Toll-like Receptor-6 Signaling Prevents Inflammation and Impacts Composition of the Microbiota During Inflammation-Induced Colorectal Cancer

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ABSTRACT

Tightly regulated immune responses must occur in the intestine to avoid unwanted inflammation, which may cause chronic sequelae leading to diseases such as colorectal cancer. Toll-like receptors play an important role in preventing aberrant immune responses in the intestine by sensing endogenous commensal microbiota and delivering important regulatory signals to the tissue. However, the role that specific innate receptors may play in the development of chronic inflammation and their impact on the composition of the colonic microbiota is not well understood. Using a model of inflammation-induced colorectal cancer, we found that Lactobacillus species are lost more quickly in wild-type (WT) mice than TLR6-deficient mice resulting in overall differences in bacterial composition. Despite the longer retention of Lactobacillus, the TLR6-deficient mice presented with more tumors and a worse overall outcome. Restoration of the lost Lactobacillus species suppressed inflammation, reduced tumor number, and prevented change in the abundance of Proteobacteria only when given to WT mice, indicating the effect of these Lactobacillus are TLR6 dependent. We found that the TLR6-dependent effects of Lactobacillus could be dissociated from one another via the involvement of IL10, which was necessary to dampen the inflammatory microenvironment, but had no effect on bacterial composition. Altogether, these data suggest that innate immune signals can shape the composition of the microbiota under chronic inflammatory conditions, bias the cytokine milieu of the tissue microenvironment, and influence the response to microbiota-associated therapies.

Introduction

Colorectal cancer is the third most common form of cancer and the second leading cause of cancer-related deaths in the United States (1). A majority of colorectal cancer cases are due to sporadic tumorigenesis, while only 10% are associated with a genetic predisposition (2, 3). The increased risk of developing colorectal cancer in people with metabolic syndrome, type 2 diabetes, and inflammatory bowel disease (IBD; ref. 4) highlights a strong role for chronic inflammation in the etiology of inflammation-associated or colitis-associated colorectal cancer (5, 6). Other common features shared between these patient populations include changes within the composition of the intestinal microbiota and the development of immune reactivity against these intestinal microbes (7, 8).

The intestine is home to a large microbial ecosystem that provides protective, structural, and metabolic functions (9). The importance of the microbiota in colorectal cancer has been illustrated in both animal models and in studies of patients with colorectal cancer (10–12). In models of inducible and sporadic colorectal cancer, the composition of conventionally raised mice changes over the course of tumorigenesis (13). Shifts in the composition of the microbiota have also been observed in human studies, which have not only found changes in the abundance of certain bacterial taxa but have identified a number of microbes thought to be drivers in the progression to malignancy. These include Fusobacterium nucleatum (14, 15), Bacteroides fragilis (16, 17), and Escherichia coli (18). The importance of the microbiota in colorectal cancer has also been demonstrated using germ-free mice, which develop less inflammation and fewer tumors than conventionally housed animals (19, 20). Despite these findings, the molecular mechanism contributing to the selection of certain bacterial families over another for an intestinal niche has not been established for colorectal cancer.

Because of the proximity of the microbiota to the intestinal epithelium and underlying immune cells, tightly regulated communication must occur to prevent abnormal tissue responses that could lead to chronic inflammation and malignancy. Coordination of intestinal responses are initiated through the recognition of both microbial-derived and host-derived ligands by innate immune receptors, such as Nod-like receptors and the Toll-like receptors (TLR; ref. 21). TLRs comprise a set of receptors that recognize conserved microbial...
motifs (e.g., lipopolysaccharide and flagellin), as well as endogenous danger signals (e.g., HSPs; ref. 22). These receptors represent a first-line of defense against invading enteric pathogens (23), but also sense and respond to our own commensal bacteria to promote epithelial cell integrity (24), localized immune responses, and even colonization of the host (25). Disruption of these signals can lead to uncontrolled inflammation and changes within the microbiota, which have significant roles in intestinal disease (26), tumorigenesis, and tumor progression (10, 27).

TLR2 recognizes di- and tri-acylated bacterial lipoproteins when heterodimerized with TLR6 (28) or TLR1 (29), respectively. In a model of inflammation-associated colorectal cancer, mice deficient for TLR2 have shown an increase in size and number of colonic tumors, dysregulation of cytokine production, and epithelial responses (30). Because both TLR1 and TLR6 require TLR2 to signal (21), deletion of TLR2 also results in the disruption of their signaling, making it difficult to delineate the role that these receptors may play in sensing and responding to microbial ligands in colorectal cancer. Using a model of inflammation-associated colorectal cancer we sought to understand how TLR6 signaling would affect a disease associated with alterations of the microbiota in a highly inflamed environment. We found that TLR6 deficiency was associated with the development of more tumors and worse survival when housed with other TLR6KO mice or when cohoused with wild-type (WT) mice. Interestingly, cohousing provided protection against tumors in the WT mice due to the presence of Lactobacillus which induced IL10 in a TLR6-dependent manner. Overall these studies suggest that innate sensing of the microbiota during inflammation can influence the local cytokine milieu, the composition of the commensals, and the response to microbiome-based immune therapies.

