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Gut microbiota protects against gastrointestinal tumorigenesis caused by epithelial injury

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Abstract

Inflammation is a critical player in the development of both colitis-associated and sporadic colon cancers. Several studies suggest that the microbiota contribute to inflammation and tumorigenesis; however, studies to understand the role of the microbiota in colon tumor development in germfree (GF) mice are limited. We therefore studied the effects of the microbiota on the development of inflammation and tumors in germfree and conventionally-raised specific pathogen-free (SPF) mice treated with azoxymethane (AOM) and dextran sulfate sodium (DSS). We discovered that GF mice developed significantly more and larger tumors compared to that in SPF mice after AOM and DSS treatment despite the lack of early acute inflammation in response to chemically-induced injury by DSS. Although the extent of intestinal epithelial damage and apoptosis was not significantly different in GF and SPF mice, there was a delay in intestinal epithelial repair to DSS-induced injury in GF mice resulting in a late onset of proinflammatory and protumorigenic responses and increased epithelial proliferation and microadenoma formation. Recolonization of GF mice with commensal bacteria or administration of LPS reduced tumorigenesis. Thus, although commensal bacteria are capable of driving chronic inflammation and tumorigenesis, the gut microbiota also have important roles in limiting chemically-induced injury and proliferative responses that lead to tumor development.

Keywords

gut microbiota; germfree; colon tumorigenesis; inflammation; injury

Colorectal cancer (CRC) is the third most common cancer in the United States. One of the major risk factors for the development of CRC is the presence of chronic inflammation as occurs in patients with inflammatory bowel disease (IBD) (1). Even in cases of sporadic colon cancer, inflammatory mediators have clearly been associated with tumor promotion within the tumor microenvironment (2, 3). Recently, there has been significant interest in the role of the gut microbiota in the development of intestinal inflammation and cancer. Epithelial barrier defects associated with adenoma formation in mice harboring the *Apc*$^{Min/+}$ mutation in CDX2-expressing colon cells result in bacterial translocation into tumors and

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enhancement of inflammatory-mediated tumor growth, suggesting that the gut microbiota promote inflammation important for tumor progression (4). Several studies also suggest that disruption of the normal microbiota that results in dysbiosis is associated with colitis and carcinogenesis (5–7). Thus, the current dogma is that the gut microbiota contributes to colitis and tumorigenesis, which is consistent with observations that inflammation and tumor development in several mouse models is abrogated in germ-free conditions or with antibiotic depletion of intestinal microbes (8–11). Notably, both IL-2-deficient and IL-10-deficient mice, which under conventional conditions develop spontaneous colitis, have significantly reduced or absent intestinal inflammation in germ-free conditions (12, 13), and furthermore, deficiency in *MyD88*, an adaptor protein downstream of Toll-like receptor (TLR) signaling that is involved in bacterial sensing, ameliorated both inflammation and tumor development in IL-10-deficient mice (8, 9). In the $Apc^{Min/+}$ mouse model of spontaneous colon tumorigenesis, deletion of the *MyD88* gene results in fewer intestinal tumors as well (14). Altogether these studies suggest a detrimental effect by the gut microbiota in promoting intestinal inflammation and tumorigenesis. However, a beneficial role for commensal bacteria in suppressing carcinogenesis has also been demonstrated. For example, *Lactobacillus* and *Bifidobacterium* have been shown to have anticarcinogenic effects through such activities as enyzmatic detoxification of carcinogens, production of short chain fatty acids that promote intestinal homeostasis and regulation of epithelial proliferation and apoptosis (15). Similarly, TLR signaling, presumably through commensal bacteria, has been implicated in increased resistance to chemically-induced colitis and promotion of intestinal epithelial repair (16, 17). In addition, mice deficient in bacterial sensors, such as members of the Nod-like receptor (NLR) family have significantly more inflammation-induced tumors than wildtype mice (5, 18–23).

To determine the role of the gut microbiota in colon tumorigenesis, we tested germfree (GF) mice in the azoxymethane (AOM)/dextran sulfate sodium (DSS) mouse model of inflammation-associated tumorigenesis. In this model, GF or conventional, specific pathogen-free (SPF) mice were given a single intraperitoneal (i.p.) injection of the carcinogen, AOM, followed by multiple rounds of DSS, which injures the intestinal epithelial and induces colitis (24, 25). In contrast to other mouse models, we found that the presence of gut bacteria was critical for suppressing tumorigenesis as GF mice developed more tumors than SPF mice. The absence of commensal bacteria in GF mice was associated with poor inflammatory responses to resolve intestinal injury, resulting initially in a hypoproliferative epithelium and delayed regeneration of the epithelium. Epithelial proliferation did eventually occur in GF mice after DSS-induced injury, but was associated with significantly elevated pro-inflammatory and protumorigenic mediators as well as abnormal epithelial restitution with microadenoma formation. The sterile inflammation that occurs in GF mice likely is mediated by MyD88-TRIF as GF mice deficient in both genes have fewer tumors. Our data suggest a critical role for the gut microbiota in promoting timely epithelial repair in response to intestinal injury to prevent dysregulated inflammation and epithelial proliferation. These findings are significant in that they demonstrate that commensal bacteria do not act solely as drivers of damaging inflammation and tumorigenesis, but highlight instead their beneficial role in maintaining intestinal health and homeostasis to prevent tumorigenesis.

Materials and Methods

Mice

SPF C57BL/6J mice were originally purchased from Jackson Laboratory and bred in-house. GF C56BL/6J mice were also originally obtained from Jackson Laboratory, rederived into GF conditions, and bred and maintained GF in the University of Michigan GF Mouse

facility. GF MyD88-TRIF-doubly-deficient mice were obtained as a kind gift from Kathy McCoy. GF mice were housed in bubble isolators and are free of all bacteria, fungi, viruses, and parasites. Sterility was verified by regular interval aerobic and anaerobic cultures as well as Gram stains of feces and bedding. Both SPF and GF mice were fed the same autoclaved chow diet. Adult (6- to 12-week old) mice were used for all experiments. All animal studies were approved by the University Committee on Use and Care of Animals.

Tumor induction

Mice were injected with 10 mg/kg AOM (Sigma-Aldrich) i.p. on day 0 followed 5 days later by a five-day course of 1% or 1.5% DSS depending on the lot of DSS in the drinking water. DSS water was sterilized by 0.2 micron filtration. Mice were then allowed to recover for 16 days with untreated drinking water. The 5 days of DSS followed by 16 days of untreated drinking water was repeated at least two times. Mice were sacrificed 3 weeks after the last cycle of DSS for tumor counting. Tumors in the colon were counted with the assistance of a magnifier and measured by calipers.

