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BASIC–ALIMENTARY TRACT

Variable Phenotypes of Enterocolitis in Interleukin 10–Deficient Mice Monoassociated With Two Different Commensal Bacteria

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See editorial on page 1122.

Background & Aims: **To explore the hypothesis that selective immune responses to distinct components of the intestinal microflora induce intestinal inflammation, we characterized disease kinetics and bacterial antigen–specific T-cell responses in ex germ-free interleukin $10^{-/-}$ and wild-type control mice monoassociated with *Enterococcus faecalis*, *Escherichia coli*, or *Pseudomonas fluorescens*.** ***Methods:*** **Colitis was measured by using blinded histological scores and spontaneous interleukin 12 secretion from colonic strip culture supernatants. Interferon γ secretion was measured from mesenteric or caudal lymph node $CD4^+$ T cells stimulated with bacterial lysate–pulsed antigen-presenting cells. Luminal bacterial concentrations were measured by culture and quantitative polymerase chain reaction.** ***Results:*** ***Escherichia coli*** **induced mild cecal inflammation after 3 weeks of monoassociation in interleukin $10^{-/-}$ mice. In contrast, *Enterococcus faecalis*–monoassociated interleukin $10^{-/-}$ mice developed distal colitis at 10–12 weeks that was progressively more severe and associated with duodenal inflammation and obstruction by 30 weeks. Neither bacterial strain induced inflammation in wild-type mice, and germ-free and *Pseudomonas fluorescens*–monoassociated interleukin $10^{-/-}$ mice remained disease free. $CD4^+$ T cells from *Enterococcus faecalis*– or *Escherichia coli*–monoassociated interleukin $10^{-/-}$ mice selectively produced higher levels of interferon γ and interleukin 4 when stimulated with antigen-presenting cells pulsed with the bacterial species that induced disease; these immune responses preceded the onset of histological inflammation in *Enterococcus faecalis*–monoassociated mice. Luminal bacterial concentrations did not explain regional differences in inflammation.**

Conclusions: **Different commensal bacterial species selectively initiate immune-mediated intestinal inflammation with distinctly different kinetics and anatomic distribution in the same host.**

Inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease, are idiopathic chronic relapsing immune-mediated inflammatory disorders. A widely accepted hypothesis suggests that genetically susceptible hosts with dysregulated immune responses, defective mucosal barrier function, or inefficient bacterial clearance develop T lymphocyte–mediated chronic intestinal inflammation when colonized with normal commensal bacteria.[1–3] Although they are not exact replicas of human disease, animal models of intestinal inflammation, particularly gene knockout and transgenic (TG) rodents, as well as inducible and lymphocyte-transfer models, have been crucial in developing new concepts of IBD pathogenesis and regulation of immunologic tolerance to commensal microflora.[1,2,4,5] Genetically susceptible or engineered rodents, including the HLA-B_{27}/β_2 microglobulin TG rat and interleukin (IL)-$10^{-/-}$, T-cell receptor $\alpha^{-/-}$, SAMP-1/Yit, and CD3ϵ TG mice, develop T lymphocyte–mediated intestinal inflammation when colonized with normal luminal bacteria yet show no inflammation or immune activation in a sterile (germ-free; GF) environment.[6–11] These results, combined with the identification of pathogenic T-cell responses to lu-

Abbreviations used in this paper: APC, antigen-presenting cell; CLN, caudal lymph node; GF, germ-free; IFN, interferon; IL, interleukin; IL-$10^{-/-}$, interleukin 10 deficient; KLH, keyhole limpet hemocyanin; MLN, mesenteric lymph node; PCR, polymerase chain reaction; SPF, specific pathogen free; TG, transgenic; WT, wild type.

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minal bacteria[10,12] and protection by antibiotics,[13] strongly implicate the enteric commensal bacterial flora in the induction and perpetuation of chronic immune-mediated intestinal inflammation. However, commensal enteric bacterial species display differential abilities to induce intestinal inflammation, as documented by selective bacterial inoculation in gnotobiotic rodents. For example, HLA-B_{27} TG rats develop colitis when monoassociated with *Bacteroides vulgatus* or when colonized with a cocktail composed of *B vulgatus* plus 5 different common enteric bacteria isolated from IBD patients (*Enterococcus faecium*, *Escherichia coli*, *Peptostreptococcus productus*, *Eubacterium contortium*, and *Enterococcus avium*).[14,15] However, inflammation is minimal or absent in TG rats monoassociated with *Escherichia coli* or colonized with the cocktail of 5 enteric bacteria without *B vulgatus*.[14,15]

The IL-10–deficient (IL-10−/−) mouse is a particularly well-characterized murine colitis model that develops intestinal inflammation when exposed to normal commensal bacteria, unlike its normal wild-type (WT) counterpart; this suggests that endogenously produced IL-10 prevents colitis.[8,9,16] IL-$10^{-/-}$ mice maintained in GF conditions remain free of intestinal inflammation and have no evidence of immune activation. However, these mice develop colitis (predominantly cecal) after colonization with specific pathogen–free (SPF) bacteria in the absence of *Helicobacter* species, with rapid progression to severe chronic colitis by 5 weeks after bacterial inoculation.[9] Of considerable importance, gnotobiotic IL-$10^{-/-}$ mice do not develop intestinal inflammation when colonized with the 6 commensal bacterial species, including *B vulgatus*, that selectively induce colitis in HLA-B_{27}/β_2 microglobulin TG rats.[9] Recent studies have used monoassociated IL-$10^{-/-}$ mice in an attempt to identify individual components of the commensal milieu that are involved in the induction of immune-mediated intestinal inflammation. Monoassociating IL-$10^{-/-}$ mice with *Helicobacter hepaticus*,[17] *Candida albicans*, *Lactococcus lactis*, a *Bifidobacterium* species, a *Bacillus* species, several species of *Lactobacillus*,[18] *Viridans* group streptococci, or *Clostridium sordellii*[19] did not induce enterocolitis in gnotobiotic IL-$10^{-/-}$ mice, although splenocytes from *Clostridium sordellii*–monoassociated IL-$10^{-/-}$ mice produced markedly increased interferon (IFN)–γ secretion when stimulated with *Clostridium sordellii* sonicates.[19,20]

Balish and Warner[18] recently showed that GF IL-$10^{-/-}$ mice selectively colonized with a human commensal *Enterococcus faecalis* strain developed colitis. This observation, in conjunction with ongoing studies investigating the effect of selectively colonizing gnotobiotic IL-$10^{-/-}$ mice with other commensal microorganisms, gave us the opportunity to address the following specific aims: (1) investigate the kinetics of mucosal inflammation and immune responses after selective inoculation with several different nonpathogenic commensal bacterial species implicated in human IBD or experimental colitis; (2) study bacterial antigen–specific T-lymphocyte responses in monoassociated IL-$10^{-/-}$ mice that develop colitis; and (3) compare and contrast the disease phenotypes induced in IL-$10^{-/-}$ mice by different bacterial species. These studies show that the kinetics and localization of colitis in *Enterococcus faecalis*– vs. *Escherichia coli*–monoassociated IL-$10^{-/-}$ mice are quite distinct and that *Enterococcus faecalis* uniquely induces obstructive duodenitis. In both sets of monoassociated mice, $CD4^+$ T-cell secretion of IFN-γ precedes the onset of histological inflammation. The selective ability of enteric bacterial species to induce disease in this model is illustrated by our finding that *Pseudomonas fluorescens*, a superantigen-expressing commensal bacterium implicated in Crohn's disease,[21,22] does not cause colitis after monoassociation in the IL-$10^{-/-}$ mouse model. These findings further our understanding of immunologic and pathogenic responses to selected bacterial species in immune-mediated colitis.

Materials and Methods

Mice and Bacteria

GF IL-$10^{-/-}$ and WT control (inbred 129S6/SvEv background) mice were derived by hysterectomy at the Gnotobiotic Laboratory (University of Wisconsin, Madison). Mice were monoassociated at 10–12 weeks of age with a human oral isolate of *Enterococcus faecalis* (strain OG1RF, provided by Mark Huycke, MD), *Pseudomonas fluorescens* (provided by Jonathon Braun, MD), or a murine strain of *Escherichia coli* (randomly isolated from WT mice raised in SPF conditions) by gavage feeding and rectal swabbing with viable cultured bacteria. Monoassociated mice were maintained in the Gnotobiotic Animal Core at the College of Veterinary Medicine (North Carolina State University, Raleigh). Bacterial monoassociation and absence of contamination by other bacterial species were confirmed by periodic aerobic culture of stool samples. WT 129S6/SvEv mice (Taconic Laboratories, Germantown, NY) maintained under SPF conditions free of *Helicobacter* species were used for the preparation of antigen-presenting cells (APCs). 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.

Histological Scoring

Mice were killed at multiple time points (between 1 and 46 weeks) after monoassociation. At necropsy, sections of colon (proximal, transverse, and distal) and cecum were fixed in 10% neutral buffered formalin. Duodenal and gastric tissue

samples were taken from representative animals. The fixed tissue was embedded in paraffin and stained with H&E. A single individual, using a well-validated scale,[9,10] assessed the severity of inflammation blindly. 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.

Colonic Tissue Fragment Cultures

Colonic tissue fragment cultures were prepared from the large intestine as previously described.[9] 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, and weighed. Colonic tissue fragments were distributed (0.05 g per well) into 24-well plates (Costar 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; GIBCO, Grand Island, NY) for 20 hours at 37°C. Supernatants were collected and stored at −20°C before use for IL-12 quantification.

