Transcription Factor NFATc2 Controls the Emergence of Colon Cancer Associated with IL-6-Dependent Colitis

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Abstract
NFAT transcription factors control T-cell activation and function. Specifically, the transcription factor NFATc2 affects the regulation of cell differentiation and growth and plays a critical role in the development of colonic inflammation. Here, we used an experimental model of colitis-associated colorectal carcinoma to investigate the contribution of NFATc2 to the promotion of colonic tumors. Compared with wild-type animals that readily presented with multiple colon tumors, NFATc2-deficient mice were protected from tumor development. This observed decrease in colonic tumor progression was associated with reduced endoscopic inflammation, increased apoptosis of lamina propria T lymphocytes, and significantly reduced levels of the critical proinflammatory cytokines interleukin (IL)-21 and IL-6. Administration of hyper IL-6 abrogated protection from tumor progression in NFATc2-knockout mice and restored tumor incidence to control levels. Taken together, our findings highlight a pivotal role for NFATc2 in the establishment of inflammation-associated colorectal tumors mediated by control of IL-6 expression. Cancer Res; 72(17); 1–11. © 2012 AACR.

Introduction
In humans, 2 major forms of inflammatory bowel disease (IBD) have been described (1). Both diseases bear the immunologic stigmata of an exaggerated CD4 T-helper (Th1) cell response associated with typical mediator proinflammatory cytokines, such as TNF-α and IL-6, which can activate NF-κB signaling, thus leading to cell proliferation, augmented angiogenesis, and inhibition of cell death furthermore promoting tumorigenesis and development of metastases (6). About 20% of all forms of cancer arise in association with chronic inflammation and likewise in those cancers that do not develop as a consequence of chronic inflammation, the tumor microenvironment may exhibit extensive inflammatory infiltrates with high levels of proinflammatory cytokine production (6).

The best characterized protumorigenic cytokine is, besides TNF-α, IL-6. It has become clear that an increased level of IL-6, which can be found in sera of patients, is linked to a higher risk of developing colorectal adenomas (7). IL-6 was shown to influence all stages of tumor development, including initiation, promotion, progression, and metastasis (8, 9) mainly resulting from its ability to activate the oncogenic transcription factor STAT3 (10). In CAC, the activation of STAT3 was shown to correlate with the amount of IL-6 secretion (11). Autocrine and paracrine factors that are produced during colitis and within the protumorigenic microenvironment control the activation of STAT3 in premalignant cells (6). Previously, it has been shown that the deletion of STAT3 in enterocytes has an inhibitory effect on adenoma development and embanks adenoma growth. At the early stages of tumor induction, most of the IL-6 in CAC is produced by lamina propria macrophages and dendritic cells (12), whereas at later stages of tumor development, it is mainly produced by CD4+ T cells (13). This is probably due to the fact that T cells persist through the high inflammatory content of tumors, whereas epithelial cells may die during tumor progression (6).

Upon activation, during an ongoing immune response, naive T cells differentiate into effector T cells consequently secreting various cytokines. This plastic process is precisely controlled by the interplay of the cytokine environment and additionally
tightly regulated by transcription factors (14). The T_{H1} transcription factors involved are T-bet, STAT1, and STAT4, which play important effector functions in experimental colitis in the gut (15, 16). On the other hand, the role of typical T_{H2}-associated transcriptional genes c-maf, GATA-3, and especially NFAT in the control of effector functions within an inflammatory environment is far from being understood in detail (17). Crohn disease and ulcerative colitis are characterized by augmented production of some T_{H1} and T_{H2} cytokines. However, it is meanwhile established that Crohn disease- and ulcerative colitis-related inflammation is also associated with enhanced secretion of IL-17 produced by a distinct subset of T_{H1} cells, termed T_{H17} cells. T_{H17} cytokines can have both tissue-protective and inflammatory effects in the gut and recent studies suggest that T_{H17} cells are able to alter their cytokine program according to the stimuli received in the local environment. It became clear that this process is controlled by inflammatory stimuli as well as by the production of IL-6 (18, 19).

