# Article

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# The Toll–Interleukin-1 Receptor Member SIGIRR Regulates Colonic Epithelial Homeostasis, Inflammation, and Tumorigenesis

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## SUMMARY

**Despite constant contact with the large population of commensal bacteria, the colonic mucosa is normally hyporesponsive to these potentially proinflammatory signals. Here we report that the single immunoglobulin IL-1 receptor-related molecule (SIGIRR), a negative regulator for Toll-IL-1R signaling, plays a critical role in gut homeostasis, intestinal inflammation, and colitis-associated tumorigenesis by maintaining the microbial tolerance of the colonic epithelium. SIGIRR-deficient ($Sigirr^{-/-}$) colonic epithelial cells displayed commensal bacteria-dependent homeostatic defects, as shown by constitutive upregulation of inflammatory genes, increased inflammatory responses to dextran sulfate sodium (DSS) challenge, and increased Azoxymethane (AOM)+DSS-induced colitis-associated tumorigenesis. Gut epithelium-specific expression of the SIGIRR transgene in the SIGIRR-deficient background reduced the cell survival of the SIGIRR-deficient colon epithelium, abrogated the hypersensitivity of the $Sigirr^{-/-}$ mice to DSS-induced colitis, and reduced AOM+DSS-induced tumorigenesis. Taken together, our results indicate that epithelium-derived SIGIRR is critical in controlling the homeostasis and innate immune responses of the colon to enteric microflora.**

## INTRODUCTION

Up to 100 trillion commensal bacteria, which are highly diverse in strains, are present in the colon, providing the host with important functions including the metabolism of nutrients and organic substrates (Sansonetti, 2004; Hooper and Gordon, 2001; Hooper et al., 2001; Berg, 1996). The beneficial effects of the commensal bacteria have evolved in parallel with the so-called microbial tolerance of the intestinal epithelial layer, in which the lack of responsiveness to this bacterial population results in the coexistence of the host and the bacteria. The intestinal epithelial layer functions not only as a physical barrier but also as an innate and adaptive immune barrier against commensal bacteria. Inappropriate activation of the immune system by commensal bacteria is thought to lead to the pathogenesis of human inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis (Podolsky, 2002).

Ulcerative colitis is associated with an elevated risk for colorectal cancer (Clevers, 2004). It is now commonly believed that the chronic inflammation process is responsible for the neoplastic transformation of the intestinal epithelium (Clevers, 2004; Balkwill and Mantovani, 2001). The proinflammatory cytokines and chemokines, such as TNFα, IL-1, IL-6, and IL-8, as well as matrix-degrading enzymes, growth factors, and reactive oxygen species (ROS), create a microenvironment that enhances cell proliferation, cell survival, cell migration, and angiogenesis, thereby promoting tumorigenesis (Coussens and Werb, 2002; Coussens et al., 2000; Dvorak, 1986). The common view is that the association of ulcerative colitis with cancer involves the inflammation of the submucosa, induced by direct contact with the intestinal microflora, promoting tumor outgrowth in the overlaying epithelium. Therefore, the microbial tolerance of the intestinal epithelial layer is important not only for controlled state of inflammation but also for preventing tumorigenesis in the colon.

This microbial tolerance of the intestinal epithelial layer is a consequence of multiple factors involved in the interaction between the host and bacteria. The Toll-like receptors (TLRs) is a family of pattern-recognition receptors that detects conserved molecular patterns of microorganisms, thereby playing a critical role in the interaction between
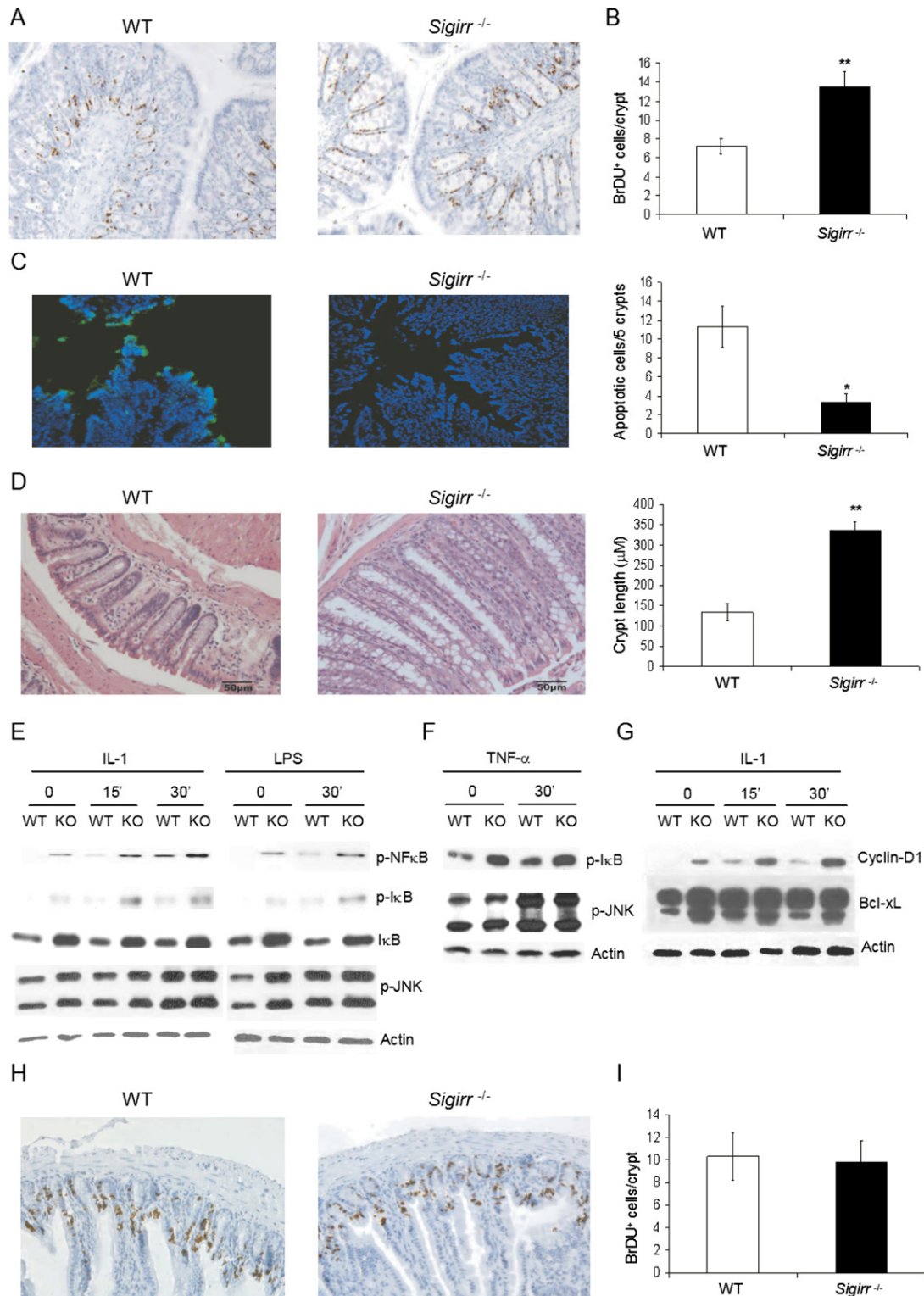
**Figure 1. Increased Cell Proliferation and Survival in _Sigirr_$^{-/-}$ Mice Colon**

(A) Bromodeoxyuridine (BrdU, 1 mg/ml) was administrated to age- and sex-matched wild-type and SIGIRR-deficient mice by i.p. injection. 24 hr after BrdU injection, the colon sections were stained for BrdU-positive cells (stained brown). Hemotoxylin (blue) was used as counter staining to visualize the whole tissue. Well-oriented crypts (at least 30 crypts) were counted on slides (at least two slides) from each mouse, and 3 pairs of mice were analyzed.

(B) Data from (A), (C), and (D) are quantified. Error bars represent ±SEM, and Student's t test was used. *p < 0.05; **p < 0.01.

host and bacteria (Medzhitov et al., 1997; Rock et al., 1998; Takeuchi et al., 1999; Chuang and Ulevitch, 2000; Hemmi et al., 2000; Zhang et al., 2004). TLR4 (a receptor for LPS, lipopolysaccharide), TLR2 (a receptor for LTA, lipoteichoic acid), and TLR9 (a receptor for CpG DNA) are sensors for bacterial infection and are critical for the initiation of inflammatory and immune defense responses. One confounding issue with this hypothesis is that TLR ligands are present on both commensal bacteria and pathogens. As such, it remains unclear how the host remains tolerant to commensal bacteria yet can initiate an effective inflammatory and immune response against pathogens when they appear. It has been shown that the spatial and temporal expression of TLRs in the mucosal surfaces of the intestinal tract contributes to the ability of the host to discriminate between pathogen and resident microflora (Melmed et al., 2003; Ortega-Cava et al., 2003). TLRs are expressed in innate immune cells, including macrophages and dendritic cells (Akira et al., 2001). The lack of pathogenic factors in commensal bacteria prevents them from crossing the physical barrier of the gut epithelium, reducing and/or excluding the contact between commensal bacteria and innate immune cells in the gut mucosa. However, recent studies suggest that the intestinal epithelium is not totally "blind" to TLR ligands. Experiments with TLR2-, TLR4-, and MyD88 (adaptor for TLRs)-deficient mice suggest that TLR signaling is required for the homeostasis of the intestinal epithelium and protects gut epithelium from injury (Rakoff-Nahoum et al., 2004). One of the major TLR-signaling pathways is the activation of the IKKβ-NF-κB-signaling cascade, which provides the survival signal for epithelial cells (Greten et al., 2004; Karin and Greten, 2005). Deletion of IKKβ in the epithelial cells reduced the colitis-associated tumor incidence in a mouse model, indicating the direct contribution of the epithelial cells in colitis-associated tumorigenesis (Greten et al., 2004; Karin and Greten, 2005).

