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TGF-β Suppresses Tumor Progression in Colon Cancer by Inhibition of IL-6 *trans*-Signaling

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Summary

Alterations of TGF-β signaling have been described in colorectal cancer, although the molecular consequences are largely unknown. By using transgenic mice overexpressing TGF-β or a dominant-negative TGF-βRII, we demonstrate that TGF-β signaling in tumor infiltrating T lymphocytes controls the growth of dysplastic epithelial cells in experimental colorectal cancer, as determined by histology and a novel system for high-resolution chromoendoscopy. At the molecular level, TGF-β signaling in T cells regulated STAT-3 activation in tumor cells via IL-6. IL-6 signaling required tumor cell-derived soluble IL-6R rather than membrane bound IL-6R and suppression of such TGF-β-dependent IL-6 *trans*-signaling prevented tumor progression in vivo. Taken together, our data provide novel insights into TGF-β signaling in colorectal cancer and suggest novel therapeutic approaches for colorectal cancer based on inhibition of TGF-β-dependent IL-6 *trans*-signaling.

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Introduction

Colorectal cancer is one of the most common fatal malignancies worldwide, with an incidence second only to lung cancer. It develops in about 5% of the adult population in the United States, and almost half of these people will die as a consequence of the disease (Weir et al., 2003). However, the molecular pathogenesis of colorectal cancer is still poorly understood. Several lines of evidence support an important role of TGF-β in the development of colorectal cancer. For instance, mutations of the TGF-β receptor II are frequently observed in patients with colon cancer, suggesting a potential role for TGF-β in preventing colon carcinogenesis (Grady et al., 1999). Furthermore, inactivation of the Smad3 gene, a downstream signaling molecule of the TGF-β receptor, may lead to the development of neoplastic lesions in the murine colon (Yang et al., 1999). However, the precise role of TGF-β signaling in colon carcinogenesis remains incompletely understood.

The cytokine interleukin (IL)-6 is a pleiotropic cytokine with a broad range of functions on immune and nonimmune cells (for a review, see Naka et al., 2002). Classic signaling of IL-6 involves binding of IL-6 to target cells bearing the membrane bound IL-6 receptor. Alternatively, IL-6 can activate cells lacking the membrane bound IL-6R when bound to a naturally occurring soluble form of the IL-6 receptor (sIL-6R) in a process called IL-6 *trans*-signaling (Jones et al., 2001). Functional studies have demonstrated both pro- and anti-inflammatory roles of IL-6 (Naka et al., 2002). In addition, IL-6 has been shown to promote hematopoiesis and terminal B cell differentiation. Finally, recent data suggest a potential role of IL-6 in colon cancer. For instance, it has been shown that levels of IL-6 are increased in the serum of patients suffering from colon carcinoma and correlated with tumor size (Chung and Chang, 2003; Galizia et al., 2002). In addition, IL-6 has been shown to promote the growth of colon cancer epithelial cells in vitro (Schneider et al., 2000). However, the molecular and immunological mechanisms underlying these observations are largely unknown.

In the present manuscript we demonstrate a novel functional link between TGF-β signaling in tumor infiltrating T cells and IL-6 *trans*-signaling for colon carcinogenesis. Specifically, our data show that carcinogenesis in the colon is highly dependent on TGF-β production in tumor infiltrating T lymphocytes via a TGF-β-dependent mechanism controlling IL-6 *trans*-signaling.

Results

In order to investigate the functional role of TGF-β signaling in colon carcinogenesis, we used a previously established murine colon carcinoma model (Okayasu et al., 1996; Tanaka et al., 2003) based on the mutagenic agent azoxymethan (AOM). Accordingly, FVB mice were treated with AOM followed by three consecutive cycles of orally administrated dextran sulfate sodium (DSS)

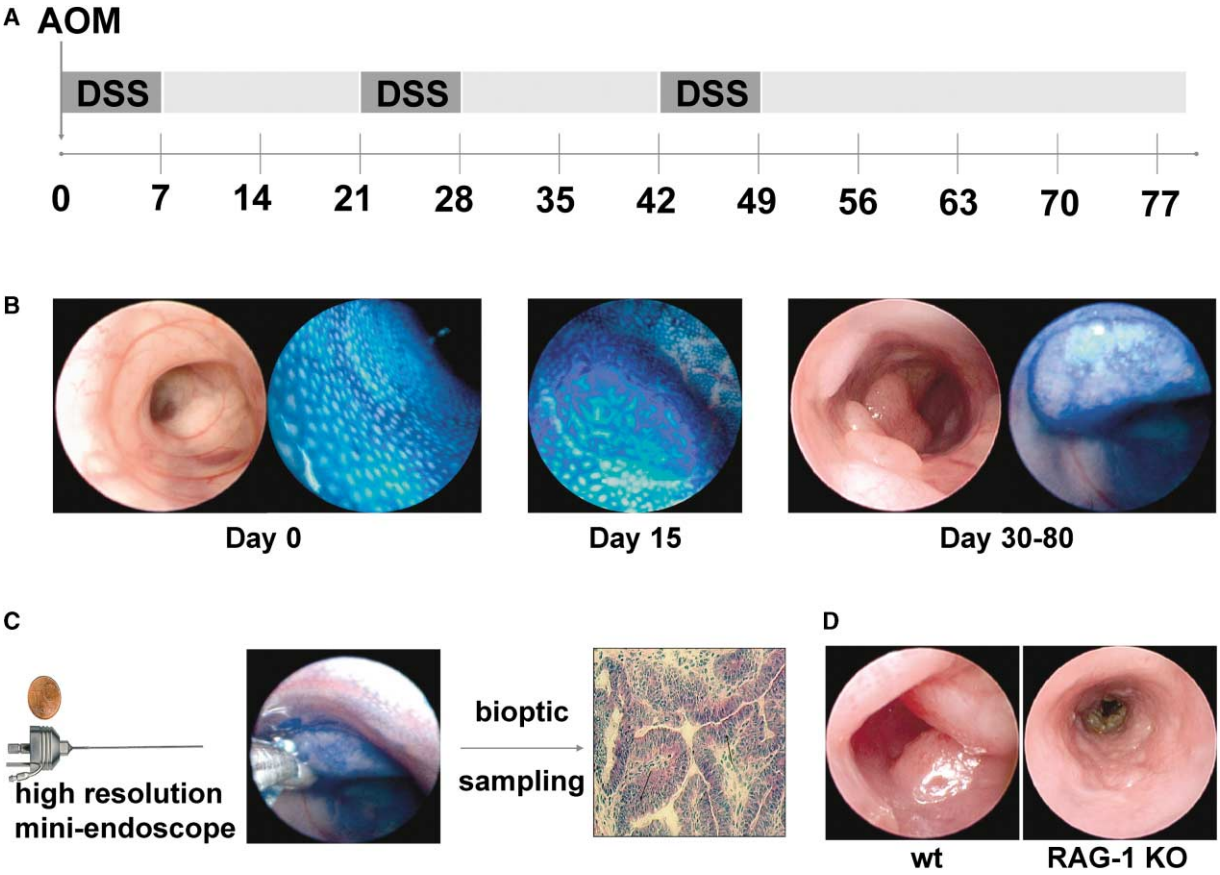

Figure 1. High-Resolution Mouse Chromoendoscopy for Monitoring of Colon Carcinogenesis

(A) Experimental procedure used to induce colon carcinomas in FVB mice. Mice were injected intraperitoneally with a single dose (7.4 mg/kg) of the mutagenic agent azoxymethan (AOM) followed by three cycles of DSS in drinking water for 1 week and normal drinking water for 2 weeks.