Materials and Methods

Animals

TLR6KO and littermate control mice were bred in-house and maintained in a specific pathogen-free facility at the University of Southern California (Los Angeles, CA). All mice used in these experiments were generated from 5–7 different founder cages where all female mice were WT for Tlr6. At the time of weaning, pups from different litters but of the same sex were mixed together for group housing to reduce founder and cage effects on the microbiome. Mice were fed a standard rodent diet AIN-78 (LabDiet) and given nonchlorinated, non-cage rinsed with ice-cold PBS and washed by submerging tissues in clean sterile PBS followed by vigorous shaking for 5 seconds. This was repeated 5 times, and the colon tissue was placed back onto the glass plate and glass microscope slides were used to gently scrape the colon tissue from the mucosa. The mucosa was added to its corresponding luminal contents and sent for 16S sequencing.

454 pyrosequencing, sequence curation, and microbiome analysis

Microbiota samples were processed and sequenced at Research and Testing Laboratory (RTL) based upon RTL protocols using a Roche FLX Titanium genome sequencer. Universal bacterial primers 28F “GAGTTTGATCNTGGCTGAG” and 519R “GNTTTACNGCGGCKGTG” were used to amplify the variable regions V1–V3 of the 16S rRNA genes. 16S rRNA gene sequences were curated using mothur v.1.35.1 (37). Briefly, sequences were denoised using a flow gram denoising algorithm (39), aligned to Silva 16S rRNA sequence database (39), and preclustered to allow up to a 2-bp difference between sequences (40). Chimeras were detected using UCHIME (41) and were culled along with chloroplast and mitochondrial sequences. Sequences were then classified using the Ribosomal Database Project version 14 with a confidence score greater than 80% (42) and phylotyped to the family level.
Prior to any data analysis the number of sequences were normalized per sample to at least 2,000 sequences. Beta diversity was calculated using the Theta YC distance metric with the family-level data and visualized using principal coordinates analysis (PCoA).

**Bacterial qPCR**

DNA was extracted from proximal colonic contents using an ISOLATE Fecal DNA Kit (Bioline). qPCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) using a SensiFAST SYBR No-ROX Kit (Bioline) with 20 ng of bacterial DNA and the following primers: *Helicobacteraceae* primers forward (5'-CCGCAAATTCCAGCAATACTT-3') and reverse (5'-TCGTTCCAAAATGACACAGGGTG-3'); *Lactobacillaceae* 16S primers LabF362 (5'-AGCAGTACGGGAATCTTCCA-3') and LabR677 (5'-CAACCGGTCAACAGTTGAGG-3'); *Porphyromonadaeaceae* forward: 5’GGTGTGCGCTTTAAGTCCAT3’ and reverse: 5’CGGGA(T/C)GTAAGGGCCGTGC3’. Ten-fold serial dilutions of plasmid-based *H. hepaticus*, *L. reuteri*, *L. johnsonii*, or *Tannerella forsythia* genomic DNA was used to generate a standard curve. Relative abundance was calculated by a ratio of the organism-specific DNA to total bacterial DNA used for the amplification.

**Lactobacillus treatments**

*L. johnsonii*, *L. reuteri*, and *L. rhamnosus GG* (LGG) were grown overnight at 37°C in Lactobacilli MRS Broth (EMD Chemicals). Cultures were diluted the next day in sterile PBS, and concentrated to 10^10 CFU/mL. On day 2 of each DSS cycle mice were administered 100 μL i.p. of *L. johnsonii*, *L. reuteri*, and *L. rhamnosus GG* per gavage.

**ELISA**

Colonic lamina propria was isolated, pelleted, and protein determination was performed by Bradford assay. An ELISA was performed according to the product instructions from BD OptEIA Kits (IFNγ and IL10) and R&D Systems Kits (IL13 and IL17).

**Quantitative reverse transcription PCR**

Colonies were flushed with PBS, opened longitudinally, and segmented into two sections. One-third of the entire length of the colon measured up from the anus was denoted as distal, while the remaining two-thirds of the colon were identified as proximal. RNA was extracted from mucosal scrapings using ISOLATE II RNA Mini Kit (Bioline) and reverse transcribed into cDNA with SensiFAST cDNA Synthesis Kit (Bioline). qPCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) using a SensiFAST SYBR No-ROX Kit (Bioline) with the following primers: *Tbet* forward 5’ GCCTACAATGCCAACAACCACA-3’, *Rorc* reverse 5’-ATTGATGAGAACCGGGCCTGTA-3’, *Foxp3* forward 5’-AGAGAGTTATTGAGGTTGG-3’, *Foxp3* reverse 5’-GCTGAGATGTCGCTCTTG-3’; and 18S forward 5’-GTAACCCGTGAACCCCAT-3’, and 18S reverse 5’-CTATCCAATCGTGTGACGG-3’. Gene expression levels for each individual sample were normalized to that of 18S. Fold changes in gene expression were relative to unstimulated controls and calculated using the ΔΔCt method.