SIGIRR (also named TIR8) represents a unique subgroup of the Toll-IL-1R superfamily, with a single immunoglobulin extracellular domain and a TIR (Toll/IL-1R) intracellular domain (Wald et al., 2003; Garlanda et al., 2004). We previously showed that SIGIRR functions as a negative regulator for IL-1 and LPS signaling, through its interaction with the TLR4 and IL-1R complex (Qin

et al., 2005). In this manuscript, we report that SIGIRR-deficient ($Sigirr^{-/-}$) mice exhibit dysregulated signaling in the gut epithelium in response to commensal bacteria, which disrupts the homeostasis of the colon epithelium and leads to hypersusceptibility of these mice to dextran sulfate sodium (DSS)-induced colitis and increased colitis-associated tumor incidence and tumor growth. The important role of SIGIRR in gut epithelium is demonstrated by the fact that gut-epithelial-specific expression of SIGIRR was able to restore the homeostasis of the colon epithelium, attenuated the severity of DSS-induced colitis, and reduced colitis-associated tumor incidence and tumor growth in the $Sigirr^{-/-}$ mice. Our results show that SIGIRR is an important modulator of intestinal epithelial homeostasis and a key regulator of mucosal immunity, maintaining microbial tolerance of the intestinal epithelial layer.

## RESULTS

### The Colon Epithelium of SIGIRR-Deficient Mice Exhibits Altered Homeostasis

Previous studies have shown that recognition of commensal bacteria by TLR2 and 4 is required for the homeostasis of intestinal epithelium (Rakoff-Nahoum et al., 2004), which is controlled by the balance of proliferation and differentiation along the crypt axis. Because SIGIRR is a negative regulator for IL-1R- and TLR4-mediated pathways and highly expressed in colonic epithelial cells (see Figure S1 in the Supplemental Data available online), we hypothesized that $Sigirr^{-/-}$ epithelial cells might be more responsive to commensal bacteria in the colon. To test this hypothesis, we examined whether the gut epithelium in the $Sigirr^{-/-}$ mice might suffer intrinsic homeostatic defects. The proliferation state of the colon crypts of the $Sigirr^{-/-}$ mice was compared with the wild-type mice by labeling the proliferating cells with BrdU administered by intraperitoneal injection. Colon sections were harvested 2 and 24 hr after BrdU injection and stained for BrdU-positive cells (Figures 1A and 1B and data not shown). There was an increased number of proliferating epithelial cells in the crypts of the $Sigirr^{-/-}$ colon as compared to that in the wild-type mice. Furthermore, although the BrdU-positive cells were only in the stem cell zone

(C) DNA fragmentation in wild-type and $Sigirr^{-/-}$ mice colon was detected by in situ TUNEL assay. Apoptotic cells were stained green and nuclei were stained blue by DAPI. Well-oriented crypts (at least 30 crypts) were counted on slides (at least two slides) from each mouse, and 3 pairs of mice were analyzed.

(D) Aged wild-type and $Sigirr^{-/-}$ mice (9 months old) were sacrificed and colon was excised. After PBS wash, the colon was cut open longitudinally and Swiss-roll was made. Sections of Swiss-roll were stained by Hematoxylin and eosin and crypt length was measured. Well-oriented crypts (at least 30 crypts) were measured on slides (at least two slides) from each mouse, and 3 pairs of mice were analyzed. Scale bar represents 100 μM.

(E–G) Colon epithelial cells from $Sigirr^{-/-}$ mice are constitutively activated and hyperresponsive to IL-1 and LPS stimulation. Colon epithelial cells prepared from wild-type or $Sigirr^{-/-}$ mice were untreated or stimulated with IL-1 (10 ng/ml), LPS (10 μg/ml), or TNFα (10 ng/ml) for 15 or 30 min. Cells were then lysed and cell lysates were resolved by SDS-PAGE. Immunoblotting was performed to detect TLR and IL-1R signaling components with anti-phospho-NF-κB (p-p65), anti-phospho-JNK, anti-IκBα, and anti-phosphor-IκBα (E and F) or proliferation and apoptosis markers with anti-Cyclin D1 and anti-Bcl-xL (G).

(H and I) Depletion of commensal bacteria abolished dysregulated cell proliferation in colon of $Sigirr^{-/-}$ mice. Wild-type and $Sigirr^{-/-}$ mice were treated with four different antibiotics in drinking water (1 g/l ampicillin, 500 mg/l vancomycin, 1 g/l neomycin sulfate, and 1 g/l metronidazole) for 4 weeks. Bromodeoxyuridine (BrdU, 1 mg/ml) was administered to antibiotic-treated mice via i.p. injection 24 hr prior to sacrifice. Colon sections from 4 pairs of mice were examined for BrdU-positive cells (brown staining).

localized at the bottom of the crypts in the wild-type colon, the proliferating epithelial cells were also found in the middle and upper regions of the crypts in the $Sigirr^{-/-}$ colon, where cells normally are differentiated and nonproliferating. Taken together, these results indicate that the epithelial cells in the $Sigirr^{-/-}$ colon exhibit a dysregulated state in their basal proliferation.

To further compare the homeostasis of the colon epithelium between $Sigirr^{-/-}$ mice and wild-type mice, we examined epithelial cell survival and apoptosis in the crypts. In situ TUNEL assay was performed on colon sections to examine the DNA fragmentation caused by apoptosis. As shown in Figure 1C, $Sigirr^{-/-}$ colon epithelium had fewer apoptotic cells at the top of the crypts as compared to the wild-type colon, which provides further evidence for imbalanced homeostasis in the $Sigirr^{-/-}$ colon epithelium.

To address the impact of this imbalance over an extended period, we examined aged $Sigirr^{-/-}$ and wild-type mice kept under identical conditions. When 9-month-old mice were examined, we noted a striking elongation in $Sigirr^{-/-}$ colon crypts, at more than twice the length of wild-type mice. In addition, goblet cells increased in both size and number in aged $Sigirr^{-/-}$ mice as compared to littermate wild-type control mice (Figure 1D). The elongation of colon crypts was observed in as early as 5-month-old $Sigirr^{-/-}$ mice as compared to wild-type mice (data not shown). These phenotypic changes in the $Sigirr^{-/-}$ colon crypts are consistent with the increased proliferation and cell survival of the $Sigirr^{-/-}$ epithelial cells.

### Constitutive Signaling in SIGIRR-Deficient Colon Epithelium Is Dependent on Commensal Bacteria

To investigate the molecular mechanism for the altered homeostasis of the $Sigirr^{-/-}$ colon epithelium, we examined signaling in these epithelial cells. Interestingly, we found that $Sigirr^{-/-}$ colon epithelial cells revealed constitutive NF-κB, IκB, and JNK phosphorylation, indicating constitutive NF-κB and JNK activation (Figure 1E). Upon IL-1 or LPS (but not TNFα) stimulation, the $Sigirr^{-/-}$ colon epithelial cells showed even higher activation, compared to the control cells (Figures 1E and 1F). Such constitutive signaling and hyperactivation probably underlie the increased proliferation and survival of the $Sigirr^{-/-}$ colon epithelial cells. Furthermore, $Sigirr^{-/-}$ colon epithelial cells had upregulated expression of Cyclin D1 and Bcl-xL compared to that in wild-type cells. IL-1 stimulation further induced the expression of Cyclin D1 in the $Sigirr^{-/-}$ colon epithelial cells, compared to the control cells (Figure 1G). These results indicate that Cyclin D1 and Bcl-xL are probably part of the effector molecules responsible for the increased cell proliferation and reduced apoptosis in the colon epithelium of the $Sigirr^{-/-}$ mice.

To test whether the constitutive signaling in $Sigirr^{-/-}$ colon epithelium is dependent on commensal bacteria-derived ligands, we removed commensal bacteria from $Sigirr^{-/-}$ and wild-type mice through oral administered antibiotics, including vancomycin, neomycin, metronidazole, and ampicillin (VNMA). The depletion of the commensal

bacteria was confirmed by counting the bacteria in the stool of untreated and antibiotic-treated mice (Figure S2 and data not shown). To compare the proliferation of the epithelial cells in the $Sigirr^{-/-}$ mice with that in wild-type mice after the removal of commensal bacteria, we injected BrdU into the antibiotic-treated $Sigirr^{-/-}$ and wild-type mice intraperitoneally. Colon sections were harvested 24 hr after BrdU injection and stained for BrdU-positive cells. As shown in Figures 1H and 1I, the number and pattern of BrdU-positive cells (proliferating epithelial cells) in the crypts were similar between the antibiotic-treated $Sigirr^{-/-}$ colon and that in wild-type mice. These results clearly showed that the removal of commensal bacteria abolished that hyperproliferative state of the $Sigirr^{-/-}$ colon epithelium, indicating that the increased proliferation of the colon epithelial cells in $Sigirr^{-/-}$ mice (Figures 1A and 1B) depends on the presence of commensal bacteria in the colon. Furthermore, colonic epithelial cells from antibiotic-treated $Sigirr^{-/-}$ and wild-type mice were also examined for signaling. Similar amounts of NF-κB and JNK phosphorylation were observed in colon epithelium cells of the $Sigirr^{-/-}$ and wild-type mice after the removal of commensal bacteria, indicating that the constitutive signaling in $Sigirr^{-/-}$ colonic epithelial cells is indeed dependent on commensal bacteria (data not shown).

### The Impact of SIGIRR Deficiency on Intestinal Inflammation

Based on these homeostatic changes, we wondered whether the microflora-mediated constitutive activation of $Sigirr^{-/-}$ colon epithelium may ultimately lead to spontaneous colitis. Comparison of the colon sections between wild-type and $Sigirr^{-/-}$ (2–12 months old) mice did not reveal significant infiltration of inflammatory cells and did not detect obvious tissue damage (data not shown), although the crypts were dramatically elongated in 5- to 9-month-old $Sigirr^{-/-}$ distal colon as compared to that in wild-type mice (Figure 1D). However, by ELISA, we did find that the $Sigirr^{-/-}$ colonic mucosa showed significantly higher expression of cytokines (TNF-α, IL-6, and IFN-γ) and chemokines (MIP-2, MCP-1, and KC) as compared to wild-type colon (Figure 2). These results suggest that although the $Sigirr^{-/-}$ mice do not develop overt spontaneous colitis, the physiologic inflammation typically found in the colonic mucosa is exaggerated in $Sigirr^{-/-}$ mice. This may make them far more susceptible to colitis when the colon is challenged by noxious stimuli.