(B) In vivo high-resolution endoscopy and chromoendoscopy of mice. Mice were anesthetized by intraperitoneal injection of avertine. Colon mucosa was stained with methylene blue to visualize the crypt pattern.

(C) For histologic evaluation, biopsies were taken from lesions during routine endoscopy.

(D) Tumorigenesis in immunodeficient RAG-1 knockout and control mice. Five mice per group were treated as above in (A) and subjected to endoscopy every week. In contrast to wild-type mice, large tumors could not be observed in RAG-1 knockout mice. One representative endoscopic picture per group is shown.

over a period of 7 days (Figure 1A). To tightly monitor tumorigenesis in living mice in vivo, we developed a high-resolution miniature endoscopic system for the murine colon (see Experimental Procedures). By using this novel system and methylene blue-aided chromoendoscopy, we were able to detect aberrant crypt foci in DSS plus AOM-treated wild-type FVB mice at day 15 before macroscopically visible lesions were seen by conventional colonoscopy (Figure 1B). Small visible lesions first appeared around day 20, which were followed by the development of large tumors until day 80. Tumor biopsies taken during endoscopy showed high-grade dysplasia and the presence of intraepithelial neoplasias (Figure 1C). To determine the role of T lymphocytes in this model, we investigated tumor development in immunocompromised RAG-1 knockout mice. In contrast to wild-type mice, RAG-1 knockout mice did not develop large tumors (Figure 1D), indicating that lymphocytes control tumor growth.

Since mutations of the TGF-β receptor (TGF-βR) have been described in colorectal cancer in humans (Grady et al., 1999), we next determined TGF-βR levels in AOM plus DSS-treated mice. Interestingly, immunohistochemical staining of colon tumors showed downregulation of TGF-βRI expression on dysplastic epithelial cells as compared to nondysplastic epithelial cells outside of the tumor and epithelial cells from untreated mice (Figure 2A). In analogy to the situation in colorectal cancer in humans, these data suggested that dysplastic epithelial cells in AOM plus DSS-treated mice prevent TGF-β signaling by downregulating TGF-βR levels.

Tumor-infiltrating cells in AOM plus DSS-treated mice were found to be largely T cells (Figure 2A). Since tumor cells in colorectal cancer are known to produce TGF-β (Coffey et al., 1986) and since TGF-β induces its own production in T cells (Seder et al., 1998), we hypothesized that such tumor-infiltrating T cells could be an important source of TGF-β. Indeed, isolated tumor-infiltrating T cells expressed large amounts of TGF-β (Figure 2B), suggesting that these cells contribute to TGF-β production in vivo. To further analyze the functional role of TGF-β production by T cells in this model of colon

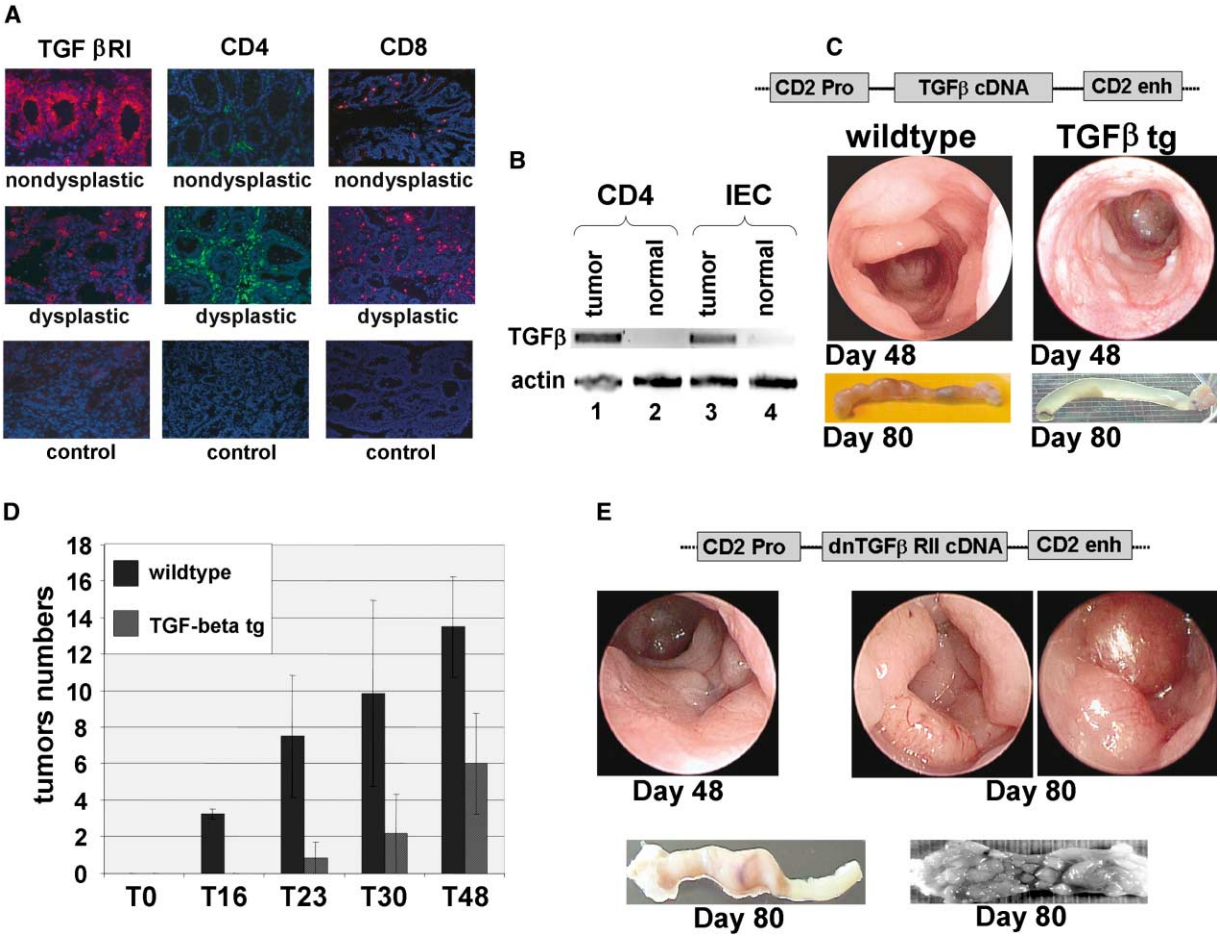

Figure 2. TGF-β Signaling in T Cells Negatively Regulates Tumorigenesis

(A) Immunohistochemistry for TGF-βR (left panels) and CD4 or CD8 (middle and right panels, respectively) on dysplastic and nondysplastic tissue from DSS plus AOM treated wild-type mice was performed by using the TSA Cy3 and FITC systems. Cryosections were fixed in acetone. Slides were then incubated with primary antibodies specific for TGFßRI or CD4/CD8, respectively. Before examination, the nuclei were counterstained with Hoechst3342.

(B) TGF-β production by tumor-infiltrating T cells. Tumor-infiltrating T cells and intestinal epithelial cells (IEC) were isolated from tumors of wild-type mice and TGF-β levels were determined by RT-PCR.

