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Cutting Edge

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Cutting Edge: STING Mediates Protection against Colorectal Tumorigenesis by Governing the Magnitude of Intestinal Inflammation

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Stimulator of IFN genes (STING) is a cytoplasmic innate immune sensor for cyclic dinucleotides that also serves a dual role as an adaptor molecule for a number of intracellular DNA receptors. Although STING has important functions in the host defense against pathogens and autoimmune diseases, its physiological role in cancer is unknown. In this study, we show that *STING*-deficient mice are highly susceptible to colitis-associated colorectal cancer. Colons of *STING*-deficient mice exhibit significant intestinal damage and overt proliferation during early stages of tumorigenesis. Moreover, *STING*-deficient mice fail to restrict activation of the NF-κB– and STAT3-signaling pathways, which leads to increased levels of the proinflammatory cytokines IL-6 and KC. Therefore, our results identified an unexpected and important role for STING in mediating protection against colorectal tumorigenesis. *The Journal of Immunology*, **2014, 193: 4779–4782.**

Colorectal cancer (CRC) is the third most common malignant tumor in the United States, with 1.5 million new cases being diagnosed each year (1). Clinical studies showed that patients diagnosed with inflammatory bowl diseases have a greater risk for developing CRC compared with healthy individuals (2). Pattern recognition receptors are key initiators of inflammation and were shown to be involved in colitis-associated CRC development (3).

Cytoplasmic nucleic acid receptors mediate the detection of DNA and RNA molecules within the cell and provide robust host immune defense against intracellular pathogens and protection against autoimmune diseases (4). Stimulator of IFN genes (STING) is a cytoplasmic receptor for cyclic dinucleotides and an adaptor for other DNA sensors (5–10). The upstream DNA sensors that signal through STING include cGAS, IFI16, and DDX41 (8–10), highlighting an important function for STING in governing multiple DNA-recognition pathways. STING recruits TBK1 to activate IRF3, the latter of which translocates into the nucleus in a dimeric form and induces transcription of type I IFNs.

Since its discovery, STING was shown to contribute to the host defense against viral, bacterial, and eukaryotic pathogens (11). Whether STING plays a role during the development of CRC or other types of cancer is unknown. Evidence for a role of a number of nucleic acid sensors in tumorigenesis is emerging. Our group and other investigators showed that NLRP3, a proposed sensor of RNA or DNA:RNA hybrid in the cytoplasm of a cell (12–14), mediates protection against CRC development by modulating IL-18 production (15, 16). The dsDNA sensor AIM2 and the dsRNA sensor RIG-I are downregulated in patients with CRC and hepatocellular carcinoma, respectively, and reduced expression of these proteins is associated with increased mortality in these patients (17, 18). Expression of LGP2, a negative regulator of RIG-I, promotes the survival of colon tumor and other cancer cell lines (19). In addition, mice lacking the DNA sensor Ku70, in combination with the $p53^{R172P}$ mutation, develop spontaneous colonic inflammation and CRC (20). These studies indicate that nucleic acid sensors may play a critical role in the regulation of tumorigenesis.

In this study, we show that *STING*-deficient mice are highly susceptible to tumor formation in a model of colitis-associated CRC. The absence of STING leads to excessive colon inflammation during early stages of tumor development, which is characterized by elevated levels of colonic and circulating proinflammatory cytokines and more proliferative intestinal epithelial cells. In addition, we observed increased phosphorylation of NF-κB and STAT3 in *STING*-deficient mice, indicating a role for STING in governing the suppression of transcription factor activities during intestinal inflammation.

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Abbreviations used in this article: AOM, azoxymethane; CRC, colorectal cancer; DSS, dextran sulfate sodium; F, forward; MIB, mouse intestinal *Bacteroides*; R, reverse; SFB, segmented filamentous bacteria; STING, stimulator of IFN genes; WT, wild type.

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Overall, these results underscore a critical role for STING in mediating host protection against colorectal tumorigenesis.

Materials and Methods

Mice

Wild-type (WT; C57BL/6) and *STING*-deficient mice [referred to as *STING*$^{gt/gt}$ in this article and described previously (21)] were all matched for age and sex. Mice were housed in a specific pathogen–free facility, and animal studies were approved by the St. Jude Children's Research Hospital Committee on the Use and Care of Animals. Mice were injected i.p. with 10 mg azoxymethane (AOM; Sigma) per kilogram of body weight. Following AOM injection, drinking water was supplemented with 3% dextran sulfate sodium (DSS; molecular mass 36-40 kDa; MP Biologicals) for 6 d, followed by a regular water supply for 2 wk. Two additional cycles of 2.5% DSS were used (Supplemental Fig. 1A). Mice were sacrificed 4 wk after the last round of DSS treatment.

