. 4A) and IL-17 (Fig. 4B) when stimulated with *B. animalis* lysate-pulsed APC, indicating a strong recall T-cell response against bacterial antigens expressed by this organism. Interestingly, these IL-10−/− T cells also secreted moderate levels of IFN-γ and IL-17 in response to APC pulsed with lysates of *B. infantis, B. bifidum*, and *E. coli,* but not to *E. faecalis* lysate-pulsed APC. Cytokine measurements from $CD4^+$ T cells from monoassociated WT mice cultured with the same lysates were consistently at or below the detection limits for these assays.

Bacterial cell lysates contain many components that could influence T-cell secretion of inflammatory cytokines

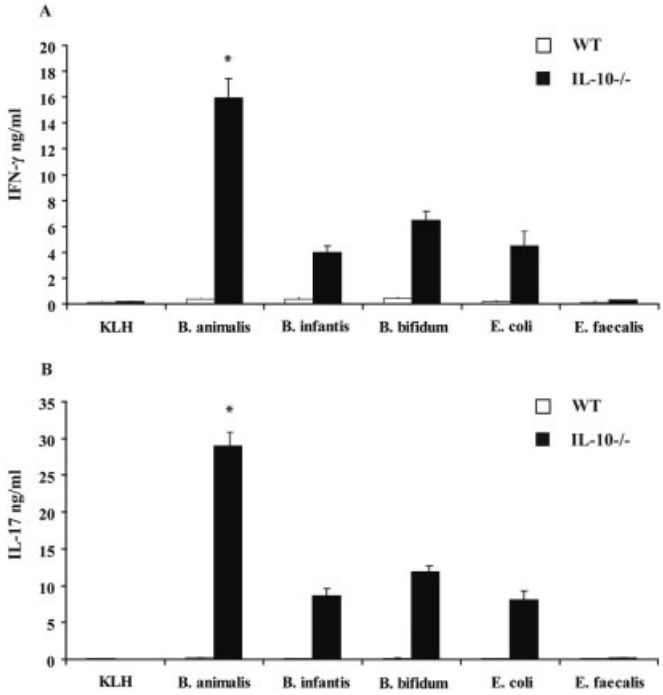

FIGURE 4. Bacterial antigen-specific $CD4^+$ T-cell responses in the MLN of *B. animalis* monoassociated mice. $CD4^+$ T cells prepared from mesenteric lymph node (MLN) cells of *B. animalis* monoassociated WT (n = 2–8 mice per stimulator) or IL-10−/− (n = 15–19 mice per stimulator) were cocultured with WT antigen-presenting cells that were pulsed with either KLH (unrelated antigen control) or with various bacterial lysates (*B. animalis; B. infantis; B. bifidum; E. coli,* or *E. faecalis*). (A) IFN-γ and (B) IL-17 were measured from cell culture supernatants collected at 72 hours. *$P < 0.0001$ *B. animalis* IL-10−/− versus *B. infantis, B. bifidum, E. coli*, and *E. faecalis* IL-10−/−.

TABLE 2. Bacterial Cell Numbers in the Distal Colon, Proximal Colon, Cecum, and Duodenum of *B. animalis* IL-10-/- and WT Mice

	Distal Colon	Proximal Colon	Cecum	Duodenum
IL-10-/-	9.4 ± 0.3	8.8 ± 0.5	9.4 ± 0.1	6.6 ± 1.4
WT	9.3 ± 0.5	9.0 ± 0.3	9.5 ± 0.2	6.7 ± 1.3

Values represent bacterial counts (mean $\log_{10}$ cfu ± SD per g tissue).