(C and D) Colon carcinogenesis in TGF-β transgenic mice: TGF-β was expressed under control of the CD2 promoter to achieve T cell specific transgene expression. Ten mice were treated according to the above carcinogenesis protocol and monitored endoscopically and macroscopically. Lesions were counted during endoscopy, and the differences between the wild-type and transgenic groups were statistically significant ($p < 0.05$). The results are expressed as mean values ± SD (D). Time points of endoscopies are indicated on the x axis.

(E) Colon carcinogenesis in transgenic mice overexpressing a dominant-negative TGF-β receptor under control of the CD2 promoter to achieve T cell specific transgene expression. Ten mice were treated according to the carcinogenesis protocol and monitored endoscopically and macroscopically. Transgenic mice showed markedly increased tumor size and number as compared to wild-type mice.

carcinogenesis, we therefore used transgenic mice overexpressing TGF-β under control of the CD2 promoter in order to obtain a T cell specific expression of the transgene (Figure 2C). The endoscopic scoring of such TGF-β transgenic mice subjected to the AOM/DSS protocol showed significant differences in terms of tumor number and size of lesions in comparison to wild-type mice (Figures 2C and 2D). Specifically, transgenic mice overexpressing TGF-β in T lymphocytes showed significantly delayed development of intraepithelial neoplasias and a smaller tumor size during the experimental protocol when compared to their wild-type littermates indicating a protective role of TGF-β production by tumor infiltrating T cells for development of colorectal cancer.

Interestingly, only few tumor-infiltrating T cells in larger tumors of wild-type mice at late stages of the AOM plus DSS protocol expressed TGF-βRI, suggesting that these cells prevent TGF-β signaling in this TGF-β rich environment (Figure 2A). To determine the functional consequences of impaired TGF-β signaling in T cells for colorectal cancer, we next analyzed mice that express a dominant-negative form of the TGFβRII chain in T lymphocytes under control of the CD2 promoter (Figure 2E). Compared to wild-type mice and TGF-β transgenic mice, the number of lesions observed in dnTGFβRII transgenic mice was higher. In addition, the size of the tumors in these mice was markedly larger, resulting in the development of stenoses in the distal colon and consecutive lethal ileus. Surviving mice sacrificed at the end of the experiment showed numerous

large tumors of the colon (Figure 2E), further supporting a key regulatory role of TGF-β signaling in T cells for colon carcinogenesis.

The possibility that T cells play a central role in the development of colon tumors led us to perform a screening of the expression of T cell-derived cytokines in colons and tumors of AOM plus DSS-treated wild-type and transgenic animals. While neither interferon-γ, IL-4, IL-10, or TNF-α were significantly up- or downregulated in these experiments (not shown), we found a markedly higher expression of IL-6 in dnTGFβRII transgenic mice when compared to wild-type and TGF-β transgenic mice (Figure 3A, left), suggesting that T cell-derived IL-6 could be responsible for the observed differences in colon carcinogenesis between wild-type and transgenic mice. To verify these findings, we isolated spleen cells from wild-type and transgenic animals and analyzed the supernatants of anti-CD3 plus anti-CD28 stimulated cells for IL-6 levels by ELISA. As shown in Figure 3A (right), cells from TGF-β transgenic mice produced significantly less IL-6 as compared to cells from wild-type mice, while cells from dnTGFβRII transgenic mice produced significantly more IL-6 as compared to cells from both wild-type and TGF-β transgenic mice. Further studies using bioptic sampling of tumors and normal tissue from wild-type mice revealed a higher expression of IL-6 mRNA in the tumor than in surrounding nondysplastic tissue (Figure 3B). Additional time course experiments demonstrated that IL-6 protein was strongly induced in colon lysates of wild-type mice during the development of colon tumors (Figure 3C). Interestingly, augmented IL-6 expression started at day 20 when the first lesions became detectable and coincided with the development of colon tumors, thus suggesting a possible correlation between IL-6 expression and tumor growth.

To analyze which cells in the lamina propria of tumors contributed to the increased expression of IL-6, we isolated intestinal epithelial cells and lamina propria mononuclear cells from a large number of tumors derived from wild-type mice and further purified these cells with immunomagnetic beads for CD4, CD8, CD11c, and B220. As shown in Figure 3D, tumor infiltrating $CD4^+$ T cells expressed IL-6, interferon-γ and TNFα mRNA implicating that the majority of $CD4^+$ T cells in the tumor stroma exhibit an activated phenotype. IL-6 was also expressed in tumor epithelial cells and dendritic cells, whereas little IL-6 was found in the macrophage enriched population and $CD8^+$ T cells. TGF-β was expressed by $CD4^+$ T cells, $CD8^+$ T cells, dendritic cells, and tumor epithelial cells, implicating possible autocrine and paracrine TGF-β-dependent signaling pathways. Furthermore, immunofluorescence staining for IL-6 and double staining for CD3 and IL-6 showed an overexpression of IL-6 in the tumor stroma as compared to nondysplastic tissue and confirmed T lymphocytes as a major source of IL-6 expression in colonic tumor tissue (Figure 3E). Finally, we determined IL-6 secretion by CD4+ T cells isolated from tumors of wild-type mice (Figure 3F). In contrast to $CD4^+$ T cells isolated from normal nondysplastic colon tissue, $CD4^+$ T cells isolated from tumors secreted large amounts of IL-6 and such IL-6 production could be reduced by adding recombinant TGF-β.

Recent data demonstrated that IL-6 could regulate the proliferation of intestinal epithelial cells (IEC) (Tebbutt et al., 2002). On the basis of these data, we next investigated a possible functional role of IL-6 in inducing growth of dysplastic lesions in vivo. Accordingly dnTGFβRII transgenic mice subjected to AOM/ DSS treatment received a weekly dose of 1 mg of a neutralizing antibody against the IL-6 receptor α chain. Mice were again monitored by colonoscopy and chromoendoscopy. Interestingly, dnTGFβRII transgenic mice treated with the anti-IL-6R antibody were protected from colon carcinogenesis (Figure 4A). In particular, the average tumor number and size were significantly reduced when compared to control transgenic mice (Figure 4B). Thus, IL-6 receptor signaling is essential for colon carcinogenesis in dnTGFβRII transgenic mice. In further experiments we tested the effects of anti-IL-6R antibody treatment in wild-type mice. As demonstrated in Figure 4C, wild-type mice treated with the anti-IL-6R antibody were also significantly protected from colon carcinogenesis. In particular, anti-IL-6R antibody treatment suppressed the growth of colon tumors. Thus, elevated IL-6 levels in wild-type mice contribute to tumor progression in experimental colon cancer.

In order to evaluate IL-6-dependent signal transduction in the colon of AOM/DSS mice, immunohistochemistry for IL-6 receptor (IL-6R) expression was performed. Epithelial cells in the colon of untreated mice and nondysplastic epithelial cells in the colon of treated mice showed strong staining for the IL-6Rα. Interestingly, however, little or no IL-6R staining was noted in tumor lesions, indicating that dysplastic epithelial cells exhibit greatly diminished surface expression of the membrane bound IL-6R (Figure 5A). Moreover, dysplastic epithelial cells exhibited strong nuclear staining for phospho-STAT-3, a known intracellular target molecule of IL-6 signaling (Figure 5B). Western blotting for the IL-6R confirmed the downregulation of the membrane bound IL-6R chain during tumorigenesis in wild-type mice (Figure 5C). In spite of the downregulation of membrane bound IL-6R on the tumor cell surface, however, RT-PCR experiments revealed that the mRNA for the membrane bound IL-6 receptor is abundantly expressed in tumor epithelial cells and, in fact, upregulated as compared to normal colon epithelial cells or stroma cells (Figure 5D). These data raised the possibility that tumor epithelial cells are a major source for the soluble IL-6R (sIL-6R) by shedding membrane bound receptor from their surface. Indeed, a second band of approx. 50 kDa corresponding to the soluble IL-6 receptor (sIL-6R) was strongly induced during tumorigenesis (Figure 5C). Furthermore, Western blot analysis of concentrated supernatants from tumor epithelial cells showed the presence of a 50 kDa band representing the soluble IL-6 receptor (Figure 5E).