The nuclear factor of activated T cells (NFAT) belongs to a transcription factor family of 5 members (NFATc1, NFATc2, NFATc3, NFATc4, and NFAT5) that all have a highly conserved DNA-binding domain (20). In T lymphocytes NFAT proteins govern gene expression, thereby regulating T-cell development, activation, differentiation as well as the induction and maintenance of T-cell tolerance. Furthermore, NFAT proteins coordinate multiple cytokines, such as IL-3, IL-4, and TNF-α, as well as impact critically the differentiation and growth of cells (21–23). Moreover, it became clear that NFAT proteins distinctly influence angiogenesis resulting from an activation of the pro-angiogenic factor VEGF (24) as well as from an induction of COX2, which was shown to impinge on migratory processes within the tumor (25). On the basis of the aforementioned characteristics, it has been suggested that NFAT transcription factors are involved in tumorigenic processes.

NFATc2 was shown to be constitutively expressed in T cells and functions as a critical player in T-cell activation (22, 23, 26). In addition, it has become evident from the analysis of NFATc2-deficient mice that in the absence of this transcription factor expression of IL-6 was significantly reduced. It was found that the aforementioned reduced IL-6 levels in NFATc2-knockout (KO) animals were causative for the protection of mice in models of colonic inflammation (17). In the present study, we therefore aimed to characterize the role of NFATc2 in the initiation and promotion of colorectal tumor formation as well as to define the involvement of critical pro-tumorigenic cytokines such as IL-6. To this end, we made use of NFATc2-deficient animals in an experimental model of colorectal tumorigenesis.

Materials and Methods

Patients

Colon resections from patients with tumors were studied and compared with respective control samples. Patients’ characteristics are reported in Supplementary Table S1. The use of surgical samples was approved by the local ethical committee.

Animals

BALB/c- and NFATc2-deficient mice were obtained from the animal care facility and described before (27). Mice used in the experimental tumor model were between 7 and 12 weeks of age and were housed under specific pathogen-free conditions. Experiments were conducted in accordance with institutional guidelines.

Induction of colitis-associated cancer

Induction of CAC was done as previously described (28). Colitis-associated tumors were induced by an intraperitoneal (i.p.) injection of a single dose of aoxymethane (AOM) with 10 mg per 1 kg body weight (Sigma) on the first day followed by 3 cycles of 2% dextran sodium sulfate (DSS; MP Biomedicals) in drinking water for 1 week and normal drinking water for 2 weeks. For the analysis of the IL-6 function, mice were injected i.p. with 2 μg of hyper IL-6 (29) in PBS on 5 consecutive days in the experiment, starting on day 20.

Miniendoscopic analysis

Tumor development was monitored with a high-resolution video endoscopic system (Storz) at indicated time points. Scoring of DSS colitis severity was conducted at end of 2% DSS-based cycle on MEICS according to a previously established scoring system (30). Scoring of tumor development was based on tumor size and the number of tumors, as previously established in detail (28, 30). For the investigation of early developing tumors during the treatment of human IL-6 (hIL-6), the colon of wild-type and NFATc2-KO mice was stained with methylene blue and then monitored by mini-endoscopy.

Analysis of apoptosis

Colon and tumor tissues were removed from mice and cryosections were prepared. To visualize proapoptotic cells, caspase-3 was stained by using a commercially available CaspACE-FITC kit (Promega) according to manufacturer’s instructions. The nuclei were counterstained with Hoechst dye 33342 (Invitrogen). Numbers of apoptotic cells were counted per field of view at ×10 magnification.

Immunohistochemistry

Colonic cryosections from normal colon and tumor tissue of wild-type and NFATc2-KO mice were obtained for hematoxin and eosin (H&E) staining and analyzed by microscopy.