TABLE 3. IL-12/IL-23 p40 Secretion from WT BMDC Stimulated with LPS or Bacterial Lysates

Stimulator	IL-12/IL-23 p40 pg/mL[a]
Media	23.5 ± 0.7
LPS	13,307.7 ± 1975.9
B. animalis	815.7 ± 76.9
B. infantis	935.0 ± 94.9
B. bifidum	1,012.0 ± 41.6
E. coli	15,307.7 ± 4137.4
E. faecalis	114.7 ± 16.8

[a]Values represent IL-12/IL-23 p40 pg/mL (mean ± standard deviation) of triplicate wells. This experiment was repeated with similar results.

through their interaction with pattern recognition receptors on APC in an antigen non-specific manner.[20] In order to evaluate the adjuvant-mediated stimulating capacity of these lysates we cultured WT BMDC in the presence of media alone, LPS (1 μg/mL), or each bacterial lysate at the same concentration used to pulse APC prior to coculture with $CD4^+$ T cells (10 μg/mL) and measured the secretion of IL-12/IL-23 p40 (Table 3). LPS and *E. coli* lysate stimulated the highest levels of IL-12/IL-23 p40 from BMDC (13,307.7 ± 1975.9 and 15,307.7 ± 4137.4 pg/mL, respectively); the 3 *Bifidobacterium* lysates stimulated ≈15-fold lower levels and *E. faecalis* stimulated only slightly more than media alone. These results indicate that the 3 different *Bifidobacterium* lysates share similar potential for adjuvant activity. Therefore, IL-10−/− mice monoassociated with *B. animalis* likely develop $CD4^+$ T-cell immune responses against antigens expressed by *B. animalis* that are either not shared or are expressed at lower levels by the other species of Bifidobacteria that we evaluated (Fig. 4).

DISCUSSION

Our study demonstrates that *B. animalis* subsp. *animalis* ATCC 25527^T causes mild colitis and marked duodenal inflammation in monoassociated gnotobiotic IL-10−/− mice. These results provide potential pathogenetic significance for our earlier study that showed an increased abundance of *B. animalis* in the colons of SPF IL-10−/− mice as cecal and colonic inflammation progressed.[10] We demonstrate that a bacterial species that is selectively expanded in SPF IL-10−/− mice with experimental colitis[10] is capable of inducing colitis and TH1 and TH17 $CD4^+$ T-cell responses in monoassociated gnotobiotic IL-10−/− mice.

Bifidobacterium animalis now joins *E. faecalis,*[7,14] *E. coli* (mouse isolate),[7] and *Enterobacter cloacae*[21] in a growing list of commensal organisms shown to initiate intestinal inflammation in IL-10−/− mice. Importantly, many commensal isolates are not able to cause intestinal inflammation in monoassociated IL-10−/− mice, including: *Pseudomonas fluorescens*,[7] *Bacteroides vulgatus* (guinea pig), *Streptococcus faecium* [Group D], *E. coli, Peptostreptococcus productus, Eubacterium contortum*, and *S. avium* (isolates from Crohn's patients),[6] *Helicobacter hepaticus*,[22] *Candida albicans, Lactococcus lactis*, a *Bifidobacterium* species, a *Bacillus* species, several species of *Lactobacillus*,[14] *Viridans* group *streptococci*, and *Clostridium sordellii*.[23] In our study, GF IL-10−/− mice monoassociated with *B. infantis* developed mild inflammation in the duodenum after 22–23 weeks, but there were no statistically significant differences in the histological scores from the ceca and colons of WT *B. infantis*-colonized animals. The studies available to date demonstrate considerable heterogeneity within bacterial genera and even within individual species in the capacity to induce intestinal inflammation in GF IL-10−/− mice. For example, a commensal strain of *E. coli* (designated NC 101) isolated from a SPF-housed 129S6/SvEv WT mouse caused moderate colitis in GF IL-10−/− mice,[7] whereas an *E. coli* strain isolated from a patient with Crohn's disease[6] and a laboratory strain, *E. coli* K12,[24] did not. Similar diversity appears to exist among various isolates/species of *Bifidobacterium*, since the *B. animalis* ATCC 25527^T strain used in our study induced marked duodenal and mild colonic inflammation in GF IL-10−/− mice, *B. infantis* 15697^T induced a low-grade response and an isolate used by Balish and Warner[14] did not cause intestinal inflammation. Indeed, significant genetic and phenotypic heterogeneity has been reported for different species and strains within the genus *Bifidobacterium*.[25,26]