As the matrix metalloproteinase TNF-α converting enzyme (TACE) is known to release soluble IL-6R by cleavage of membrane bound IL-6 receptor on the cell surface (Jones et al., 2001), we next analyzed TACE expression on tumor epithelial cells. As shown in Figure 6A, TACE was highly expressed on tumor epithelial cells as compared to controls, suggesting that matrix metalloproteinases such as TACE may control IL-6R shedding in colon carcinogenesis. To further test this concept, we used an organ culture system in which tumor tissue was

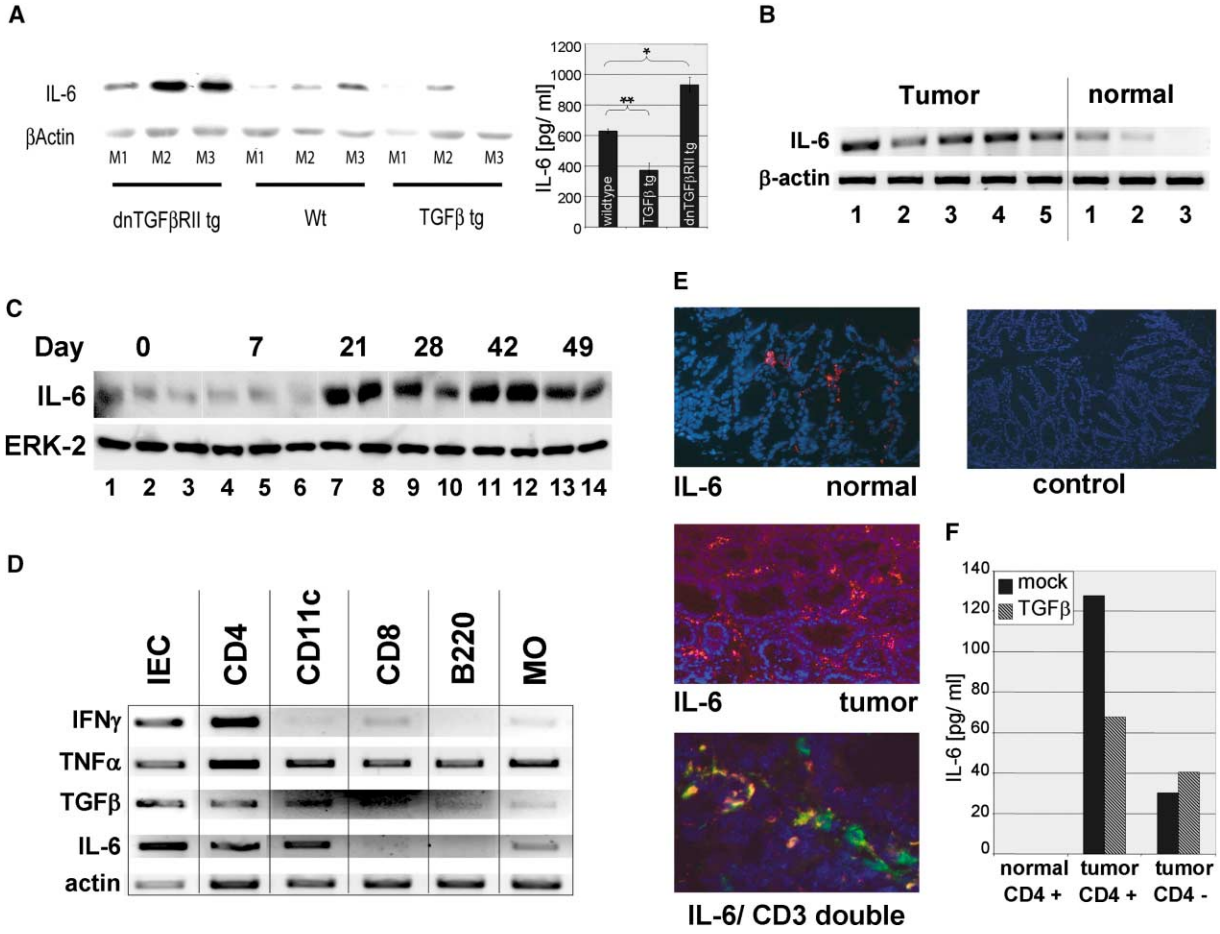

Figure 3. IL-6 Is Highly Expressed by T Cells in the Tumor Stroma

(A) Left: Western blotting of tumors taken at day 80 of the experiment. Three representative tumors were taken from wild-type, TGFβ transgenic or dnTGFβRII transgenic mice. Tumors were lysed, and 30 μg of protein was loaded on a 10% polyacrylamide gel. After transfer, membranes were incubated with an antibody specific for IL-6. Right: ELISA for IL-6 levels in supernatants of isolated spleen cells from wild-type, TGF-β transgenic, or dnTGFβRII transgenic mice upon stimulation with anti-CD3 plus anti-CD28 antibodies. The data represent three independent experiments ± SD (*$p < 0.05$, **$p < 0.01$).

(B) RT-PCR for IL-6 on biopsies taken from tumors or surrounding nondysplastic tissue in wild-type mice. Biopsies were frozen in liquid nitrogen. RNA isolation and cDNA synthesis was performed as described in methods. Data show results from five tumors and three tumor-free samples.

(C) Western blotting of colon samples taken at indicated time points throughout the course of the experiment in wild-type mice. Experimental procedures were as described above.

(D) RT-PCR for different cytokines of purified intestinal epithelial cells (IEC), LP $CD4^+$ and $CD8^+$ T cells, $CD11c^+$ cells, $B220^+$ B cells, and macrophage-enriched cells (MO) from tumors of wild-type mice. RNA isolation and cDNA synthesis was performed as described in the Experimental Procedures.

(E) Immunohistochemistry for IL-6 was performed with the TSA Cy3 and FITC system. Cryosections of tumor and tumor-free samples were fixed in acetone. Slides were then incubated with primary antibodies specific for IL-6 or CD3. The nuclei were counterstained with Hoechst3342.

(F) IL-6 production by 2×10^5 mock or TGF-β (10 ng/ml) treated $CD4^+$ T cells or CD4 negative mononuclear cells isolated from tumors or tumor-free control tissue obtained from wild-type mice. A second independent experiment gave similar results.

coincubated with an inhibitor of matrix metalloproteinases (TAPI-TNF-α protease inhibitor) followed by analysis of membrane bound IL-6R expression. Interestingly, TAPI treatment led to a marked upregulation of the expression of membrane bound IL-6 receptor on the tumor cell surface (Figure 6B). These data suggested that matrix metalloproteinases induce a shift from membrane bound IL-6R expression on the tumor cell surface toward release of soluble IL-6R, thereby allowing IL-6 *trans*-signaling.