Staining of CD4+ T cells was done using monoclonal L3T4 antibody to CD4 at a concentration of 1:200 (BD Biosciences). Afterwards, slides were incubated with a biotinylated anti-rat secondary antibody (BioLegend) and stained with a TSA-Cy3 system (PerkinElmer) according to the manufacturer’s guidelines.

For intracellular staining of Foxp3, an anti-rat antibody (BD Biosciences) was chosen. Goat-anti-rat as biotinylated secondary antibody was used for subsequent staining in combination with TSA-Cy3 signal amplification kit. Cytokine staining of IL-6 was done with a rat antibody (eBioscience) in combination with a secondary goat anti-rat antibody and the TSA-Cy3 kit. For intracellular pSTAT3 staining, monoclonal rabbit antibody to pSTAT3 (Cell Signaling) was used together with biotinylated
goat anti-rabbit secondary antibody and finally stained with TSA system. Nuclei were counterstained with Hoechst dye 33342 (Invitrogen). For staining of NFATc2 in patients, a specific antibody (ImmunoGlobe) was used in combination with the biotinylated secondary goat-anti-rabbit antibody (Dianova) and for subsequent staining with TSA-Cy3 signal amplification kit. Quantification of positive cells was conducted by counting positive cell in 6 to 10 high-power fields.

**Preparation of cytopsins**

Intestinal epithelial cells were isolated and stained for CD326, CD11c, and IL-6. For detailed description, see Supplementary Material and Methods.

**Isolation of mRNA and real-time PCR**

Total RNA was isolated from tissue of mice using the RNA micro kit (MACHERY-NAGEL) according to manufacturer’s guidelines. cDNA was subsequently generated with Affinity Script Multi-Temp RT (Stratagene). Quantitative real-time PCR was conducted with the SYBR Green Kit (Bio-Rad) in combination with specific primers for IL-6 (Qiagen). Using β-actin as a reference gene, the relative expression level of cytokine mRNA was calculated.

**Cell isolation of spleen mononuclear cells and lamina propria mononuclear cells**

Mononuclear cells were isolated from spleen specimens of the AOM/DSS-treated BALB/c and NFATc2-KO mice. Therefore, the MACS system (Miltenyi Biotec) was used to isolate CD4⁺ T cells according to the manufacturer’s instructions. The cells were cultured at a density of 2.5 × 10⁶/mL in Iscove’s Modified Dulbecco’s Medium. CD4⁺ T cells were incubated in the presence or absence of anti-CD3/CD28 antibodies. For preparation of lamina propria mononuclear cells (LPMC), see Supplementary Material and Methods (31).

**Cytokine measurements**

Cell culture supernatants were taken 48 hours after stimulation of cells. Cytokine concentration was measured in supernatants of mononuclear spleen cells, colonic pieces, or isolated splenic CD4⁺ T cells. In addition, sera of AOM/DSS-treated BALB/c and NFATc2-KO mice were tested. Quantification of IL-6 was conducted using ELISA analysis according to the manufacturer’s instructions (eBioscience).

**Statistics**

Statistical differences between groups were determined using the Student t test. P values <0.05 were considered as statistically significant and identified with 1 asterisk or <0.001 with 2 asterisks or even 3 asterisks. Results are expressed as mean values. The error bars in histogram figures represent SEM.

**Results**

**Enhanced expression of NFAT family members in patients with IBDs**

NFATc1 was found to play a fundamental role in controlling calcium-dependent T-cell activation (20–23). Furthermore, we previously showed that NFATc2-expressing T cells have major functions in IBD. To characterize the impact of NFATc2 in the development of inflammation-associated cancer, in a first set of experiments, we analyzed the expression of NFATc2 in patients with IBD by immunohistochemistry.

In these studies, colonic cryosections from patients with and without colorectal tumors were stained with NFATc2-specific antibodies. As shown in Fig. 1A, NFATc2⁺ cells were found in the lamina propria of patients, and the number was significantly increased in tumor tissue (Fig. 1B) when compared with cryosections of control tissue, thereby supporting the concept that NFATc2-expressing cells accumulate within colonic tumors in IBD.

