Increased Susceptibility to Colitis-Associated Cancer of Mice Lacking TIR8, an Inhibitory Member of the Interleukin-1 Receptor Family

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Abstract

TIR8 (also known as SIGIRR) is a member of the interleukin-1/Toll-like receptor family with inhibitory activity on inflammatory reactions and high expression in intestinal mucosa. Here, we report that Tir8-deficient mice exhibited a dramatic intestinal inflammation in response to dextran sulfate sodium salt (DSS) administration in terms of weight loss, intestinal bleeding, and mortality and showed increased susceptibility to carcinogenesis in response to azoxymethane and DSS. Increased susceptibility to colitis-associated cancer was associated to increased permeability and local production of prostaglandin E2, proinflammatory cytokines, and chemokines. Thus, these results are consistent with the hypothesis that TIR8, by negatively regulating intestinal inflammation, plays a nonredundant role in the control of the protumor activity of chronic inflammation in the gut. [Cancer Res 2007; 67(13):6017–21]

Introduction

Several lines of evidence, based on epidemiologic studies as well as animal models, are consistent with the view that chronic local inflammation plays an important role in malignant progression (1–3). The inflammatory environment of tumors is characterized by the presence of mediators and cells that act as tumor promoters (1). Soluble mediators such as cytokines, chemokines, enzymes, and growth factors are released by tumor cells and stromal cells and are responsible of leukocyte recruitment in the tumor environment, survival, and differentiation. Leukocytes infiltrating the stroma, in particular tumor-associated macrophages, promote tumor proliferation and progression, stroma deposition and remodeling, and angiogenesis and inhibit effective antitumor T-cell–dependent immunity (1, 4). A pivotal role in these pathophysiologic processes is played by the transcription factor nuclear factor κB (NF-κB), which has dual functions in promoting tumor growth. NF-κB activated in inflammatory cells in response to microbial stimuli, inflammatory cytokines, and danger signals released by necrotic cells regulates the production of cytokines, chemokines, and growth and angiogenic factors; in tumor cells, activation of NF-κB leads to the production of cell cycle genes, antiapoptotic genes, and invasive proteases (5–7).

The activation of the signaling cascade leading to NF-κB activation by interleukin-1 receptors (IL-1R) and Toll-like receptors (TLR) is tightly regulated at different levels, extracellularly and intracellularly, and more than 20 pathways of negative regulation of IL-1R/TLR signaling have been described (8, 9).

The IL-1R family member TIR8 (also known as single immunoglobulin IL-1R-related molecule, SIGIRR), an orphan receptor, inhibits signaling from the IL-1R/TLR complexes, possibly by trapping IRAK-1 and TRAF-6 (8, 10). TIR8 is characterized by the presence of a single immunoglobulin domain in its extracellular region, a conserved TIR domain, and a 95-amino-acid long tail with inhibitory properties (11, 12). TIR8 is expressed in several tissues, especially in the digestive tract, and cell-type expression is particularly high in epithelial cells (12, 13). Accordingly, there is evidence for a nonredundant regulatory role of this molecule in inflammation involving the gastrointestinal mucosa (13).

Colitis-associated cancer is a colorectal disease that arises in patients suffering from chronic inflammatory bowel disease, in particular ulcerative colitis (14). In mice, colitis-associated cancer can be induced by injection of the procarcinogen azoxymethane, followed by three cycles of exposure to dextran sodium sulfate (DSS; ref. 15), which causes chronic inflammation, mimicking inflammatory bowel disease.

Given the regulatory role of TIR8 in the intestinal tract, it was important to assess its potential involvement in colitis-associated cancer. Here, we report that Tir8 deficiency resulted in increased susceptibility to colitis-associated cancer, which was associated to higher local production of proinflammatory cytokines and chemokines. These results strongly support the hypothesis that TIR8 plays a nonredundant, tuning role in gastrointestinal inflammation–associated cancer and underline its potential as target for cancer prevention and therapy.

Materials and Methods

Animals. Tir8-deficient (Tir8<sup>−/−</sup>) mice were generated as described (13). Mice used were 8 to 12 weeks old on a mixed (C57BL/6J × 129/SvJ) or inbred (backcrossed for 11 generations with C57BL/6J) background. Littermates of Tir8<sup>−/−</sup> mice or C57BL/6J obtained from Charles River Laboratories were used as wild-type controls (Tir8<sup>+/+</sup>). Mice were housed in a specific pathogen-free animal facility of the Istituto Clinico Humanitas in individually ventilated cage systems. Procedures involving animals and their care conformed with institutional guidelines in compliance with national (D.L. N.116, G.U., suppl. 40, 18-2-1992) and international (EEC Council Directive 86/609, OF L 358,12-12-1987; NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council 1996) laws and policies. All efforts were made to minimize the number of animals used and their suffering.
Azoxy methane/DSS–induced colon cancer. To induce colon tumors, mice were treated, as described (15), with a single dose (10 mg/kg) of the mutagenic agent azoxymethane (Sigma) followed by three cycles of 3%, 2%, or 1.5% DSS (molecular mass, 40 kD; ICN) dissolved in sterile, distilled drinking water, resulting in a 60-day experimental period. At the end of treatment, mice were sacrificed for histologic analysis of the intestine. For cytokine production analysis, animals were sacrificed at the end of the first DSS cycle. The determination of clinical scores was done as described (13).