Next, we investigated whether IL-6 *trans*-signaling could induce proliferation of dysplastic epithelial cells. Therefore, we injected a daily dose of hyper-IL-6, a designer cytokine consisting of IL-6 and a covalently linked soluble IL-6 receptor (Fischer et al., 1997), for 1 week into dnTGFβRII mice. Biopsies were taken from the same tumors before and after hyper-IL-6 treatment. Indeed, mice that received hyper-IL-6 displayed a stronger proliferation of dysplastic epithelial cells as measured by counting KI-67 positive cells (Figure 6C; left panels). Furthermore, epithelial cells in hyper-IL-6 treated animals showed a stronger nuclear staining of phospho-STAT-3 (Figure 6C; right panels). Finally, specific blockade of signaling via the sIL-6R using gp130-Fc sup-

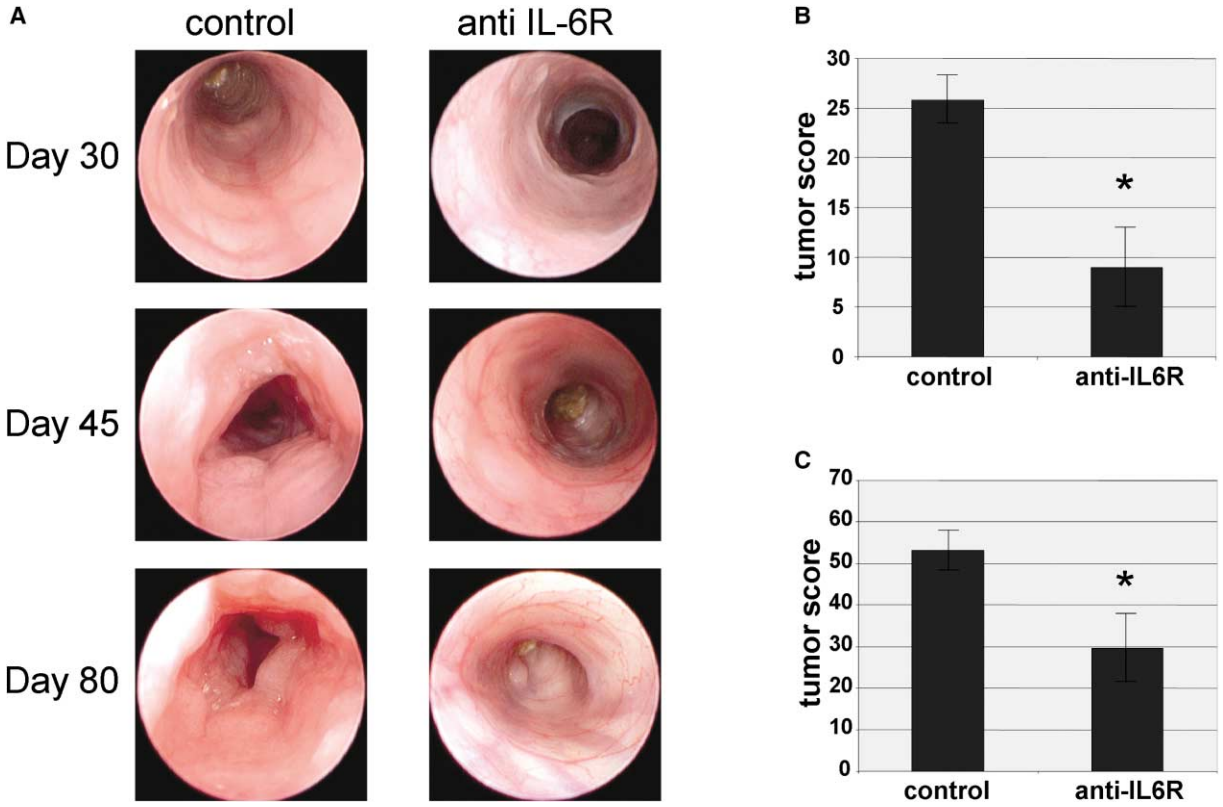

Figure 4. Blocking IL-6 Receptor Signaling Interferes with Colon Carcinogenesis in Wild-Type Mice and Mice Carrying a Dominant-Negative TGF-β Receptor in T Cells

Wild-type (C) and dnTGFβRII transgenic mice (A and B) were treated with azoxymethane and DSS to induce colon carcinogenesis. Ten dnTGFβRII transgenic and ten wild-type mice were separated into two groups. One group received a weekly dose of a neutralizing antibody against the IL-6 receptor α chain by intraperitoneal injection. The other group was injected with PBS as a control. Tumor development was monitored by endoscopic screening of the mice at indicated time points upon AOM administration (A). Tumors were counted and average tumor scores were compared between the groups (B and C). *$p < 0.05$.

pressed colon carcinogenesis in dnTGFVRII mice (Figure 6D) implicating IL-6 *trans*-signaling via the sIL-6R as being responsible for epithelial cell growth in tumor lesions. Thus, IL-6 *trans*-signaling is essential for colon carcinogenesis in dnTGFVRII mice and can induce phosphorylation of STAT-3 and cell cycle progression in dysplastic IEC. Taken together, our data indicate that IL-6 *trans*-signaling triggers the growth of dysplastic epithelial cells and that this cascade can be modulated by TGF-β signaling in tumor infiltrating T cells.

Discussion

In the present manuscript, we have identified a novel mechanism whereby tumor infiltrating T lymphocytes control tumor growth in a murine model of colon cancer. Specifically, we found that TGF-β production in tumor infiltrating T lymphocytes suppresses tumor growth in the colon via inhibition of IL-6 production and subsequent IL-6 signal transduction. Interestingly, IL-6 signal transduction was mediated by the soluble rather than the membrane bound IL-6R, indicating that tumor growth is controlled by IL-6 *trans*-signaling via the soluble form of the IL-6Rα. Such TGF-β-dependent IL-6 *trans*-signaling provides a molecular explanation for regulation of tumor cell growth by tumor infiltrating lymphocytes. Furthermore, blockade of IL-6 *trans*-signaling emerges as a novel therapeutic approach for colorectal cancer.

IL-6 is a cytokine involved in numerous functions within the immune system (Glimcher and Murphy, 2000; Hurst et al., 2001; Jones et al., 2001; Murphy and Reiner, 2002; Naka et al., 2002; Rengarajan and Szabo, 2000) and there is growing evidence that it is involved in normal development of the intestinal epithelium. In fact, recent data indicate that IL-6 regulates the proliferation of intestinal epithelial cells (IEC) (Tebbutt et al., 2002). Further findings implicate a role for IL-6 in the pathogenesis of colon cancer: IL-6 serum levels have been shown to be strongly elevated in patients with colon cancer and were correlated to the tumor load suggesting the use of IL-6 serum levels as a prognostic factor in colorectal cancer (Chung and Chang, 2003; Galizia et al., 2002). Furthermore, IL-6 has been shown to enhance colony formation of human colon carcinoma cells in vitro in a dose-dependent manner, suggesting that IL-6 may drive cancer growth (Schneider et al., 2000).