**A key regulatory role of NFATc2 in CAC**

To analyze the functional role of NFATc2 in CAC, we made use of NFATc2-deficient mice. In these studies, NFATc2-KO and wild-type mice were subjected to cycles of AOM/DSS, which is characterized by the induction of adenomas with dysplasia appearing within 8 to 10 weeks.

Wild-type mice readily developed multiple colonic tumors (Fig. 2A). Mini-endoscopic analyses revealed significantly reduced tumor numbers in NFATc2-deficient mice when compared with wild-type mice. Consistently, histologic analyses showed mucosal hyperplasia, crypt distortions, and rarification in wild-type mice subjected to cycles of DSS/AOM in contrast to NFATc2-deficient animals, which nearly revealed normal histologic profiles. Furthermore, the tumor scores evaluated by a pathologist showed significantly reduced scores for NFATc2-deficient mice when compared with wild-type animals. The tumor score, which takes into account not only the number of tumors but also their size, was significantly lower in NFATc2-KO mice than in wild-type mice (Fig. 2B).

In additional experiments, mice were subjected to cycles of DSS and development of colitis severity was monitored by mini-endoscopy (Supplementary Fig. S1A). It became clear from these experiments that NFATc2-KO mice were significantly protected from the development of DSS-mediated colitis compared with wild-type mice (Supplementary Fig. S1B).

As discussed above, NFATc2-deficient mice were significantly protected from the development of colonic tumors in the AOM/DSS model. The observed protection from the development of inflammation in NFATc2-KO mice might be explained by the significantly decreased levels of proinflammatory cytokines. However, mRNA levels of some proinflammatory cytokines such as IFN-γ and IL-1β were comparable between wild-type and knockout animals (Supplementary Fig. S2A and S2B), and IL-11 mRNA was only upregulated in tumor tissue from wild-type animals (Supplementary Fig. S3A). On the other hand, immunomodulatory cytokines such as IL-17, IL-21, and IL-22 might control the proinflammatory and protumorigenic role of NFATc2 in the experimental CAC model. Indeed, IL-22 production by splenic T cells and IL-21 production by LPMCs were significantly reduced in the absence of NFATc2 as compared with controls (Supplementary Figs. S3B and S4A). As IL-21 has been recently shown to augment inflammation and tumorigenesis in the AOM/DSS model (32), these data are
consistent with the idea that NFATc2 deficiency, at least partially, controls colitis and tumor development by regulating IL-21 production. Further analysis of IL-17A production showed significantly increased production by splenic T cells but no changes in lamina propria cells in the absence of NFATc2 (Supplementary Fig. S4B).

We next assessed the production of IL-6 in the CAC model. It was found that IL-6 levels were significantly reduced in sera from NFATc2-KO mice in comparison with wild-type mice (Fig. 2C). Splenic CD4⁺ T cells from NFATc2-deficient mice produced significantly lower amounts of IL-6 than T cells from wild-type mice (Fig. 2D). Furthermore, we analysed LPMCs from NFATc2-deficient and wild-type mice by real-time PCR and normalized IL-6 levels to the housekeeping gene β-actin. It became apparent from these studies that LPMCs from NFATc2-KO mice produced significantly lower amounts of IL-6 in normal tissue as well as in tumors (Fig. 2E). Further characterization of intestinal epithelial cells and myeloid cells by cytospin analyses showed no marked reduction of IL-6 production in the absence of NFATc2 (Supplementary Fig. S5A and S5B), suggesting that this transcription factor mainly regulates IL-6 production by CD4⁺ T cells, as previously reported (17).