Bacterial Lysates

Lysates of bacteria were prepared from individual colonies of *Escherichia coli*, *Enterococcus faecalis*, and *B vulgatus*. The guinea pig *B vulgatus* strain was originally obtained from Andrew Onderdonk, PhD (Harvard University)[23] and had previously been used in HLA-B_{27} TG rat monoassociation studies.[14] After a single colony was selected, the organisms were grown under aerobic or anaerobic conditions in brain–heart infusion broth for 48 to 72 hours. The bacterial cultures were centrifuged at 10,000 rpm for 10 minutes, and the bacterial pellets were washed twice in RPMI. Freshly prepared MD solution (composed of 0.02 mg/mL $MgCl_2$ plus 0.1 mg/mL deoxyribonuclease in sterile water) was added (0.25 mL per 1.0 mL of bacterial suspension) and then vortexed. The bacteria were disrupted with 0.1-mm glass microbeads by using a Mini Bead Beater (Biospec Products, Bartsville, OK) for 3 minutes and were centrifuged at 7500 rpm for 5 minutes. The resulting supernatants were collected and filtered by using a 0.45-μm filter. Lysate sterility was confirmed by culture. Protein concentrations were measured according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Lysates were aliquoted and stored at −80°C until use.

Determination of Bacterial Colonization of Different Sections of the Intestinal Tract

Culture of luminal contents and lymph nodes. Bacteria were cultured from the cecum, the duodenum, and the distal colon and mesenteric lymph nodes (MLNs) of representative IL-$10^{-/-}$ *Enterococcus faecalis*– and *Escherichia coli*–monoassociated mice. Cecal and colonic luminal contents were collected into preweighed sterile tubes and then serially diluted (at 10^{-4}, 10^{-6}, and 10^{-8}) in sterile PBS. Single-cell suspensions of MLN cells were prepared by teasing in sterile PBS and plated (at 10^{0} to 10^{-1}). These diluted samples were plated on sheep blood or colistin-nalidixic acid agar plates (*Enterococcus faecalis*) and MacConkey agar plates (*Escherichia coli*) incubated at 37°C for 2 days under aerobic conditions. The number of colonies on plates was counted to determine final bacterial counts.

Quantitative real-time polymerase chain reaction. Bacterial DNA was isolated from overnight cultures of *Escherichia coli* and *Enterococcus faecalis* by using a DNAeasy Tissue Kit (Qiagen), and DNA from samples was extracted by using a QIAamp DNA Stool Mini Kit (Qiagen, Alameda, CA). Species-specific oligonucleotide primers and fluorescent probes were designed with Primer Express software (Applied Biosystems, Foster City, CA) to target *Escherichia coli*–specific and *Enterococcus faecalis*-specific regions of the 16S ribosomal RNA gene. Primers and probes for *Escherichia coli* were ECO445F (5′-GGGAGGAAGGGAGTAAAGTTAATACC-3′), ECO524R (5′-CTGCTGGCACGGAGTTAGC-3′), and ECO472T (5′-6-FAM-TTGCTCATTGACGTTACCCGCAGAAGAA-3′ BHQ). For *Enterococcus faecalis*, they were EF132F (5′-CGTGGGTAACCTACCCATCAGA-3′), EF230R (5′-AAAGCGCCTTTCACTCTTATGC-3′), and EF164T (5′-6-FAM-ACTTGGAAACAGGTGCTAATACCGCATAACAGT-3′ BHQ). The *Escherichia coli* polymerase chain reaction (PCR) mixture consisted of PCR buffer, 1.5 mmol/L $MgCl_2$, 200 μmol/L each deoxynucleoside triphosphate, 0.3 μL 10× Rox Reference Dye (Invitrogen, Carlsbad, CA), 900 nmol/L ECO445F, 300 nmol/L ECO524R, 100 nmol/L ECO472T, 0.375 U of *Taq* DNA Polymerase (Promega, Madison, WI), and 5 μL of template DNA in a final volume of 15 μL. The *Enterococcus faecalis* PCR mixture consisted of 7.5 μL of 2× ABsolute QPCR ROX Mix (Abgene, Rochester, NY), 900 nmol/L of each primer, 100 nmol/L probe, 5 μL of template DNA, and the previously described concentrations of deoxynucleoside triphosphate and *Taq*.

Real-time PCR assays were performed with genomic DNA from cultured bacteria and DNA extracted from samples of luminal contents. A negative (no-template) control was included in every run. Amplification, detection, and analyses were performed in an ABI Prism 7900 Sequence Detection System (ABI/PE, Foster City, CA) with a cycle profile of 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. C_T was defined as the cycle at which the fluorescence became detectable above the background fluorescence. By using standard curves constructed from serial dilutions of purified bacterial DNA, the quantity of *Escherichia coli* or *Enterococcus faecalis* DNA in samples was calculated.

$CD4^+$ T-Cell Isolation

$CD4^+$ T cells were enriched from MLN or from caudal lymph node (CLN) cells harvested from IL-$10^{-/-}$ or WT mice by negative selection. B cells and $CD8^+$ T cells were depleted by using antibody-coated magnetic beads and cell-sorting techniques according to the manufacturer's instructions (Miltenyi-Biotec, Auburn, CA) as previously described.[10] The MLN and CLN $CD4^+$ T cell–enriched cell populations both contained 95%–98% $CD4^+$ T cells. $CD4^+$ T cells from CLNs

of *Escherichia coli*–monoassociated IL-10$^{-/-}$ mice were not evaluated because the CLN had insufficient cell numbers to allow CD4$^+$ T-cell isolation.

Antigen-Presenting Cell Preparation and CD4$^+$ T-Cell Stimulation

Splenic APCs were obtained from SPF 129S6/SvEv mice using methods previously described.[10] Briefly, after lysis of red blood cells, the splenocytes were depleted of T cells by rabbit complement–mediated lysis of Thy1.2-positive cells. T cell–depleted splenocytes were composed of 88%–95% B cells, 1%–6% CD4$^+$ T cells, and 1%–6% CD8$^+$ T cells. The cells were resuspended in RPMI complete medium (5% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 5×10^{-5} mol/L 2-mercaptoethanol, and 50 μg/mL gentamicin) and pulsed with 10 μg/mL of *Enterococcus faecalis*, *Escherichia coli*, or *B vulgatus* lysate or keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL) as an unrelated antigen control. After overnight incubation at 37°C, antigen-pulsed APCs were collected, washed twice, and cocultured with CD4$^+$ T cells (3×10^5 APCs per well plus 2×10^5 CD4$^+$ T cells per well) in flat-bottomed 96-well cell culture plates (Costar 3595) at 0.2 mL per culture. Supernatants were collected after 72 hours and frozen at −20°C until use for IFN-γ and IL-4 measurements.

Cytokine Measurements

We used commercially available monoclonal anti-mouse IFN-γ, IL-4, and IL-12 capture and detection reagents (BD Biosciences Pharmingen, San Diego, CA) in our well-validated enzyme-linked immunosorbent assay protocols to measure amounts of IFN-γ and IL-4 secreted by stimulated CD4$^+$ T cells and IL-12 secreted constitutively in colon tissue fragment cultures.[9,10] Cytokine levels were measured in triplicate supernatants and compared with standard curves generated by using recombinant murine cytokines.

Flow Cytometry

Cells requiring analysis (splenic APC, CLN, and MLN cells before and after negative selection) were incubated for 30 minutes at 4°C with fluorochrome-conjugated antibodies, including anti-CD4 (Caltag, Burlingame, CA), CD8 (Caltag), and B220 (BD Biosciences Pharmingen). After washing with fluorescence-activated cell sorter buffer (RPMI plus 1% bovine serum albumin), cells were analyzed by flow cytometry by using the FACScan (BD Biosciences, Mountain View, CA).

Statistical Analysis

Parametric data were analyzed with the paired Student *t* test. Nonparametric data (histological scores) were analyzed with the Kruskal–Wallis test (SAS Institute, Cary, NC) by statisticians in the Biostatistics Core of the Center for Gastrointestinal Biology and Disease. Statistical significance was defined as $P < .05$ for the comparisons indicated.

Results

Interleukin 10–Deficient Mice Monoassociated With *Enterococcus faecalis* or *Escherichia coli* Develop Progressive, Chronic Colitis With Regional Variability

IL-10$^{-/-}$ mice monoassociated with *Enterococcus faecalis* or *Escherichia coli* developed histological evidence of intestinal inflammation, but the 2 commensal microorganisms induced distinctly different disease kinetics and regional variability. *Enterococcus faecalis*–monoassociated mice had predominantly distal colitis, with mild histological inflammatory changes first noted 10–12 weeks after bacterial inoculation (Figure 1*A*). The distal colon was the first region of the intestinal tract to show inflammatory changes and progressed to moderate colitis by 16 weeks of monoassociation. By 42 weeks, disease was severe, with near-maximal inflammatory scores (3.75 ± 0.5, with a maximum score of 4.0). All other segments of the lower intestinal tract—cecum, proximal, and transverse colon—developed inflammation, but at a slower pace that progressed to moderate disease (eg, a cecal score of 2.1 ± 0.2) by 42 weeks. The cecum was the region that showed the least inflammation; this did not reach statistically significant differences compared with WT mice until 42 weeks after colonization. The distal colon showed a significantly greater degree of inflammation compared with the cecum at all time points studied ($P < .05$).