Assessment of inflammation

Colons were harvested from mice, flushed free of feces, and jelly-rolled for formalin fixation and paraffin embedding. Five-micron sections were used for H&E staining. Histologic assessment was performed in a blinded fashion using a previously described scoring system, but modified as follows (19). Sections were scored on a 3–4 point scale for three parameters, inflammation/cellular infiltration, epithelial lesions and epithelial regeneration, that were summed together. For inflammation, severity and distribution were separately assessed and combined into one score; assessment of the epithelium was evaluated by averaging the severity of crypt loss or ulceration over 15 fields; epithelial hyperplasia was scored based on severity and distribution.

Apoptosis and Proliferation

Colon sections from formalin-fixed, paraffin-embedded were assessed for apoptotic cells by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using the ApoAlert DNA fragmentation assay kit (Clontech Laboratories, Inc.). For tumors, number of apoptotic cells were counted and averaged over 3–5 high power fields (HPF), and for colon tissue sections of mice treated with DSS, the number of apoptotic surface epithelial cells per crypt was counted over approximately 150 crypts. Epithelial proliferation was assessed by Ki67 staining and proliferation index was assessed by counting the number of Ki67+ cells per crypt in approximately 50 well-aligned crypts.

Cytokine expression

Colonic tissue was homogenized and total RNA isolated using the Nucleospin RNA kit (Machery-Nagel). cDNA synthesis was performed using iScript (Bio-Rad), and cDNA was used for quantitative PCR using the SYBR Green Master Mix (Applied Biosystems) on the ABI 7900HT (Applied Biosystems). Ct values were normalized to the housekeeping gene β-actin. Primer sequences are available in Supplementary Methods.

Treatment of mice with lipopolysaccharide

GF mice were administered sterilely filtered lipopolysaccharide (LPS, *E. coli* O26:B6, Sigma) at 1 mg/ml in the drinking water beginning at 1 week prior to the administration of DSS (day -4 AOM/DSS protocol) and continued throughout the duration of the experiment. This concentration was selected based on the results of Rakoff-Nahoum *et al*., which demonstrated decreased mortality of commensal-depleted SPF mice with this concentration of LPS (16).

Statistical Analysis

Data are represented as means ± SEM. Comparison of tumor counts, cytokine expression, proliferation and apoptosis between SPF and GF mice were performed using Student's unpaired t test. The presence or absence of adenomas in SPF and GF mice was assessed by Fisher's exact test. P values <0.05 were considered statistically significant.

Results

The gut microbiota is important for epithelial-injury-associated colon tumor suppression

To directly interrogate the role of the gut microbiota in colon tumorigenesis, we used a well-established inflammation-associated colon cancer model (24), which mimics human colitis-associated colon cancer, but also has features resembling sporadic colon cancer, namely the prevalence of mutations affecting the Wnt signaling pathway and the progression of adenomatous polyps to carcinomas (26, 27). In this model, mice are injected with a single i.p. injection of the experimental carcinogen AOM followed by repeated rounds of water containing DSS which causes epithelial injury, increased intestinal permeability, resulting in bacterial translocation into the mucosa, and commensal-driven inflammatory responses. GF C57BL/6 mice are particularly susceptible to DSS-induced injury, and we observed 100% mortality with 5 days of 2.5% or 2% DSS together with AOM and was associated with complete loss of crypts in a significant proportion of the distal colon observed microscopically in moribund GF mice (Fig. S1). However, with lower concentrations of DSS, 100% survival of germfree mice can be achieved. After treatment with AOM/DSS, GF mice developed significantly more adenomatous tumors that were larger in size than that in conventionally-housed SPF mice (Fig. 1A–C). As described previously with tumors associated with the AOM/DSS model, tumors in GF mice were premalignant adenomatous polys associated with nuclear beta-catenin localization similar to that observed in SPF mice (Fig. 1D and S2)(26). Altogether these results strongly suggest that the gut microbiota can protect the host against the development of colon tumors secondary to chemically-induced epithelial injury and challenges to genomic integrity by chemical carcinogenesis.

Increased tumorigenesis in GF mice is not associated with alterations in levels of epithelial apoptosis

Increased host susceptibility to inflammation-induced tumorigenesis has been associated with increased epithelial destruction that promotes excessive proinflammatory, pro-tumorigeneic responses (19, 22, 23). Alternatively, enhanced cellular survival may also lead to increased tumor development (18). To investigate the first possibility, we assessed levels of DSS-induced apoptosis along the surface epithelium of the colon during the first round of DSS (day 8), which precedes the development of mucosal erosion and ulceration (19) and upon completion of DSS (day 10). At both of these timepoints, we observed similar numbers of apoptotic cells within the surface epithelium, suggesting no differences in early DSS-induced damage in SPF and GF mice (Fig. 2A). Consistently, GF mice did not have significant losses in weight compared with SPF mice during the initial rounds of DSS (Fig. S3). Similar to the early lesions, evaluation of tumors on day 98 after 4 rounds of DSS in SPF and GF mice also demonstrated no significant differences in levels of apoptotic cells within tumors (Fig. 2B), suggesting that the gut microbiota does not suppress tumor development by affecting epithelial apoptosis either before or after tumorigenesis.

GF mice exhibit impaired early inflammatory responses to intestinal injury followed by delayed inflammation and production of pro-inflammatory, pro-tumorigenic mediators

The development of tumors typically correlates with the extent of inflammation during the acute inflammatory response after the first round of DSS (19, 21, 23). We, therefore,

examined the colons of AOM/DSS-treated SPF and GF mice immediately after the first round of DSS at the peak of inflammatory responses and 1–2 weeks following when the epithelium has typically undergone restitution in mice in this model (19, 23). Inflammation was scored histologically based on the extent of inflammatory cell infiltration, mucosal erosion and extent of regenerating gland formation, or hyperplasia (see Methods). During the acute inflammatory phase (days 12–13), SPF mice had significantly higher histologic scores (Fig. 3A). Consistently, SPF mice exhibited increased recruitment of inflammatory cells compared with that in GF mice, particularly Gr1+ and CD11b+ cells, representative of both neutrophils and macrophages, within the colon lamina propria (Fig. S4), consistent with previous reports (17). The increased histologic score and inflammatory cell infiltration in SPF mice was accompanied by an elevation in the production of inflammatory cytokines and chemokines within the colon that are important for immune cell recruitment and wound repair, such as CXCL1, MIP-2, IL-6, IL-22, and Reg3γ as assessed by real-time PCR (Fig. 4). In contrast, upregulation of these cytokines and chemokines are significantly impaired during the acute inflammatory phase in GF mice on day 12 (Fig. 4).