In the next set of experiments, we stained CD4⁺ cells in tumor and normal tissue to clarify whether the deficiency of NFATc2 would affect the number of T cells in the mucosal tissue. No significant differences in the number of tumor-infiltrating T cells could be observed between both groups (Fig. 3A). Furthermore, we assessed whether NFATc2 had a direct effect on the tolerance mechanisms and analyzed Foxp3 expression in cryosections from wild-type and knockout mice. No differences in Foxp3 expression could be found in tumor and normal tissue between both groups (Fig. 3B).

As discussed above, IL-6 production was shown to be lower in splenic cells, in serum, and in LPMCs from NFATc2-deficient mice when compared with wild-type mice. In further experiments, we conducted analyses of mucosal pSTAT3 expression, which mainly regulates IL-6 expression. Increased pSTAT3⁺ cells could be observed in tumor cryosections as compared with the normal mucosa from wild-type mice (Fig. 3C). In addition, the number of pSTAT3⁺ cells was significantly reduced in tumor tissue from NFATc2-KO mice as compared with tumor tissue from wild-type mice. Likewise, IL-6 expression levels were significantly enhanced in tumor and normal tissue of wild-type mice when compared with NFATc2-deficient mice (Fig. 3D).
Figure 2. Tumor-promoting role of NFATc2 in an experimental CAC. A, NFATc2-KO mice were analyzed in the AOM/DSS model of CAC. The tumor evaluation was monitored using mini-endoscopy. In comparison to wild-type (wt) animals, NFATc2-KO mice were found to be protected in this experimental model and developed few colonic tumors. For this experiment, 12 wild-type mice and 11 NFATc2-KO mice were used. Histologic sections (bottom) were H&E stained. Wild-type mice showed big tumors with increased cell density and lesions. In contrast, in the absence of NFATc2, mice revealed little signs of tumor development and showed nearly a normal colon structure. B, tumor numbers and tumor scores of NFATc2-KO and wild-type mice in the AOM/DSS model. C, IL-6 concentrations in sera of NFATc2-KO mice and wild-type mice analyzed by ELISA. D, analysis of IL-6 secretion in splenic CD4⁺ T cells. CD4⁺ T cells from knockout and wild-type were stimulated for 48 hours with anti-CD3/CD28 antibodies, and the supernatants were analyzed by IL-6 ELISA. E, real-time PCR analysis of IL-6 mRNA expression in tumor/normal tissue of wild-type and NFATc2-KO. Total RNA was isolated from tissue, cDNA was generated, and real-time PCR was done as described. Significant differences are indicated.
Figure 3. NFATc2 has no influence on T-cell numbers but regulates IL-6 and pSTAT3 expression. Immunohistochemical stainings of normal and tumor colon tissue. Representative stainings are shown. Negative controls showed no positive cells. Data represent mean values ± the SD per high-power field. A, immunohistochemical stainings of CD4⁺ T cells in colonic cryosections. Tissues were incubated with a CD4⁺-specific antibody, and nuclei were counterstained with Hoechst dye. Quantitative analysis of positive cells revealed no significantly different numbers of CD4⁺ cells in normal and tumor tissue between NFATc2-deficient mice and wild-type mice. B, impact of NFATc2 deficiency on Foxp3⁺ regulatory T cells. Foxp3⁺ stainings of cryosections showed a similar distribution of Foxp3⁺ cells in normal tissues of wild-type and NFATc2-KO mice. Arrows represent Foxp3⁺ cells. Quantitative analysis of positive cells revealed no significantly different numbers of Foxp3⁺ cells in normal and tumor tissue of NFATc2-deficient mice compared with wild-type mice. C, pSTAT3 expression in the colon. Cryosections were stained with a pSTAT3-specific antibody and the nuclei were counterstained with Hoechst dye. Quantitative analysis of positive cells revealed a significantly increased number of pSTAT3⁺ cells in tumor tissue of wild-type mice as compared with NFATc2-deficient mice. D, IL-6 stainings in cryosections of wild-type and NFATc2-KO mice. Shown are immunohistochemical stainings of normal and tumor tissues with an IL-6-specific antibody, and the nuclei were counterstained with Hoechst dye. Negative controls showed no positive cells. Quantitative analysis of positive cells revealed a significantly increased number of IL-6⁺ cells in tumor tissue of wild-type mice as compared with NFATc2-deficient mice.
Increased apoptotic rate of NFATc2-deficient lamina propria T cells in CAC