Our observation that *B. animalis* causes intestinal inflammation in this genetically susceptible model is of considerable interest, since this species is widely utilized as a probiotic organism. Much of the clinical research has been done using the strain *B. animalis* DN-173 010.[12] This strain has a high postgastric survival rate that is greater than another commercially available *Bifidobacterium* strain.[27,28] *B. animalis* DN-173 010 also reduced colonic transit time when consumed in *Bifidobacterium*-fermented milk.[29] Van der Meulen et al[30] reported *B. animalis* DN-173 010 to be unique among *B. animalis* isolates in its preferential metabolism of certain short-chain fructans, suggesting it may have a competitive advantage over other microorganisms in the human gut, where oligo- and polysaccharides are the main sugar substrates. Recently, *B. animalis* has been reclassified to include the 2 subspecies *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis* based on molecular and phenotypic characteristics.[26] Strains of the *lactis* subspecies including BB12 and HN019 have variable probiotic effects in animals and humans.[31–35] Following our previous observation that *B. animalis* forms an increased proportion of the gut microbiota of colitic IL-10−/− mice compared with WT mice,[10] we selected strain ATCC 25527^T to colonize GF animals because

of its designation as the type strain for *B. animalis*. Our results suggest that the strain ATCC 25527^T possesses unique phenotypic traits that allow it to initiate an inflammatory response under certain circumstances, including monoassociation of IL-10-deficient rodents.

The mechanisms underlying the ability of some commensal bacterial strains to cause inflammation in susceptible hosts, while others remain innocuous, are currently unknown. One possibility is that detrimental species colonize genetically susceptible individuals to a greater extent than nonsusceptible individuals, thereby providing increased stimulus for the innate and adaptive immune systems.[2] However, quantitative bacterial culture from different intestinal segments revealed no difference in *B. animalis* colonization patterns between monoassociated WT and IL-10−/− mice. Furthermore, *B. animalis* colony counts from the duodena of both WT and IL-10−/− mice were 2–3 orders of magnitude lower than those obtained from the colon. We previously reported moderate duodenal inflammation with functional obstruction in IL-10−/− mice monoassociated with *E. faecalis*, but not with *E. coli* NC101 despite higher duodenal concentrations of *E. coli* versus *E. faecalis*, thus demonstrating a similar disassociation of regional intestinal inflammation and luminal bacterial concentrations.[7] The quantitative bacterial culture technique used in the current study measures both luminal and adherent bacteria[18] but does not distinguish the relative proportion of adherent organisms, which has been reported to correlate with regional intestinal inflammation in colonic biopsies.[4]

The extensive inflammation and possible partial obstruction observed in the duodena of *B. animalis*-monoassociated IL-10−/− mice is noteworthy. IL-10−/− mice housed in our SPF facility lack duodenal inflammation,[6] although Kuhn et al[36] described extensive duodenal and small bowel intestinal inflammation in IL-10−/− mice housed under conventional, but not SPF conditions. The moderate duodenitis observed by Kim et al[7] in *E. faecalis*-monoassociated IL-10−/− mice was not noted until after 30 weeks of colonization, whereas distal colitis was first observed at 10–12 weeks and was well advanced by 22 weeks. In contrast, *B. animalis* colonized IL-10−/− mice exhibit extensive duodenal inflammation after 20–24 weeks with only mild inflammation in the colon. GF HLA-B27 transgenic rats develop mild/moderate antral and proximal duodenal inflammation in addition to severe colitis 1 month after colonization with SPF fecal microbiota, but only mild/moderate colitis and no duodenal inflammation after monoassociation with *Bacteroides vulgatus*.[15] Dohi et al[37] described a model of chronic gastroduodenitis in TCR−/− mice housed under SPF conditions that were transfer recipients of IL-4−/− CD4$^+$CD45RB^{hi} T cells. These mice developed gastroduodenitis and colitis without obvious changes in the jejunum or ileum. Interestingly, the gastroduodenitis was only partially blocked with antibiotic treatment, whereas colitis was completely inhibited, suggesting a role for nonviable antigens in the duodenal compartment.[37] This is consistent with the observations that SAMP-1/Yit Fc mice continue to exhibit ileitis[38] and IL-2-deficient mice have gastroduodenitis[39] in GF conditions, although mice of both types have attenuated colonic inflammation in the absence of viable bacteria. The explanation for more active inflammation in the duodenum compared to other intestinal compartments that we evaluated remains unclear. One possibility is that incompletely degraded food components and fecal bacteria ingested through coprophagia could increase exposure of the duodenal mucosa to nonviable *B. animalis* antigens and adjuvants, thereby recruiting pathogenic immune cells to this region. As stated above, it is also possible that there is a difference in the extent of mucosal epithelial adherence of *B. animalis* in the duodenum compared to the colon, which warrants further study.