$Sigirr^{-/-}$ mice has been reported to be more sensitive to DSS-induced colitis (Garlanda et al., 2004). We also employed the DSS-induced colitis model to investigate the mechanism by which SIGIRR regulates intestinal inflammation. Both $Sigirr^{-/-}$ and wild-type mice were continuously treated with 3% DSS in drinking water. 9 days after DSS treatment, the $Sigirr^{-/-}$ mice began to die, and by the end of day 13, all of the mice (n = 15) had died. In contrast, the entire cohort of wild-type mice was still alive even at day 16 (Figure 3A). These results clearly indicate that the $Sigirr^{-/-}$ mice were much more susceptible to DSS treatment as compared to wild-type mice. The DSS-induced
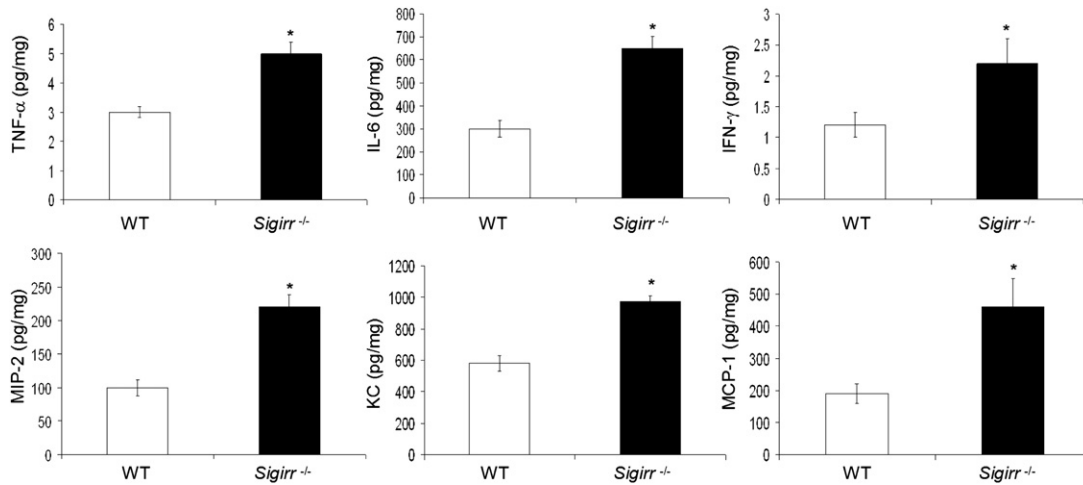
**Figure 2. Constitutive Upregulation of Proinflammatory Cytokines and Chemokines in Colon of *Sigirr*<sup>−/−</sup> Mice**

Same amount of colon tissue from wild-type and *Sigirr*<sup>−/−</sup> mice (ranging from 200 to 300 mg) was cut into small pieces and incubated in serum-free RPMI medium for 24 hr, and secreted cytokines (IL-6, TNFα, and IFNγ) and chemokines (KC, MIP-2, and MCP-1) in the medium were measured by ELISA. Error bars represent ±SEM, and Student's t test was conducted. *p < 0.05.

colitis phenotype in *Sigirr*<sup>−/−</sup> mice appeared to be much stronger than that described by Garlanda et al. (2004) for the *Sigirr*<sup>−/−</sup> mice, which could be due to strain difference or difference of the microflora spectrum in the colon.

The higher mortality of the *Sigirr*<sup>−/−</sup> mice was associated with increased gut injury caused by DSS treatment. Histology analysis of colon sections showed increased damage in *Sigirr*<sup>−/−</sup> epithelial layer as compared to that in wild-type mice after the DSS treatment (Figure 3B and Figure S3). By immunofluorescent staining for infiltrating leukocytes, we found that colon sections of DSS-treated *Sigirr*<sup>−/−</sup> mice had highly increased numbers of infiltrated inflammatory cells as compared to those in wild-type mice (Figure S4). Furthermore, inflammatory cytokine gene and protein expression were also clearly induced to much higher amounts in DSS-treated *Sigirr*<sup>−/−</sup> colon than the wild-type mice, including IL-12 p40, IFN-γ, IL-17, IL-6, and IL-1β (Figure 3C and Figure S5). Taken together, the above results indicate that severe damage of colonic epithelium in DSS-treated *Sigirr*<sup>−/−</sup> mice is due to increased inflammation.

We then examined whether hypersensitivity of the *Sigirr*<sup>−/−</sup> mice to DSS challenge is dependent on the presence of commensal bacteria. Antibiotic-treated *Sigirr*<sup>−/−</sup> and wild-type mice were treated with 3% DSS and followed by histology analyses. As shown in Figure 3D and Figure S6, antibiotic treatment reduced the sensitivity of the *Sigirr*<sup>−/−</sup> mice to DSS treatment. The antibiotic-treated *Sigirr*<sup>−/−</sup> mice showed similar inflammatory response to DSS treatment as the antibiotic-treated wild-type mice. These results demonstrate that the regulatory role of SIGIRR in DSS-induced colitis is commensal bacteria dependent, probably through its negative regulation on TLR-IL-1R-mediated-signaling induced directly or indirectly by commensal bacteria.

## The Impact of SIGIRR Deficiency on Colitis-Associated Cancer

We next examined the impact of SIGIRR deficiency on colitis-associated cancer. The link between chronic inflammation and tumorigenesis has been well established for colorectal cancer (Clevers, 2004; Balkwill and Mantovani, 2001). Because SIGIRR plays an important role in maintaining colonic epithelial homeostasis and controlling intestinal inflammation, we predict that SIGIRR should have an impact on inflammation-induced cancer in the colon tissues. To test this hypothesis, we employed a mouse model of colitis-associated cancer (CAC), injecting mice with procarcinogen AOM (Azoxymethane) followed by three cycles of oral administration of DSS (Okayasu et al., 1996). Although AOM treatment introduces genetic instability and mutation of oncogenes in epithelia, mice developed chronic colitis after repeated treatment with DSS, accelerating tumor promotion and progression in colon. *Sigirr*<sup>−/−</sup> and wild-type mice were subjected to this AOM+DSS-induced colitis-associated cancer model. Whereas 63% of the wild-type mice developed macroscopic polyps, all the *Sigirr*<sup>−/−</sup> mice developed colon tumors (Figure 4A). The tumors detected in both wild-type and *Sigirr*<sup>−/−</sup> mice were exclusively located in distal colon. Some of *Sigirr*<sup>−/−</sup> mice showed severe rectal bleeding, diarrhea, or loss of weight toward the end of treatment. Furthermore, the average tumor number per mouse (17 ± 4.03) in *Sigirr*<sup>−/−</sup> mice was twice more than that in wild-type mice (7 ± 3.3) (Figure 4B). These results showed that tumor incidence was increased in *Sigirr*<sup>−/−</sup> mice, indicating that SIGIRR deficiency enhances colitis-associated tumor promotion. SIGIRR deficiency also had impact on the size of the tumors. About 60% of the polyps developed in wild-type mice were small adenomas (<2 mm diameter), whereas the size of the other 40% polyps were 2–5 mm in diameter (Figure 4C and Figure S7). Remarkably, about
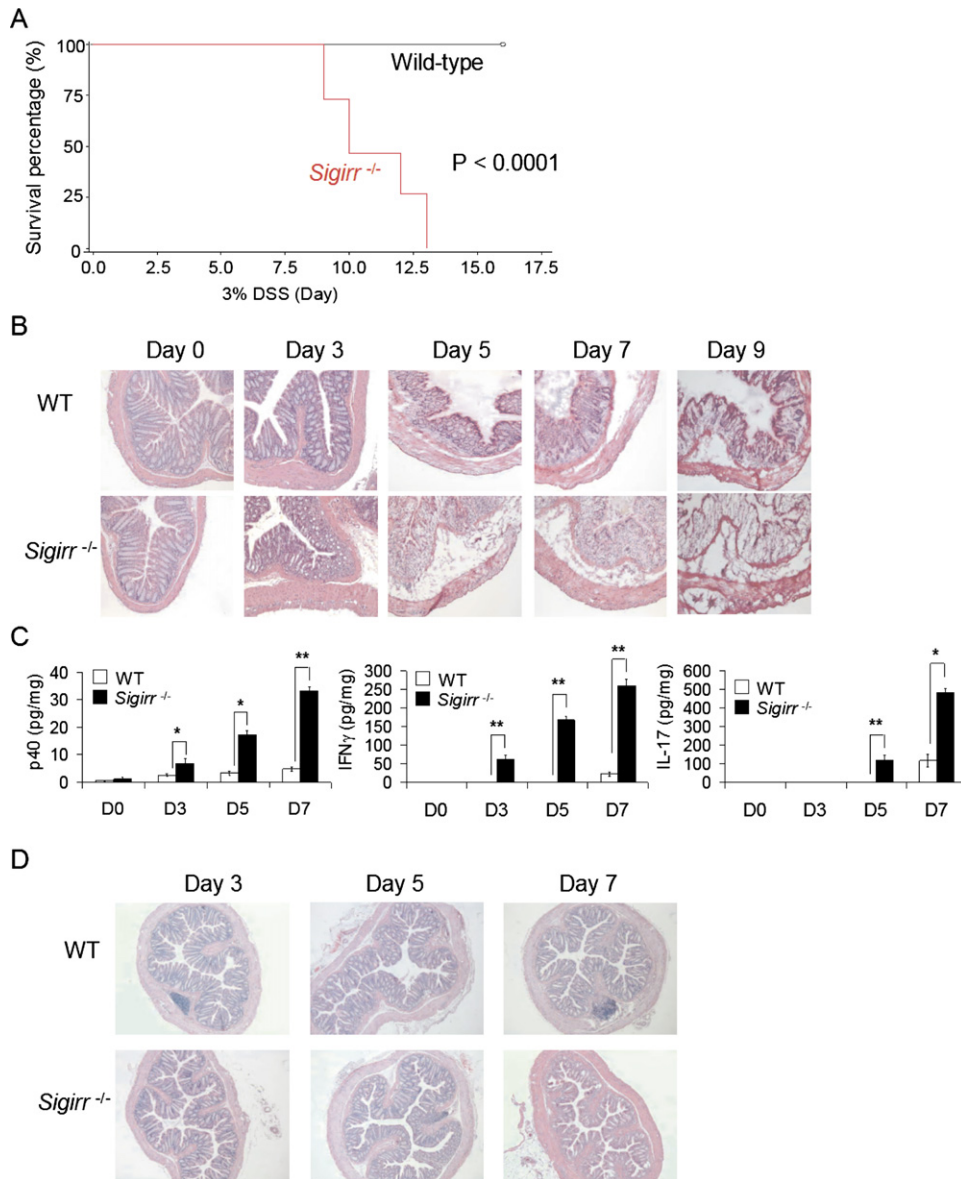
**Figure 3. *Sigirr*$^{-/-}$ Mice Are Highly Susceptible to Develop DSS-Induced Colitis**
SIGIRR-deficient and wild-type control mice were treated continuously with 3% DSS in drinking water.
(A) Kaplan-Meier plot of survival of wild-type and *Sigirr*$^{-/-}$ mice (n = 15) after DSS treatment for 16 days.
(B) Hematoxylin and eosin staining of colonic cross sections of mice treated with 3% DSS for various days (original magnification 100×).
(C) Increased cytokine production in colon of *Sigirr*$^{-/-}$ mice after DSS treatment. 200 mg of colon tissue was removed from *Sigirr*$^{-/-}$ and wild-type mice. Whole-colon culture was collected on various days after treatment with 3% DSS, and cytokine production was examined by ELISA. Error bars represent ±SEM, and Student's t test was conducted. *$p < 0.05$, **$p < 0.01$.
(D) Removal of commensal bacteria abolished the hypersensitivity of *Sigirr*$^{-/-}$ colon to DSS treatment. Wild-type and *Sigirr*$^{-/-}$ mice were treated with four different antibiotics as described in Experimental Procedures for 4 weeks and followed by DSS (3%) treatment for 3–7 days. H&E staining was conducted to examine the histology of cross-sections from *Sigirr*$^{-/-}$ and WT mice. Magnification 50×.