**Dendritic cell isolation and stimulation**

To purify dendritic cells, mesenteric lymph nodes were digested with 400 units per mL of collagenase type IV (Sigma-Aldrich). After filtering, the cells were resuspended in 22.5% Optiprep (Sigma-Aldrich), overlaid with Hank’s Balanced Salt Solution Saline (HBSS, Sigma-Aldrich) and centrifuged at 670 g for 30 minutes. Dendritic cells were harvested from the interface of the HBSS and Optiprep, incubated with anti-CD11c (Miltenyi Biotech), and positively selected for CD11c using Automacs (Miltenyi Biotech). After isolation, the cells were stimulated overnight with bacterial lysates at 37°C before performing the ELISAs.

**Recombinant IL10 and cIL10 treatments**

In some experiments, mice were treated with probiotic bacteria and/or corresponding to the days of probiotic therapy, were administered 200 μg of anti-IL10 or anti-IgG1 (BioXCell) intraperitoneally. In other experiments mice were administered 1 ng of recombinant IL10 (rIL10; R&D Systems) diluted in 500 μL sterile PBS given intraperitoneally 3 times during every DSS cycle: on day 2, day 4, and the day after discontinuation of the DSS.

**Immune fluorescence**

Murine colons were rolled using the Swiss roll technique and fixed in 10% neutral buffer formalin (VWR International) overnight. Paraffin-embedded (without formalin) tissues were cut 5-μm thick. H&E staining was performed by AML Laboratories or in house using the following antibodies: Rabbit polyclonal IgG, (Abcam, ab27472), rabbit monoclonal IgG (Abcam, ab172730), anti-bcl2 (clone 3F11). Bromodeoxyuridine (BrdU) staining was performed as per the manufacturer’s instructions (Abcam).

For antigen retrieval, slides were placed into tubes containing sodium citrate buffer (pH 6.0; Sigma-Aldrich) and heated at 99°C in water bath. The slides were washed and blocked before staining with primary antibody. The slides with primary antibody were incubated overnight at 4°C in a humid chamber and the following day, washed and incubated with secondary antibody at 37°C for 1 hour. The slides were mounted with DAPI and confocal images were acquired using a Nikon Eclipse C1 Laser-Scanning Microscope (Nikon) fitted with a 60 Nikon objective (PL APO, 1.4NA) and Nikon image software.

**Western blot analysis**

Colonies scrapings of the whole colon were placed in RIPA buffer (VWR International) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). Samples were...
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normalized for protein content using Bradford Reagent (Bio-Rad, 500-0205). Protein was separated by size by SDS-PAGE using Mini-Protein tris-glycine gels (4%–15%; Bio-Rad, 456-1083) and transferred to polyvinylidene difluoride membrane for blotting. Membranes were blocked with 3% dry nonfat milk in TBS containing 0.05% Tween-20 and incubated with primary antibodies overnight at 4°C: anti-cleaved caspase-3 (clone 3F11, Abcam), anti-pAkt thr308 (clone 244F9, Abcam), and anti-pStat3 Y705 (clone S727, Abcam). Membranes were washed with TBS containing 0.05% Tween-20 and incubated with goat anti-rabbit-HRP secondary antibody (Santa Cruz Biotechnology) for 2 hours at room temperature. Membranes were developed using SuperSignal West Femto Maximum Sensitivity Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions and imaged using a Licor Odyssey.

Statistical analysis
Data are expressed either as the mean value ± SEM or as individual values. Specific statistical tests used for each experiment are described in the figure legends and performed in GraphPad PRISM (GraphPad Software LLC). P < 0.05 was considered significantly different.

Results
TLR6 deficiency exacerbates inflammation-associated colorectal cancer, while cohousing protects WT mice
TLR6-deficient (TLR6KO) and littermates (WT) were weaned from their mothers and separately housed by genotype (SH) or cohoused (CH) together, 2 weeks later the mice were treated with the mutagen azoxymethane followed by three rounds of DSS over 66 days (Fig. 1A). Despite little difference in weight loss (Fig. 1B), TLR6-deficient mice had an overall worse survival outcome compared with the SH-WT mice regardless of whether they were cohoused or housed with only other TLR6KO’s (Fig. 1C). Both SH- and CH-TLR6KO mice showed an increase in the size and number of tumors (Fig. 1D and E) compared with both the SH- and CH-WT mice. To our surprise, WT mice cohoused with TLR6KO mice exhibited smaller and less tumors than their CH-TLR6KO cage mates and compared with SH-WT mice (Fig. 1F and G). Analysis of H&E sections of the colon from these mice indicated CH-WT mice had less pathologic changes in the colon, less inflammation, and a reduction in overall pathology score than both their CH-TLR6KO cage mates and SH-WT mice (Fig. 1F and G). Consistent with their increased tumor number and mortality, the TLR6KO mice had significantly worse dysplasia (P = 0.02) and epithelial hyperplasia (P = 0.03) compared with SH-WT mice (Fig. 1G). Despite no significant differences in inflammatory parameters, hyperplasia, dysplasia, or adenomas, the SH-TLR6KO mice had more crypt loss, high-grade adenocarcinomas, and carcinomas than SH-WT mice (Fig. 1F and G). Compared with SH-WT mice, cohousing significantly reduced epithelial hyperplasia (P = 0.050) and dysplasia (P = 0.02) in WT mice. In contrast, the CH-TLR6KO mice had significantly more hyperplasia, dysplasia, a greater extent of disease, and more adenomas than the CH-WT mice (Fig. 1F and G).