Histology

Histological analysis and scoring, as well as Ki-67 staining techniques, were performed as described (22, 23).

Cytokine analysis

Levels of cytokines from colon and sera samples were measured by ELISA, according to the manufacturers' instructions (IL-18 from eBioscience, IFN-β from BioLegend, and all other cytokines from Millipore).

Western blotting

Proteins were extracted using RIPA buffer supplemented with protease and phosphatase inhibitors (Roche). Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Primary Abs used were anti-ERK, anti-IκBα, anti–p-ERK, anti–p-IκBα, anti-STAT3, anti–p-STAT3 (all from Cell Signaling Technology), and anti–caspase-1 p10 (Santa Cruz).

Quantitative real-time PCR

RNA was extracted using TRIzol reagent (Life Technologies). Isolated RNA was reverse transcribed into cDNA using the First-Strand cDNA Synthesis Kit (Life Technologies). Real-time PCR was performed on an ABI 7500 real-time PCR instrument with 2× SYBR Green (Applied Biosystems). For PCR analysis of intestinal bacteria, fecal DNA was extracted using a QIAamp DNA Stool Mini Kit (QIAGEN). Primers used were *STING*-forward (F) 5′-CAT TGG GTA CTT GCG GTT-3′, *STING*-reverse (R) 5′-CTG AGC ATG TTG TTA TGT AGC-3′, *β-actin*–F 5′-CAG CTT CTT TGC AGC TCC TT-3′, *β-actin*–R 5′-CAC GAT GGA GGG GAA TAC AG-3′, eubacteria (Universal)-F 5′-ACT CCT ACG GGA GGC AGC AGT-3′, eubacteria (Universal)-R 5′-ATT ACC GCG GCT GCT GGC-3′, Prevotellaceae-F 5′-CCA GCC AAG TAG CGT GCA-3′, Prevotellaceae-R 5′-TGG ACC TTC CGT ATT ACC-3′, *Bacteroides*-F 5′-GGT TCT GAG AGG AGG TCC C-3′, *Bacteroides*-R 5′-GCT GCC TCC CGT AGG AGT-3′, mouse intestinal *Bacteroides* (MIB)-F 5′-CCA GCA GCC GCG GTA ATA-3′, MIB-R 5′-CGC ATT CCG CAT ACT TCT C-3′, segmented filamentous bacteria (SFB)-F 5′-GAC GCT GAG GCA TGA GAG CAT-3′, SFB-R 5′-GAC GGC ACG GAT TGT TAT TCA-3′, TM7-F 5′-GCA ACT CTT TAC GCC CAG T-3′, and TM7-R 5′-GAG AGG ATG ATC AGC CAG-3′. The level of *STING* expression was normalized to *β-actin*. The level of the 16S rRNA gene from each bacterial population was normalized to the 16S rRNA gene of eubacteria.

Statistical analysis

Statistical differences were determined by the Student *t* test; $p < 0.05$ was considered statistically significant.

Results and Discussion

STING is expressed in a variety of tissues and organs in mice (Fig. 1A). The spleen and small and large intestines expressed the highest levels of STING mRNA (Fig. 1A). To investigate the role of STING in colon tumor development, we used a colitis-associated CRC model to induce colon tumorigenesis in mice (Supplemental Fig. 1A). We injected AOM i.p. into WT and *STING*$^{gt/gt}$ mice, followed by three rounds of DSS treatment. Eighty days post-AOM injection, we observed that *STING*$^{gt/gt}$ mice had significantly increased tumor burden compared with WT controls (Fig. 1B, 1C). Although the number of tumors doubled in *STING*$^{gt/gt}$ mice, the size of tumors in *STING*$^{gt/gt}$ and WT mice was similar (Fig. 1D). Histological analysis revealed that *STING*$^{gt/gt}$ mice exhibited more severe pathological damage after tumor development, which was characterized by increased colonic inflammation and hyperplasia (Fig. 1E, 1G). Histological observations were quantified, and the scores revealed that *STING*$^{gt/gt}$ mice showed enhanced intestinal pathology compared with WT mice (histological scores: 17.6 ± 1.2 for *STING*$^{gt/gt}$, 10.6 ± 0.9 for WT; Fig. 1F). All regions of the colon from *STING*$^{gt/gt}$ mice, especially the distal region, displayed more severe pathology compared with WT mice (Supplemental Fig. 1B). Notably, dysplasia was observed exclusively in *STING*$^{gt/gt}$ mice, with 75% of the colon samples from *STING*$^{gt/gt}$ mice showing hallmarks of low-grade (50% of the samples) or high-grade (25%) dysplasia (Fig.