In the AOM/DSS model, resolution of intestinal damage and inflammation typically occurs 1–2 weeks after the first round of DSS (23), just prior to the second round of DSS as reflected in the decreasing histologic scores in SPF mice (Fig. 3A) and evidence of regenerating epithelium with hyperplasia on day 13 (Fig. 3B). GF mice, on the other hand, continue to demonstrate evidence of persistent intestinal damage on day 13 (Fig. 3B) with loss of crypts and absence of hyperproliferative epithelium. By the second week on day 18 or day 26 just prior to the second round of DSS, SPF mice have nearly restituted their epithelium back to baseline; however, the colons of GF mice continue to have persistent mucosal damage and delayed formation of regenerating glands, resulting in higher histologic scores compared to SPF at these later timepoints (Fig. 3A and B). Associated with the higher histologic scores for GF mice, there is also a delayed, but significantly higher upregulation in pro-inflammatory mediators as well as factors involved in epithelial remodeling and growth such as the matrix metalloproteinase (MMP)-12, c-myc, and the epidermal growth factor family member epiregulin compared to that in SPF mice on day 17 (Fig. 4).

Delayed hyperproliferation in GF mice is associated with early microadenoma formation

We next examined levels of epithelial proliferation in the colons of SPF and GF mice early (day 12) and late (day 26) after the first round of DSS by Ki67 staining. During the acute inflammatory phase immediately after completion of the first round of DSS (day 12), when upregulation of inflammatory cytokines and recruitment of immune cells occurred in SPF mice, there was an increased number of Ki67+ epithelial cells associated with epithelial regeneration and subsequent near-complete resolution of inflammation by day 26 (Fig. 5A). In contrast, the colons of GF mice were in a hypoproliferative state with no evidence of any epithelial regeneration immediately after completion of the first round of DSS on days 12 and 13 (Fig. 5A), consistent with previous reports (17). However, on day 26, more than two weeks after completion of the first cycle of DSS and just prior to start of second round of DSS, when the colons of SPF mice have essentially normalized morphologically, we observed instead significantly elevated levels of epithelial proliferation in GF mouse colons as demonstrated by increased Ki67 staining within the epithelium (Fig. 5A). More importantly, the delayed hyperplasia in GF mice was not associated with normalization of the epithelium; rather, we observed formation of microadenomas within the mucosa of GF mice by day 26 in the distal rectum that were not present in SPF mice (Fig. 5B), specifically with no microadenomas present in the SPF mice group and microadenomas present in 100% of the GF mice group ($p<0.05$, Fisher's exact test, N=5 mice/group). However, in established tumors, there were no differences in proliferative activity between SPF and GF tumors (Fig. S5). Together, these results suggest that the gut microbiota is important for

promoting normal inflammation necessary for repair of damaged epithelium to prevent aberrant and delayed inflammatory and epithelial growth responses that lead to tumorigenesis.

GF mice deficient in TLR receptor signaling have reduced tumorigenesis

Despite the absence of bacterial-driven inflammatory responses in GF mice, inflammation and the upregulation of proinflammatory mediators still occur albeit late. In GF mice, this upregulation is clearly commensal-independent, and therefore must arise from endogenous signals that may be produced during tissue injury. TLRs, although primarily recognized as bacterial sensors, are also capable of recognizing endogenous ligands that are released during cell death and injury to mediate sterile inflammation (28–30). Moreover, MyD88 signaling is associated with induction of tumor promoting factors and promotes spontaneous intestinal tumorigenesis in $Apc^{Min/+}$ mice (14). We therefore hypothesized that in GF mice, pathologic activation of TLR signaling during sterile inflammation by persistent tissue injury results in increased tumorigenesis. To test this hypothesis, we treated B6 GF mice deficient in both MyD88 and TRIF (MyD88-TRIF DKO), adaptor proteins downstream of all TLRs, with AOM/DSS. Downregulation of all TLR signaling in GF MyD88-TRIF DKO was associated with reduced number and size of tumors compared to that in GF wildtype (WT) mice (Fig. 6A–C) although was not sufficient to limit tumor development to the same extent as that in SPF WT mice (Fig. 6A and B), suggesting that other pathways are also involved in tumor suppression.

The gut microbiota and its products limit AOM/DSS-induced tumorigenesis in GF mice

We next determined whether recolonization of GF mice with commensal bacteria by co-housing with SPF mice was sufficient to protect mice from DSS-induced injury and tumorigenesis. After cohousing GF mice with SPF mice for three weeks followed by AOM/DSS treatment, 100% survival of conventionalized GF mice was achieved with 2% DSS that was previously associated with 100% mortality in GF mice (Fig. S1 and S6A), and weight changes in conventionalized GF mice with AOM/DSS treatment more closely followed that of SPF mice (Fig. S6B). Importantly, the number of tumors that developed in recolonized GF mice after AOM/DSS was no longer significantly different from that in SPF mice (Fig. 7A). Furthermore, tumors were similar in size between recolonized GF and SPF mice (Fig. 7B). The similarity in tumor development between conventionalized GF mice and SPF mice was likely due to similar recovery times from DSS-induced injury as observed by insignificant differences in histologic scores after the first cycle of DSS (Fig. S7A and B) and in the kinetics of proinflammatory/proliferative marker induction as measured by real-time PCR between SPF and conventionalized GF mice (Fig. S7C). These results suggest that colonization of GF mice by microbiota is sufficient to limit DSS-induced injury and promote normal inflammatory responses to restore epithelial restitution and protect against tumorigenesis.

It has previously been demonstrated that lipopolysaccharide (LPS) produced by commensal bacteria increases resistance to DSS-induced injury by promoting inflammation and epithelial repair (16). We therefore wanted to determine whether administering LPS to GF mice would also protect against AOM/DSS-induced tumorigenesis. Indeed, continuous administration of LPS in the drinking water of GF significantly reduced the number of tumors in GF mice although the size of tumors that ultimately developed was not significantly different (Fig. 7E and F).

Discussion

In this study, we used GF mice to determine the importance of the gut microbiota in suppressing colon tumorigenesis using the AOM/DSS model. We demonstrated that in GF mice devoid of any microbiota, there is delayed upregulation of inflammatory responses associated with poor healing and restitution of DSS-induced epithelial damage. Despite the initial hypoproliferative state observed in GF mice, there is eventually, even in the absence of bacteria, a delayed induction of proinflammatory mediators and growth factors that leads to dysregulated epithelial proliferation and microadenoma formation without complete epithelial restitution. This delayed upregulation of proinflammatory and proliferative factors during sterile inflammation in GF mice likely occurs in part through MyD88 and/or TRIF as GF MyD88-TRIF DKO mice developed fewer tumors than GF WT mice. GF mice can also be rescued from these defects by recolonization with commensal bacteria or administration of the bacterial product LPS that has been previously demonstrated to be important for promoting epithelial repair (16).