TGF-β has been shown to suppress Th2 cytokine production via inhibition of key transcription factors such as STAT-6 and GATA-3 (Heath et al., 2000). The findings

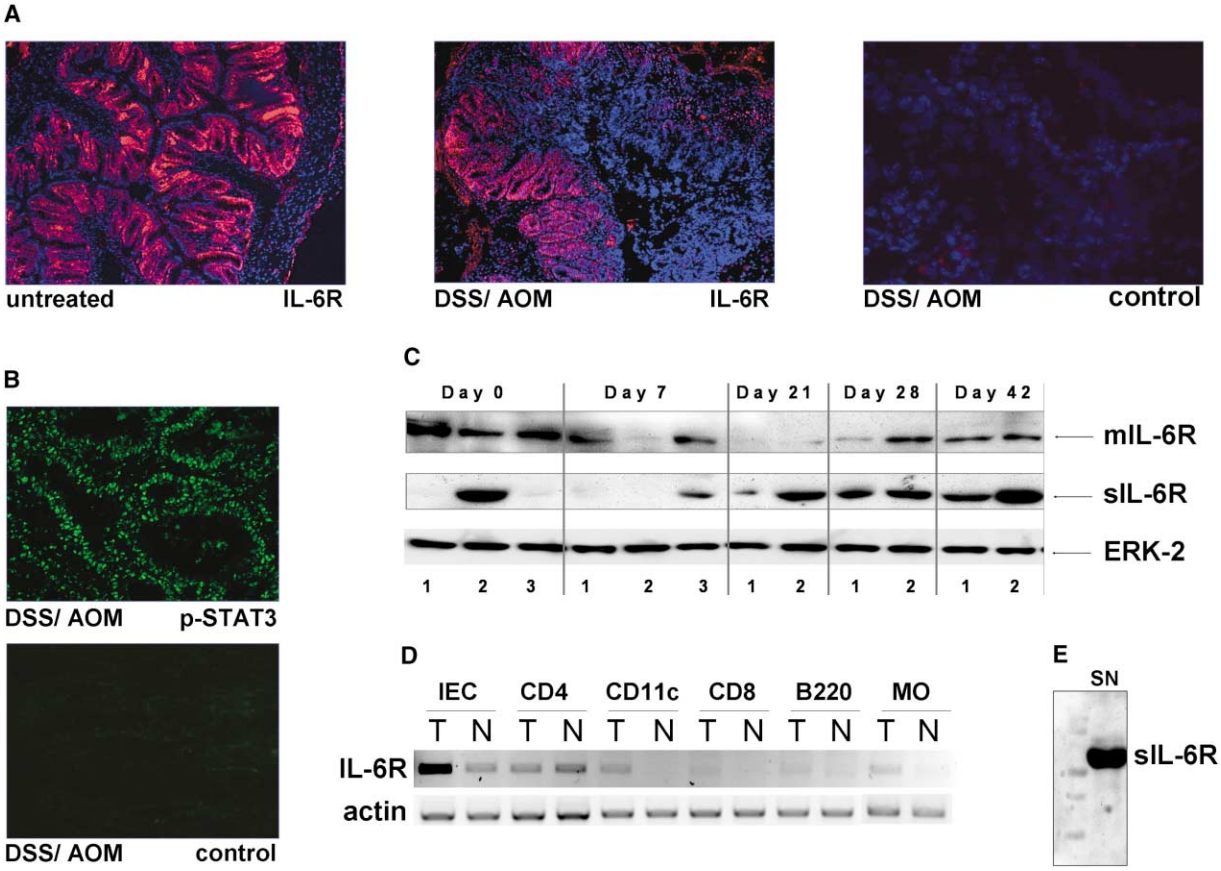

Figure 5. The Soluble IL-6 Receptor, but Not the Membrane Bound Receptor, Is Strongly Expressed in Colon Tumors

(A and B) Immunohistochemistry for the IL-6 receptor (A) and phospho-STAT-3 (B) was performed with the Cy3 and FITC systems, respectively. Cryosections of colon tumors from DSS plus AOM-treated wild-type mice and colonic tissue from untreated control mice were fixed in acetone. Slides were then incubated with primary antibodies specific for the IL-6 receptor or phospho-STAT-3. Before examination, the nuclei were counterstained with Hoechst3342.

(C) Western blot for the IL-6 receptor α chain and the sIL-6R. Colon samples from wild-type mice were taken on days 0, 7, 21, 28, and 42 of the experiment. Tissue was lysed with a homogenizer, and 30 μg of each sample was loaded on a polyacrylamide gel. After transfer onto nitrocellulose, the membrane was incubated with an antibody specific for the IL-6 receptor. As a control, membranes were probed with an antibody against the housekeeping gene ERK-2.

(D) RT-PCR for the IL-6R α chain was performed by using cDNA from purified cells derived from tumors (T) and tumor-free control tissue (N) of wild-type mice. Intestinal epithelial cells (IEC), $CD4^+$ T cells, $CD11c^+$ cells, $CD8^+$ T cells, $B220^+$ B cells, and macrophage-enriched cells (MO) were isolated as described in the Experimental Procedures.

(E) Western blot with supernatants of tumor epithelial cells cultured for 24 hr in serum-free medium. Supernatants were concentrated by using acetone precipitation, and 50 μg of protein sample was loaded on a polyacrylamide gel. The membrane was probed with an antibody specific for the IL-6 receptor α chain. A strong band at 50 kDa corresponding to the sIL-6R was seen.

in the present manuscript suggest that TGF-β signaling in tumor infiltrating T cells regulates IL-6 production in colorectal cancer. Furthermore, we observed that suppression of TGF-β signaling in T cells of transgenic mice expressing a dominant-negative TGF-β receptor II led to an augmented and accelerated tumor cell growth in vivo in an IL-6-dependent fashion. IEC-derived tumor cells produced large amounts of IL-6R mRNA but lacked the membrane bound IL-6R. Organ culture studies showed that this finding was due to shedding of the membrane bound IL-6R on tumor cells with subsequent release of the soluble IL-6R (sIL-6R) via matrix metalloproteinases. Furthermore, the matrix metalloproteinase TACE, which is known to induce IL-6R shedding (Jones et al., 2001), was upregulated in tumor cells suggesting a model in which proteases produced by tumor cells lead to sIL-6R production and subsequent sIL-6R-dependent tumor growth. Consistent with a role for matrix metalloproteinases in controlling tumor progression, TACE is known to activate epidermal growth factor (EGF) ligands such as TGF-α and thereby controls EGFR-dependent growth of breast tumor cells in vivo (Borrell-Pages et al., 2003; Sunnarborg et al., 2002).

Subsequent studies showed that the growth of IEC-derived tumor cells was dependent on IL-6 *trans*-signaling via the sIL-6R. Such IL-6 *trans*-signaling has recently emerged as the molecular consequence of heat responses in T cells and plays a pivotal role in amplification of immune responses during fever reactions (Chen et al., 2004; Rose-John and Neurath, 2004). Furthermore, it has been previously shown to induce chronic inflammation and mediates recruitment of leukocytes to in-