It has been established previously that NFATc2-deficient mice harbor a defect in the apoptosis of lymphocytes. To clarify a possible involvement of dysregulated apoptosis in mediating the protection from CAC in the absence of NFATc2 in the next set of experiments, we assessed apoptosis in mucosal tumors and normal tissue. Accordingly, cryosections of wild-type and NFATc2-KO mice were stained by caspase-3 assays (Fig. 4A). It became apparent from these studies that control tissue from wild-type mice had only low amounts of proapoptotic cells. In contrast, tumors and control tissue of NFATc2-KO mice presented with significantly higher numbers of proapoptotic cells. Furthermore, immunohistochemical analyses of CD4 and caspase-3 (Fig. 4B) revealed a higher number of apoptotic T cells in the tumor tissue of NFATc2-deficient mice compared with wild-type mice. These results indicated that T cells are mainly affected by NFATc2 deficiency. The number of apoptotic T cells decreased in the rescue experiment, where application of hIL-6 to NFATc2-KO mice during the induction of CAC was conducted. Our data therefore supported the notion that external factors such as reduced IL-6 levels could result in increased mucosal T-cell apoptosis in tumors in the absence of NFATc2. Additional analyses supported the concept that NFATc2 deficiency may also inhibit tumor induction via downregulation of COX2 and VEGF. COX2 and VEGF mRNA levels were found to be expressed at markedly lower levels in tumor tissue from NFATc2-KO mice as compared with wild-type mice (Supplementary Fig. S6A and S6B). In accordance with the decreased IL-6 production in NFATc2-KO mice, the proliferation rate of
IL-6 reverses the protumorigenic effect of NFATc2 deficiency in a model of colorectal tumorigenesis

The aforementioned results therefore suggested that NFATc2 acts as an important regulatory factor normally supporting the induction of IL-6. IL-6 in turn supports the development of inflammation and tumors. To test whether activation of IL-6 signaling in vivo could overcome the reduced susceptibility of NFATc2-deficient mice to develop CAC, we next treated NFATc2-deficient mice during the experimental tumor model with hyper IL-6. In these experiments, NFATc2-deficient mice were injected i.p. with hyper-IL-6 once a week over a time period of 5 weeks. Strikingly, NFATc2-deficient mice given hyper-IL-6 showed induction and development of colorectal tumors comparable with wild-type mice, as shown by mini-endoscopic analysis (Figs. 5A and B), histopathologic analyses (Fig. 5C), and tumor scores (Fig. 5D). Administration of hyper IL-6 abrogated the protection of NFATc2-KO mice in the colon tumor model and restored tumor induction in NFATc2-KO mice to levels observed in wild-type mice treated with hIL-6.

In summary, the results of the present study strongly suggest that the protumorigenic role of NFATc2 in CAC model is mainly caused by the regulation of the critical proinflammatory cytokine IL-6.

Discussion

In the present study, we established a critical tumor-promoting role of NFATc2 in an experimental model of colorectal tumorigenesis. The clinical relevance of the presented findings is further supported by the fact that in human patients with colitis-associated cancer, NFATc2 expression levels were found to be significantly elevated when compared with samples from control patients. These data underline an important protumorigenic impact of NFATc2 in CAC. In line with this notion, in the absence of NFATc2, mice were almost completely protected from the development of colorectal tumors in DSS/AOM model, in contrast to wild-type mice, which readily developed colonic tumors. The protumorigenic role of NFATc2 in the initiation of colorectal cancer could result from its impact on cytokine production, cell-cycle regulation, apoptosis regulation, and activation of calcium signaling (33). Promotion of angiogenesis via induction of VEGF (24) and initiation of tumor migration upon regulation of COX2 (25) could further account for the tumor-promoting capacities of NFATc2 (34).