Although the microbiota has been implicated in cancer prevention through its ability to detoxify potential carcinogens, an increase in AOM metabolism in GF mice due to the absence of bacteria is unlikely to be a reason for their increased susceptibility to tumorigenesis. This is because, in addition to metabolism of AOM by the liver to its active metabolites, bacterial β-glucuronidase also contributes to the conversion of AOM to its active metabolite methylazoymethanol rather than its detoxification (31), and therefore, the absence of bacteria would be expected to result in decreased metabolism of AOM and fewer tumors. The increased proliferative responses and inflammation that manifest late after initial DSS administration in GF mice are also unlikely to be due to differences in levels of DSS-induced intestinal epithelial damage as epithelial apoptosis and resultant epithelial damage early after the initial DSS treatment were not statistically different between SPF and GF mice (Fig. 2 and 3). Rather, the persistence of intestinal epithelial damage associated with impaired activation of inflammatory, wound repair pathways likely results in inappropriate proliferative responses later on that are further fueled by repeated DSS-induced damage and inflammation from additional cycles of DSS.

In our tumor studies with our colony of C56BL/6J GF mice, we reduced the concentration of DSS to enable GF mice to survive multiple rounds of DSS. At these lower concentrations of DSS, our analysis of colons at multiple timepoints reveal an early defect in inflammatory, wound-healing responses in GF mice that may have not been evident in other studies with other colonies of GF mice where higher concentrations of DSS resulted in significant damage and inflammation (32–34). With our colony, concentrations above 2% resulted in 100% mortality, but examination of their colons histologically showed significant mucosal damage and submucosal edema (Fig. S1). It would be interesting to determine whether specific bacterial populations previously demonstrated to have protective effects against colitis or bacterial products are effective in increasing survival in our colony of GF mice with higher concentrations of DSS, and whether the mechanism involves decreasing inflammation and damage, or promoting timely epithelial repair.

Our studies demonstrate an essential function for commensal bacteria in the prevention of colon tumorigenesis by facilitating epithelial repair. These results are in contrast to earlier reports of decreased inflammation-associated tumorigenesis in other mouse models such as the *Il-10*$^{-/-}$/AOM or *Apc*$^{Min/+}$ mouse model in which under GF conditions, inflammation and tumorigenesis are abrograted in the absence of bacteria (9, 35). The difference in outcome between these two models may be due to epithelial injury as a prominent feature of the AOM/DSS model, resulting in dependence on wound repair pathways for limiting tumor development. Thus, with the AOM/DSS model, in the context of chronic epithelial injury,

intestinal bacteria are critical for triggering “normal” inflammatory responses necessary for timely repair of injury and inhibition of tumorigenesis. Consistently, after the first cycle of DSS, GF mice exhibited decreased levels of recruitment of inflammatory cells (both Gr1+ and CD11b+), representing both neutrophils and macrophages, which have been demonstrated to be associated with effective wound repair and are poorly recruited in GF and *MyD88*$^{-/-}$ mice after DSS-induced intestinal injury (17, 36). Furthermore, GF mice that are recolonized with commensal bacteria demonstrate upregulation of factors involved in epithelial repair and restitution of the epithelium similarly to SPF mice (Fig. 7 and S7). LPS, a major component of intestinal bacteria that is recognized by TLR4 and signals through the downstream adaptor MyD88, has been previously demonstrated to promote the induction of cytoprotective factors, such as CXCL1, TNF-α, and IL-6, during physiologic inflammatory responses to DSS-induced injury (16), and was also capable of reducing tumor development in AOM/DSS-treated GF mice (Fig. 7D–F). It is also important to note, however, that the LPS used in this study was not highly purified, and may contain contaminating bacterial components that signal through other pattern recognition receptors (37). Furthermore, although the difference in tumors numbers in GF mice treated with LPS followed by AOM and 1% DSS was not statistically significantly different from that in SPF mice with the number of experimental mice used, tumors still developed whereas SPF mice developed none (Fig. 7E). It is therefore possible that other bacterial activities will also contribute to epithelial repair and tumor suppression. For example, other bacterial sensing mechanisms such as through the NLRs are also important for promoting wound repair and curtailing aberrant inflammatory responses during colitis-associated tumorigenesis (5, 6, 19, 20, 22, 23, 38, 39). Alternatively, the gut microbiota may also help promote intestinal epithelial homeostasis through the production of metabolic byproducts such as short-chain fatty acids, rather than through its direct immuostimulatory activities. This is consistent with studies demonstrating that short chain fatty acids ameliorate DSS-induced colitis when administered to GF mice (33).

Despite the absence of bacterial-driven inflammatory responses in GF mice, sterile inflammation can still occur. However, this results in pathologic proliferation and early microadenoma formation rather than epithelial restitution. This phenomenon is associated with upregulation of inflammatory mediators, such as CXCL1, MMP12, IL-6, that although are important initially for wound repair, are also implicated in tumor promotion (40–43). Similarly, IL-22, which was poorly induced in germ free mice and is important for repair, is significantly upregulated at later timepoints, which has been associated with tumor promotion (44). In addition, the aberrant, late inflammatory response is associated with upregulation of factors such as c-myc and epiregulin, which are involved in proliferation and tumorigenesis. In GF mice, this upregulation must arise from endogenous signals that may be produced during tissue injury in the absence of bacteria, resulting in sterile inflammation. Our results suggest that these sterile inflammatory responses that may predispose to tumor development are mediated through MyD88 and TRIF as GF MyD88-TRIF DKO mice developed fewer tumors than GF WT mice. MyD88 and TRIF are adaptor proteins that are downstream of the TLRs, which in addition to recognizing bacteria, also respond to molecules released during cell death, as can occur with DSS-induced injury (28–30). Moreover, MyD88 signaling is associated with induction of tumor promoting factors (14). Thus, our data suggests that in GF mice, persistent tissue damage results in inappropriate, pathologic activation of the MyD88 and/or TRIF signaling pathway that promote sterile inflammation, epithelial proliferation, and tumorigenesis. Although MyD88 is downstream of TLRs, the IL-1R/IL-18R pathways also utilize MyD88 as an adaptor protein(45–47), and therefore these non-TLR pathways may also be involved in promoting inflammation and tumorigenesis in GF mice. In addition, since GF MyD88-TRIF DKO mice still develop more tumors compared to SPF WT mice, it is also likely that other pathways that remain to be identified contribute to tumorigenesis. It is also interesting to note that SPF MyD88 KO

mice have been previously reported to have more tumors than SPF WT mice with a higher concentration of DSS than used in the current study(48), and may be explained in part by the presence of commensal bacteria driving inflammation and tumorigenesis in SPF MyD88 KO mice.