Conversely, *Escherichia coli*–monoassociated IL-10$^{-/-}$ mice initially developed mild inflammation, which was most apparent in the cecum, by 3 weeks after bacterial inoculation (Figure 1*B*). The remaining colonic segments developed mild inflammation with gradual progression in all areas. The cecum was moderately inflamed by 16 weeks (2.7 ± 0.2) but never achieved the degree of severe inflammation seen with *Enterococcus faecalis*–monoassociated mice at the late time points. The distal colonic segment of *Escherichia coli*–monoassociated IL-10$^{-/-}$ mice developed significant colitis (1.8 ± 0.9) by 16 weeks after colonization relative to WT mice. The cecum had significantly higher histological scores than the distal colon until the advanced (16–24 weeks) stages of disease. Histological scores for the proximal and transverse colonic segments were intermediate between the cecum and distal colonic scores for both *Enterococcus faecalis*– and *Escherichia coli*–monoassociated IL-10$^{-/-}$ mice (data not shown).

IL-10$^{-/-}$ *Enterococcus faecalis*–monoassociated mice studied at advanced stages of inflammation (>30 weeks) also developed upper gastrointestinal tract involvement manifested by duodenal thickening and moderate histo-

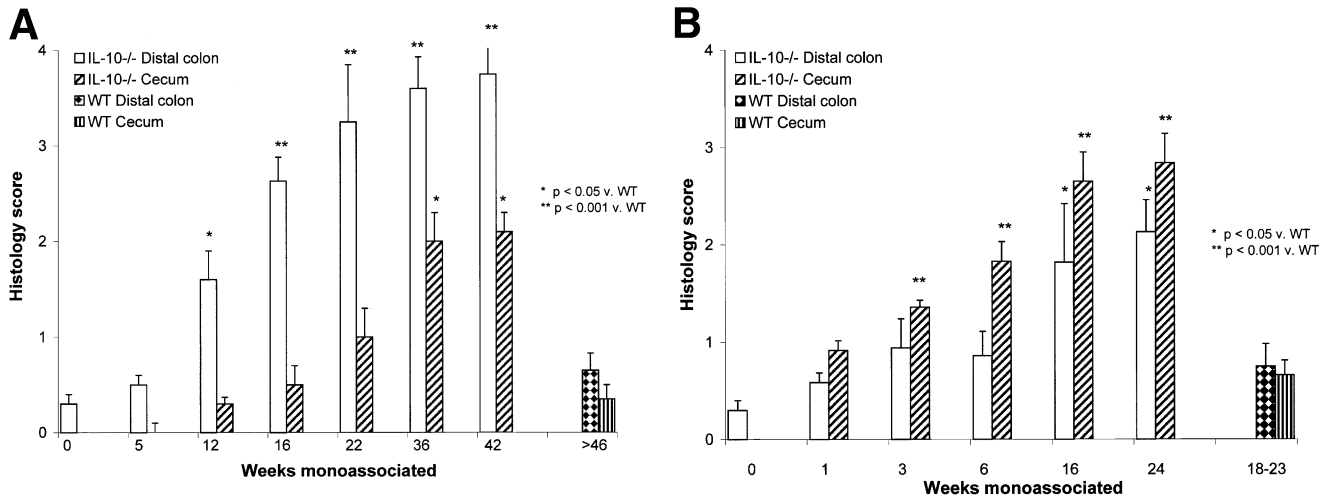


Figure 1. Kinetics and disease localization in IL-10$^{-/-}$ mice. (*A*) *Enterococcus faecalis*–monoassociated mice and (*B*) *Escherichia coli*–monoassociated mice. Histological scores (mean score ± SD) of the distal colon (open bars) and cecum (striped bars) from IL-10$^{-/-}$ *Enterococcus faecalis*–monoassociated mice (*A*) or *Escherichia coli*–monoassociated mice (*B*) are shown at different time points (n = 5–8 mice per time point) after initial bacterial inoculation and from representative wild-type (WT) mice (n = 6–7) studied ≥46 weeks after *Enterococcus faecalis* monoassociation or 18–23 weeks after *Escherichia coli* monoassociation. *$P < .05$ and **$P < .001$ vs. the histological score for the indicated region of the intestine from WT mice.

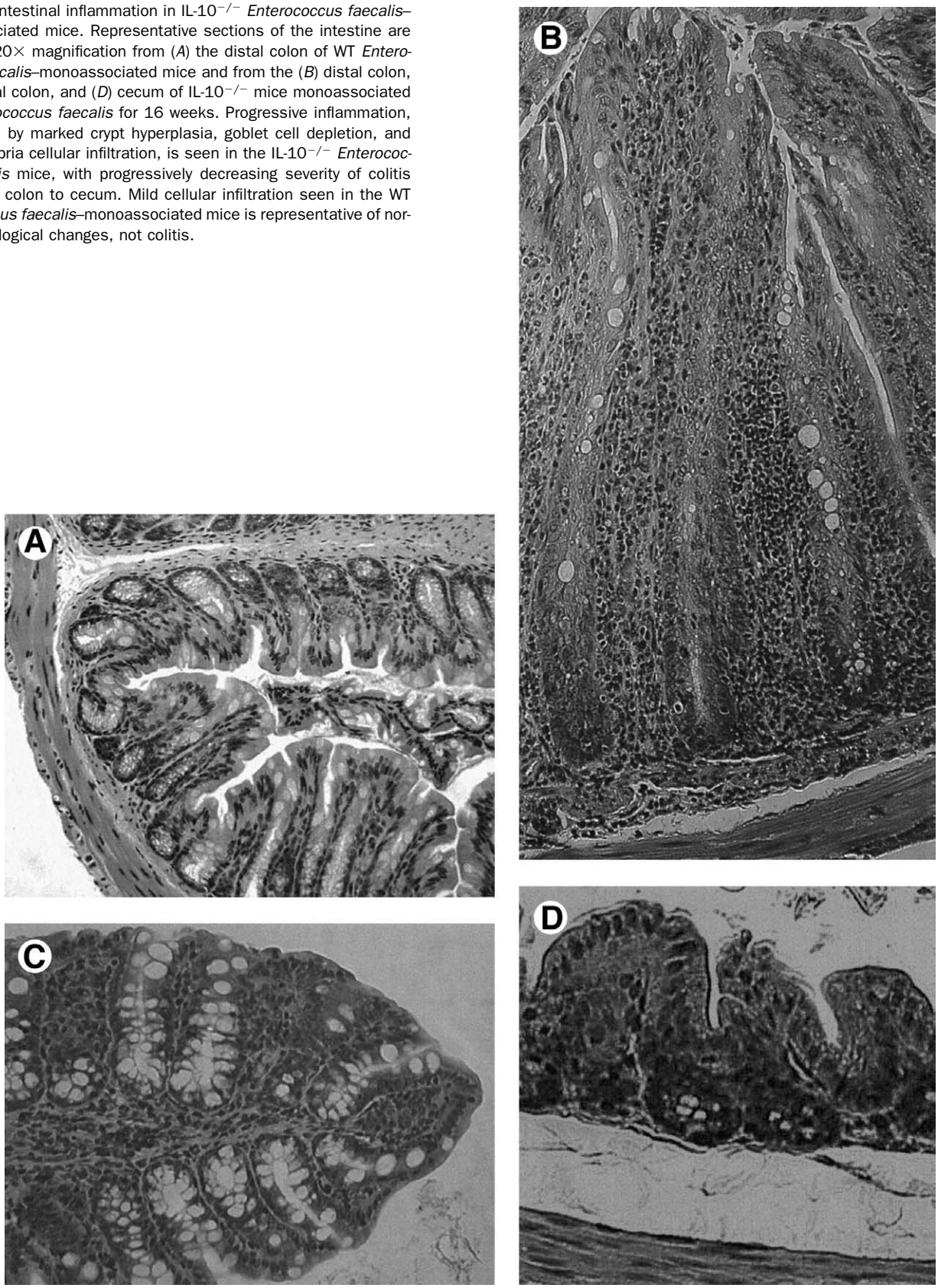
logical duodenal inflammation (n = 9; histological score, 1.9 ± 0.2) when compared with WT *Enterococcus faecalis*–monoassociated mice (n = 7; histological score, 0.4 ± 0.3; $P < .03$). Approximately 50% of these IL-10$^{-/-}$ mice colonized with *Enterococcus faecalis* for >30 weeks manifested clinical signs of duodenal obstruction, including gastric and proximal duodenal distention with fluid retention and weight loss. The distal small intestine, however, was not affected grossly or histologically at any time point studied. Neither IL-10$^{-/-}$ nor WT mice monoassociated with *Escherichia coli* developed duodenitis.

No inflammation was detectable in any segment of the intestinal tract in IL-10$^{-/-}$ mice after monoassociation with *Pseudomonas fluorescens*. Luminal cultures showed appropriate colonization with this organism.