70% of the polyps formed in *Sigirr*$^{-/-}$ mice were big tumors (>2 mm diameter), and each mouse developed one or two large tumor (>5 mm diameter) with histology characteristics of adenocarcinoma (Figures 4C and 4D). These results indicate that SIGIRR deficiency not only enhances tumor promotion but also increases tumor progression. Histology analyses showed that all the tumors formed in

wild-type mice were tubular adenomas, with no sign of invasion to mucosa or muscular layer (Figure 4D). However, the large tumors formed in *Sigirr*$^{-/-}$ mice showed high-grade dysplasia, less differentiation, and obvious infiltration of inflammatory cells (Figure 4D). We frequently observed crypts penetrating into the mucosa or muscular mucosa (Figure 4D).
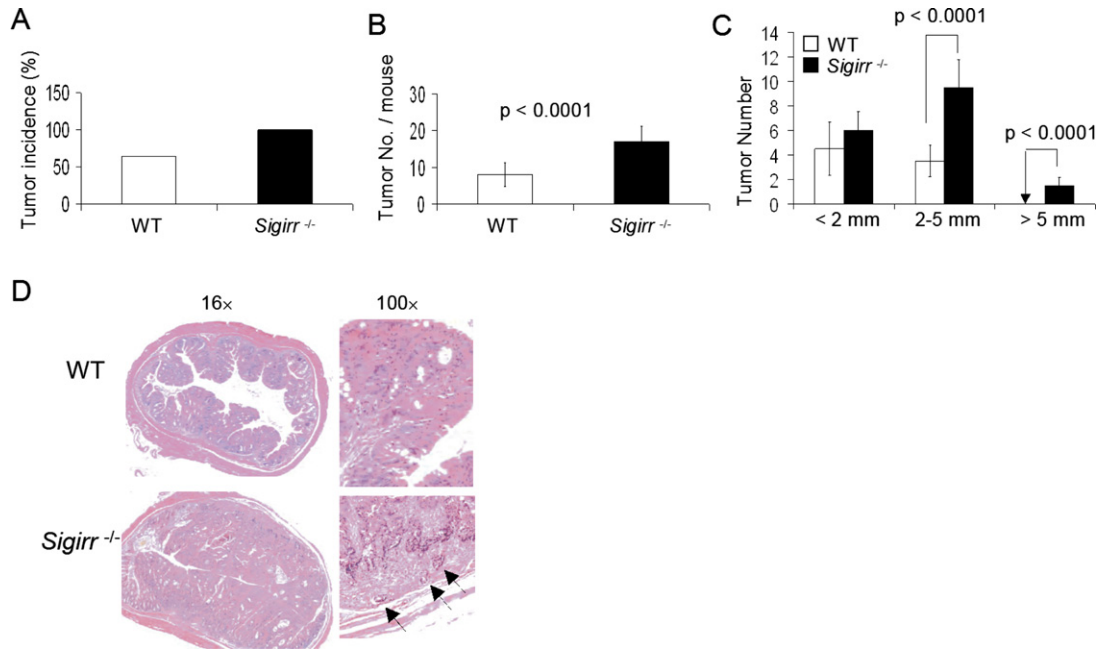
**Figure 4. SIGIRR Deficiency Enhances Tumorigenesis in Mouse Colon**

(A) Tumor incidence induced by AOM plus DSS regime in $Sigirr^{-/-}$ and WT mice. Wild-type, n = 13; $Sigirr^{-/-}$, n = 14.

(B) Total colonic polyps formed in colorectal of $Sigirr^{-/-}$ and WT mice.

(C) Size distribution of colonic tumors formed in $Sigirr^{-/-}$ and WT mice. The data shown are the mean ± SD (WT, n = 13; KO, n = 14), and p < 0.0001 according to ANOVA analysis.

(D) Histology of tumors formed in $Sigirr^{-/-}$ and WT mice. Photomicrograph of hematoxylin and eosin-stained sections of $Sigirr^{-/-}$ and WT mice are shown. Magnification 16× or 100×. Arrows point to crypts found in the mucosa and muscular mucosa.

## Hyperactivation of NF-κB and STAT3 Contribute to Increased Tumorigenesis in SIGIRR-Deficient Mice

To examine how SIGIRR deficiency leads to increased tumor promotion and progression, we first compared the proliferation status of colon epithelium of the $Sigirr^{-/-}$ mice with that of wild-type control mice at the early stage of tumorigenesis upon AOM and DSS treatment. After AOM injection and the first cycle of DSS treatment, we detected increased Ki-67-positive cells in the colon epithelium of $Sigirr^{-/-}$ mice as compared to that in wild-type control mice, indicating that cell proliferation is increased in $Sigirr^{-/-}$ epithelium (Figure 5A). Previous studies have shown that NF-κB activation in colon epithelial cells has important tumor-promoting function (Karin and Greten, 2005). The NF-κB pathway was indeed activated at a higher amount in the colon tissue of $Sigirr^{-/-}$ mice (with more phosphorylation of IKK, IκB, and NF-κB) after AOM+DSS treatment as compared to that in wild-type control mice (Figure 5B). NF-κB nuclear accumulation was increased in epithelium of $Sigirr^{-/-}$ mice, confirming the activation of NF-κB (data not shown). Consequently, expression of NF-κB target genes Bcl-xL and Cyclin D1 (which are important for cell survival and proliferation) was highly induced in colon tissues of $Sigirr^{-/-}$ mice (Figures 5B and 5C). In addition to increased cell proliferation, inflammation and damage of crypt cells were much more evident in $Sigirr^{-/-}$ mice at the early stage of tumorigenesis after the initial AOM and DSS treatment. We detected

much stronger induction of inflammatory gene COX2 (Figure 5B) and cytokines (IL-17, TNF-α, and IL-6) in colon tissues of $Sigirr^{-/-}$ mice as compared to that in wild-type mice (Figure 5D). In particular, IL-6 has been shown to promote tumor progression in inflammation-associated cancer models through the activation of oncogene STAT3 (Becker et al., 2004). Consistent with this, nuclear accumulation of phospho-STAT3 was indeed increased in $Sigirr^{-/-}$ epithelium as compared to that in wild-type mice (Figure 5E), which probably contributed to the increased cell proliferation observed in the $Sigirr^{-/-}$ epithelium. Taken together, the above results showed that NF-κB and STAT3 were highly activated during the early stage of tumorigenesis in $Sigirr^{-/-}$ colon epithelium, which in turn promote tumor formation through the expression of their target genes (including Cyclin D1 and Bcl-xL) important for cell survival and proliferation.