Our group and others have demonstrated a role for TLR6 and its binding partner, TLR2, in modulating inflammation via the induction of IL10. Analysis of the lamina propria of the colon of AOM/DSS-treated mice showed that TLR6KO mice, regardless of whether they were co- or single housed, had significantly higher levels of IL17, IFNγ, and corresponding transcription factors Rorc and Tbet, compared with WT mice housed similarly (Fig. 2A and B). Interestingly, only the CH-WT mice, which developed less tumors, had significantly elevated levels of IL10 and higher expression of the transcription factors Gata3 and Foxp3 compared with SH-WT mice, and their cohoused TLR6KO counterparts (Fig. 2A and B). Taken together, these data indicate that loss of TLR6 signaling causes an increase in tumor number and worsens survival, while cohousing protects WT mice from inflammation and developing tumors.

The exacerbated inflammatory response following AOM/DSS observed in the TLR6KO mice could be a result of deficient immune-modulatory mechanisms or mediated by a hyperinflammatory microbiota associated with the TLR6KO mice. To begin to address both of these questions we measured IL10 and IL12p40 production from naive WT and TLR6KO bone marrow–derived dendritic cells (BMDC) after stimulation with colonic contents harvested from cohoused and separately housed mice at day 66 of AOM/DSS treatment, or naive age-matched controls. IL10 production was significantly higher in WT BMDCs cultured with colonic contents from either naive or AOM/DSS-treated cohoused mice compared with TLR6KO BMDCs stimulated with the same contents (Fig. 2C). In contrast, separately housed WT and TLR6KO contents elicited much more IL12p40 and very little IL10 (Fig. 2C). When the BMDCs were derived from TLR6KO mice, we observed a poor IL10 response and elevated IL12p40 levels from all samples except naive WT and TLR6KO colonic contents (Fig. 2C). Taken together, these data indicate that the colonic contents of cohoused TLR6KO and WT mice seemed to induce an IL10-dominant response and dampen the levels of IL12p40, while the colonic contents of both separately housed WT and TLR6KO promoted a more inflammatory response with high IL12p40 and low IL10. These data also suggest that in the context of TLR6 deficiency, colonic contents normally associated with high IL10 instead induce a robust IL12p40 response.

Both genotype and housing influence dysbiosis associated with AOM/DSS
We hypothesized that both genotype and housing would contribute to alterations in the composition of the microbiota after AOM/DSS treatment. 16S rRNA sequencing was performed by scraping the colon with glass slides to capture both luminal- and mucosal-associated microbiota in naive age-matched mice and day 66 AOM/DSS-treated mice. Analysis of the operational taxonomic unit (OTU) at the phylum level.

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revealed that cohousing significantly affected the composition of WT and TLR6KO mice, both naïvely and after AOM/DSS treatment (Fig. 3A). Prior to AOM/DSS treatment, the colonic contents of SH-WT and SH-TLR6KO mice were dominated by taxa within Firmicutes, while cohousing WT and TLR6KO mice together lead to a dominance of Bacteroidetes (Fig. 3A). Following AOM/DSS treatment there were major shifts in the phylum level, regardless of genotype or housing (Fig 3A). Despite the absence of TLR6, cohoused mice shared a similar microbiome, dominated by the families Lactobacillaceae, Clostridiaceae, and Erysipelotrichaceae (Fig. 3B). In contrast, genotype seemed to impact the composition of the separately housed mice after AOM/DSS treatment, in which we observed a significant increase in Helicobacteraceae and a small increase in Porphyromonadaceae in SH-WT mice (Fig. 3B). This compositional phenotype was reversed in SH-TLR6KO mice, which had much a higher abundance of Porphyromonadaceae and only a slight increase in Helicobacteraceae (Fig. 3B).
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Figure 2.
Colonic lysates from cohoused mice are less inflammatory and induce IL10 in a TLR6-dependent manner. A, Levels of indicated cytokines from colonic lysates measured by ELISA. B, Relative mRNA transcript levels of indicated transcription factors from colonic lysates were assessed by qRT-PCR using naive WT and TLR6KO (6KO) mice as controls. C, IL10 (left) and IL12p40 (right) protein production from BMDC generated from indicated naive mouse (WT or TLR6KO) and stimulated with colonic lysates from indicated donors for 24 hours (N, naive; +, present; –, absent). A–C, Data are expressed as mean ± SEM of 6–8 mice from two independent experiments. *, P < 0.05; **, P < 0.01 two-way ANOVA with Bonferroni post hoc test.

taken pre- or post-AOM/DSS treatment, whereas CH-WT mice have a microbiota more similar to naive mice regardless of AOM/DSS treatment (Fig. 3C). PCoA revealed a higher degree of likeness pre-AOM/DSS treatment in cohoused WT and TLR6KO mice than to their respective single house counterparts, suggesting that housing exerted a strong influence on composition in a steady state (Fig. 3D). This influence was maintained under inflammatory conditions as the cohoused WT and TLR6KO mice clustered separately from both SH-WT and SH-TLR6KO following AOM/DSS treatment (P = 0.006, AOM/DSS SH-WT vs. CH-WT; Fig. 3D). However, the compositional changes that occurred in the SH-WT and SH-TLR6KO during AOM/DSS treatment were highly influenced by genotype as they showed distinct clusters in the PCoA (Fig. 3D).