We demonstrate that CD4$^+$ MLN T cells from *B. animalis* monoassociated IL-10−/− mice, but not WT mice, secrete IFN-γ and IL-17 when restimulated in vitro with APC previously incubated with *B. animalis* bacterial lysate. These T cells also secreted the same 2 cytokines, albeit to a lesser extent, in response to APC pulsed with lysates of *B. infantis, B. bifidum,* and *E. coli*, but not *E. faecalis* bacterial lysate or an unrelated protein antigen, KLH. Therefore, there appears to be some immunologic crossreactivity and/or shared bacterial antigens among the 3 *Bifidobacterium* species and *E. coli*. The higher recall response to *B. animalis* than to *B. infantis* or *B. bifidum* suggests that mucosal CD4$^+$ T cells react to unique antigens expressed by *B. animalis* that are not shared by other *Bifidobacterium* species. Thus, antigenic heterogeneity among different bacterial isolates may influence their potential to initiate an inflammatory response. However, the presence of an immunogenic bacterial antigen may not be sufficient to cause inflammation. Sydora et al[23] demonstrated that IL-10−/− mice monoassociated with *Bacteroides vulgatus* did not develop intestinal inflammation, but did generate a strong systemic T-cell response to *B. vulgatus* antigens. The authors recently went on to show that disruption of the intestinal epithelial barrier of *B. vulgatus* monoassociated IL-10−/− mice with indomethacin allowed the bacteria to initiate and sustain an intense intestinal inflammatory response.[40] These studies suggest that in order to cause inflammation, bacteria may require the capacity to breach the epithelial barrier to interact with lamina propria antigen presenting cells such as dendritic cells. Whether *B. animalis* ATCC 25527^T is more invasive than other Bifidobacteria remains to be determined. We evaluated the adjuvant stimulating capacity of our bacterial lysates and found no difference in the ability of the 3 *Bifidobacterium* lysates to stimulate IL-12/IL-23 p40 secretion by BMDC, further supporting our conclusion that the strong T-cell recall response to *B. animalis* represents true bacterial antigen specificity.

The inflammation initiated by *B. animalis* subsp. *animalis* ATCC 25527^T is characterized by a mild pancolitis and marked duodenitis. The fact that duodenal inflammation is not observed in IL-10−/− mice housed under SPF conditions[6] suggests that either the *B. animalis* that expand in these animals with disease progression are in some way different from strain ATCC 25527^T, that the microbiota in the proximal gut of SPF mice prevent *B. animalis* from colonizing, attaching to or invading the duodenum, or that other members of the microbial community stimulate or inhibit the inflammatory response in a manner that is not reproduced by simple monoassociation. In conventional mice, the duodenum is populated by high numbers of lactobacilli[41] and these organisms can prevent inflammation in various animal models of IBD.[11] Therefore, it is possible that lactobacilli prevent *B. animalis* from colonizing, attaching to the epithelium or invading the duodenum in SPF animals or may exert an anti-inflammatory effect at this site to prevent visible signs of duodenitis.

In summary, we have demonstrated that *B. animalis*, which has previously been shown to represent an increased proportion of the gut microbiota of colitic IL-10−/− mice, can induce intestinal inflammation and recall TH1 and TH17 immune responses after these animals have been monoassociated. This suggests that *B. animalis* may contribute to the colitis observed in SPF IL-10−/− mice. We do not suggest that our study implicates *B. animalis* as an unsafe probiotic organism for human consumption. Most strains utilized in this manner have undergone rigorous testing for safety and have no documented adverse effects.[13,42] However, our results indicate that even enteric bacterial species that are traditionally viewed to have probiotic activities also possess the potential to contribute to intestinal inflammation in a susceptible host.

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