Histologic analysis. The large intestine was removed, rolled up, fixed in 10% neutral buffered formalin, and embedded in paraffin. H&E-stained serial tissue sections were used for pathologic evaluation in a blinded fashion by a pathologist (E.S.). Proliferative and neoplastic lesions were classified as gastrointestinal intraepithelial neoplasia, low-grade and high-grade adenoma, or adenocarcinoma. The area of each proliferative lesion was determined with an image analysis software (Win Roof, version 3.6, Mitani).

Immunohistologic analysis. Eight-micrometer-thick consecutive frozen sections were cut and mounted on Superfrost slides (Bio-Optica). Immunohistochemistry was done with the following antibodies: biotinylated anti-CD68 monoclonal antibody (mAb; clone FA-11, HyCult Biotechnology), rabbit anti-human/mouse CD3 polyclonal antibody (DakoCytoMation), rat anti-mouse/rat FoxP3 mAb (clone FJK-16s, eBioscience), and rat anti-mouse CD88 monoclonal antibody (mAb; clone FA-11, HyCult Biotechnology), rabbit anti-human/mouse CD3 polyclonal antibody (DakoCytomation), biotinylated antiammunoglobulin G (Vector Laboratories), and ZyMax streptavidin-HRP conjugate (Zymed). The chromogen was 3,3' diaminobenzidine–free base. A quantitative evaluation of immunostaining was applied using WinRec Programme (Image Pro Plus) by counting immunopositive cells per field at ×20 magnification.

Whole colon organ culture and colon tissue lysate. Colon segments (100–200 mg of tissue) were washed in cold PBS supplemented with penicillin and streptomycin and either homogenized in PBS containing (100–200 mg of tissue) were washed in cold PBS supplemented with penicillin and streptomycin and either homogenized in PBS containing protease inhibitors cocktail Complete (Roche Diagnostic) and stored at −80°C or cultured in 24-well flat-bottomed culture plates in RPMI 1640 with 10% FCS. After incubation at 37°C for 24 h, supernatants were centrifuged at 13,000 rpm at 4°C for 5 min and stored at −80°C until analyzed.

ELISA. Murine cytokines and chemokines (IL-6, tumor necrosis factor α, IL-1β, IL-10, IFNγ, KC/CXC, JE/CCL2, and MIP1α/CCL3) were measured in whole colon culture supernatants and colon tissue lysates by ELISA (R&D DuoSet ELISA Development Systems). The sandwich ELISA for PTX3 was done with the anti-murine PTX3 mAbs 2C3 and 6B11. Prostaglandin E2 (PGE2) was measured by enzyme immunoassay (Cayman Chemical Company). Levels were standardized to the content of total protein by quantification by bicinchoninic acid analysis (Pierce) and presented as nanograms or picograms of cytokine per milligram of protein.

Evaluation of colonic epithelial permeability. Colonic epithelial permeability was assessed by the penetration of Evans blue (Sigma) from the lumen into the wall of colon on day 7 after administration of 2% DSS as described (16).

Results

Deletion of Tir8 does not perturb normal gastrointestinal development, morphology, or function in nonchallenged mice. Analysis of body weight, intestinal histology in terms of morphology and number of lymphoid follicles at different time points (2, 6, 10, and 12 months of age), and cytokine and chemokine production at the intestinal level in untreated mice did not reveal differences between Tir8−/− and Tir8+/+ mice on both genetic backgrounds examined (C57BL/6 and C57BL/6×129/Sv; data not shown). These results suggest that, under homeostatic conditions in the absence of epithelial barrier erosion, Tir8 deficiency is not sufficient to result in spontaneous intestinal inflammation triggered by endogenous intestinal flora.

To assess the role of TIR8 in colitis-associated cancer, the protocol of a single azoxymethane injection followed by three
cycles of DSS for 7 days and normal drinking water for 14 days was applied. Mice treated only with azoxymethane did not develop tumors during the observation period, suggesting that in the absence of induced inflammation, Tir8 deficiency per se does not increase susceptibility to cancer development.