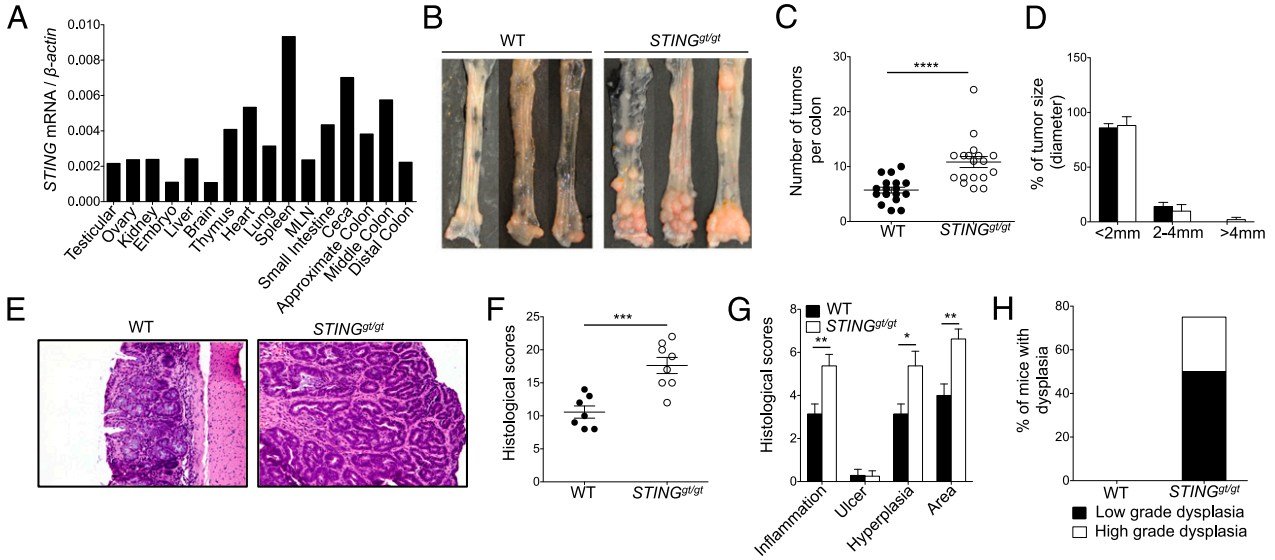

FIGURE 1. STING is critical for mediating protection against colon tumor development. (**A**) Relative *STING* mRNA levels in different tissues of WT C57BL/6 mice. (**B**) Colons from WT and *STING*$^{gt/gt}$ mice on day 80 post-AOM injection. Tumor number (**C**) and size (**D**) in WT and *STING*$^{gt/gt}$ mice. (**E**) Colon tissue sections (H&E, original magnification ×20). Total histological scores (**F**) and scores for different parameters (**G**) on day 80. (**H**) Percentage of WT and *STING*$^{gt/gt}$ mice with dysplasia. Data are mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

1H). Therefore, these data suggest a crucial role for STING in suppressing colorectal tumorigenesis in colitis-associated CRC.

We observed that *STING*$^{gt/gt}$ mice lost significantly more body weight than did WT mice (Fig. 2A). Indeed, the colon length was greatly reduced in *STING*$^{gt/gt}$ mice on day 14 (Fig. 2B). H&E staining revealed an increase in cellular infiltration, crypt thickness, and hyperchromatin in colon tissues of *STING*$^{gt/gt}$ mice on day 14 (Fig. 2C). In agreement, all of the histologic parameters assessed—inflammation, ulceration, and hyperplasia—were significantly higher in *STING*$^{gt/gt}$ mice compared with WT mice (Fig. 2D, 2E). To assess whether STING mediates neoplastic changes that predispose the host to increased tumorigenic events, we performed Ki-67 staining on colon tissue sections and quantified the magnitude of intestinal epithelial proliferation. In line with our histological analysis, the colon of *STING*$^{gt/gt}$ mice had significantly increased numbers of Ki-67$^+$ cells/crypt (58.7 ± 2.1; $n = 137$) compared with WT mice (39.6 ± 1.3; $n = 153$; $p < 0.0001$) on day 14 (Fig. 2F). Taken together, these results suggest that STING plays an important function in controlling disease initiation and susceptibility to hyperproliferation during early stages of colitis-associated CRC.