Genotype and housing influence the kinetics and composition of the gut microbiota during AOM/DSS

The genus-level bacteria that were significantly affected by the interaction of housing and genotype by ANOVA, included Porphyromonas (P < 0.0001), Tannerella (P < 0.0001), Prevotella (P = 0.0003), Lactobacillus (P < 0.0001), and Clostridium (P = 0.0122; Supplementary Fig. S1A). To more fully understand the changes in the composition, we collected colonic samples over the course of AOM/DSS treatment and performed PCR using primers that bind within conserved areas of 16S rDNA to identify bacterial families (43). We found that the housing status had a significant impact on levels of bacterial DNA in the colon (Supplementary Fig. S1B). In naïve cohoused mice, roughly 50% of the total 16S rDNA was identified as Porphyromonadaeae, compared with only 15% in naïve single-housed mice of both genotypes (Supplementary Fig. S1B). During AOM/DSS treatment, the levels of Porphyromonadaeaeae decreased in the cohoused mice regardless of genotype, increased in the SH–TLR6KO, and stayed relatively the same in SH–WT mice (Supplementary Fig. S1B). In all untreated mice, 30%–40% of the total 16S DNA belonged to Lactobacillaceae. Upon AOM/DSS treatment we found that the SH–TLR6KO mice were able to maintain levels of Lactobacillaceae DNA until the second round of DSS, while these levels dropped significantly after the first round of DSS in SH–WT mice (Supplementary Fig. S1B). In the cohoused mice we found
that housing status had a positive effect on the amount of Lactobacillaceae DNA, as neither CH-WT nor CH-TLR6KO mice exhibited a reduction after the first or second rounds of DSS, and both genotypes maintained relatively consistent levels of Lactobacillaceae DNA throughout the experiment (Fig. 4B; Supplementary Fig. S1B). After the first round of DSS, when the levels of Lactobacillaceae DNA dropped in the SH-WT mice, we found that Helicobacteraceae DNA increased from 1% to 15% (Supplementary Fig. S1B). Unlike the SH-WT, we saw no increase in Helicobacteraceae in the cohoused mice or the SH-TLR6KO mice (Supplementary Fig. S1B). Taken together, these data demonstrate the absence of TLR6 signaling affects the compositional changes during AOM/DSS and that cohousing allows a maintenance of probiotic Lactobacillaceae, suggesting that both genotype and housing influence the kinetics and composition of the gut microbiota during AOM/DSS.

Restoring commensals ameliorates disease and reduces inflammatory cytokines in WT mice via a TLR6-dependent mechanism

Many Lactobacillus exist within our gastro-intestinal tract and confer health benefits, thus the overall reduction in the abundance of Lactobacillus observed in SH-WT and SH-TLR6KO during inflammation-associated colorectal disease.
Figure 4.
Restoration of Lactobacillus reduces tumor burden and suppresses inflammation in a TLR6-dependent and -independent manner. A, Method. Mice received an oral inoculation of sham (media; n = 9), L. johnsonii and L. reuteri (Lj/Lr; n = 11), or LGG (n = 6) at the time points indicated in the text and Materials and Methods section. Percent weight change (B) and number of macroscopic tumors from mice (C) at the time points indicated in the text and Materials and Methods section. Levels of indicated cytokines from colonic lysates as measured by ELISA. D, Levels of indicated cytokines from colonic lysates as measured by ELISA. E, Concentration of IL10 and IL12p40 produced from WT or TLR6KO (6KO) BMDC stimulated for 24 hours with 10^6 cfu indicated Lactobacillus species. F, Relative DNA levels of indicated bacterial families to total 16S rDNA from colon at day 66. Same method as in (A, above) except WT mice were given an intraperitoneal injection of rIL10 instead of Lactobacillus (G) and the number of tumors (H) and the relative DNA levels of indicated bacterial families to total 16S rDNA from colon at day 66 (I). A–I, Data are the mean ± SEM of 7–12 mice pooled from three independent experiments. *P < 0.05; **P < 0.01, Student’s t-test (C–F); Two-way ANOVA with Bonferroni post hoc tests (H and I).
cancer led us to hypothesize that a recolonization strategy using Lactobacillus could be efficacious in treating disease. To accomplish this, mice were administered a mixture of two taxa that were the most reduced as determined by OTUs in our tumor model (L. johnsonii and L. reuteri) or a species that was not found either naively or post-AOM/DSS (L. rhamnosus GG). To ensure that any effect on tumor development was not due to the metabolism of AOM by the Lactobacillus, the recolonization was performed 5 days post-AOM injection, beginning simultaneously with DSS administration, and given every other day for a total of four feedings. This regimen was repeated for each round of DSS (Fig. 4A). Physical observations after administration of L. johnsonii/L. reuteri (Lj/Lr) revealed no effect on weight (Fig. 4B), however the SH-WT mice treated with Lj/Lr had significantly fewer tumors than the media control–treated SH-WT mice (Fig. 4C). Furthermore, the reduction in tumor number seemed to be dependent upon TLR6 expression as Lj/Lr treatment failed to reduce tumor number or size in the SH-TLR6KO mice (Fig. 4C). In contrast, treating SH-WT mice with L. rhamnosus (LGG) failed to suppress both tumor number and size (Fig. 4C).

