Transcription factor NFATc2 controls the emergence of colon cancer associated with IL-6-dependent colitis

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Key words: T-cells, colitis, cancer, transcription factors

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The authors have no conflicting financial interests.
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 pro-inflammatory cytokines 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.
INTRODUCTION

In humans two major forms of inflammatory bowel disease (IBD) have been described (1). Both diseases bear the immunological stigmata of an exaggerated CD4-T-helper cell response associated with typical mediator profiles. In CD a Th1-mediated inflammation predominates with increased production of the Th1-signature cytokine IFN-γ. The increased production of IL-5 in UC results from a mostly Th2-mediated disease (2). The two chronic types of colonic inflammation are associated with an increased risk for the development of colitis-associated colorectal cancer (CAC) (3), however the risk for developing CAC is markedly higher in patients suffering from UC as compared to CD (4). Considering the pathogenesis of CAC, a stepwise progression from inflamed and hyperplastic epithelia to flat dysplasia and adenocarcinoma has been suggested (5).

The development of CAC is linked to an overproduction of pro-inflammatory cytokines, such as TNF-α and interleukin-6 which can activate NF-κB signalling, 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 pro-inflammatory cytokine production (6). The best characterized pro-tumorigenic cytokine is, beside 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 pro-tumorigenic 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 naïve 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 Th1 transcription factors involved are T-bet, STAT-1 and STAT-4 which play important effector functions in experimental colitis in the gut (15), (16). On the other hand, the role of typical Th2-associated transcriptional genes c-maf, GATA-3 and especially NFAT in the control of effector functions within an inflammatory environment are far from being understood in detail (17). CD and UC are characterized by augmented production of some Th1 and Th2 cytokines. However, it is meanwhile established that CD- and UC-related inflammation is also associated with enhanced secretion of IL-17 produced by a distinct subset of Th cells, termed Th17- cells. Th17 cytokines can have both tissue-protective and inflammatory effects in the gut and recent studies suggest that Th17 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 five 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), (22, 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). Based on 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 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.
Material and Methods

Patients:
Colon resections from patients with tumors were studied and compared with respective control samples. Patients' characteristics are reported in supplementary Table 1. 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 - 12 weeks of age and were housed under specific pathogen-free conditions. Experiments were performed 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 Axoxymethane (AOM) with 10 mg per 1 kg body weight (Sigma) on the first day followed by 3 cycles of 2% 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 five consecutive days in the experiment, starting on day 20.

Mini-endoscopic analysis
Tumor development was monitored with a high-resolution video endoscopic system (Storz) at indicated time points. Scoring of DSS-colitis severity was performed 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 hIL-6 the colon of wt and NFATc2 KO mice was stained with methylene blue and then monitored by miniendoscopy.

**Analysis of Apoptosis**

Colon and tumor tissues were removed from mice and cryosections were prepared. In order to visualize pro-apoptotic 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 10x magnification.

**Immunohistochemistry**

Colonic cryosections from normal colon and tumor tissue of wt and NFATc2 KO mice were obtained for hematoxylin 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 pSTAT-3 staining monoclonal rabbit antibody to pSTAT-3 (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 performed by counting positive cell in 6-10 high power fields.

**Preparation of cytospins**

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 performed with the SYBR Green Kit (BioRad) in combination with specific primers for IL-6 (Qiagen). Using β-actin as reference gene, the relative expression level of cytokine mRNA was calculated.

**Cell isolation of spleen mononuclear cells and LPMCs**

Mononuclear cells were isolated from spleen specimens of the AOM/DSS-treated BALB/c and NFATc2 knockout 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 $\times$ 10$^6$/ml in IMDM medium. CD4$^+$ T-cells were incubated in the presence or absence of Anti-CD3/CD28 antibodies. For preparation of lamina propria mononuclear cells see Supplementary Material and Methods and (31).

**Cytokine measurements**

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

Statistics

Statistical differences between groups were determined using the Student's $t$ test. P values
<0.05 were considered as statistically significant and identified with one asterisk or <0.001
with two asterisks or even three 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 inflammatory bowel diseases

NFAT was found to play a fundamental role in controlling calcium-dependent T-cell activation (20), (21), (22), (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 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 Figure 1A, NFATc2⁺-cells were found in the lamina propria of patients and the number was significantly increased in tumor tissue (Fig. 1B) when compared to 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 8-10 weeks.