Because inflammation is one of the most important drivers in the development of CRC (3), we hypothesized that STING controls inflammation during early stages of AOM-DSS treatment, which ultimately determines host susceptibility to colorectal tumorigenesis. To analyze the magnitude of colonic inflammation in *STING*$^{gt/gt}$ mice, we measured levels of the proinflammatory cytokines IL-6 and KC on day 14 (in the absence of any tumors) and day 80 (when tumors were fully developed). At 14 d post-AOM injection, we observed a significant increase in the levels of IL-6 and KC in colon tissues of *STING*$^{gt/gt}$ mice compared with WT mice (Fig. 3A). We confirmed this finding and observed elevated levels of circulating IL-6 and KC in the serum of *STING*$^{gt/gt}$ mice (Fig. 3B). Consistently, we found elevated phosphorylation of ERK and IκBα in colon tissues of *STING*$^{gt/gt}$ mice compared with WT mice on day 14 (Fig. 3C). On day 80, when tumors were fully developed, the levels of IL-6 and KC remained elevated in *STING*$^{gt/gt}$ mice (Supplemental Fig. 1C). Previous reports showed that myeloid cell–derived IL-6 signals through IL-6R and gp130 to activate cytoplasmic STAT3 in intestinal epithelial cells (24, 25). STAT3 is frequently activated in premalignant and cancer cells to promote colon tumorigenesis by amplifying inflammation and tumorigenic transformation (26). Indeed, colon tissues from *STING*$^{gt/gt}$ mice showed increased STAT3 phosphorylation compared with WT mice

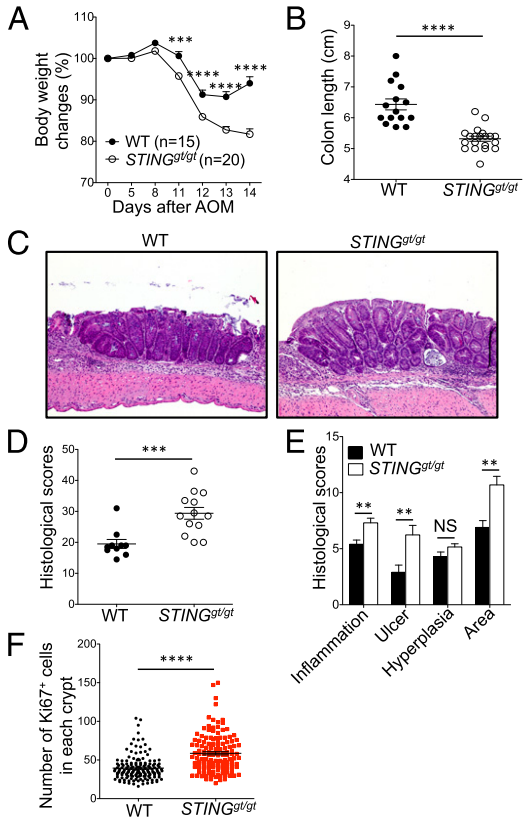

FIGURE 2. STING dampens early intestinal damage and overt proliferation. (**A**) Body weight changes in WT and *STING*$^{gt/gt}$ mice during early stages of colorectal tumorigenesis. (**B**) Colon length on day 14 post-AOM injection. (**C**) H&E staining of colon tissue sections on day 14 (original magnification ×10). Total histological scores (**D**) and scores for different parameters (**E**) on day 14. (**F**) Number of Ki-67$^+$ cells in each crypt of each animal on day 14 (≥27 crypts/mouse). WT, $n = 5$; *STING*$^{gt/gt}$, $n = 5$. Data are mean ± SEM of two independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