As shown in Fig. 1, the protocol of colitis-associated cancer resulted in dramatic susceptibility to colon inflammation in Tir8−/− mice compared with Tir8+/+ mice, with high rate of mortality associated to intestinal bleeding and weight loss, in particular in mice on C57BL/6 background, a more susceptible strain to DSS-induced colitis than 129/Sv. In C57BL/6 Tir8−/− mice fed with 3% or 2% to 1.5% DSS, mortality was 100% in 10 days or 80% at the end of the experimental period, respectively, compared with 30% or 14% in Tir8+/+ mice (Fig. 1A). In Tir8−/− mice treated with 2% to 1.5% DSS, the body weight loss was 26.5% at day 10 and 11% at day 50, compared with 7.8% and +3%, respectively, in Tir8+/+ mice ($P < 0.001$ and $P < 0.05$ at the two time points; Fig. 1B). Bleeding scores were more severe in Tir8−/− mice throughout the period analyzed (Fig. 1C). In mice on a mixed background fed with 3% DSS, mortality was 33% in Tir8−/− mice and 20% in wild-type littermates at the end of the experimental period, and the clinical scores, in terms of body weight loss and in particular of intestinal bleeding, were less severe (data not shown).

The permeability to Evans blue albumin was used as an index of colon epithelial permeability. Colonic epithelial permeability to Evans blue was significantly increased in Tir8−/− DSS-fed mice compared with Tir8+/+ mice ($P = 0.001$; Fig. 1D).

As shown in Fig. 2A, which reports results obtained in mice on a mixed background fed with 3% DSS at the end of the 60-day experimental period, the deficiency of Tir8 significantly increased the incidence of tumors ($P = 0.007$). When the severity of lesions was examined, a significant difference was also observed in the number of gastrointestinal intraepithelial neoplasia ($P = 0.01$) and of low-grade adenomas ($P = 0.04$), but not of high-grade adenomas ($P = 0.3$). Although there was no difference in incidence, high-grade adenomas tended to be bigger in Tir8−/− mice (2.67 ± 0.8 versus 1.72 ± 0.28 mm²). Given the high susceptibility of C57BL/6 mice to DSS-induced colitis, a modified protocol was used (2% in the first cycle and 1.5% in the following two cycles or 1.5% for three cycles) with increased incidence of lesions, mostly low-grade adenomas in Tir8−/− mice ($P = 0.01$; data not shown).

Similarly to ulcerative lesions, most of the proliferative lesions were in the distal colon and rectum, projecting into the lumen, and eventually almost completely obliterating the rectal lumen (Fig. 2B–D).

To address the mechanisms underlying increased susceptibility to inflammation and colon cancer in Tir8-deficient mice, NF-κB-regulated genes encoding proinflammatory factors involved in colon inflammation and tumor growth were measured by ELISA in lysates and supernatants of colon segments collected from mice at the end of the first DSS cycle. As shown in Table 1, increased levels of IL-1β, IL-6, transforming growth factor β (TGFβ), and of chemokines JE/CCL2, KC/CXC, and MIP1α/CCL3 were observed in colon homogenates from Tir8−/− mice compared with wild-type littermates. Similar results were obtained when supernatants from colon specimens were considered (22 ± 4 and 38 ± 5 ng/mg KC/CXC in Tir8−/− and Tir8+/+ mice, respectively). In addition, IL-10 and PGE2 levels were higher in Tir8−/− supernatants ($P = 0.01$). Interestingly, a significant decrease of IFNγ in Tir8−/− supernatant was observed (4.2 ± 4.2 versus 25 ± 7 pg/mg in controls; $P = 0.01$).

Finally, tumors arising in Tir8−/− mice were investigated in terms of leukocyte infiltration (Fig. 3). The leukocyte infiltrate of Tir8−/− tumors was characterized by a more prominent infiltration of CD68+ cells ($P = 0.01$) and, intriguingly, of FoxP3+ regulatory T cells ($P = 0.01$; Fig. 3A and B). The percentages of FoxP3+ cells over CD3+ cells were 54% and 85% in Tir8+/+ and Tir8−/− tumors,
respectively, whereas the percentages of CD8+ cells over CD3+ cells were 28% and 8% in Tir8+/+ and Tir8-/- tumors.

**Discussion**

Colon carcinoma represents a paradigm for the connection between inflammation and cancer (6). The present study was designed to explore the regulatory role of TIR8, an inhibitory member of the IL-1/TLR family, on intestinal carcinogenesis. Results obtained confirm and extend previous observations on the increased susceptibility to intestinal inflammation of Tir8-deficient mice. Moreover, Tir8-deficient mice showed increased susceptibility to carcinogenesis in response to azoxymethane and DSS.