Wild-type mice readily developed multiple colonic tumors (Fig. 2A). Miniendoscopic analyses revealed significantly reduced tumor numbers in NFATc2 deficient mice when compared to wild-type mice. Consistently, histologic analyses showed mucosal hyperplasia, crypt distortions and rarefaction in wild-type mice subjected to cycles of DSS/AOM in contrast to NFATc2 deficient animals which nearly revealed normal histological profiles. Furthermore, the tumor scores evaluated by a pathologist showed significantly reduced scores for NFATc2 deficient mice when compared to 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 knockout 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 miniendoscopy (Fig. S1A). It became clear from these experiments that NFATc2 knockout mice were significantly protected from the development of DSS-mediated colitis compared to wild-type mice (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 knockout mice might be explained by significantly decreased levels of pro-inflammatory cytokines. However, mRNA levels of some proinflammatory cytokines such as IFN-γ and IL-1β were comparable between wild-type and knockout animals (Figs. S2A, S2B) and IL-11 mRNA was only upregulated in tumor tissue from wild-type animals (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 lamina propria mononuclear cells were significantly reduced in the absence of NFATc2 as compared to controls (Fig. S3B, Fig. S4A). As IL-21 has been recently shown to augment inflammation and tumorigenesis in the AOM/DSS model (32), these data are consistent with 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 (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 knockout mice in comparison to 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 realtime PCR and normalized IL-6 levels to the house keeping gene beta-actin. It became apparent from these studies that LPMCs from NFATc2 knockout 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 cytopsin analyses showed no marked reduction of IL-6 production in the absence of NFATc2 (Suppl. 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 in order 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 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 demonstrated to be lower in splenic cells, in serum and in LPMCs from NFATc2 deficient mice when compared to wt mice. In further experiments, we performed analyses of mucosal p-STAT3 expression, which mainly regulates IL-6 expression. Increased p-STAT3⁺-cells could be observed in tumor cryosections as compared to the normal mucosa from wild-type mice (Fig. 3C). In addition, the number of p-STAT3⁺-cells was significantly reduced in tumor tissue from NFATc2 knockout mice as compared to 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 to NFATc2 deficient mice (Fig. 3D).

**Increased apoptotic rate of NFATc2 deficient lamina propria T-cells in CAC.**

It has been established previously that NFATc2 deficient mice harbour a defect in apoptosis of
lymphocytes. In order 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 knockout 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 pro-apoptotic cells. In contrast, tumors and control tissue of NFATc2 knockout mice presented with significantly higher numbers of pro-apoptotic 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 to 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 knockout mice during the induction of CAC was performed. 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 knockout mice as compared to wild-type mice (Fig. S6A and S6B). In accordance with the decreased IL-6 production in NFATc2 knockout mice the proliferation rate of intestinal epithelial cells in these mice was reduced as compared to wild-type controls, as determined by Ki-67 staining (Fig. S7A and S7B).

**Interleukin-6 reverses the pro-tumorigenic 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. In order to test whether activation of IL-6 signalling *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 to wild-type mice, as shown by miniendoscopic analysis (Figure 5A and 5B), histopathological analyses (Figs. 5C) and tumor scores (Fig. 5D). Administration of hyper-IL-6 abrogated the protection of NFATc2 knockout mice in the colon tumor model and restored tumor induction in NFATc2 knockout mice to levels observed in wild-type mice treated with hIL-6.

In summary, the results of the present study strongly suggest that the pro-tumorigenic role of NFATc2 in CAC model is mainly caused by regulation of the critical pro-inflammatory 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 to samples from control patients. These data underline an important pro-tumorigenic 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 pro-tumorigenic 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 signalling (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 et al. 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 production of IL-17A, an important tumor regulating cytokine (37), (38), no changes were observed between lamina propria cells from NFATc2 knockout 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 wt but not from NFATc2 KO mice. These data could be explained by the fact, that the IL-11 promotor possesses two 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 knockout 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 to 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 pro-apoptotic marker caspase-3
were performed, we observed significantly enhanced numbers of pro-apoptotic T-cells in
tumors from NFATc2 knockout 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
pro-apoptotic rate of T-cells within the tumor tissue when compared to 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
knockout 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 knockout mice to compensate for loss of T-cells due to 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 to wild-type
controls.

Colitis results in elevated expression levels of multiple cytokines and pro-inflammatory
cytokines critically contributes to the process of initiating tumors (43). IL-6 was found to
protect normal cells from apoptosis and its genotype was demonstrated 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 regulation of IL-6 expression since it has been shown that application of NFAT
inhibitors such as Cyclosporine 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 supported the notion that NFAT functions as an important co-factor 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 pro-tumorigenic 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 STAT-3. The fact that increased levels of the activated form p-STAT3 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 et al. 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 signalling. Upon activation of IL-6 signalling 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 pro-inflammatory cytokine IL-6.