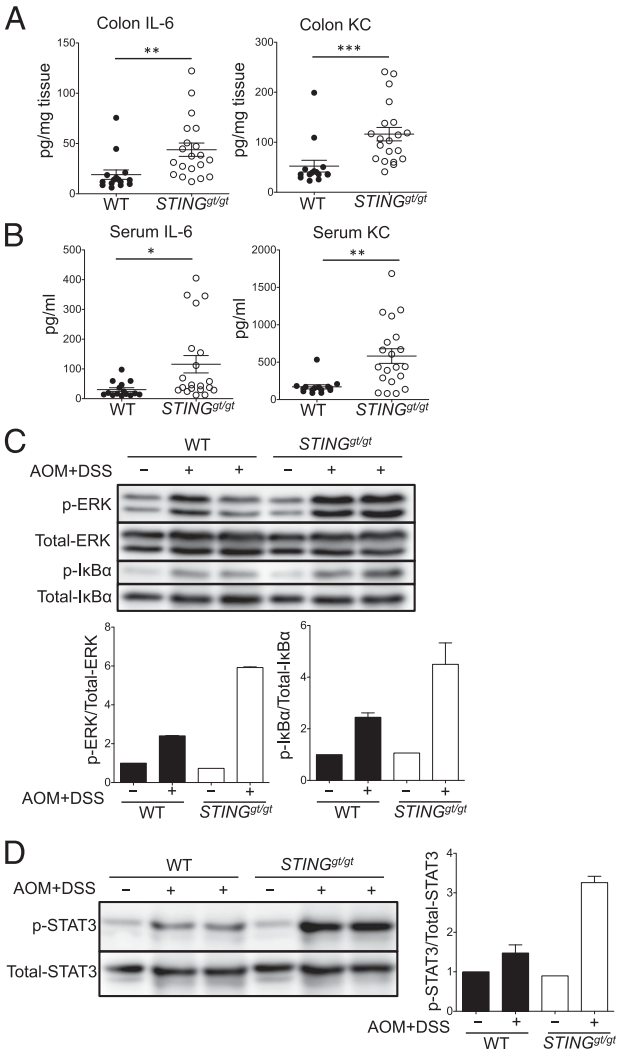

FIGURE 3. STING suppresses overt colon inflammation during tumor development. Levels of IL-6 and KC in colon tissues (**A**) and serum (**B**) on day 14 post-AOM injection. Phosphorylation of ERK and IκBα (**C**) and STAT3 (**D**) in colon tissues on day 14, quantified by densitometric analysis. Data are mean ± SEM of two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(Fig. 3D). We also measured type I IFN production 14 d post-AOM injection and detected only basal levels of IFN-β in WT or *STING*$^{gt/gt}$ mice (Supplemental Fig. 2A). Caspase-1–mediated release of IL-18 was shown to play a protective role in AOM-DSS–induced colorectal tumorigenesis (16). We found reduced levels of caspase-1 activation and IL-18 release in colon tissues of *STING*$^{gt/gt}$ mice compared with WT mice after 14 d (Supplemental Fig. 2B, 2C). Therefore, it is possible that the cross-talk between STING and caspase-1 could mediate protection at the intestinal barrier. These findings highlight that STING restricts NF-κB– and STAT3-signaling pathways to control colon inflammation and tumorigenesis.

The gut microbiota plays a critical role in modulating susceptibility to CRC. Dysbiosis is associated with increased susceptibility to colitis and CRC development (3). To investigate whether an altered gut microbiota profile contributed to the elevated incidence of colorectal tumorigenesis in *STING*$^{gt/gt}$ mice, we performed real-time quantitative PCR analysis to detect the abundance of five major intestinal bacterial populations. We found that WT and *STING*$^{gt/gt}$ mice harbored similar levels of Prevotellaceae, *Bacteroides*, MIB, SFB, and TM7 (Supplemental Fig. 2D–H). Therefore, it is unlikely that differences in these bacterial populations contributed to the protective role of STING during CRC development.

A recent study revealed that administration of multiple doses of the STING ligand cyclic di-GMP, alone or in combination with an adjuvant, reduced the number of metastases, tumor weight, and tumor size in a mouse model of metastatic breast tumor (27). The ability of cyclic di-GMP to enhance T cell responses, amplify IL-12 production by myeloid-derived suppressor cells, and activate caspase-3–dependent cell death in tumor cells was reported to play a role in reducing metastatic breast cancer (27). The antitumorigenic properties of cyclic di-GMP in breast cancer indicate a need to evaluate the therapeutic value of this STING ligand in CRC. It is possible that STING itself, or in concert with an upstream DNA sensor, recognizes endogenous DNA released by dying cells during intestinal barrier damage. In addition, oxidative DNA damage was shown to accumulate in intestinal epithelial cells and activate STING signaling (28, 29). It is possible that recognition of endogenous DNA by STING mediates suppression of inflammation, which reduces the likelihood of uncontrolled inflammation leading to tumorigenesis. Overall, our results underscore a novel role for STING in modulating activation of NF-κB and STAT3 signaling and production of IL-18 during the development of CRC. These inflammatory mediators may modulate the tumorigenic properties of intestinal cells, which ultimately regulate proliferation and tumorigenesis. Collectively, our data indicate that STING plays a critical role in mediating protection against inflammation-associated CRC development. Therapeutic modulation of STING in susceptible individuals may have the potential to prevent and treat CRC.

Disclosures

The authors have no financial conflicts of interest.

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