In contrast to the progressive colitis that developed in the IL-10$^{-/-}$ mice colonized with either bacterial species, control WT mice selectively colonized with the same *Enterococcus faecalis* or *Escherichia coli* strains did not develop inflammation in any portion of the intestinal tract, thus signifying that these bacteria were not traditional pathogens. *Enterococcus faecalis*–monoassociated IL-10$^{-/-}$ mice had no inflammation in any region of the duodenum, colon, or cecum 4–5 weeks after bacterial colonization relative to WT mice monoassociated for >46 weeks (Figure 1*A*) or GF IL-10$^{-/-}$ mice, whereas *Escherichia coli*–monoassociated IL-10$^{-/-}$ mice did not display any inflammatory changes at 1 week of bacterial colonization (Figure 1*B*). Representative histological sections of the distal colons from WT mice monoassociated with *Enterococcus faecalis* (Figure 2*A*) showed normal physiologic cellularity, whereas sections of the distal colon, proximal colon, and cecum from IL-10$^{-/-}$ mice monoassociated with *Enterococcus faecalis* for 16 weeks (Figure 2*B–D*) showed a progressively decreasing gradation of inflammation from distal colon to cecum. Inflammation was manifested in the distal colon by crypt hyperplasia, infiltration of predominantly mononuclear cells with focal neutrophils, and goblet cell depletion, similar to the histological features of SPF IL-10$^{-/-}$ mice.[9] WT *Escherichia coli*–monoassociated mice were not inflamed in any region of the intestinal tract, including the cecum, even after >20 weeks of bacterial monoassociation (Figure 3*A*). IL-10$^{-/-}$ mice monoassociated with *Escherichia coli* for ≥3 weeks, however, developed cecal-predominant histological inflammation and a lesser degree of proximal and distal colitis (Figure 3*B–D*). Representative histological sections are shown at the same magnification (note similar muscularis mucosae thickness in all sections) from IL-10$^{-/-}$ and WT mice monoassociated with *Enterococcus faecalis* or *Escherichia coli*. Although the kinetics and location of disease were different in IL-10$^{-/-}$ mice monoassociated with *Enterococcus faecalis* or *Escherichia coli*, the histological features of disease were similar; however, less crypt hyperplasia was noted in the cecum than in the distal colon.

Differences in epithelial responses and extracolonic manifestations of disease were also noted between IL-10$^{-/-}$ mice monoassociated with *Enterococcus faeca-*

Figure 2. Intestinal inflammation in IL-10$^{-/-}$ *Enterococcus faecalis*–monoassociated mice. Representative sections of the intestine are shown at 20× magnification from (*A*) the distal colon of WT *Enterococcus faecalis*–monoassociated mice and from the (*B*) distal colon, (*C*) proximal colon, and (*D*) cecum of IL-10$^{-/-}$ mice monoassociated with *Enterococcus faecalis* for 16 weeks. Progressive inflammation, manifested by marked crypt hyperplasia, goblet cell depletion, and lamina propria cellular infiltration, is seen in the IL-10$^{-/-}$ *Enterococcus faecalis* mice, with progressively decreasing severity of colitis from distal colon to cecum. Mild cellular infiltration seen in the WT *Enterococcus faecalis*–monoassociated mice is representative of normal physiological changes, not colitis.



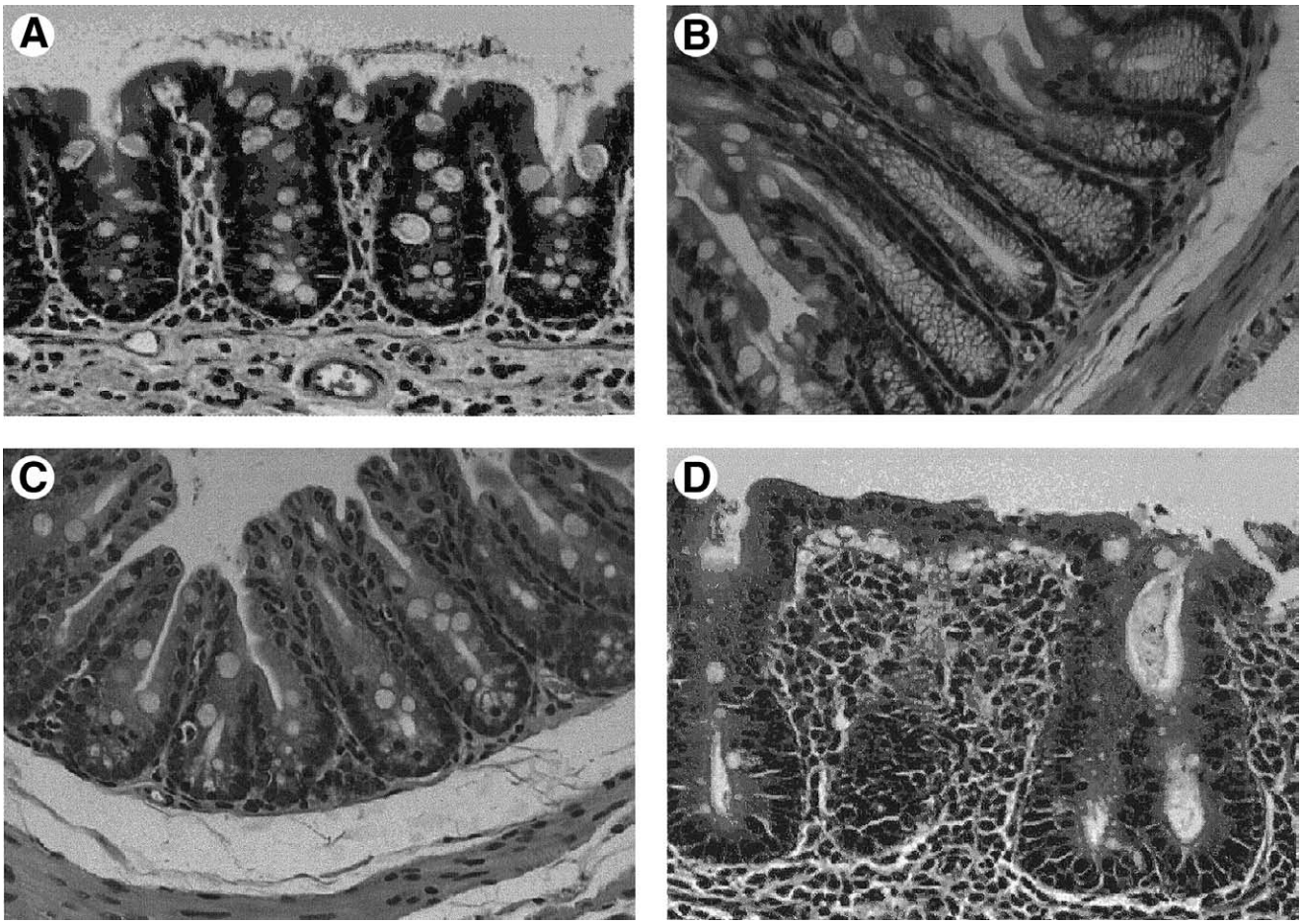


Figure 3. Intestinal inflammation in IL-10$^{-/-}$ *Escherichia coli*–monoassociated mice. Representative sections of the intestine are shown at 20× magnification from (*A*) cecum of WT *Escherichia coli*–monoassociated mice and of (*B–D*) IL-10$^{-/-}$ mice monoassociated with *Escherichia coli* for 3 weeks: (*B*) distal colon, (*C*) proximal colon, and (*D*) cecum. Disease is cecal predominant, with lesser inflammatory changes noted in the distal and proximal colon.

lis vs. *Escherichia coli*. Histological sections of the distal colon from 2 of 12 IL-10$^{-/-}$ *Enterococcus faecalis* mice monoassociated for >30 weeks showed signs of severe reactive atypia associated with underlying extensive active and chronic distal colitis, but no dysplastic changes or invasive carcinoma were noted. Because reactive atypia occurred in only a small proportion of the animals studied here, it is clear that evaluation of more animals is required before firm conclusions are drawn. Epithelial atypia was not seen at earlier time points in these mice, and neither atypia nor dysplasia was noted in any intestinal region in mice monoassociated with *Escherichia coli*. Representative sections from several regions of the small intestine were taken from mice at advanced stages of disease (>30 weeks). Moderate duodenitis, manifested by mucosal thickening and crypt hyperplasia (Figure 4*A*), was noted in IL-10$^{-/-}$ *Enterococcus faecalis* mice, but the remainder of the small intestine was spared. No evidence of duodenitis was observed in *Enterococcus faecalis*–monoassociated WT mice or *Escherichia coli*–monoassociated IL-10$^{-/-}$ mice (Figure 4*B* and *C*).