Our findings highlight the importance of commensal-driven inflammatory responses to properly initiate intestinal repair responses in the presence of chemically-induced injury that is critical for preventing late tumorigenesis. What will be important to determine is whether specific bacterial populations or delivery of bacterial products aside from LPS are also capable of limiting tumorigenesis by promoting wound repair and the context by which these occur. Our germfree model system will enable us to address these questions and also allow us to develop strategies that harness the beneficial activities of the gut microbiota to prevent the development of dysregulated inflammation and colon cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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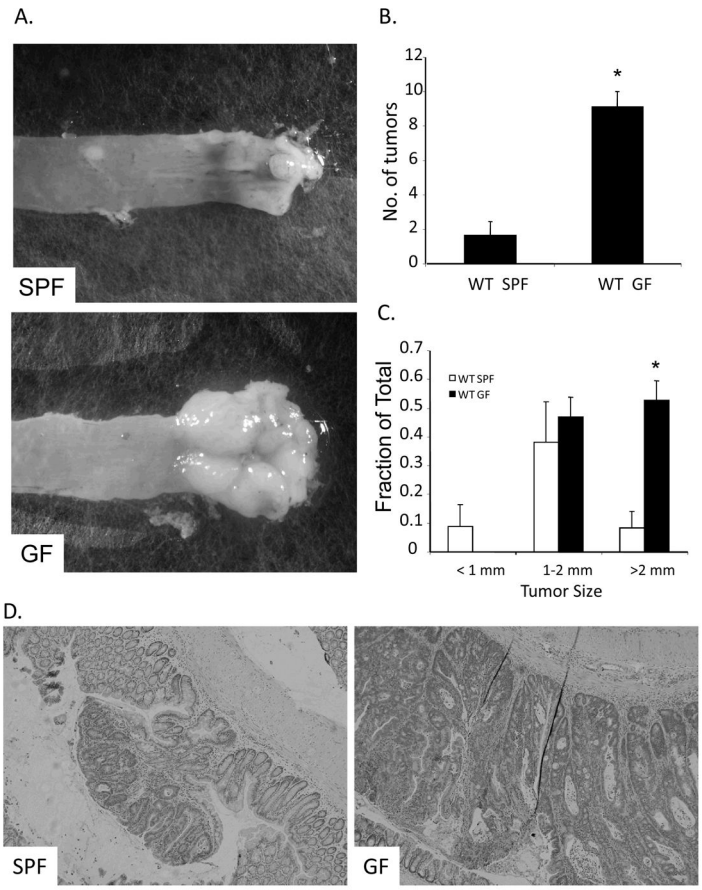

Figure 1.

GF mice develop more tumors compared to SPF mice. A. Representative photographs of the distal rectum and anus of SPF and GF mice after treatment with AOM and 4 cycles of 1.5% DSS. B. Number of tumors in age- and sex-matched B6 GF (N=14) and SPF mice (N=20). *p< 0.05. Data expressed as means ± S.E.M. C. Graph of tumor size in GF and SPF mice after AOM/DSS treatment. Data expressed as means ± S.E.M. D. Representative micrographs of adenomatous tumors in SPF and GF mice after AOM/DSS treatment. Magnification 200X.

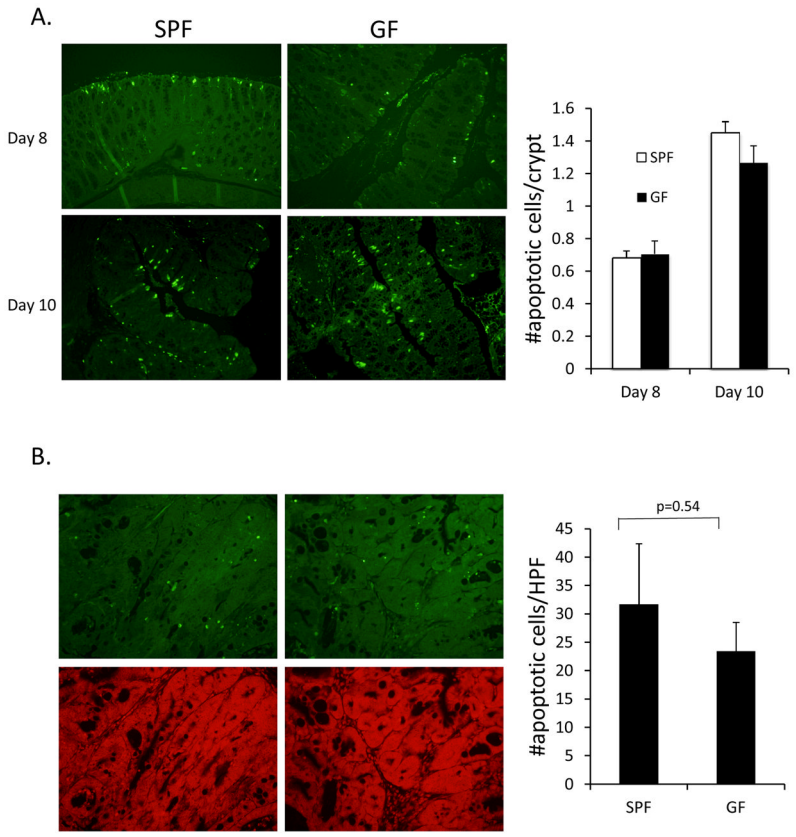

Figure 2.

Chemically-induced early epithelial apoptosis and tumor apoptosis are not affected by the gut microbiota. A. (left) Representative micrographs (200X) of colon sections from SPF and GF mice on days 8 and 10 of AOM/1.5% DSS treatment (3 and 5 days after start of first round of DSS, respectively) after TUNEL staining. (right) Graph of number of apoptotic cells/crypt with approximately 150 crypts counted B. (right) Representative micrographs (200X) of tumor sections at the end of AOM/DSS treatment stained by TUNEL assay (top) and with propidium iodide as a counterstain (bottom). (left) Graph of average number of TUNEL+ cells per high power field (5 fields counted) in GF and SPF tumors (N=4–5 mice/each). Data expressed as means ± S.E.M.