Analysis of the mucosal cytokines associated with Lactobacillus treatment revealed distinct cytokine patterns. SH-WT mice treated with Lj/Lr had a significant increase in anti-inflammatory IL10 and subsequent suppression of inflammatory cytokines, IFNγ and IL17, compared with media–treated WT mice (Fig. 4D). The observed cytokine effects were also mediated by TLR6 as Lj/Lr–treated SH-TLR6KO mice were unable to induce IL10 or suppress IFNγ or IL17 (Fig. 4D). In contrast, treatment with LGG had no effect on cytokine production and seemed to be independent of TLR6 signaling as WT and TLR6KO mice had similar cytokine patterns (Fig. 4D).

In addition to their ability to secrete antimicrobial peptides (44) and shift the pH of the colonic environment to kill pathogenic bacteria (45), Lactobacillus are also well known for their anti-inflammatory properties (46). Our group and others have shown that TLR6 signaling in antigen-presenting cells promotes IL10 production (47) and polarize anti-inflammatory T cells (48). As we observed IL10 production in SH-WT mice treated with Lj/Lr, but not by LGG, we wanted to assess the ability of a panel of lactic acid–producing bacteria to induce IL10 and IL12p40 and determine their dependency on TLR6 by using BMDCs from naive WT and TLR6KO mice. L. johnsonii, L. reuteri, and L. casei induced IL10 and IL12p40 from WT BMDCs, but only IL10 was dependent upon the expression of TLR6 (Fig. 4E). LGG-stimulated BMDC were able to produce IL10 regardless of TLR6 expression and had a robust TLR6-independent IL12p40 response (Fig. 4E). These data suggest that LGG induces low amounts of IL10 through a TLR6-independent mechanism and could explain its inability to protect WT mice.

We next wanted to determine whether the Lj/Lr treatment had any effect on the microbiota by screening the colonic contents of mice at the end of the AOM/DSS treatment by qPCR using the same primers used in Fig. 4. Confiming our 16S and qPCR data, we detected high amounts of Helicobacteraceae DNA in colons from SH-WT mice, while SH-TLR6KO mice had higher amounts of Porphyromonadaeae DNA and only a small amount of DNA from Helicobacteraceae (Fig. 4F).

Treatment with Lj/Lr was able to reduce the levels of Helicobacteraceae and Porphyromonadaeae in both the SH-WT and SH-TLR6KO mice, respectively (Fig. 4F). Despite the lack of any antitumor activity in our model, we found that LGG treatment was also able to reduce the amount of Helicobacteraceae and Porphyromonadaeae DNA recovered from the colons of both SH-WT and SH-TLR6KO mice. Interestingly, treatment by either Lj/Lr or LGG resulted in an increase in Lactobacillaceae DNA in both WT and TLR6KO mice (Fig. 4F).

IL10 is responsible for the partial reduction in tumor number but is dispensable for changes in the microbiota

The molecular pathobiology of colorectal cancer has implicated inflammation in the promotion of tumor progression, invasion, and metastasis (49). A clear example of this is the finding that patients with IBD are at higher risk of colorectal cancer (50). One mechanism that could account for the reduction in tumor number observed in SH-WT mice given Lj/Lr may be the ability to limit inflammation via the production of IL10. To evaluate whether IL10 alone was sufficient to suppress tumor number and limit colonization by Helicobacteraceae, we substituted Lj/Lr treatments with direct intraperitoneal administration of rIL10 (Fig. 4G). This protocol allowed us to determine whether IL10 could mediate any of the phenotype observed in Lj/Lr–treated WT mice. Furthermore, it would allow us to determine the requirement for TLR6, as administration of rIL10 to TLR6KO mice would bypass the need for TLR6 signaling, and these mice should behave more similarly to the WT mice treated with AOM/DSS. Similar to treatment with Lj/Lr, rIL10 provided a partial reduction in tumor number in SH-WT mice (Fig. 4H), but it failed to reduce the amount of Helicobacteraceae DNA (Fig. 4H and I). As expected, the TLR6KO mice treated with rIL10 treatment also had a significant reduction in the number and size of tumors compared with control–treated TLR6KO mice given AOM/DSS (Fig. 4H). However, the number of tumors was still significantly higher in the TLR6KO mice treated with rIL10 than WT mice given rIL10 (Fig. 4H) suggesting that there may be other molecular pathways contributing to the development of tumors in the TLR6KO mice. Similar to WT mice, we found no effect on the level of Helicobacteraceae or Porphyromonadaeae DNA in the colons of the rIL10–treated TLR6KO mice.