Other organs, including spleen and liver, were normal in appearance, without gross or histological signs of inflammation, infiltrative masses, or fibrosis. MLNs were moderate to large in size, with increased cellularity, in mice monoassociated for 11–46 weeks with *Enterococcus faecalis* ($63.3 \pm 22.9 \times 10^6$ cells) or for 5–26 weeks with *Escherichia coli* ($42.0 \pm 16.7 \times 10^6$ cells) compared with those found in WT-monoassociated controls (*Enterococcus faecalis*, $5.7 \pm 1.7 \times 10^6$ cells; *Escherichia coli*, $19.4 \pm 5.0 \times 10^6$ cells). Of interest, CLNs of *Enterococcus faecalis*–monoassociated

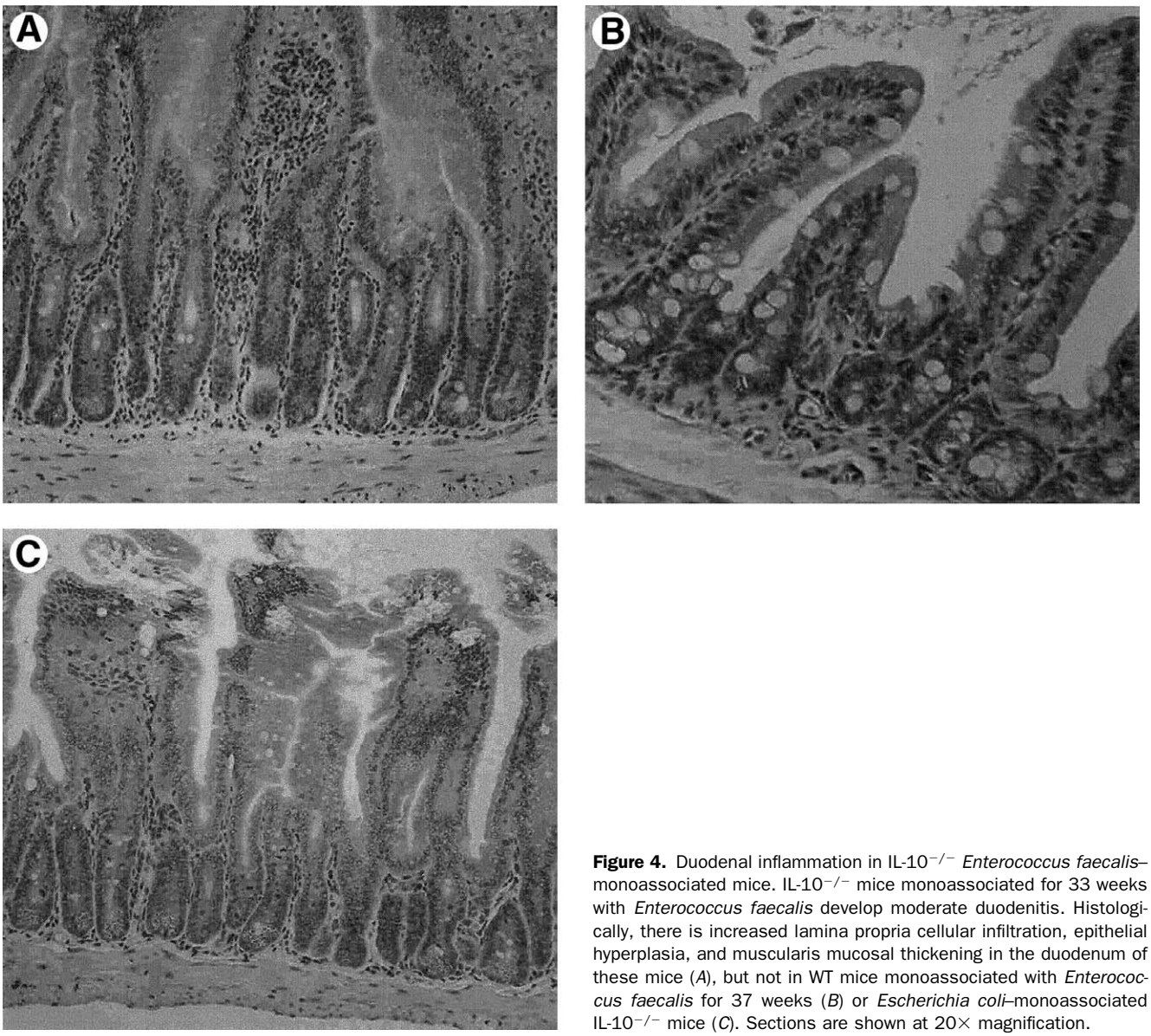


Figure 4. Duodenal inflammation in IL-10$^{-/-}$ *Enterococcus faecalis*–monoassociated mice. IL-10$^{-/-}$ mice monoassociated for 33 weeks with *Enterococcus faecalis* develop moderate duodenitis. Histologically, there is increased lamina propria cellular infiltration, epithelial hyperplasia, and muscularis mucosal thickening in the duodenum of these mice (*A*), but not in WT mice monoassociated with *Enterococcus faecalis* for 37 weeks (*B*) or *Escherichia coli*–monoassociated IL-10$^{-/-}$ mice (*C*). Sections are shown at 20× magnification.

IL-10$^{-/-}$ mice, which drain the distal colon, were enlarged, with increased cellularity ($22 \pm 5 \times 10^6$ cells). However, IL-10$^{-/-}$ *Escherichia coli*–monoassociated GF mice and WT *Escherichia coli*– or *Enterococcus faecalis*–monoassociated mice did not have visible CLNs.

Together, these results show that *Enterococcus faecalis*– and *Escherichia coli*–monoassociated IL-10$^{-/-}$ mice develop colitis with different kinetics of onset, different locations of predominant involvement, and different complications (obstruction and epithelial atypia). These findings suggest that different nonpathogenic commensal enteric bacterial species can induce different phenotypes of disease in the same genetically susceptible host.

Luminal Bacterial Concentrations Do Not Explain Variable Distribution of Disease

We then quantified luminal bacterial concentrations in a representative group of monoassociated mice with advanced colitis to determine whether variable luminal bacterial concentrations accounted for the regional distribution of disease. We used the following 2 complementary methods: (1) aerobic culture of serially diluted weighed luminal contents from the cecum, distal colon, and duodenum or of MLNs from *Enterococcus faecalis*– and *Escherichia coli*–monoassociated IL-10$^{-/-}$ mice and (2) real-time PCR of DNA extracts from weighed luminal contents or MLNs by using 16S ribosomal probes specifically recognizing

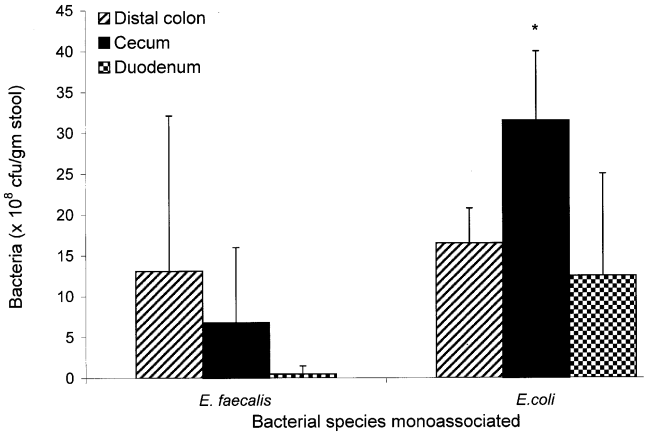


Figure 5. Luminal bacterial concentrations in IL-10$^{-/-}$ monoassociated mice. Luminal bacterial concentrations quantified by routine culture are shown for the distal colon (*striped bars*), cecum (*solid bars*), and duodenum (*checkered bars*) of IL-10$^{-/-}$ mice monoassociated with *Enterococcus faecalis* or *Escherichia coli* (n = 4 mice per group). There was no statistically significant difference in bacterial counts between the distal colon vs. cecum of IL-10$^{-/-}$ mice monoassociated with *Enterococcus faecalis* or *Escherichia coli*. There was a statistically significant difference in the growth of bacteria in the cecum of IL-10$^{-/-}$ mice monoassociated with *Enterococcus faecalis* compared with the growth of bacteria in the cecum of *Escherichia coli*–monoassociated mice (*$P < .02$ vs. bacterial growth in the cecum of *Enterococcus faecalis*–monoassociated IL-10$^{-/-}$ mice). cfu, colony-forming units.

each bacterial species. No statistically significant differences in bacterial growth were seen by standard culture techniques (Figure 5) or by real-time PCR (data not shown) between the distal colon vs. the cecum of IL-10$^{-/-}$ mice monoassociated with either *Enterococcus faecalis* or *Escherichia coli*. Duodenal concentrations of *Enterococcus faecalis* were quite low. Concentrations of *Enterococcus faecalis* vs. *Escherichia coli* in the cecum (*Enterococcus faecalis*, 6.8×10^8 colony-forming units of bacteria per gram of stool; *Escherichia coli*, 31.5×10^8 colony-forming units of bacteria per gram of stool; $P < .02$) were significantly different. Of interest, MLNs of all 4 *Enterococcus faecalis*–monoassociated IL-10$^{-/-}$ mice, but only 1 of 4 *Escherichia coli*–monoassociated mice, contained translocated bacteria, detected by both aerobic culture and real-time PCR.

Mucosal Cytokine Production in *Enterococcus faecalis*–Monoassociated Interleukin 10$^{-/-}$ Mice Precedes Onset of Inflammation

We then asked whether immune responses preceded or followed the onset of histological inflammation, to determine whether these responses were etiologically significant or secondary phenomena. Proinflammatory cytokine levels (IL-12 secretion from colonic culture supernatants and IFN-γ from MLN CD4$^+$ T cells stimulated with *Enterococcus faecalis*–pulsed APCs) were significantly increased in monoassociated IL-10$^{-/-}$ mice compared with WT controls at both early (4–5 weeks after colonization) and late time points (Table 1). In contrast, no histological inflammation was detected 4–5 weeks after *Enterococcus faecalis* monoassociation, thus indicating that immune activation, manifested by spontaneous colonic and stimulated MLN T-cell cytokine secretion, preceded the onset of detectable inflammation. Both spontaneous colonic IL-12 secretion and bacterial antigen–induced IFN-γ production by CD4$^+$ T cells from *Enterococcus faecalis*–monoassociated IL-10$^{-/-}$ mice increased with time (up to 46 weeks) after colonization but remained low in WT mice monoassociated with the same bacterial species for the same length of time. These results indicate that pathogenic immune responses to *Enterococcus faecalis* precede the onset of disease and suggest a pivotal role for bacterial antigen–stimulated responses in the pathogenesis of intestinal inflammation.