Human colon cancers often have mutation and/or loss of heterozygosity in the key components of APC (adenomatous polyposis coli)-β-catenin pathway, indicating an essential role for β-catenin in carcinogenesis of the colon (Radtke and Clevers, 2005; Gregorieff and Clevers, 2005). It has been reported that β-catenin, rather than APC, is often mutated in AOM-induced murine colon tumors (Greten et al., 2004; Guda et al., 2004). β-catenin nuclear accumulation was indeed detected by immunohistochemistry showing a shift of β-catenin from the membrane toward a cytoplasmic and nuclear localization in the tumor tissues
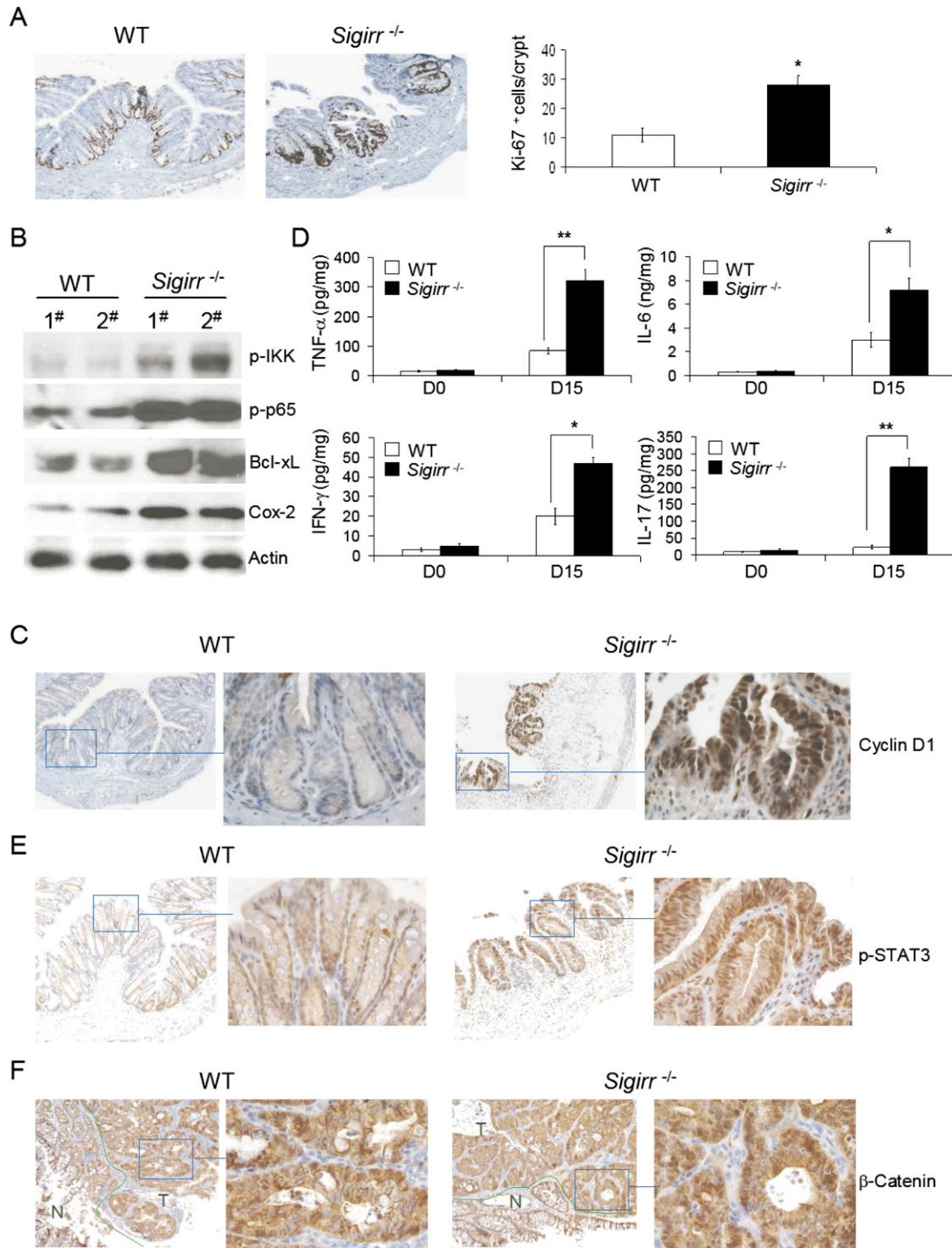
**Figure 5. Increased Cell Proliferation and Enhanced Inflammatory Response in *Sigirr*^{−/−} Mice during Early Stage of Tumorigenesis Induced by AOM+DSS Treatment**

(A) Increased cell proliferation of colon epithelia of *Sigirr*^{−/−} mice. Immunohistochemical staining was performed to examine Ki-67-positive cells on cross-sections of colons from *Sigirr*^{−/−} and WT mice on day 15 under AOM plus DSS regime (magnification 100×). Ki-67-positive cells were quantitated from 6 cross-sections of two pairs of mice. Error bars represent ±SEM, *$p < 0.05$ according to Student's t test.

(B) NF-κB pathway is highly activated in *Sigirr*^{−/−} mice. Protein lysates were prepared from whole colon tissue from two pairs of *Sigirr*^{−/−} and WT mice on day 15 of AOM plus DSS regime. Western blotting was performed with antibodies against p-IKKα/β, p-p65, COX2, and Actin. 1[#] and 2[#] represent samples from two individual mice.

from both wild-type and $Sigirr^{-/-}$ mice (Figure 5F), indicating that β-catenin pathway was activated in this tumor model. β-catenin nuclear accumulation probably resulted from AOM-induced mutations in components of the β-catenin pathway in both wild-type and $Sigirr^{-/-}$ mice. The hyperactivation of NF-κB and STAT3 in $Sigirr^{-/-}$ colon epithelium after AOM+DSS treatment are probably responsible for the increased tumor incidence and tumor growth in $Sigirr^{-/-}$ mice by promoting tumor formation of the transformed cells (with activated β-catenin) mutated by AOM.

### The Impact of Gut-Epithelial Cell-Specific Expression of SIGIRR on the Homeostasis of Colon Epithelium, Intestinal Inflammation, and Tumorigenesis

One important question is what cell type is mainly responsible for the regulatory role of SIGRR in gut mucosal immunity. Although SIGIRR is highly expressed in colon epithelial cells, substantial amounts of SIGIRR expression were also detected in T cells (Figure S1). Therefore, it is important to determine the relative contribution of bone marrow-derived versus non-bone marrow-derived cells for SIGIRR's action in the gut. Interestingly, we found that the $Sigirr^{-/-}$ mice that received bone marrow from either wild-type or $Sigirr^{-/-}$ mice died much faster than wild-type mice transplanted with wild-type or $Sigirr^{-/-}$ bone marrow upon DSS treatment, indicating the main contribution of the non-bone marrow-derived tissue cells to SIGIRR's function in intestinal inflammation (Figure S8). The fact that wild-type or $Sigirr^{-/-}$ mice transplanted with bone marrow from $Sigirr^{-/-}$ mice had somewhat stronger phenotype as compared to those transplanted with wild-type bone marrow also implicated some degree of contribution of bone marrow-derived cells to SIGIRR's function in regulating DSS-induced colitis.

To examine the function of SIGIRR in gut epithelial cells, we generated a gut-epithelial-specific SIGIRR-transgenic mouse by constitutively expressing SIGIRR in the distal small intestinal and colonic epithelium. We chose to express flag-tagged SIGIRR under the control of transcriptional regulatory elements derived from a fatty acid-binding protein gene, a gut-epithelial-specific gene (Figures S9A and S9B; Saam and Gordon, 1999). The expression of SIGIRR transgene was examined by immunoprecipitation with Flag antibody followed by immunoblot analysis with anti-SIGIRR. The SIGIRR transgene was specifically expressed in intestine and colon but not in other tissues (Figure S9C).

To address the function of SIGIRR in gut epithelial cells, the gut-epithelial-specific SIGIRR-transgenic mouse was bred to SIGIRR-deficient mice to create a mouse in which SIGIRR is expressed only in gut epithelial cells and not in other tissues or cell types. We named this mouse SIGIRR-TG–KO. The expression of SIGIRR transgene in the colon of SIGIRR-TG–KO mice was comparable to that in wild-type mice (Figure 6A). Because SIGIRR deficiency leads to increased cell proliferation and survival in the colon epithelium, we examined the impact of gut-epithelial expression of SIGRR transgene on homeostasis of colon epithelium. We compared cell survival and apoptosis of the colon epithelium between SIGIRR-TG–KO and $Sigirr^{-/-}$ mice by in situ TUNEL assay. Interestingly, we found that more apoptotic cells were detected in SIGIRR-TG–KO colon epithelium as compared to $Sigirr^{-/-}$ mice (KO) (Figure 6B), suggesting that gut-epithelial cell-specific expression of SIGIRR transgene reduced cell survival.

To examine the effect of gut-epithelial cell-specific expression of SIGIRR on intestinal inflammation, the SIGIRR-TG–KO mice were compared with $Sigirr^{-/-}$ mice for their susceptibility to DSS-induced colitis. Both SIGIRR-TG–KO and $Sigirr^{-/-}$ mice were continuously treated with 3% DSS in drinking water. Although all of the $Sigirr^{-/-}$ mice died 14 days after DSS treatment, only 45% of the SIGIRR-TG–KO mice died at day 16 after DSS treatment (Figure 6C). These results clearly showed that the SIGIRR-TG–KO mice are more resistant to DSS treatment as compared to $Sigirr^{-/-}$ mice.

In support of the lethality curve, histology analysis of colon sections detected much reduced inflammation and less tissue damage in epithelial layer of the SIGIRR-TG–KO mice as compared to that in $Sigirr^{-/-}$ mice (Figure 6D and Figure S10). Consistent with the histology data, the inflammatory gene expression was induced at much lower amounts in DSS-treated SIGIRR-TG–KO colon as compared to that in $Sigirr^{-/-}$ colon, including IL-6, TNF-α, and IFN-γ (Figure 6E). Taken together, the above results indicate that the specific expression of SIGIRR in gut epithelial cells can rescue the $Sigirr^{-/-}$ mice from developing severe DSS-induced colitis.