The requirement for Lactobacillus–induced IL10 was also further determined by coadministering a neutralizing antibody against IL10 beginning 1 day prior to treatment with Lj/Lr and continuing for 2 days posttreatment (Fig. 5A). Coadministration of an IL10–neutralizing antibody with Lj/Lr had little effect on the weight of SH-WT or SH-TLR6KO mice (Fig. 5B). Neutralizing IL-10 in the absence of Lj/Lr had no effect on the number of tumors observed in WT or TLR6KO mice.
mice (Fig. 5C). Coadministration of anti-IL10 with Lj/Lr only partially reversed the reduction in tumor number and had no effect on the levels of Helicobacteraceae DNA in WT mice (Fig. 5C and D). As expected, neutralizing IL10 during Lj/Lr treatment in TLR6KO mice had little effect, likely due to the inability of TLR6KO mice to produce IL10 during treatment (Fig. 5B–D). Analysis of the mucosal cytokines demonstrated that neutralizing IL10 was able to reverse the reduction in the levels of IFNγ and IL17 observed in Lj/Lr-treated SH-WT mice (Fig. 5E). Taken together, these results demonstrate that IL10 production by Lj/Lr can partially explain the reduction in tumor size and number. While IL10 did play a partial role in the reduction of tumors, it had no impact on the amount of Helicobacteraceae, Porphyromonada-ceae, or Lactobacillaceae DNA. These data thereby dissociate the anti-inflammatory effects of IL10 from the regulation of the microbiota in inflammation-associated colorectal cancer.

Impaired apoptosis during AOM/DSS in TLR6-deficient mice

While the data above provide evidence as to how cohousing would lead to reduced tumors in the WT mice, it does not
address why both single and cohoused TLR6KO mice have more tumors than the SH-WT mice. We hypothesized that in the absence of TLR6 an important cellular regulatory pathway may be altered leading to an environment more sensitive to mutagenesis. We also hypothesized that this effect would be independent of a specific microbiota signature, as the CH-TLR6KO and SH-TLR6KO have distinct colonic microbial compositions.

One of the early steps in colorectal cancer progression is the increase in epithelial cell proliferation and/or an inhibition of apoptosis. To assess the proliferation of enterocytes, we performed IHC staining on colon sections of AOM/DSS-treated WT and TLR6KO mice for Ki67 and BrdU. Ki67 is a commonly used marker that identifies cells that have recently undergone proliferation by labeling S-, G1-, and G2-phases of the cell cycle, while BrdU incorporates into newly synthesized DNA in actively replicating cells during S-phase. There was an increase in the number of Ki67+ cells found in both the cohoused and single-genotype–housed TLR6KO mice compared with WT mice (Fig. 6A). Two hours following injection of BrdU into AOM/DSS-treated mice the colons were removed and stained for actively proliferating cells. In contrast to the Ki67 staining, we identified on average 3–4 BrdU+ cells per crypt, regardless of housing or genotype (Fig. 6A). These data indicate that the disruption of TLR6 signaling or the housing status was not impacting the turnover of intestinal stem cells. To assess whether the TLR6KO mice may have defects in apoptosis (51), we performed IHC on AOM/DSS colons looking for the antiapoptotic factor Bcl-2. Analysis of the stained tissue revealed many Bcl-2+ cells within the epithelium, as well as in the lamina propria of SH-TLR6KO and CH-TLR6KO mice compared with WT mice (Fig. 6B). TLR2 signaling has been shown to signal through the PI3K/Akt pathway (52). PI3K/Akt has been shown to phosphorylate Stat3, which has been shown to influence apoptosis and proliferation signals in enterocytes (51, 52). We performed Western blot analysis on the colonic enterocytes at day 28 during AOM/DSS to assess whether signaling pathways upstream and downstream of Bcl-2 were disturbed in the TLR6KO mice, thereby driving less apoptosis and making these mice more sensitive to AOM/DSS. Both SH- and CH-TLR6KO mice had lower levels of cleaved caspase-3 compared with WT mice (Fig. 6C). Despite the reduction in cleaved caspase-3 and the increase in Bcl-2, the TLR6KO mice had similar levels of phosphorylated Akt and phosphorylated Stat3 (Fig. 6C). Taken together, these data suggest that the deficiency in TLR6 may lead to an antiapoptotic response that is Stat3- and Akt-independent, but involves Bcl-2 and cleaved caspase-3.