Table 1. Immune Activation and Histological Inflammation in IL-10$^{-/-}$ Mice Monoassociated With *Enterococcus faecalis* (*Ef*)

Mouse type	No. of mice	IL-12[a] ($\times 10^3$ pg/0.05 g tissue)	IFN-γ[b] ($\times 10^3$ pg/mL)	DC score[c] (0 to 4)
IL-10$^{-/-}$ *Ef* (4–5 wk)[d]	8	0.9 ± 0.2[e]	7.5 ± 1.7[e]	0.7 ± 0.2
IL-10$^{-/-}$ *Ef* (16–25 wk)	6–10[f]	2.6 ± 0.2[e]	27.5 ± 3.4[e]	3.2 ± 0.3[e]
IL-10$^{-/-}$ *Ef* (30–46 wk)	4–13[g]	2.7 ± 0.1[e]	53.6 ± 21.4[e]	3.5 ± 0.3[e]
WT *Ef* (5–7 wk)	10	0.1 ± 0.01	0.35 ± 0.5	0.6 ± 0.1
WT *Ef* (>46 wk)	7	0.1 ± 0.02	1.1 ± 0.8	0.6 ± 0.2

[a]Values represent mean pg/0.05 g tissue $\times 10^3$ ± SEM of IL-12 detected by enzyme-linked immunosorbent assay (ELISA) in supernatants of colonic tissue fragment cultures. Duplicate measurements were performed in each animal.
[b]Values represent mean pg/mL $\times 10^3$ ± SEM of IFN-γ detected by ELISA in supernatants of CD4$^+$ mesenteric lymph node T cells collected on day 3 after stimulation with *E faecalis* lysate–pulsed APCs. Triplicate measurements were performed in each animal.
[c]Values represent mean ± SD of blinded histological scores for distal colon (DC).
[d]Numbers in parentheses indicate time elapsed between monoassociation with *E faecalis* and killing the mice.
[e]$P < .01$ vs. *Ef* WT mice (early and late time point).
[f]n = 10 for IL-12 and distal colon histological score; n = 6 for IFN-γ.
[g]n = 10 for IL-12; n = 13 for DC score; n = 4 for IFN-γ.

Table 2. Immune Activation and Histological Inflammation in IL-10$^{-/-}$ Mice Monoassociated With *Escherichia coli* (*Ec*)

Mouse type	No. of mice	IL-12[a] ($\times 10^3$ pg/0.05 g tissue)	IFN-γ[b] ($\times 10^3$ pg/mL)	Cecum score[c] (0 to 4)
IL-10$^{-/-}$ *Ec* (1 wk)	5	0.5 ± 0.07	8.5 ± 1.6	0.9 ± 0.3
IL-10$^{-/-}$ *Ec* (3 wk)	9	0.5 ± 0.06	7.6 ± 1.0	1.4 ± 0.4[d]
IL-10$^{-/-}$ *Ec* (5–7 wk)	8	1.4 ± 0.3[d]	24.2 ± 6.7[d]	1.8 ± 0.4[d]
IL-10$^{-/-}$ *Ec* (16–18 wk)	6	3.0 ± 0.2[d]	58.1 ± 15.1[d]	2.7 ± 0.4[d]
WT *Ec* (1 wk)	5	0.1 ± 0.01	6.5 ± 1.3	0.5 ± 0.1
WT *Ec* (18–23 wk)	6	0.1 ± 0.02	4.2 ± 0.7	0.7 ± 0.2

[a]Values represent mean pg/0.05 g tissue × 10^3 ± SEM of IL-12 detected by enzyme-linked immunosorbent assay (ELISA) in supernatants of colonic tissue fragment cultures. Duplicate measurements were performed in each animal.
[b]Values represent mean pg/mL × 10^3 ± SEM of IFN-γ detected by ELISA in supernatants of CD4$^+$ mesenteric lymph node T cells collected on day 3 after stimulation with *Escherichia coli* lysate–pulsed APCs for *E coli*–monoassociated mice. Triplicate measurements were performed in each animal.
[c]Values represent mean ± SD of blinded histological scores for cecum.
[d]$P < .01$ vs. *Ec* WT mice (early and late time point).

In *Escherichia coli*–monoassociated IL-10$^{-/-}$ mice, histological inflammation was first evident at 3 weeks (Table 2). IFN-γ from MLN CD4$^+$ T cells stimulated with *Escherichia coli*–pulsed APCs was higher at 5–7 weeks and 16–18 weeks in IL-10$^{-/-}$ *Escherichia coli*–monoassociated mice compared with WT *Escherichia coli*–monoassociated mice at early (1 week) and late (18–23 weeks) time points. Of note, however, is the moderately increased production of IFN-γ by WT MLN CD4$^+$ T cells stimulated with *Escherichia coli*–pulsed APCs compared with the very low amounts of IFN-γ (90 ± 20 pg/mL IFN-γ) produced by same cells stimulated with the unrelated protein antigen control (KLH-pulsed APCs). Levels of spontaneous IL-12 secreted in colonic culture supernatants were not significantly increased until mild to moderate inflammation was seen 5–7 weeks after monoassociation with *Escherichia coli*, although concentrations were 5 times higher at 1 and 3 weeks compared with WT values. However, the longer duration of *Escherichia coli* colonization is accompanied by dramatically increased IFN-γ responses by cells from IL-10$^{-/-}$ mice, but not by cells from WT mice.

Both bacterial strains used to inoculate IL-10$^{-/-}$ and WT mice were nonpathogenic, as shown by the lack of clinical abnormalities, the absence of intestinal inflammation, and detection of only low levels of spontaneous IL-12 secretion in colonic cultures from monoassociated WT mice.

Interleukin 10$^{-/-}$ *Enterococcus faecalis*– and *Escherichia coli*–Monoassociated Mice Show Bacterial Antigen–Specific CD4$^+$ T-Cell Responses

To determine whether activated mucosal CD4$^+$ T cells from monoassociated IL-10$^{-/-}$ mice show bacterial antigen–specific responses, we cultured MLN-derived CD4$^+$ T cells with APCs that had been pulsed in vitro with *Enterococcus faecalis*, *Escherichia coli*, or *B vulgatus* lysates or with KLH (as an unrelated protein antigen control). CD4$^+$ T cells from MLNs of IL-10$^{-/-}$ *Enterococcus faecalis*–monoassociated mice with active disease secreted substantially more IFN-γ and IL-4 after stimulation with *Enterococcus faecalis*–pulsed APCs compared with stimulation with *Escherichia coli*, *B vulgatus*, or KLH-pulsed APCs (Table 3), thus indicating bacterial antigenic specificity in IL-10$^{-/-}$ *Enterococcus faecalis*–monoassociated mice. Both *Enterococcus faecalis*–stimulated IFN-γ and IL-4 concentrations were significantly higher in supernatants of CD4$^+$ MLN cells stimulated with *Enterococcus faecalis*–pulsed APCs compared with

Table 3. Bacterial Antigen Specificity of Cytokine Production by CD4$^+$ Mesenteric Lymph Node T Cells Isolated From *Enterococcus faecalis*– or *Escherichia coli*–monoassociated IL-10$^{-/-}$ Mice

Bacterial lysate	IFN-γ (*pg/mL*)	IL-4 (*pg/mL*)
Enterococcus faecalis-monoassociated[a]		
E faecalis	53,644 ± 21,384[b]	3.5 ± 7.1[b]
E coli	1589 ± 712	1.7 ± 0.6
Bacteroides vulgatus	196 ± 104	<1
KLH	185 ± 78	<1
Escherichia coli-monoassociated[c]		
E faecalis	453 ± 121	1.8 ± 0.4
E coli	58,069 ± 15,110[b]	9.6 ± 1.9[b]
B vulgatus	704 ± 16	2.1 ± 0.7
KLH	146 ± 35	1.9 ± 0.5

NOTE. IFN-γ and IL-4 were measured in supernatants of CD4$^+$ T cells stimulated with bacterial lysate–pulsed APCs. Values represent mean pg/mL ± SEM of cytokines measured in supernatants collected on day 3 after culture initiation.
[a]CD4$^+$ T cells were isolated from MLN of IL-10$^{-/-}$ mice (n = 4; 3 separate experiments) analyzed 32–36 weeks after monoassociation with *E faecalis*.
[b]$P < .05$ vs. KLH stimulation.
[c]CD4$^+$ T cells were isolated from MLN of IL-10$^{-/-}$ mice (n = 6; 2 separate experiments) analyzed 16–18 weeks after monoassociation with *E coli*.

supernatants of the same cells stimulated with KLH-pulsed APCs. The ratio of IFN-γ levels to IL-4 levels was similar during the preclinical (5 weeks: IFN-γ/IL-4 = 1305) and advanced (>30 weeks: IFN-γ/IL-4 = 1750) phases of colitis. $CD4^+$ T cells from MLNs of both WT *Enterococcus faecalis*–monoassociated and SPF WT mice did not produce detectable IFN-γ or IL-4 when stimulated by APCs pulsed with any of the same bacterial lysates (data not shown).