Lastly, we examined the effect of gut-epithelial cell-specific expression of SIGIRR on colitis-associated cancer. We first injected the SIGIRR-TG–KO mice with AOM followed by three cycles of oral administration of DSS. In comparison with $Sigirr^{-/-}$ mice, re-expression of SIGIRR exclusively in intestinal epithelia reduced the tumor incidence to 68% (Figure 6F), which is close to that of wild-type mice. Furthermore, the average total and big tumors (>2 mm) per mouse was greatly reduced in SIGGIR-TG–KO mice (Figures 6G and 6H), indicating that SIGIRR's expression in epithelial cells has the ability to suppress colitis-associated tumorigenesis.

(C) Immunochemical staining was performed to examine the nuclear accumulation of cyclin D1 on colonic cross-sections from $Sigirr^{-/-}$ and WT mice on day 15 under AOM plus DSS regime.

(D) Cytokine production in $Sigirr^{-/-}$ and WT mice. On day 15 under the AOM plus DSS regime, 200 mg of colon tissue was removed from $Sigirr^{-/-}$ and wild-type mice. Whole colon cultures were subsequently prepared as described in Experimental Procedures. ELISA was conducted to measure cytokine production. Error bars represent ±SEM, and Student's t test was conducted. *p < 0.05.

(E) Immunohistochemical staining of p-STAT3 on colonic cross-sections of $Sigirr^{-/-}$ and WT mice on day 15 of AOM plus DSS regime.

(F) Nuclear β-catenin staining in tumors from WT and $Sigirr^{-/-}$ mice after the completion of the AOM plus DSS regime. Note the line separating tumor from adjacent normal tissue. Magnification 100× or 400×.
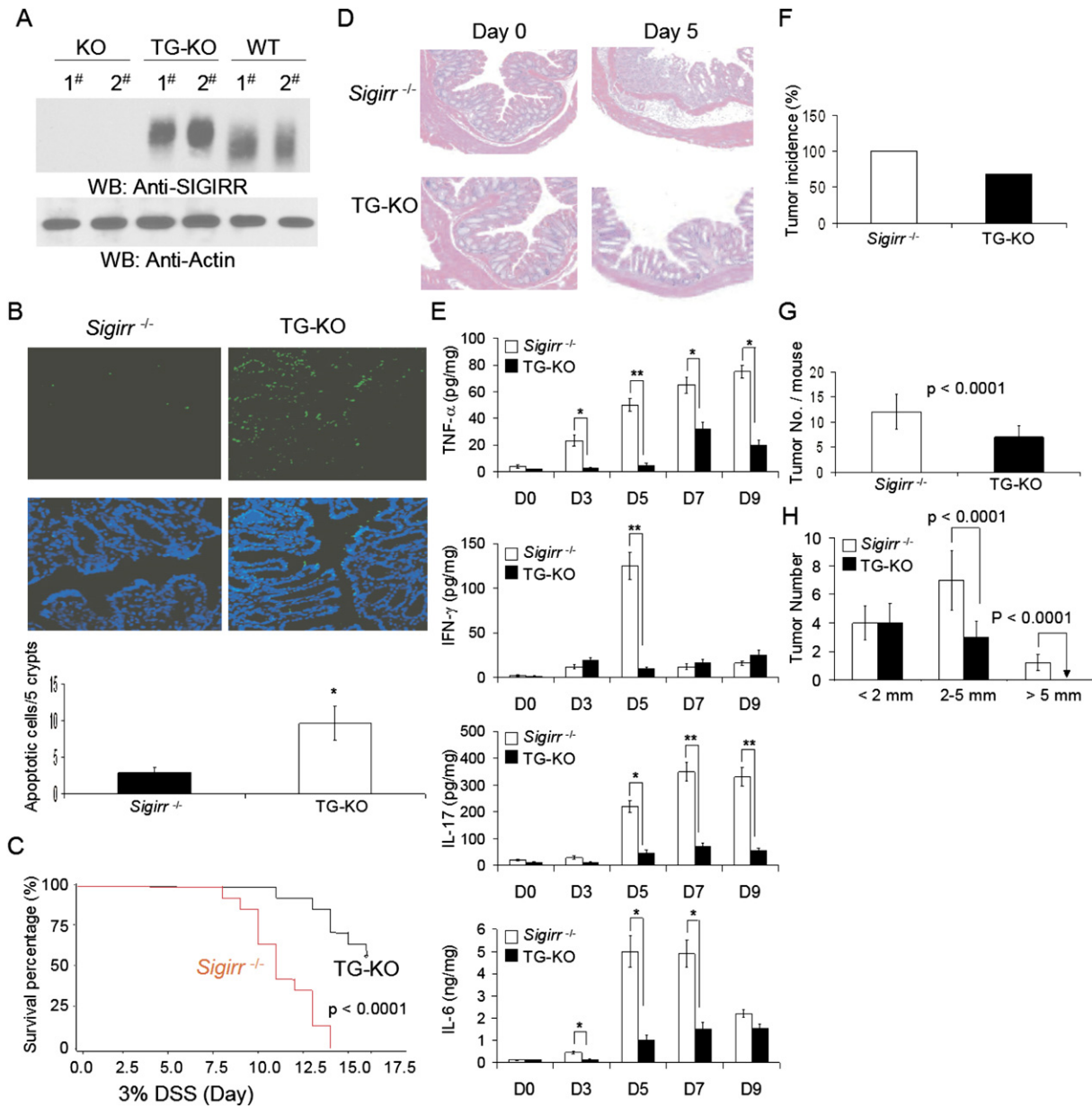
**Figure 6. Gut-Epithelial-Specific Expression of SIGIRR Transgene Rescued the _Sigirr_$^{-/-}$ Mice from Severe DSS-Induced Colitis**

(A) Protein lysates were prepared from colon crypts of the _Sigirr_$^{-/-}$, SIGIRR-TG–KO, and wild-type mice and followed by western analysis with anti-SIGIRR and anti-Actin (R&D).

(B) DNA fragmentation was detected on frozen colon sections by in situ TUNEL assay. At least 30 intact crypts were counted on each slide of three pairs of mice. The data shown is the mean ±SEM. *p < 0.05 compared to _Sigirr_$^{-/-}$ mice, Student's t test.

(C) Kaplan-Meier plot of survival study of SIGIRR-TG–KO and _Sigirr_$^{-/-}$ mice under 3% DSS treatment (p < 0.0001 and n = 14).

(D) Hematoxylin and eosin staining of colonic cross-sections of untreated or DSS-treated mice (magnification 200×).

(E) Whole-colon cultures were prepared from colons of SIGIRR-TG–KO or _Sigirr_$^{-/-}$ mice under DSS treatment for indicated days. The same segments of colon with the weight of 250 mg were cut into small pieces and incubated with 1 ml of serum-free RPMI medium for 24 hr, and the secreted cytokines and chemokines in the medium were then measured by ELISA. Data shown are the mean ± SEM from three experiments. Student's t test was conducted. *p < 0.05, **p < 0.01.

(F–H) Intestine-specific transgene of SIGIRR suppress colonic carcinogenesis in _Sigirr_$^{-/-}$ mice. The data shown are the mean ± SD (_Sigirr_$^{-/-}$, n = 13; SIGIRR-TG–KO, n = 11), p < 0.0001 according to ANOVA analysis.
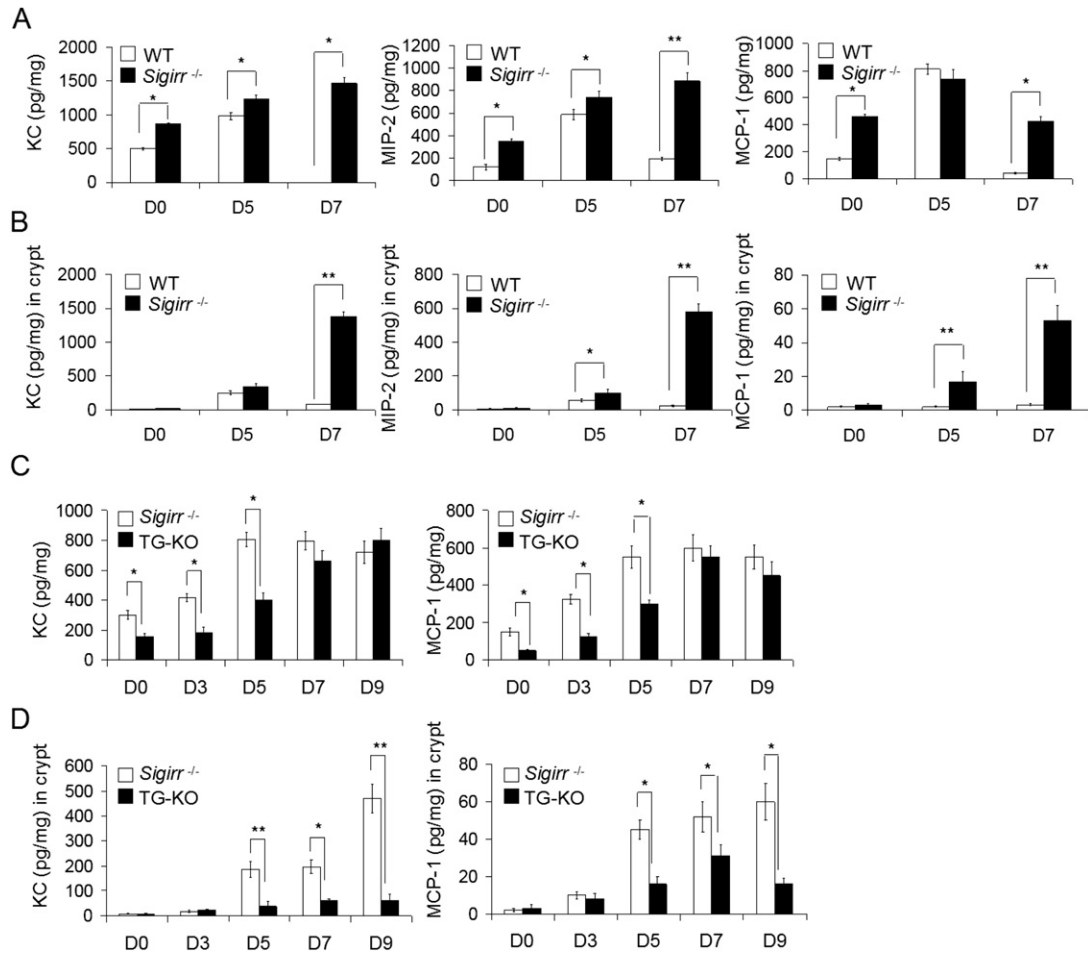
**Figure 7. Chemokine Production Was Highly Induced in *Sigirr*$^{-/-}$ Colon Tissue and Crypt Cells after DSS Treatment**

(A and B) Chemokine production from whole-colon tissue culture (A) or protein lysate of isolated crypt cells (B) from *Sigirr*$^{-/-}$ and WT mice was measured by ELISA.