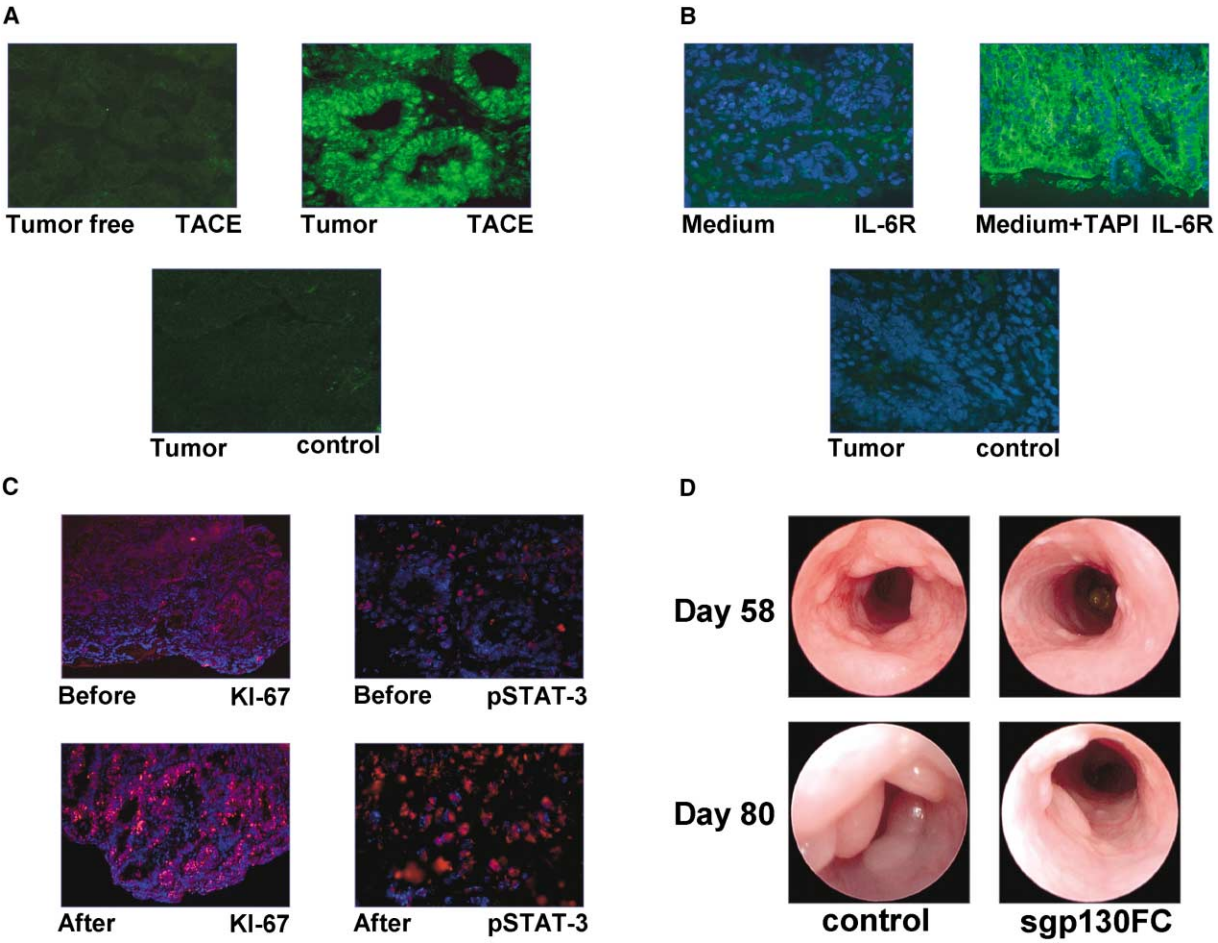

Figure 6. The sIL-6R Induces Phospho-STAT-3 and KI-67 Expression in Dysplastic Epithelial Cells

(A) Immunohistochemistry for TACE expression on tumor epithelial cells (upper right) as compared to tumor-free tissue (upper left) from wild-type mice.

(B) Detection of the membrane bound IL-6 receptor α chain expressed on the surface of tumor epithelial cells. Organ culture of tumor tissue was performed in the presence or absence of the matrix metalloproteinase inhibitor TAPI, as indicated. TAPI treatment led to a marked upregulation of IL-6R expression on tumor cells.

(C) Biopsies from wild-type mice were taken during endoscopy of three individual tumors at day 80 of the experiment. The same mice were then intraperitoneally injected daily with 2 μg of hyper-IL6 (designer cytokine consisting of IL-6 and sIL-6R) to induce IL-6 *trans*-signaling. Biopsies were then taken from the same tumors, as shown by video endoscopy. Biopsies were frozen in liquid nitrogen. Cryosections of these biopsies from the same tumors before and after hyper-IL-6 treatment were subjected to immunohistochemistry for KI-67 (left panels) and phospho-STAT-3 (right panels). Hyper-IL-6 treatment induced STAT-3 phosphorylation and proliferation of colonic tumors in vivo. (D) AOM plus DSS-treated dnTGFbRII transgenic mice were treated with gp130-Fc to block IL-6 *trans*-signaling in vivo. Endoscopic monitoring showed suppression of tumorigenesis upon administration of gp130-Fc.

flammatory lesions in vivo (Atreya et al., 2000; Hurst et al., 2001; Romano et al., 1997; Rose-John and Heinrich, 1994). Hereby, IL-6 *trans*-signaling induced STAT-3 activation and production of the anti-apoptotic proteins bcl-xl and bcl-2 in T cells. Furthermore, it induced expression of adhesion molecules such as L-selectin, resulting in increased adhesion of T lymphocytes.

In addition to the previously described role of IL-6 *trans*-signaling in acute and chronic inflammatory responses, our data define a novel role for such signaling in the control of cancer growth. A role for the *trans*-signaling mechanism of action was implicated by the finding that mice treated with DSS/azoxymethan showed increased levels of soluble IL-6 receptor around day 20 of the experiment and such increased expression coincided with the appearance of lesions in these animals and the activation of the IL-6-dependent transcription factor STAT-3 (Akira, 2000; Neurath et al., 2002) in dysplastic epithelial cells. Furthermore, suppression of IL-6 signal transduction via anti-IL-6R antibodies prevented tumor growth in wild-type and transgenic mice. These findings demonstrated that the effects upon blockade of TGF-β signaling in T cells are critically dependent on IL-6 signal transduction. Finally, the *trans*-signaling mechanism of action was revealed operationally by the finding that treatment of animals with gp130-Fc (which inhibits signaling via the sIL-6R, but not the membrane bound IL-6R) suppressed cancer growth in vivo. These findings provide strong evidence that signaling via the soluble rather than the membrane bound

IL-6R controls tumor growth in vivo and allow novel insights into the molecular pathogenesis of colon cancer.

Tumor infiltrating T lymphocytes have been associated with a better prognosis of colorectal cancer (Funada et al., 2003). Our data suggest that TGF-β production in tumor infiltrating T lymphocytes strongly suppresses tumor growth in the colon via inhibition of IL-6 production by T cells in an autocrine or paracrine fashion, thereby providing a novel mechanism for regulation of tumor cell growth by tumor-infiltrating lymphocytes. In contrast, suppression of TGF-β signaling in these cells augmented and accelerated tumor cell growth in an IL-6-dependent fashion. IEC-derived tumor cells lacking the membrane bound IL-6R were dependent on IL-6 *trans*-signaling via the sIL-6R. The potential relevance of these findings for human colorectal cancer is highlighted by the findings that a large subgroup of colorectal cancers shows decreased expression of the membrane-bound IL-6 receptor but increased expression of the sIL-6R (C.B., M.C.F., and M.F.N., unpublished data). Interestingly, it has been recently suggested that the sIL-6R controls the adherence of colon tumor cells to the vascular endothelium, thereby supporting the formation of metastases (Dowdall et al., 2002). Thus, the soluble IL-6 receptor emerges a key molecule at different stages of colon cancer pathogenesis. Furthermore, targeting of the sIL-6R and IL-6 *trans*-signaling can be used for therapy of colon cancer.