$CD4^+$ T cells from MLNs of IL-$10^{-/-}$ *Escherichia coli*–monoassociated mice with active colitis (colonized for 16–18 weeks) secreted significantly higher levels of IFN-γ and IL-4 after stimulation with *Escherichia coli*–pulsed APCs vs. stimulation with *Enterococcus faecalis*, *B vulgatus*, or KLH-pulsed APCs (Table 3). These findings show bacterial antigen–specific immune responses in both *Escherichia coli*– and *Enterococcus faecalis*–monoassociated IL-$10^{-/-}$ mice.

Differential Bacterial Antigen–Specific $CD4^+$ T-Cell Responses in Mesenteric Versus Caudal Lymph Nodes Do Not Explain the Regional Colitis Observed in *Enterococcus faecalis*–Monoassociated Interleukin $10^{-/-}$ Mice

To address whether the regional distribution of histological inflammation (distal vs. proximal colon) was due to differences in the degree of immune activation in the lymph nodes that drain different segments of the colon, $CD4^+$ T cells isolated both from MLNs that drain the proximal colon and cecum and from CLNs that drain the distal colon of *Enterococcus faecalis*–monoassociated IL-$10^{-/-}$ mice with either mild to moderate (14 weeks of monoassociation) or advanced (25–36 weeks of monoassociation) intestinal inflammation were stimulated with *Enterococcus faecalis* lysate–pulsed APCs. As shown in Table 4, both MLNs and CLNs contained $CD4^+$ T cells that produce IFN-γ after stimulation with *Enterococcus faecalis* lysate–pulsed APCs. However, the IFN-γ levels were higher in supernatants of MLN $CD4^+$ T-cell/APC cocultures compared with supernatants of CLN $CD4^+$ T-cell/APC cocultures at both disease stages (this reached statistical significance at the later stage). Differences in histological colitis in different areas of the colons of these mice did not account for the different amounts of IFN-γ produced (Figure 1*A*). These results show that the intensity of bacterial antigen–induced immune responses in the draining regional lymph nodes of *Enterococcus faecalis*–monoassociated mice did not correlate with the site of colonic inflammation. $CD4^+$ T cells from CLNs of *Escherichia coli*–monoassociated IL-$10^{-/-}$ mice were not evaluated, because the CLNs had insufficient cell numbers to allow $CD4^+$ T-cell isolation.

Table 4. IFN-γ Production by $CD4^+$ T Cells Isolated From Mesenteric Lymph Nodes and From Caudal Lymph Nodes of IL-$10^{-/-}$ Mice Monoassociated With *Enterococcus faecalis* for 14 or 25–36 Weeks, Time Points Corresponding With Mild–Moderate and Severe Colitis, Respectively

Lymph node	IFN-γ (*pg/mL*)[a]	Total cells ($\times 10^7$)[b]
14 wk monoassociated		
Mesenteric	25,653 ± 9656	4.0 ± 0.5[c]
Caudal	16,898 ± 4756	0.4 ± 0.03
25–36 wk monoassociated		
Mesenteric	48,667 ± 13,630[c]	7.0 ± 1.0[c]
Caudal	19,463 ± 652	2.2 ± 0.5

NOTE. Results were obtained from individual IL-$10^{-/-}$ mice monoassociated with *E faecalis* for 14 weeks (n = 5) or 25–36 weeks (n = 6). Mice were analyzed in 4 separate experiments.

[a]Values represent mean ± SEM of IFN-γ (pg/mL) detected by enzyme-linked immunosorbent assay in supernatants collected 72 hours after stimulation of lymph node $CD4^+$ T cells with *E faecalis* lysate–pulsed APCs.

[b]Values represent total number of cells ($\times 10^7$) obtained from mesenteric or caudal lymph nodes of *E faecalis*–monoassociated IL-$10^{-/-}$ mice.

[c]$P < .05$ vs. CLN.

Discussion

These results show that 2 distinct nonpathogenic bacterial species induce different phenotypes of enterocolitis with regional disease specificity in a single genetically defined host. Previous studies have documented the importance of normal commensal bacteria in this model; GF IL-$10^{-/-}$ mice on the susceptible 129S6/SvEv background do not develop intestinal inflammation but rapidly develop predominantly cecal inflammation after colonization with SPF enteric bacteria.[9] Unlike the rapid onset of predominantly cecal and proximal colonic inflammation observed in IL-$10^{-/-}$ mice that were born GF and colonized with SPF bacteria,[9] *Enterococcus faecalis*–monoassociated mice developed progressive inflammation that was most active in the distal colon. Of considerable interest, antibiotics with different narrow spectra of bactericidal activity preferentially treated different colonic regions in SPF IL-$10^{-/-}$ mice. Ciprofloxacin had dominant effects in the cecum, whereas metronidazole preferentially ameliorated inflammation in the distal colon.[13] In addition, the onset of disease is delayed in *Enterococcus faecalis*–monoassociated IL-$10^{-/-}$ mice compared with SPF animals. GF IL-$10^{-/-}$ mice moved into SPF housing develop severe cecal inflammation by 5 weeks after colonization.[9] Colitis developed much more slowly in *Enterococcus faecalis*–monoassociated mice, with

the onset of distal intestinal inflammation apparent by 12 weeks after initial bacterial inoculation. However, *Enterococcus faecalis*–monoassociated IL-$10^{-/-}$ mice ultimately developed severe transmural inflammation with ulceration and crypt abscesses. *Escherichia coli*–monoassociated IL-$10^{-/-}$ mice, in contrast, developed a predominantly right-sided colitis similar in distribution to SPF IL-$10^{-/-}$ mice, with a more rapid onset but a less intense peak than the disease observed in *Enterococcus faecalis*–monoassociated mice. Thus, both the location and kinetics of colitis were variable; they were dependent on the specific nonpathogenic commensal bacterial species used to selectively colonize a susceptible mouse strain.

The duodenum was moderately inflamed at later stages (>30 weeks) of *Enterococcus faecalis* monoassociation, with functional obstruction manifested by severe gastric distention noted in some mice, but the ileum was spared at all time points studied. The duodenal lumen contained relatively low bacterial concentrations, as enumerated by routine bacterial culture, but coprophagia could increase exposure of the duodenal mucosa to nonviable fecal bacterial antigens and adjuvants. However, no gastritis was observed in these mice in response to ingested bacteria. Duodenal inflammation was not noted in IL-$10^{-/-}$ mice monoassociated with *Escherichia coli*, despite higher duodenal concentrations of *Escherichia coli* vs. *Enterococcus faecalis*, although proximal small-intestinal inflammation with rapid progression to lethal enterocolitis has been reported in conventionally housed IL-$10^{-/-}$ mice.[8] We have not observed duodenitis in IL-$10^{-/-}$ mice colonized with SPF bacteria.[9] Several other rodent models develop ileitis, but not duodenitis, when exposed to commensal bacteria, including the SAMP-1/Yit mouse[24] and the TNFΔARE mouse.[25] SPF HLA-B_{27} TG rats develop antral gastritis, proximal duodenitis, colitis, and arthritis[14] but show only colitis when monoassociated with *B vulgatus*.[15] However, our current observations that a genetically engineered rodent IBD model developed clinical luminal obstruction are unique, although SAMP-1/Yit mice were reported to have ileal luminal narrowing with no obstruction.[26]

The precise mechanisms explaining the regional differences in intestinal inflammation remain unclear, because differences in luminal concentrations of *Enterococcus faecalis* and *Escherichia coli* do not account for differential locations of disease. Bacterial colonization in the cecum vs. distal colon was not significantly different in either group of IL-$10^{-/-}$ monoassociated mice, although *Escherichia coli* did achieve higher cecal concentrations than did *Enterococcus faecalis*. Duodenal concentrations of *Enterococcus faecalis* were quite low despite the presence of inflammation and stricturing in this region. Regional differences in intestinal inflammation may not depend on absolute bacterial counts but, rather, the relative proportion of adherent bacterial organisms in each region[27] or alterations of bacterial virulence factors in different areas of the intestinal tract. It is possible that differences in regionally specific epithelial receptors, bacterial virulence factors, or both may result in selective regional epithelial binding by *Enterococcus faecalis* vs. *Escherichia coli*. Of potential relevance to our results, *Enterococcus faecalis* strains isolated from patients with chronic urinary tract infections efficiently adhere in vitro to urinary epithelial cells (T24 cell line), but *Enterococcus faecalis* strains isolated from healthy controls do not.[28] Furthermore, some *Enterococcus faecalis* strains show preferential adherence to epithelial cell lines originating from different areas of the intestinal tract. *Enterococcus faecalis* strains possessing an aggregation substance preferentially adhere to colonic (HT-29 and T84) and duodenal (Hutu 80), but not ileal (HCT-8), epithelial cell lines.[29] These results suggest that *Enterococcus faecalis* may, in part, induce disease through preferential adherence to the intestinal epithelia in different regions of the intestine.

Bacterial translocation from the intestinal tract to MLNs was shown by both routine culture and real-time PCR quantification in 100% of a representative group of IL-$10^{-/-}$ *Enterococcus faecalis*–monoassociated mice. In contrast, bacterial translocation to MLN was documented in only 25% of *Escherichia coli*–monoassociated IL-$10^{-/-}$ mice. These findings correlate with previous murine studies. *Enterococcus faecalis* translocation to MLNs was observed in mice with *Enterococcus faecalis* bacterial overgrowth (induced by broad-spectrum antibiotic administration) in the presence of an intact intestinal epithelium.[30] Whether this preferential translocation is a property of *Enterococcus faecalis* or is secondary to the severity or location of colitis remains to be determined.