(C and D) Chemokine production from whole-colon tissue culture (C) or protein lysate of isolated crypt cells (D) from *Sigirr*$^{-/-}$ and SIGIRR-TG–KO mice was measured by ELISA. 200 mg of colon tissue was used for whole-colon tissue culture or isolation of crypt cells as described in Experimental Procedures. Error bars represent ±SEM, and Student's t test was conducted. *p < 0.05, **p < 0.01.

## The Role of Epithelial-Derived SIGIRR in the Regulation of Chemokine Gene Expression

The above results showed that epithelial-derived SIGIRR can specifically rescue DSS-induced colitis and suppress colitis-associated tumorigenesis. The question was then how SIGIRR modulates intestinal inflammation and tumorigenesis through its function in epithelial cells in response to intestinal microflora. Considering that the epithelium can play an important role in attracting inflammatory and immune cells, we examined the expression of several chemokine genes within the inflamed colon. By RT-PCR and ELISA, it was found that the DSS-treated and AOM+DSS-treated *Sigirr*$^{-/-}$ colon had much greater expression of chemokine genes that play critical roles in the recruitment of T cells (IP-10 and MIG), neutrophils (KC and MIP-2), and macrophages (Rantes and MCP-1) as compared to that in wild-type mice (Figure 7A and Figure S11). To address the specific role of the colonic

epithelium in the DSS-induced inflammatory response, we isolated crypt epithelial cells from wild-type and *Sigirr*$^{-/-}$ colon untreated or treated with DSS. Interestingly, the *Sigirr*$^{-/-}$ crypt epithelial cells produced much higher amounts of chemokines after DSS treatment as compared to wild-type crypt cells, including MCP-1, MIP-2, and KC (Figure 7B). These results indicate that much of the increased chemokine expression found in the DSS-treated *Sigirr*$^{-/-}$ mice was mediated by epithelial cells, and the epithelial response probably plays a critical role in DSS-induced colitis.

We then further addressed the role of epithelial-derived SIGIRR in the regulation of chemokine gene expression by using the SIGIRR-TG–KO mice. Interestingly, the colon tissues from SIGIRR-TG–KO had reduced basal amounts of chemokines and also reduced induction of chemokines after DSS treatment as compared to *Sigirr*$^{-/-}$ mice (Figure 7C). These results suggest that the expression of

SIGIRR transgene in gut-epithelial cells suppressed the constitutive activation of chemokine genes and also DSS-induced chemokine gene expression. Furthermore, the crypt cells from DSS-treated SIGIRR-TG–KO mice produced much lower amounts of chemokines as compared to the crypt cells from DSS-treated *Sigirr*$^{-/-}$ mice (Figure 7D). These results indicate that the expression of SIGIRR transgene in gut epithelial cells suppressed the production of chemokines, which is likely to be the main mechanism for how the SIGIRR transgene rescued the *Sigirr*$^{-/-}$ mice from developing severe DSS-induced colitis, thereby suppressing colitis-associated cancer. Taken together, our results clearly indicate that the impact of SIGIRR on the intestinal inflammation and tumorigenesis is mainly through its function in gut-epithelial cells.

## DISCUSSION

The results presented here reveal an important role of SIGIRR in regulating the interaction between commensal microflora and colonic epithelium. The recent study by Rakoff-Nahoum et al. suggests that activation of TLR signaling by commensal microflora is required for the homeostasis of the gut epithelium (Rakoff-Nahoum et al., 2004). Mice deficient in TLR signaling have dysregulated intestinal homeostasis, causing them to be more sensitive to DSS-induced injury. The results in this manuscript indicate that excessive commensal bacteria-induced TLR-IL-1R-mediated signaling in the colon is also detrimental. This suggests that there is a fine balance of pro- and anti-inflammatory signals in the colonic epithelium and that this balance critically involves SIGIRR. Deletion of SIGIRR, the negative regulator of TLR-IL-1R signaling, leads to exaggerated commensal bacteria-induced TLR-IL-1R signaling disrupting the homeostatic regulation of the proliferative and inflammatory responses of the colonic epithelium to commensal bacteria, resulting in enhanced colitis-associated tumorigenesis.

The fact that there were more proliferating cells in the *Sigirr*$^{-/-}$ colon crypts than in control mice indicates an intrinsic proliferative dysregulation in SIGIRR-deficient colonic epithelial cells. Importantly, dysregulated proliferation in the *Sigirr*$^{-/-}$ colonic epithelial cells was no longer detected after removal of commensal bacteria from SIGIRR-deficient mice. These results support the hypothesis that the TLR ligands (such as LPS) carried by commensal bacteria are recognized by Toll-like receptors in the colon epithelial layer, mediating the proliferation and survival of the epithelial cells to maintain the homeostasis of the epithelium. SIGIRR probably modulates the levels of TLR signaling in the epithelial cells either directly through its interaction with the Toll-like receptors that are activated by commensal bacteria or indirectly through its impact on pathways regulated by TLR signaling.

It has been reported that mice deficient in TLR signaling were more sensitive to DSS-induced injury (Rakoff-Nahoum et al., 2004). It was clearly shown that the colon damage and associated mortality in TLR-deficient mice after DSS treatment was not due to overt inflammatory cell infiltration. However, our results showed that inflammation was the primary cause for the increased tissue damage and high mortality in *Sigirr*$^{-/-}$ mice after DSS treatment. Importantly, gut-epithelial-specific expression of SIGIRR transgene rescued the *Sigirr*$^{-/-}$ mice from developing severe DSS-induced colitis, indicating that epithelial-derived SIGIRR has a major impact on intestinal inflammation.

Previous studies showed that chronic inflammation and cancer are closely associated in the intestine (Clevers, 2004; Balkwill and Mantovani, 2001). Indeed, we found that *Sigirr*$^{-/-}$ mice were much more susceptible to develop colitis-associated cancer induced by carcinogen AOM plus DSS as compared with wild-type mice, indicating that the *Sigirr*$^{-/-}$ mice are an excellent model to study the link between inflammation and tumorigenesis. Tumorigenesis can be divided into three mechanistic stages: initiation (genomic alteration), promotion (proliferation of genetically altered cells), and progression (tumor growth) (Karin and Greten, 2005). Inflammatory cells and the innate immune system are shown to be important mediators of tumor promotion and progression, but not for tumor initiation. In our colitis-associated cancer model, carcinogen AOM introduces genomic instability and mutation of oncogenes in the epithelia, whereas repeated DSS treatment creates a microenvironment of chronic inflammation in the colon. Mutations in the APC or β-catenin gene that lead to stabilization and nuclear accumulation of β-catenin and transcriptional activation with TCF-4 play a critical role in the initiation of colorectal tumorigenesis. The activation of β-catenin pathway (β-catenin nuclear accumulation) was detected in the tumor tissues from both wild-type and *Sigirr*$^{-/-}$ mice, implying that AOM probably introduced mutations to the key components of the β-catenin pathway in these mice. When the *Sigirr*$^{-/-}$ mice were subjected to AOM injection or repeated DSS treatment alone, colon tumors were not developed (data not shown), indicating that both AOM-induced tumor initiation and DSS-induced chronic inflammation are necessary for this tumor model.

NF-κB links inflammation and immunity to cancer development and progression (Karin and Greten, 2005). As discussed above, the constitutive activation of TLR signaling (activation of NF-κB and JNK) in the *Sigirr*$^{-/-}$ epithelial cells led to upregulation of genes for cell survival and proliferation (Cyclin D1 and Bcl-xL), resulting in increased cell survival and cell proliferation. Enterocyte-specific ablation of IKKβ was shown to decrease tumor incidence markedly, indicating that IKKβ-dependent NF-κB-activation in intestinal epithelial cells operates during early tumor promotion (Greten et al., 2004). Therefore, the constitutive NF-κB activation in *Sigirr*$^{-/-}$ colon epithelium is likely to contribute to the increased tumor incidence in the *Sigirr*$^{-/-}$ mice.

In addition to increased NF-κB activation, we also detected hyperactivation of STAT3 in colon epithelium of *Sigirr*$^{-/-}$ mice upon AOM+DSS treatment. IL-6, which has been shown to promote cancer growth in inflammation-associated cancer models, was highly induced in

*Sigirr*$^{-/-}$ mice treated with AOM+DSS. The elevated IL-6 production is probably responsible for the increased nuclear phospho-STAT3 detected in *Sigirr*$^{-/-}$ epithelium. Hyperactivation of STAT3 has been shown to promote tumor progression in gastric and colonic cancers (Becker et al., 2004). Colorectal tumors result from accumulation of multiple changes that lead to activation of oncogenes combined with the inactivation of tumor suppressor genes (Radtke and Clevers, 2005). Although deletion of SIGIRR probably does not directly introduce mutations to the colon epithelium, it does lead to the activation of two important transcription factors, NF-κB and STAT3, during the early stage of colitis-associated tumorigenesis, which in turn promotes cell proliferation and cell survival through the upregulation of their target genes (including Cyclin D1 and Bcl-xL) in *Sigirr*$^{-/-}$ epithelium, leading to tumor promotion and progression.