Recently, it has been shown that cytokines control tumorigenesis (32, 35, 36). Interestingly, the transcription factor NFATc2 influences proinflammatory cytokines such as IL-21 that may contribute to carcinogenesis by regulating tumor growth. Data from Stolﬁ and colleagues showed that IL-21 was critically involved in the regulation of CAC (32). In the present study, IL-21 was found to be expressed at lower levels in supernatants from lamina propria cells. This observation suggests that NFATc2-dependent IL-21 may play an important role in the local environment of developing tumors. However, it should be noted that NFATc2 deficiency did not abrogate proinflammatory cytokine production in general, as other cytokines with important functions in colorectal tumorigenesis such as TNF and IFN-γ (35, 36) were not affected by the absence of NFATc2. With regard to the production of IL-17A, an important tumor-regulating cytokine (37, 38), no changes were observed between lamina propria cells from NFATc2-KO and wild-type mice suggesting that this cytokine does not play a major role in controlling NFATc2-dependent tumor growth. Furthermore, IL-11 is known to bind to its low-affinity receptor and pg130 with subsequent signal activation of STAT3. Here, we established that production of IL-11 is only increased in tumors from wild-type but not from NFATc2-KO mice. These data could be explained by the fact that the IL-11 promoter possesses 2 NFAT-binding sites (39) and supporting the notion that regulation of IL-11 is obsolete in NFAT-KO mice. In support of the suggested role of NFATc2 in the development of inflammation-associated colorectal cancer, it was shown that NFAT functions as an important player in the regulation of CD4+ T-cell proliferation and activation (40). In the present set of experiments, immunohistochemical analyses of colonic cryosections from NFATc2-KO and wild-type control animals did, however, not reveal any significant differences in the number of colon-residing CD4+ T cells. In the absence of NFATc2, mice showed no differences in T-cell numbers and regulatory T-cell numbers when compared with wild-type mice.

Another hallmark of tumorigenesis is the ability of cancer cells to evade programmed cell death. Apoptosis was found to be critically regulated at both the cellular and molecular levels and to take part in every cell type (40). When analyses of the proapoptotic marker caspase-3 were conducted, we observed significantly enhanced numbers of proapoptotic T cells in tumors from NFATc2-KO mice. Previous studies suggested a critical function of NFAT in mediating the expression of the membrane-bound death receptor ligand FasL (41). Data from our study clearly support the notion that deficiency of NFATc2 results in an increased proapoptotic rate of T cells within the tumor tissue when compared with wild-type mice. Thus, our data further support the concept that NFATc2 exerts important functions in regulating programmed cell death. Consistent with previous studies (42), we could show that NFATc2-KO mice have higher apoptotic rates of T cells in tumor tissue. As the numbers of tumor-infiltrating T cells were similar between wild-type and knockout mice, however, alternative mechanisms for local T-cell accumulation (e.g., via accelerated recruitment) must exist in NFATc2-KO mice to compensate for the loss of T cells because of apoptosis. In any case, the augmented rate of T-cell apoptosis and reduced rate of epithelial cell proliferation may explain the significantly lower number of tumors in NFATc2-deficient mice as compared with wild-type controls.