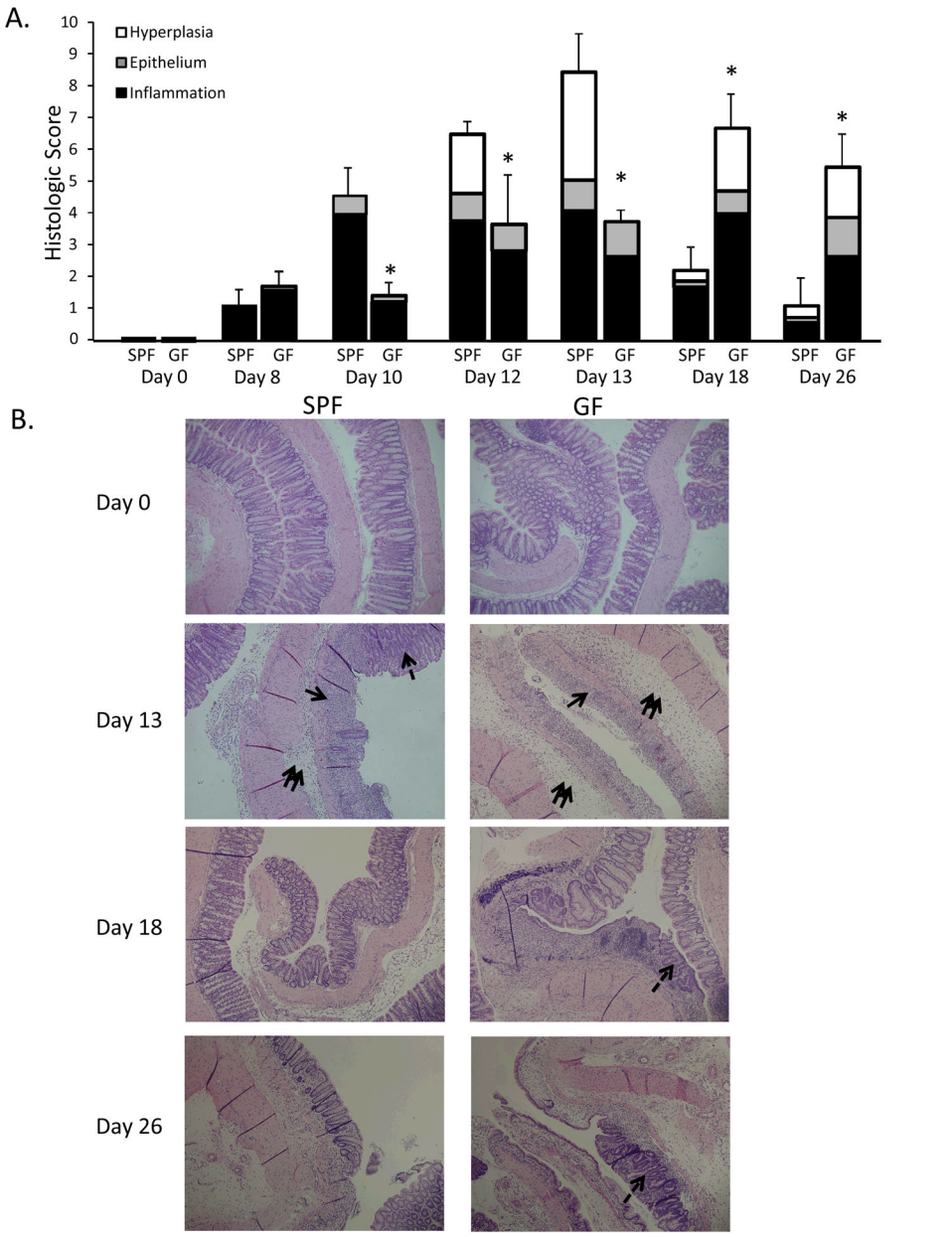

Figure 3.

GF mice exhibit delayed colonic inflammation. A. Histologic scores encompassing extent of inflammation, epithelial damage, and epithelial hyperplasia in GF and SPF mice (N=5 mice/group/timepoint) at various timepoints during AOM/DSS treatment. B. Representative micrographs (200X) of colons harvested from age- and sex-matched GF and SPF mice at various timepoints during AOM/DSS treatment. Day 13=3 days after completion of first round of DSS and Day 26=start of second round of DSS. Arrows depict mucosal erosion with inflammatory cell infiltration; double arrows point to submucosal edema; dashed arrows depict regenerating epithelium/hyperplasia. *$p<0.05$; Data expressed as means ± S.E.M.

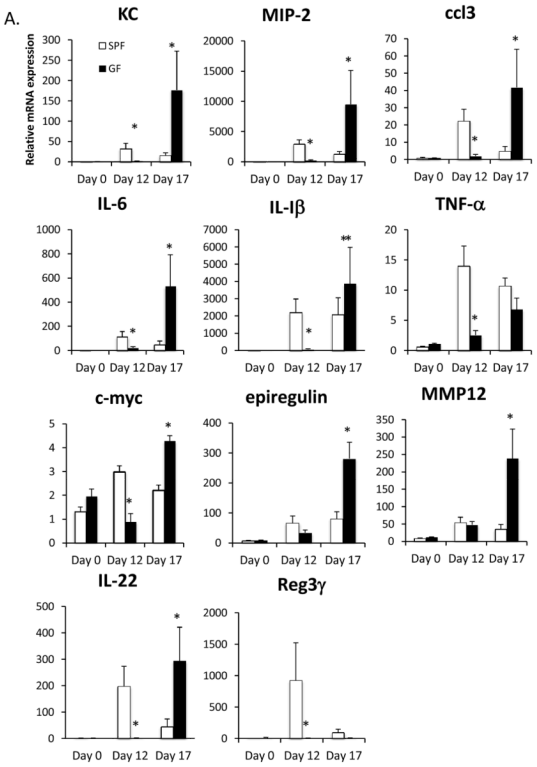

Figure 4.

GF mice have impaired initial inflammatory responses followed by delayed increased in proinflammatory, protumorigenic factors. A. Relative mRNA expression of various factors associated with intestinal repair and inflammation on day 12 (2 days after completion of first round of DSS) and day 17 (1 week after completion of first round of DSS) of AOM/1.5% DSS treatment in age- and sex-matched GF and SPF mice as measured by real-time PCR. Expression values were normalized with respect to the housekeeping gene β-actin (N=5–6 mice/group). Data expressed as means ± S.E.M. *,$p<0.05$, **, $p=0.09$.