Experimental Procedures

Animals

Specific pathogen free FVB/N, C57 BL/6 and RAG-1 knockout mice (2–4 months old) were obtained from the central animal facility (ZVTE, University of Mainz, Germany). To induce colon carcinomas, mice were injected intraperitoneally with a single dose (7.4 mg/kg) of the mutagenic agent azoxymethane (AOM) followed by three cycles of 3% dextran sodium sulfate (DSS) in drinking water for 1 week and normal drinking water for 2 weeks. Transgenic mice were generated as previously described (Schramm et al., 2003). In some experiments, mice were given weekly doses (1 mg) of antibody against mouse IL-6R (MR-16-1; kindly donated by Chugai Pharmaceuticals, Shizuoka, Japan), 500 μg gp130-Fc, or phosphate buffered saline (PBS), by intraperitoneal injection.

Endoscopic Procedures

For the continuous monitoring of tumorigenesis, a high-resolution mouse video endoscope, denoted Coloview, was developed (2 mm outer diameter). Mice were anesthetized by intraperitoneal injection of avertine (Sigma Chem., St. Louis). The experimental setup consisted of a miniature endoscope, a xenon light source, and an air pump to achieve a regulated inflation of the mouse colon. The endoscopic procedure was viewed on a color monitor and digitally recorded on tape by using a triple chip camera. This novel technique was combined with a whole colon chromoendoscopic staining with methylene blue in order to visualize the crypt pattern and to detect aberrant crypt foci in vivo. Therefore, the colon was flushed with 500 μl of a 1% solution of methylene blue by using a syringe mounted to the Luer lock cones of the examination sheath of the endoscope. Tumor sampling in living mice was performed by taking biopsies that were then frozen immediately in liquid nitrogen.

Western Blot Analysis

Western blotting was performed as previously described (Becker et al., 2003). In some experiments, culture supernatants were used and concentrated by acetone precipitation. Equal amounts of extract (30 or 50 μg) were added to 10 μl electrophoresis sample buffer. After boiling, the proteins were separated by 10% SDS-PAGE, then transferred to nitrocellulose membranes and detected with a specific antibody against IL-6, ERK-2, β-actin (Santa Cruz Biotechnology, Santa Cruz, California), or IL-6R alpha (MR-16-1; kindly donated by Chugai Pharmaceuticals, Shizuoka, Japan and Santa Cruz Biotechnology) and the ECL Western blotting analysis system (Amersham).

ELISA

Cells were seeded at a concentration of 1,000,000 cells/ml (spleen cells) or 200,000 cells/ml (tumor-infiltrating T cells). After 24 hr supernatants were taken for ELISA. For the detection of IL-6 in supernatants, an ELISA kit was used (R&D Systems) according to the manufacturer's instructions.

Isolation and Culture of Tumor-Infiltrating Cells and Spleen Cells

Lamina propria mononuclear cells (LPMC) were isolated as follows: the colon was opened longitudinally and washed several times in PBS to remove feces and debris. Tumors and tumor-free colon pieces were incubated at 37°C in PBS supplemented with 0.145 mg/ml DTT and 0.37 mg/ml EDTA for 15 min to separate epithelial cells. The tissue was then digested in RPMI 1640 containing 0.15 mg/ml type II collagenase (Worthington, Munich, Germany) and 0.1 mg/ml DNase (Roche Molecular Biochemicals, Mannheim, Germany) for 75–90 min at 37°C on a shaking platform. $CD4^+$ T, $CD8^+$ T, and $B220^+$ B lymphocytes as well as $CD11c^+$ dendritic cells were subsequently isolated by using microbeads and MACS techniques (Miltenyi Biotech, Bergisch-Gladbach, Germany). Remaining cells were used as macrophage-enriched fraction. In addition, spleen cells were isolated as previously described (Atreya et al., 2000). Tumor epithelial cells and T cells were incubated in X-vivo 15 (Bio Whittaker). In some experiments, recombinant murine TGF-β was added at a concentration of 10 ng/ml.

In Vitro Organ Culture

Intestinal tumors were dissected from the colon of mice after 80 days of DSS/AOM treatment. The tumors were cut in two parts (2–3 mm) and placed on steel grids in an organ culture chamber at 37°C in a 5% CO_2/95% O_2 atmosphere in complete RPMI1640 medium. TAPI (TNFα protease inhibitor; obtained from Calbiochem, San Diego, CA) was added to a final concentration of 50 μM. After 24 hr, tumor specimens were collected and frozen in OCT compound for subsequent immunohistochemical analysis.

Isolation of mRNA and RT-PCR

Total RNA was isolated with the High Pure RNA isolation kit (Roche) according to the manufacturer's recommendations. Reverse transcription into cDNA was performed with the Superscript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's recommendations. PCR was performed by using the following primers derived from previously published sequence data: murine IL-6, 5′-ACACACTGGTTCTGAGGGAC-3′ and 5′-TACCACAAGGTTGGCAG GTG; murine TGFβ1, 5′-TGCTGCTTTCTCCCTCAACCT-3′ and 5′-CAC TGCTTCCCGAATGTCTGA-3′; murine IFN-γ, 5′-ACACTGCATCTTG GCTTTGC-3′ and 5′-CGGATGAGCTCATTGAATGCT-3′; murine TNFα, 5′-AACTGGCAGAAGAGGCACTC-3′ and 5′-TTGGGCAGATT GACCTCAGC-3′; murine IL-6Rα, 5′-ACACACTGGTTCTGAGGGAC-3′ and 5′-TACCACAAGGTTGGCAGGTG-3′; β-actin, 5′-TGACGGG GTCACCCACACTGTGCCCATCTA-3′ and 5′-CTAGAAGCATTTGC GGTGGACGATGGAGGG-3′. PCR products were analyzed on 1% agarose gels.

Histologic Analysis of Colon Cross-Sections

Tissues were removed from mice and sections were made and stained with hematoxylin and eosin. The degree of dysplasia on microscopic cross-sections of the colon was graded by the same pathologist (H.A.L.) in a blinded fashion.

Immunohistochemistry

Immunofluorescence was performed by using the TSA Cy3 and FITC systems (Perkin Elmer) and a fluorescence microscope (Olympus, Melville, NY) (Becker et al., 2003). In brief, cryosections were fixed in ice-cold acetone for 10 min followed by sequential incubation with methanol, avidin/biotin (Vector Laboratories, Burlingame, CA),

and protein blocking reagent (DAKO, Wiesbaden, Germany) to eliminate unspecific background staining. Slides were then incubated overnight with primary antibodies specific for CD4, CD3, CD8, pSTAT-3, IL-6, IL-6R alpha, TGFβRI, TACE (all from Santa Cruz), or KI-67 (DAKO). Subsequently, the slides were incubated for 30 min at room temperature with biotinylated secondary antibodies (Dianova, Darmstadt, Germany). All samples were finally treated with streptavidine-HRP and stained with Tyramide (Cy3 or FITC) according to the manufacturer's instructions (PerkinElmer, Heidelberg, Germany). Before examination, the nuclei were counterstained with Hoechst3342 (Molecular probes, Eugene, OH).

Construction of gp130-Fc and Preparation of Hyper-IL-6

The gp130-Fc fusion protein was made by linking cDNA coding for the extracellular portion of human gp130 to cDNA coding for the Fc portion of human IgG1. gp130-Fc was expressed in COS-7 cells or stably transfected CHO cells and was purified with protein A sepharose followed by gel filtration (Atreya et al., 2000). Hyper-IL-6 was made, as previously described (Fischer et al., 1997).

Statistical Analysis

Data were analyzed by the Student's t-test with the program Microsoft Excel.

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