Colitis results in elevated expression levels of multiple cytokines and proinflammatory cytokines critically contributes to the process of initiating tumors (43). IL-6 was found to protect normal cells from apoptosis and its genotype was shown to impact the risk of CAC (44). Moreover, IL-6-deficient
mice were shown to possess decreased COX2 expression levels leading to decreased tumors (12). It became evident that in the absence of NFATc2 T cells, purified from spleen and lamina propria, produce lower levels of IL-6. Likewise, sera of NFATc2-deficient animals presented with reduced IL-6 levels. NFAT expression was found to be linked to the regulation of IL-6 expression, as it has been shown that application of NFAT inhibitors such as cyclosporin A resulted in a decrease of IL-6 expression. Likewise, overexpression of NFAT blockers caused a reduction in IL-6 mRNA (45). In addition, previous data

Figure 5. Abrogation of the tumor-promoting effect of NFATc2 upon administration of hIL-6. A, wild-type and NFATc2-KO mice were additionally treated with hIL-6 during the normal procedure of DSS/AOM cycles. Mini-endoscopic analyses of mice showed normal tumor development in wild-type mice, whereas NFATc2-KO mice were protected from the development of colorectal tumors. The application of hyper IL-6 restored induction of tumors in NFATc2-KO mice. B, miniendoscopic analysis with methylene blue staining further supported the abrogation of a tumor-promotive effect in NFATc2-deficient mice in the presence of hIL-6 administration. C, H&E staining of colon sections showed multiple large tumors in wild-type mice. In contrast, no tumors developed in NFATc2-KO mice, whereas NFATc2-KO mice treated with hIL-6 showed tumor numbers to a similar extent as wild-type mice. D, pathologic analysis of tumor development in control and NFATc2-deficient mice upon administration of hIL-6 during cycles of DSS/AOM. Significant changes are indicated.
supported the notion that NFAT functions as an important cofactor in NF-κB-dependent IL-6 expression (46), providing further explanations for decreased IL-6 levels in the absence of NFATc2 as observed in our present study. Furthermore, IL-6 possesses critical protumorigenic functions thereby increasing the risk for the development of colorectal tumors (7). These findings are consistent with the here-described decrease in tumor incidence in NFATc2-deficient mice when subjected to DSS/AOM model.

IL-6 expression levels correlate with the expression of the associated transcription factor STAT3. The fact that increased levels of the activated form pSTAT3 could mainly be found in cryosections from tumors and normal tissues of wild-type mice rather than in NFATc2-deficient mice further underlines the proposed scenario that NFATc2 activity critically impinges on IL-6 gene expression. We suggest that the observed protection of NFATc2-deficient animals from the development of CAC results from a significantly lowered production of IL-6. Further studies indicated that IL-6 and STAT3 were required for the development of CAC and increased activity of this pathway was found in gastrointestinal cancers (12, 47). In the present experimental setting, IL-6 was found to be mainly produced by T cells and some myeloid cells. However, also other cells such as fibroblasts may produce IL-6. In fact, Quante and colleagues recently proposed the concept of myofibroblasts producing IL-6 in tumor tissue thereby facilitating tumor progression (48).

As a proof-of-principle experiment, the application of hyper IL-6 was shown to be sufficient to abrogate the protective capacities in NFATc2-deficient animals in experimental CAC model. Upon injection with hyper IL-6 during DSS/AOM cycles, NFATc2-deficient mice readily developed tumors to a similar degree as observed in wild-type mice. These findings are in lines with studies from Bromberg and Wang (49), which likewise noted enhanced tumor multiplicity in the presence of increased IL-6 signaling. Upon activation of IL-6 signaling, during early stages of colorectal cancer development, increased tumor incidence could be observed paralleled by increased phosphorylation of STAT3 (12). Thus, IL-6 signaling emerges as a key link between inflammation and tumor growth (50) that results in T-cell resistance against apoptosis and augmented epithelial cell proliferation.

To conclude, in the present study, we provide evidence that the transcription factor NFATc2 functions as a critical player in the regulation of tumor progression and show for the first time that in the absence of NFATc2, mice are almost completely protected from the development of colorectal tumors. When subjected to injections with hyper IL-6, the protective effect of NFATc2-deficient mice was abrogated further underlining the proposed scenario that the protection from CAC in the absence of NFATc2 is mainly caused by a reduction of the critical proinflammatory cytokine IL-6.

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

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