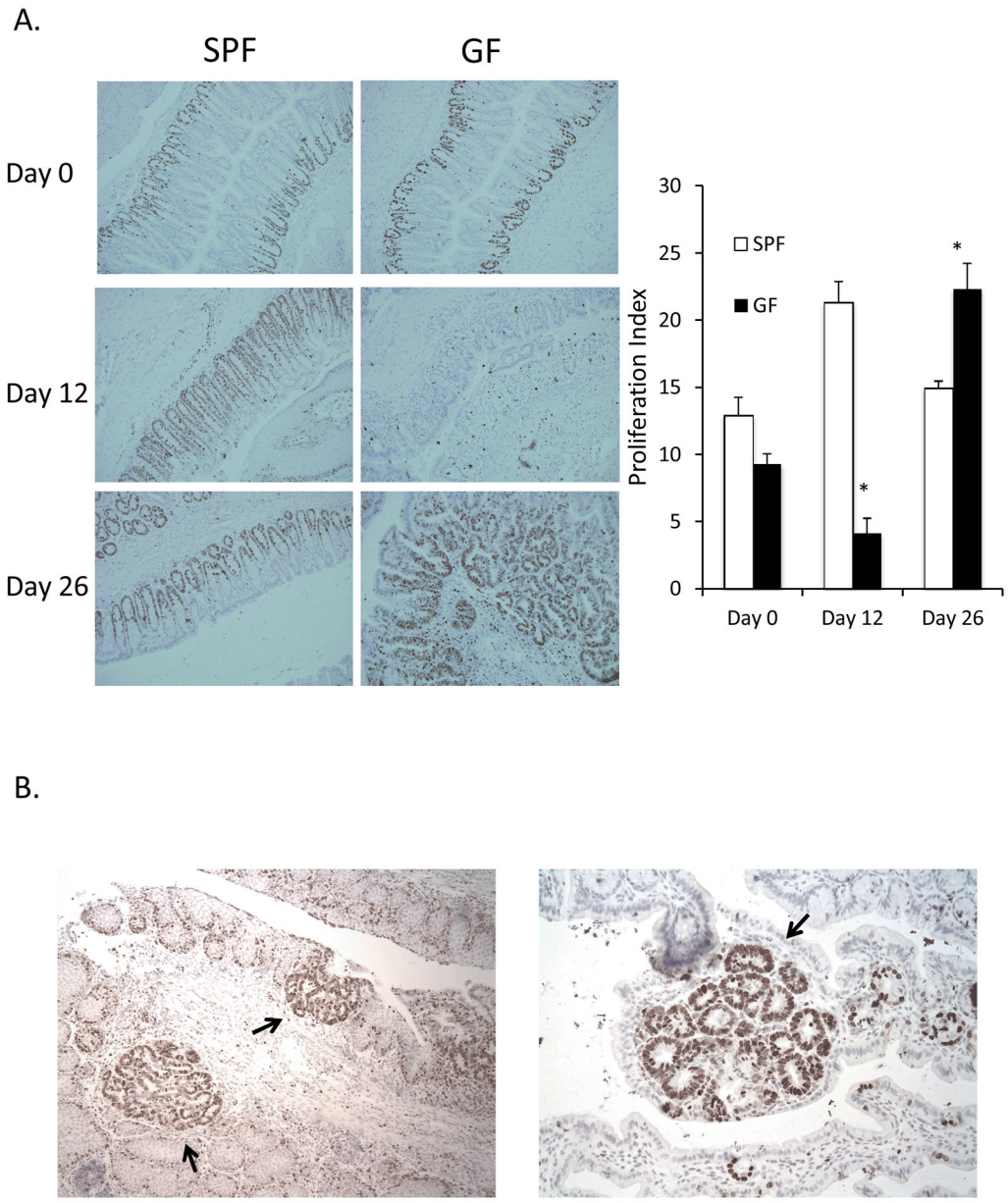

Figure 5.

Delayed proliferative responses in GF mice are associated with early microadenoma formation. A. (left) Representative micrographs of Ki67 imunoreactivity in colon sections (200X) from age- and sex-matched GF and SPF mice at timepoints early (day 12) and late (day 26) after completion of first round of 1.5% DSS (N=4–5 mice/group/timepoint). (right) Graph of number of Ki67+ cells per crypt (approximately 30–50 crypts counted) in GF and SPF mice at various timepoints after the first round of DSS during AOM/DSS tumor induction protocol. Student's t-test was used to determine significance. *, $p < 0.05$; Data expressed as means ± S.E.M. B. Representative micrographs of day 26 (just prior to start of second round of DSS) Ki67-stained GF colon sections at 100X (left) and 200X (right) magnification. Arrows depict microadenomas.