Our results indicate bacterial species–specific induction of colitis, because IL-$10^{-/-}$ mice monoassociated with *Pseudomonas fluorescens*, which contains the I2 gene sequence identified within lymphoid tissue of patients with colonic Crohn's disease,[21,22,31] did not develop colonic or small-intestinal inflammation. Prior attempts to induce colitis in IL-$10^{-/-}$ mice selectively colonized with other bacterial species have not been successful. Gnotobiotic IL-$10^{-/-}$ mice monoassociated with *Helicobacter hepaticus*, *Candida albicans*, *Lactococcus lactis*, several *Lactobacillus* species, *Viridans* group *Streptococcus* species, and *Clostridium sordellii* do not develop disease.[17–20] Indeed, *Enterococcus faecalis* and our randomly isolated murine *Escherichia coli* strain are the only nonpathogenic bacterial species reported to induce both chronic intes-

tinal inflammation and bacterial antigen–specific cytokine production (IFN-γ and IL-4) in monoassociated IL-$10^{-/-}$ mice. The nonpathogenic nature of the 2 resident bacterial strains used in our experiments was confirmed by the lack of intestinal inflammation or pathogenic immune responses in WT monoassociated controls. In another murine model, Jiang et al[32] showed colonic inflammation in *Helicobacter muridarum*–monoassociated severe combined immunodeficiency disease mice reconstituted with CD45 RB^{hi} T lymphocytes. Increased $CD4^+$ T-cell numbers were observed, but the antigen specificity of these cells was not evaluated in that study. Of considerable interest, various bacterial species can induce disease and alter the degree of inflammation in different susceptible hosts. Waidmann et al[33] showed that monoassociating gnotobiotic IL-$2^{-/-}$ mice with a nonpathogenic *Escherichia coli* strain induced colitis; however, dual association with *B vulgatus* and *Escherichia coli* prevented the development of intestinal inflammation without decreasing the concentration of *Escherichia coli* in the colon. HLA-B_{27} TG rats monoassociated with *B vulgatus* develop only mild to moderate intestinal inflammation, in contrast to severe colitis in SPF TG rats; *Escherichia coli*–monoassociated HLA-B_{27} TG rats have no disease.[15] However, onset of disease (1 month after colonization) was similar with both simplified (monoassociated or 6 defined species) and complex (SPF) flora. In *B vulgatus*–monoassociated HLA-B_{27} TG rats, disease was not progressive, and the location of colitis (predominantly cecal) was anatomically similar whether the rats were colonized with *B vulgatus* or multiple organisms. Of note, SPF TG rats developed antral and duodenal inflammation, whereas *B vulgatus*–monoassociated rats did not.[14,15] To date, all reported monoassociated rodent models show less aggressive disease than GF rodents colonized with SPF enteric bacteria. Thus, although several enteric bacterial species can selectively induce intestinal inflammation in several genetically susceptible monoassociated hosts, additional members of the complex commensal bacterial populations potentiate inflammation because of additive or synergistic activities.

Our monoassociated IL-$10^{-/-}$ mice developed bacterial antigen–specific T-helper type 1 and 2 lymphocyte responses. $CD4^+$ T cells isolated from IL-$10^{-/-}$ *Enterococcus faecalis*–monoassociated mice secreted high levels of IFN-γ and more modest absolute levels of IL-4 when stimulated with *Enterococcus faecalis*–pulsed APCs but showed only minimal responses to *Escherichia coli* lysates. Similarly, IL-$10^{-/-}$ *Escherichia coli*–monoassociated mice produced high amounts of IFN-γ when stimulated with *Escherichia coli*, but not *Enterococcus faecalis*– or *B vulgatus*–pulsed APCs. Likewise, IL-4 production by T cells from *Escherichia coli*–monoassociated mice was detected only in cocultures with *Escherichia coli*–pulsed APCs. Spencer et al[34] reported that IL-4 production by concanavalin A–stimulated lamina propria lymphocytes increased in parallel with IL-12 and IFN-γ in SPF IL-$10^{-/-}$ mice, but unlike IL-12 and IFN-γ, which waned during the late stages of disease (>30 weeks), IL-4 and IL-13 responses remained high. We did not observe similar decreases in colonic IL-12 secretion or MLN T-cell IFN-γ production in IL-$10^{-/-}$ mice monoassociated with either *Enterococcus faecalis* or *Escherichia coli* at the later time points evaluated.

T lymphocytes are required for the induction of intestinal inflammation in multiple rodent models.[10,12,24,35,36] In our study, we observed spontaneous colonic secretion of IL-12 and IFN-γ production by MLN $CD4^+$ T cells stimulated with bacterial lysate–pulsed APCs before the onset of histological and clinical intestinal inflammation in IL-$10^{-/-}$ mice monoassociated with *Enterococcus faecalis*. These findings suggest that bacterial-specific immune responses are associated with the onset of colitis in these mice rather than being a secondary response. However, the progressive increase in bacterial antigen–specific T-cell activation as inflammation progresses indicates that T-cell responses and inflammation are interrelated, possibly because of enhanced luminal antigen uptake with increased intestinal permeability during inflammation, clonal expansion of pathogenic T cells responding to bacterial stimulation in the absence of IL-10 immunosuppression, or positive feedback loops between IL-12 secretion and T-lymphocyte activation.

In contrast to findings from *Enterococcus faecalis*–monoassociated WT mice, IFN-γ levels measured from T cells of noninflamed *Escherichia coli*–monoassociated WT control mice (at 1 week or 18–23 weeks after colonization) were slightly higher after stimulation with *Escherichia coli*–pulsed APCs compared with KLH-stimulated values, although spontaneous IL-12 secretion from colonic strip culture supernatants was not increased, and histological evidence of colitis was not seen. Monoassociation with gram-negative organisms can stimulate proinflammatory cytokine secretion at ≤1 week in normal hosts, but subsequent induction of immunomodulatory molecules dampens this response.[37] Together, these data indicate that gram-negative bacteria and the lipopolysaccharide they produce can transiently stimulate innate and possibly cognate immune responses in WT mice. However, in WT mice, endogenous IL-10 and TGF-β production suppresses pathogenic immune responses, thus preventing the progression of inflammation.

It is possible that region-specific T cells induce variable disease in hosts colonized with different bacterial

species. Regional T-cell specificity could be due to selective recruitment of T cells through locally produced endothelial adhesion molecules, chemokines, or other signaling molecules. For example, T-cell expression of the ligand for the adhesion molecule P-selectin can be up-regulated by IL-12, which is locally stimulated in the intestinal tract by *Enterococcus faecalis* or *Escherichia coli* colonization.[38] However, we were unable to document differences in bacterial antigen–specific $CD4^+$ T-cell responses in MLNs vs. CLNs. Therefore, our results fail to support the concept of variable T-cell activation and recruitment in the distal vs. proximal colon, although we did not investigate lamina propria T-cell responses.

Consistent with observations in rodent models, IBD patients mount pathologic responses to resident enteric bacteria. Duchmann et al[39,40] showed a loss of tolerance to luminal bacteria in mucosal lymphocytes isolated from patients with active IBD and showed that $CD4^+$ T-cell receptor $\alpha\beta^+$ T-cell clones isolated from peripheral blood and intestinal biopsy samples from IBD patients preferentially responded to *Escherichia coli*, among several commensal bacteria. More recently, Swidsinski et al[27] showed high concentrations of enteric bacteria adherent to the intestinal mucosa of individuals with human IBD, especially Crohn's disease, with bacterial concentrations correlating with the degree of intestinal inflammation. Predominant mucosally associated organisms were *Bacteroides* and Enterobacteriaceae (including *Escherichia coli*) species, with lower concentrations of *Enterococcus/Streptococcus*, *Clostridia*, and *Peptostreptococcus* species. *Escherichia coli* have been further implicated in Crohn's disease by Darfeuille-Michaud et al,[41] who recovered enteroadherent/invasive *Escherichia coli* strains in greater frequency from the ileal mucosa of patients with postoperative recurrence of disease compared with either Crohn's disease patients with chronic ileal lesions or healthy controls. Finally, Cartun et al[42] showed that invasive *Escherichia coli* and enterococci were present in Crohn's disease tissue samples in areas immediately adjacent to ulcers and fistulas.

Our observations that different bacterial species can cause inflammation in different intestinal regions in the same host have important implications for human IBD, because both ulcerative colitis and Crohn's disease have heterogeneous clinical patterns of disease that respond differently to therapy. For example, metronidazole, either alone or in combination with ciprofloxacin, is more active in colonic vs. ileal Crohn's disease, perhaps because of different bacterial antigenic stimulation in the colon vs. the small bowel.[43–45] However, no single organism has been proven to induce IBD, although multiple pathogens and commensal enteric bacterial species have been investigated as potential etiologic agents.[1]

This study shows that different nonpathogenic commensal organisms can induce distinct disease phenotypes in the same genetically susceptible host and that only certain bacterial species induce disease within a given host. In addition, bacterial antigen–specific cellular immune activation precedes histological evidence of colitis in monoassociated IL-$10^{-/-}$ mice and progressively increases relative to WT controls. These results indicate that genetically susceptible hosts with immune dysregulation can selectively respond to specific components of the complex luminal microflora and can eventually lead to chronic intestinal inflammation. These findings can help guide investigations of distinct genetic and phenotypic subtypes of human IBD by offering insights into possible mechanisms of disease pathogenesis and providing potential targets for diagnostic tests and selective therapeutic manipulation of different subsets of IBD patients.

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