**Acknowledgements**

The authors would like to thank Professor Laurie Glimcher for generously providing NFATc2 knockout mice. The authors like to thank Mrs. Anne von Berg and Mrs. Alexandra Wandersee for technical assistance.

The research of B. Weigmann was supported by the German Research Council (DFG) WE 4656/1-1. S. Rose-John was funded by the DFG within the Sonderforschungsbereich SFB415.
References


Figure legends

**Fig. 1:** Higher expression levels of NFATc2 in human tumor sections. (A) Representative immunohistochemical stainings for NFATc2 are shown. Cryosections of the colonic specimens were incubated with anti-NFATc2 antibody and secondary biotinylated antibody followed by TSA-amplification. Cells were counterstained with Hoechst dye. (B) Quantitative analysis of positive cells revealed a significantly increased number of NFATc2⁺-cells in tumor tissue of patients. Data represent mean values ± the SD per high power field.

**Fig. 2:** Tumor-promoting role of NFATc2 in an experimental CAC. (A) NFATc2 knockout mice were analyzed in the AOM/DSS model of CAC. The tumor evaluation was monitored using miniendoscopy. In comparison to wild-type animals, NFATc2 knockout 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 knockout mice were used. Histological sections (lower panel) were HE 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 demonstrated nearly a normal colon structure. (B) Tumor numbers and tumor scores of NFATc2 knockout and wild-type mice in the AOM/DSS model. (C) IL-6 concentrations in sera of NFATc2 knockout 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 48h 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 knockout mice. Total RNA was isolated from tissue, cDNA was generated and real-time PCR was done as described. Significant differences are indicated.

**Fig. 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 knockout 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 to wild-type mice. (C) pSTAT-3 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 p-STAT3\(^+\)-cells in tumor tissue of wild-type mice as compared to NFATc2 deficient mice. (D) IL-6 stainings in cryosections of wild-type and NFATc2 knockout 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 to NFATc2 deficient mice.

**Fig. 4: Enhanced apoptosis in tumors of NFATc2 knockout mice.** (A) Cryosections of normal and tumor tissues from both groups were stained with caspase-3/FITC conjugate. The nuclei were counterstained with Hoechst dye. Representative stainings are shown for wild-type and NFATc2 knockout mice in normal tissue as well as tumor-containing colonic tissue. Cryosections were analyzed quantitatively by fluorescence microscopy of caspase-3\(^+\)-cells.
Data represent mean values of positive cells ± SD per high power field. A marked increase of caspase-3\(^{+}\)-cells in control tissue of NFATc2 deficient mice as compared to wild-type mice was observed. In addition to apoptotic mononuclear cells, some intestinal epithelial cells in tumor bearing NFATc2 knockout mice were caspase-3 positive. Arrows represent epithelial caspase-3\(^{+}\)-cells. (B) To determine the number of apoptotic T-cells, cryosections of tumor tissues from wild-type mice and NFATc2 knockout mice were prepared and double stained using anti-CD4 antibodies and caspase-3 staining reagent. The nuclei were counterstained with Hoechst dye. More double positive cells could be found in tumor tissue of NFATc2 knockout mice as compared to wild-type controls. Upon administration of hIL-6 apoptotic CD4\(^{+}\) T-cells became less apparent in tumor tissue of NFATc2 knockout mice suggesting protection from apoptosis. Arrows represent double positive caspase-3/CD4 T-cells.

**Fig. 5: Abrogation of the tumor-promoting effect of NFATc2 upon administration of hIL-6.** (A) Wild-type and NFATc2 knockout mice were additionally treated with hIL-6 during the normal procedure of DSS/AOM cycles. Miniendoscopic analyses of mice showed normal tumor development in wild-type mice, whereas NFATc2 knockout mice were protected from the development of colorectal tumors. The application of hyper IL-6 restored induction of tumors in NFATc2 knockout mice. (B) Mini-endoscopic 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) HE-staining of colon sections showed multiple large tumors in wild-type mice. In contrast, no tumors developed in NFATc2 knockout mice, whereas NFATc2 knockout mice treated with hIL-6 showed tumor numbers to a similar extent as wild-type mice. (D) Pathological analysis of tumor development in control and NFATc2 deficient mice upon administration of hIL-6 during cycles of DSS/AOM. Significant changes are indicated.
Fig. 3
Fig. 4
Fig. 5