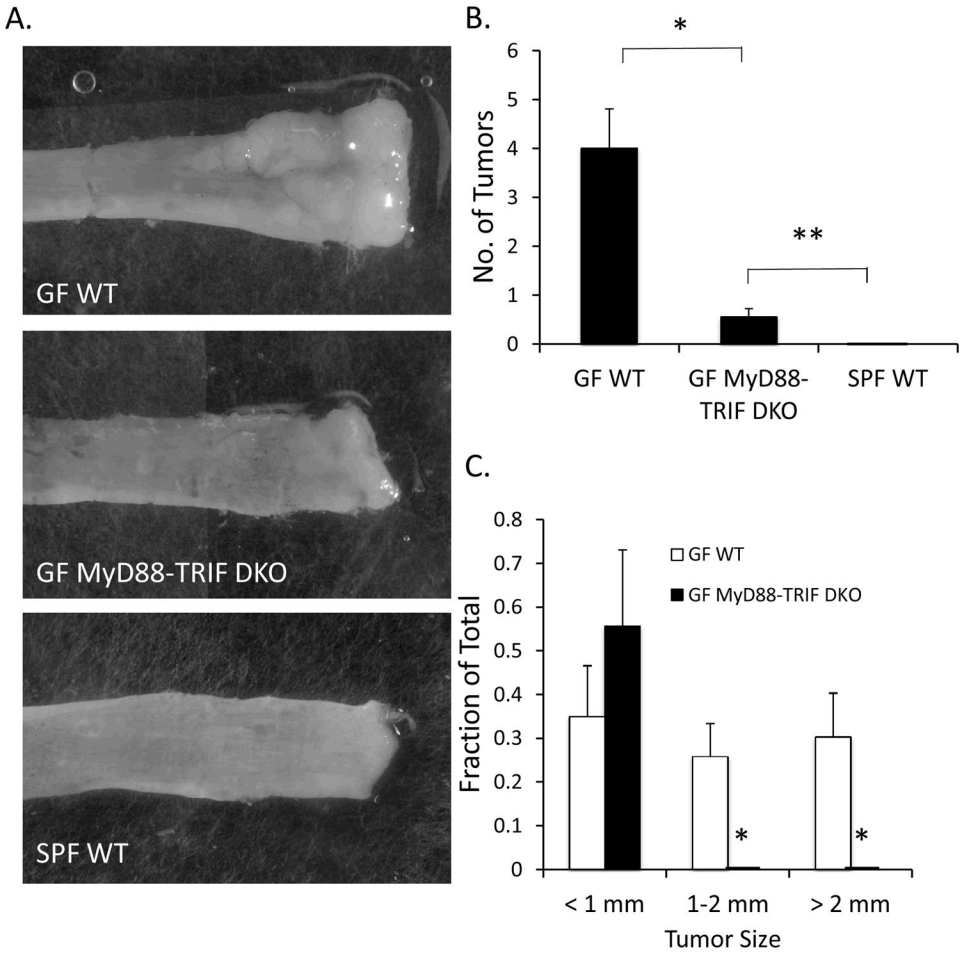

Figure 6.

GF MyD88-TRIF DKO mice develop fewer tumors than GF mice. A. Representative photographs of the distal rectum and anus of C57BL/6 SPF WT (N=9), GF WT (N=11), and GF MyD88-TRIF DKO mice (N=9) after treatment with AOM and 3 cycles of 1% DSS. B. Number of tumors in age- and sex-matched SPF WT, GF WT, and GT MyD88-TRIF DKO mice with AOM/DSS treatment. C. Graph of tumor size in SPF WT, GF WT, and GF MyD88-TRIF DKO mice. Data expressed as means ± S.E.M. *, ** $p < 0.05$.

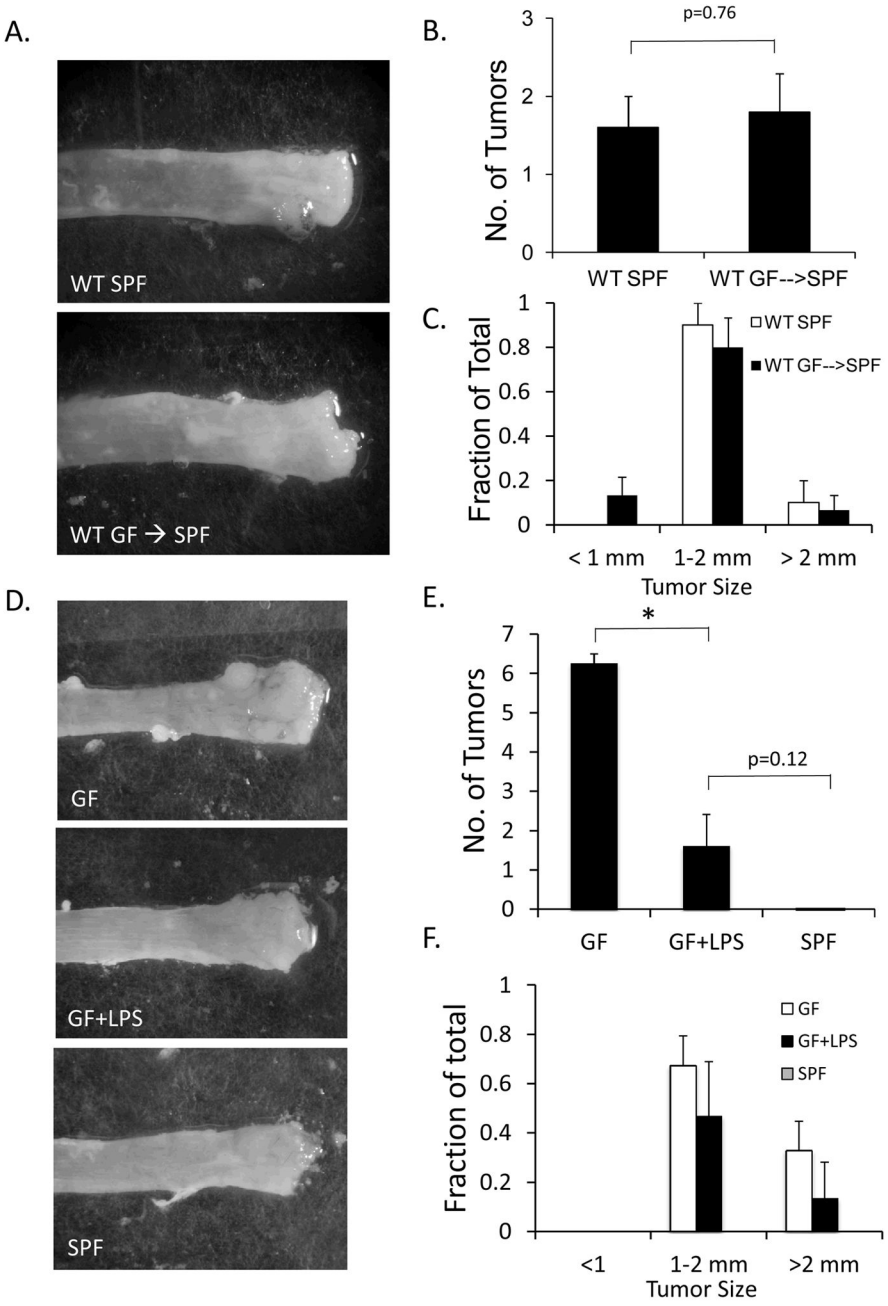

Figure 7.

Recolonization of GF mice with the gut microbiota or treatment with LPS alone can limit tumorigenesis. A. Representative photographs of the distal rectum and anus of SPF and GF WT recolonized with commensal bacteria after 3 weeks co-housing with SPF mice followed by treatment with AOM and 4 cycles of 1.5% DSS. B. Number of tumors in age- and sex-matched GF and SPF mice (N=5/group) after AOM/DSS treatment. C. Graph of tumor sizes in GF and SPF mice after AOM/DSS treatment. D. Representative photographs of the distal rectum and anus of SPF, GF, and GF mice treated with 1 mg/ml LPS daily beginning at day -4 followed by 3 cycles of 1% DSS. E. Number of tumors in age- and sex-matched SPF (N=5), GF (N=4) and GF treated with LPS (N=5) after AOM/DSS treatment. C. Graph of

tumor sizes in SPF, GF, and GF treated with LPS after AOM/DSS treatment. Data expressed as means ± S.E.M. *p< 0.05.