Our studies also implicate that SIGIRR-regulated chemokine gene expression plays a critical role for epithelial-derived SIGIRR to modulate intestinal inflammation and colitis-associated cancer. Importantly, epithelial-specific expression of SIGIRR transgene reduced the expression levels of chemokines before or after DSS stimulation, confirming the critical role of epithelial-derived SIGIRR in the regulation of chemokine gene expression. We hypothesize that the DSS-induced chemokines produced by the *Sigirr*$^{-/-}$ epithelial cells play critical roles in the recruitment of the inflammatory cells to the colon mucosa, leading to severe inflammation, which in turn promotes tumorigenesis in the AOM+DSS model. Although our studies demonstrate a critical role for SIGIRR expression by the colonic epithelium, it is also clear that we can not rule out modest regulatory roles of SIGIRR in other cell types. In addition to its high-expression colon epithelial cells, SIGIRR is also expressed in T cells and has low level of expression in dendritic cells. Future experiments are required to dissect the specific function of SIGIRR in each cell type.

Despite the density of commensal bacteria and their products, the colonic mucosa maintains a controlled state of inflammation (physiologic inflammation). What is the role of SIGIRR in the microbial tolerance of the colonic epithelial layer? Based on the findings in this manuscript, we propose that in addition to its function as a physical barrier, the colon epithelial layer also functions as an active innate immune barrier. SIGIRR is an important modulator to regulate the interaction between commensal bacteria and gut epithelium to maintain the innate immune tolerance of the colon epithelial layer. SIGIRR plays a critical role in preventing overreaction of the colon epithelial layer to commensal bacteria, contributing to the microbial tolerance of the colon. While loss of SIGIRR was insufficient on its own to cause spontaneous colitis in these mice, our findings suggest that SIGIRR is a strong candidate for regulating inflammatory responses against real or perceived luminal threats, with its dysregulation possibly a predisposition to exaggerated or even chronic forms of infectious, idiopathic colitis or colitis-associated cancer.

## EXPERIMENTAL PROCEDURES

### Construction of SIGIRR-Transgenic Mouse

To generate the *Fabpl*$^{4x\ at\ -132}$/*Sigirr* construct, DNA encoding SIGIRR was placed under the control of transcriptional regulatory elements derived from a fatty acid-binding protein gene (Saam and Gordon, 1999) followed by the human growth hormone reporter gene (hGH). *Fabpl*$^{4x\ at\ -132}$ include elements consist of nucleotides –596 to +21 of rat *Fabpl* with 4 additional copies of a 35 bp sequence (spanning nucleotides –177 to –133, that has been inserted at nucleotide –132). Earlier light and EM immunohistochemical studies of transgenic mice demonstrated that *Fabpl*$^{4x\ at\ -132}$ can direct the expression of a human growth hormone (hGH) reporter throughout the epithelium of crypts in the distal small intestine, cecum, and colon of adult mice (Saam and Gordon, 1999). A Flag tag was included at the N terminus of SIGIRR to distinguish the transgene from the endogenous gene. *Fabpl*$^{4x\ at\ -132}$/*Sigirr* was sent to the Transgenic Mouse Service in the University of Cincinnati and injected into the pronucleus of the fertilized eggs, followed by implantation into the oviduct of a 0.5 day p.c. pseudopregnant female mouse. The founders that carry the *Sigirr* transgene were identified by genomic Southern analysis with hGH reporter cDNA and *Sigirr* cDNA as probes. The *Sigirr* transgenic founder lines were bred to generate F1. *Sigirr* transgenic mice were bred to *Sigirr*$^{-/-}$ mice to generate mice express SIGIRR only in gut-epithelial cells. SIGIRR-TG–KO mice were maintained on C57BL/6×129/SvJ background. All of the mice utilized in this manuscript were housed in animal facility (with SPF condition) at the Cleveland Clinic Foundation in compliance with the guidelines set by Institutional Animal Care and Use Committee.

### DSS-Induced Colitis

Experimental colitis was induced by giving 3% (w/v) DSS (M.W. 40,000 kDa; MP Biomedicals Inc., Solon, OH) in drinking water ad libitum. Mice (6–8 weeks) were treated for 16 days for survival studies. For histological, gene expression, and cytokine production studies, mice were sacrificed after DSS treatment for indicated days.

### TUNEL Assay

Frozen sections obtained from untreated mice were fixed with 4% paraformadehyde and permeabilized by 0.1% Triton X-100. TUNEL assay kit (Roche) was used to detect apoptotic cells and DAPI was used to stain the nuclei.

### BrdU Staining

1 mg/ml of BrdU in PBS was injected to mice via i.p. Mice were sacrificed in 24 hr after BrdU injection. The same segment of distal colon was fixed in 10% neutral formalin and paraffin embedded. Proliferating cells were detected with BrdU detection kit (BD Bioscience). Tissues were counterstained with hematoxylin. The number of BrdU-positive cells was quantified by number of cells in intact, well-orientated crypts.

### Whole-Colon Culture

200–300 mg of colon tissue was washed in cold PBS supplemented with penicillin and streptomycin. These segments were cut into small pieces and cultured in 12-well flat bottom culture plates (Falcon) in serum-free RPMI medium. High concentration of penicillin and streptomycin was supplemented to prevent bacteria growth. After incubation at 37°C for 24 hr, medium was collected and stored at −80°C until use.

### Colon Crypt Isolation and Treatment

Mice colon was washed with cold PBS and cut longitudinally. After incubation with 0.04% sodium hypochlorite (Sigma) for 30 min, colon was cut into small pieces and shaken continuously in PBS buffer with 1 mM EGTA and 1 mM EDTA at room temperature for 30 min. Crypts in the supernatant were collected. Collected crypt cells were lysed directly (for ELISA) or after treatment with 10 ng/ml IL-1 (National Cancer Institute), 10 μg/ml LPS (Sigma), or 10 ng/ml TNFα (R&D System) (for western blotting).

### ELISA

Whole-colon culture or crypt lysate from DSS-treated mice was examined for cytokine and chemokine production with ELISA kits obtained from R&D Systems, according to the manufacturer's instruction. Cytokine and chemokine production is normalized by total colon tissue weight (whole-colon culture) or total protein amount (crypt protein lysate) measured by BCA analysis (Pierce). 3–4 pairs of mice were used for each time point.

### Western Blot

Crypt protein lysates were resolved by SDS-PAGE and transferred to PVDF membrane. Blots were probed with phospho-IκBα, phospho-p65, phospho-JNK (Cell Signaling), anti-IκBα, Cyclin-D1, Bcl-xL, and β-Actin (Santa Cruz Biotechnology, CA). After incubation with HRP-conjugated secondary antibody, ECL (Amersham, Arlington Heights, IL) was used to develop.

### Commensal Depletion

6- to 8-week-old mice were treated with ampicillin (A, 1 g/l, Sigma), vancomycine (V, 500 mg/l), neomycin sulfate (N, 1 g/l), and metronidazole (M, 1g/l) in drinking water for 4 weeks. Stool collected from antibiotic-treated and untreated mice was diluted and ground in 1.5 ml PBS. The ground stool was diluted 10 times with PBS and fixed with formalin. 2 μl of fixed bacteria was diluted in 1 ml PBS. This dilution was filtered through 0.2 μm Whatman Anodisc 25 filter (VWR) with the help of low vacuum. Attached bacteria were incubated in 100 μl of SYBR green solution (SYBR Green 1 nucleic acid gel stain, Invitrogen). The stained filters were dried and covered with mounting medium on a slide. Bacteria stained on the filter were counted under fluorescence microscope. After commensal depletion, mice were then either sacrificed for BrdU staining or treated with 3% DSS in drinking water for indicated days and then sacrificed for histological study.

### Tumorigenesis Procedure

8-week-old mice ($Sigirr^{-/-}$ and WT littermates on C57BL/6 background and $Sigirr^{-/-}$ mice and SIGIRR-TG–KO littermates on mixed C57BL/6×129/SvJ background) were injected with AOM (Sigma) dissolved in 0.9% NaCl intraperitoneally at a dose of 12.5 mg/kg body weight. 5 days after injection, mice were treated with 2.5% DSS in drinking water, then followed by regular water for 16 days. This cycle was repeated twice (at the third cycle, mice were treated with 2.0% DSS for 4 days) (Greten et al., 2004). 2 weeks after DSS treatment, mice were sacrificed and murine colon was removed and flushed carefully with PBS buffer. Colon was then cut longitudinally and fixed flat in 10% neutral buffered formalin overnight. All of the colon tumors were counted and measured under a stereomicroscope. Representative tumors were paraffin embedded and sectioned at 5 μm. Histology analysis was carried out on H&E-stained tumor sections.

### Immunohistochemistry

Formalin-fixed and paraffin-embedded colon sections or tumor samples were deparaffined, rehydrated, and pretreated with 3% hydrogen peroxidase in PBS buffer for 20 minutes. Antigen retrieval in DAKO's antigen retrieval buffer was conducted in a steam cooker for 20 minutes at 96°C, followed by slowly cooling down at room temperature. After blocking with DAKO's block buffer, avidin/biotin block, sections were incubated with anti-Ki67 (1:150, Dako, USA), anti-β-Catenin (1:1000, BD Pharmingen), anti-CyclinD1 (1:50, Santa Cruz), anti-p65 (1:1000, Abcam), or anti-p-STAT3 (1:50, Abcam) for 1 hr at room temperature. After incubation with biotin-conjugated secondary antibody and streptavidin-HRP, positive signals were visualized by DAB kit (BD pharmingen) and counterstained with Harris hematoxylin (Fisher Scientific).

### Statistic Analysis

Kaplan-Meier study was performed for analyzing mouse survival under DSS treatment. ANOVA was used to characterize the tumorigenic studies under AOM plus DSS regime. Student's t test was used for all other studies and $p < 0.05$ is considered significant.

### Supplemental Data

Supplemental Data include 11 figures and Experimental Procedures and can be found with this article online at http://www.immunity.com/cgi/content/full/26/4/461/DC1/.

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