Discussion

In this study we established a potential treatment for colorectal cancer using a microbiome-associated therapy that restores commensal species lost during inflammatory disease. Compositional data generated by 16S rRNA sequencing of colons from AOM/DSS-treated WT mice revealed a distinct microbiota from their naive counterparts. Most noticeably, was an increase in the abundance of cancer-promoting Helicobacteraceae, and a loss of beneficial Lactobacillaceae, restoring Lactobacillus dampened inflammation and reduced tumor number. These data underscore the need for a more personalized approach in using probiotics to treat or ameliorate disease. The rationale “not all probiotics are created equal” has been used to explain the failure of probiotics in clinical trials, focusing on differences between bacterial species, strain, or dose. However, data from this study and others (53, 54) suggest that the personal relationship established between the innate immune system, intestinal cells, and the resident commensals determine whether a given probiotic will be effective. Our study also demonstrates that the effects on inflammation-associated colorectal cancer by L. johnsonii and L. reuteri (Lj/Lr) are mediated by multiple mechanisms that can be dissociated from each other by the involvement of IL10. While Lactobacillus has been shown to treat disease via IL10-dependent (55, 56) and IL10-independent (57–60) mechanisms, our study demonstrates that the most efficient antitumor effects were associated with inhibiting inflammation and restoring microbial ecology by reducing the abundance of Proteobacteria.

Genetics play an important part in disease susceptibility and response to treatment, yet their impact on the microbiota is still not understood. Here, genetic ablation of Tlr6 in mice was associated with an increased incidence of tumors, and insensitivity to Lj/Lr therapy. Our data suggests that expression of TLR6 is critical for the production of IL10 during treatment with Lj/Lr, confirming our previous studies linking TLR6 signaling with IL10 (61) and further implicating the role of TLR6 in the sensing of endogenous microbial signals. TLR6 signaling did have an effect on the kinetics associated with the loss of Lactobacillus, with a more gradual decrease observed in the TLR6KO mice. The prolonged presence of the Lactobacillus had no beneficial effect on tumor number in TLR6KO mice, likely due to their inability to sense immune-modulatory ligands through TLR6 and induce IL10. However, the prolonged presence of Lactobacillus in AOM/DSS-treated TLR6KO mice had a protective effect on WT mice when cohoused together. It may be that the WT mice were able to maintain Lactobacillus levels due to ingestion/exposure of Lactobacillus in TLR6KO stool through coprophagy and this helped prevent tumors in these mice. Indeed, our data shows that restoring Lactobacillus species during DSS exposures was able to reduce the tumor burden in SH-WT mice in part through production of IL10.

Compositional analysis of the colonic microbiota by 16S sequencing and qPCR revealed that SH-WT mice had an increase in Helicobacteraceae, while the TLR6KO mice had an increase in Porphyromonadaeae. Each of these increases occurred when Lactobacillaceae was lowest after the first or second round of DSS in the WT and TLR6KO mice, respectively. It is plausible that Helicobacteraceae has only a small window of opportunity when a comfortable niche is created by
Proliferation and apoptosis of colonocytes are altered in TLR6KO (6KO) mice. A, Pictures of colons from mice at day 28 during AOM/DSS treatment stained with Ki67 or from mice injected with BrdU 2 hours before the end of the experiment with summary and quantitation of positive-stained cells. Representative image from 1 of 3–5 mice per group. Quantification of crypts was performed in a blinded manner on 7 mice per group with at least five crypts per mouse assessed. B, Histologic sections of the colon from mice at day 28 stained with anti-Bcl-2 antibody or isotype control. Representative image from 1 of 3–6 mice per group. C, Western blot of whole colon from individual mice at day 28 post-AOM/DSS blotted for phosphorylated Akt, phosphorylated Stat3, and cleaved caspase-3 and Actin as a loading control. A, B, P < 0.05. Two-way ANOVA with Bonferroni post hoc test.
the absence of Lactobacillus. If this window is missed than other commensals, such as Porphyromonadaceae, may fill that niche. Our data would suggest that innate immune sensing of the endogenous microbiome impacts dysbiosis through altering the kinetics, in which bacterial species are reduced, and by contributing to the inflammatory milieu of the local tissue microenvironment. These data imply that the genetics of the host are linked to dysbiosis through expression of such receptors and could possibly explain why many of the polymorphisms associated with IBD involve innate immune sensing.

The contribution of innate signaling to the cytokine milieu of the local tissue microenvironment must also not be overlooked. Here, IL10 production by the Lactobacillus was almost completely dependent on TLR6 as BMDCs deficient in TLR6 failed to produce IL10 after stimulation by these bacteria, and also by the finding that the beneficial effects of Lactobacillus are lost in TLR6KO mice. Importantly, we observed that after treatment with Lactobacillus, decreases in Helicobacteraceae and Porphyromonadaceae were both IL10 and TLR6 independent. While this study did not identify the mechanism by which Lj/Lr modulates the microbiota, treatment with rIL10 or neutralization of IL10 during Lj/Lr administration had little effect on the levels of Helicobacteraceae and Porphyromonadaceae, thus ruling out a role of IL10 in dysbiosis and indicating an IL10-independent role for Lactobacillus in maintaining microbiotal ecology.

Regardless of housing, TLR6KO mice had higher tumor numbers and higher mortality compared with the WT mice. This increased tumor number is likely independent of the microbiota as single and cohoused TLR6KO mice had very different microbiomes at both the induction of the AOM/DSS microbiota. First, microbiota-associated therapies can protect against inflammation and its effects on the microenvironment. The strong influence of genetics and environment on the microbiota and the response to microbiota-associated therapy suggest that a personalized approach to understanding the microbiota during disease will be critical to the development of new microbiota-associated therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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Cancer Prevention Research

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