Activation of NF-κB has been reported in epithelial cells and macrophages from inflammatory bowel disease patients as well as in colon cancers, and anti-inflammatory therapy with inhibitors of cyclooxygenases and NF-κB signaling pathway reduces the risk of colitis-associated cancer (1, 6). TIR8 acts in vitro and in vivo as a negative regulator of NF-κB activation in response to TLR and IL-1R agonists (10, 13). In the present study, Tir8-deficient mice showed increased production of prostaglandins, inflammatory cytokines (e.g., IL-1 and IL-6), and chemokines (KC/CXC, JE/CCL2, and MIP1α/CCL3) downstream of NF-κB. These have been shown to promote inflammation-propelled neoplasia, in particular in the gastrointestinal tract (6, 15). For instance, chemokines produced by tumor cells and by recruited leukocytes influence the extent and phenotype of leukocyte infiltrate, tumor cell and endothelial cell growth and migration, and, finally, are involved in recruiting polarized T helper 2 cells and T regulatory cells (17). In particular, CXC chemokines mediate angiogenesis downstream of prostaglandins in colon cancer (18, 19).

A predominant T helper 2 inflammatory response has been implicated in colon cancer and IFNγ deficiency increased the frequency of colonic neoplasms (20). The low levels of IFNγ observed in Tir8-/- colons could be indicative of a T helper 2 polarized environment. Higher levels of IL-10 and TGFβ, associated to higher FoxP3+ regulatory T-cell infiltrate in the tumors of Tir8-deficient mice, possibly reflect the development of an immunosuppressive response inhibiting effective antitumor T-cell-dependent immunity (17).

The results presented here show that TIR8 acts as a negative regulator of intestinal inflammation and inflammation-promoted activation of NF-κB has been reported in epithelial cells and macrophages from inflammatory bowel disease patients as well as in colon cancers, and anti-inflammatory therapy with inhibitors of cyclooxygenases and NF-κB signaling pathway reduces the risk of colitis-associated cancer (1, 6). TIR8 acts in vitro and in vivo as a negative regulator of NF-κB activation in response to TLR and IL-1R agonists (10, 13). In the present study, Tir8-deficient mice showed increased production of prostaglandins, inflammatory cytokines (e.g., IL-1 and IL-6), and chemokines (KC/CXC, JE/CCL2, and MIP1α/CCL3) downstream of NF-κB. These have been shown to promote inflammation-propelled neoplasia, in particular in the gastrointestinal tract (6, 15). For instance, chemokines produced by tumor cells and by recruited leukocytes influence the extent and phenotype of leukocyte infiltrate, tumor cell and endothelial cell growth and migration, and, finally, are involved in recruiting polarized T helper 2 cells and T regulatory cells (17). In particular, CXC chemokines mediate angiogenesis downstream of prostaglandins in colon cancer (18, 19).

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The results presented here show that TIR8 acts as a negative regulator of intestinal inflammation and inflammation-promoted activa

| Table 1. Inflammatory mediators in colon lysates from DSS-fed mice (N = 8) |
|-----------------------------|-----------------------------|-----------------------------|
|                            | Tir8+/+ (pg/mg protein)     | Tir8-/- (pg/mg protein)     | P  | Fold increase |
| IL-1β                      | 171.3 ± 39.03               | 304.3 ± 28.44               | 0.01 | 1.8          |
| IL-6                       | 30 ± 19.55                  | 158.6 ± 57.3                | 0.02 | 5.3          |
| MCP-1/CCL2                 | 48.75 ± 12.88               | 243.3 ± 95.7                | 0.03 | 4.6          |
| KC/CXC                     | 390 ± 59.22                 | 2,501 ± 1,110               | 0.03 | 6.4          |
| MIP1α/CCL3                 | 65.0 ± 10.41                | 186 ± 57.93                 | 0.05 | 2.9          |
| PTX3                       | 1,431 ± 203.9               | 3,216 ± 1,108               | 0.05 | 2.2          |
| TGFβ                       | 80 ± 4.08                   | 151.4 ± 25                  | 0.03 | 1.9          |
| IL-10-1                    | 58 ± 18                     | 155 ± 34                    | 0.01 | 2.7          |
| IFNγ                       | 257 ± 7                     | 4.2 ± 4.2                   | 0.01 | 0.17         |
| PGE2-1                     | 8.58 ± 1.79                 | 19.45 ± 3.6                 | 0.01 | 2.3          |

*Values are presented as mean ± SE.

Unpaired one-tailed Student’s t test was used.

Measures were done in supernatants.

PGE2 values are in nanograms per milligram of protein.
carcinogenesis in mice. It will be important to assess whether polymorphisms at the TIR8 locus impinge on human colon cancer and whether TIR8 can be a target for pharmacologic intervention.

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References

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