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IL-6 and Stat3 Are Required for Survival of Intestinal Epithelial Cells and Development of Colitis-Associated Cancer

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SUMMARY

Colitis-associated cancer (CAC) is the most serious complication of inflammatory bowel disease. Proinflammatory cytokines have been suggested to regulate preneoplastic growth during CAC tumorigenesis. Interleukin 6 (IL-6) is a multifunctional NF-κB-regulated cytokine that acts on epithelial and immune cells. Using genetic tools, we now demonstrate that IL-6 is a critical tumor promoter during early CAC tumorigenesis. In addition to enhancing proliferation of tumor-initiating cells, IL-6 produced by lamina propria myeloid cells protects normal and premalignant intestinal epithelial cells (IECs) from apoptosis. The proliferative and survival effects of IL-6 are largely mediated by the transcription factor Stat3, whose IEC-specific ablation has profound impact on CAC tumorigenesis. Thus, the NF-κB-IL-6-Stat3 cascade is an important regulator of the proliferation and survival of tumor-initiating IECs.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common fatal malignancies worldwide (Weir et al., 2003). CRC develops in ∼5% of the adult population in the United States, and almost half of the affected individuals will die from this disease (Weir et al., 2003). In patients with inflammatory bowel disease (IBD), such as ulcerative colitis (UC), the risk of CRC development is much higher than in the general population (Langholz et al., 1992). Long-standing UC predisposes to development of colitis-associated cancer (CAC), the major cause of death in UC patients (Eaden et al., 2001). It has been proposed that noxious compounds released during chronic colonic inflammation damage DNA and/or alter cell proliferation or survival, thereby promoting oncogenesis (Meira et al., 2008). While chronic inflammation may contribute to oncogenic mutagenesis through production of reactive oxygen and nitrogen species (Hussain et al., 2003), experimental evidence suggests that it mainly acts as a tumor promoter rather than as an initiator (Greten and Karin, 2005).

The tumor-promoting effect of inflammation is now widely recognized and better understood (Coussens and Werb, 2002; Karin et al., 2006). Immune cells, which often infiltrate tumors and preneoplastic lesions, produce a variety of cytokines and chemokines that propagate a localized inflammatory response and also enhance the growth and survival of premalignant cells

SIGNIFICANCE

Tumor development and growth are driven in many cases by inflammatory cells, which produce cytokines that stimulate the growth and survival of malignant cells. Identification of such cytokines and their mechanism of action is of importance because inhibition of protumorigenic cytokine action may offer therapeutic and preventive avenues. In previous work, we have shown that NF-κB activation in myeloid cells stimulates the proliferation of premalignant intestinal epithelial cells (IECs) in colitis-associated cancer (CAC). Here we identify IL-6 as a critical NF-κB-dependent protumorigenic cytokine produced by lamina propria myeloid cells that stimulates the survival and proliferation of premalignant IECs. These effects of IL-6 are mediated by the oncogenic transcription factor Stat3. Therefore, IL-6 and Stat3 may be useful targets for prevention and treatment of CAC.

by activating transcription factors such as NF-κB (Lin and Karin, 2007; Pikarsky et al., 2004). We have found that NF-κB-driven cytokine production by myeloid cells is instrumental in CAC tumor growth, whereas NF-κB activation in intestinal epithelial cells (IECs) promotes the survival of newly emerging premalignant cells (Greten et al., 2004).

These studies suggested that cytokines or growth factors produced upon NF-κB activation in intestinal myeloid cells stimulate the proliferation of premalignant IECs generated during early stages of CAC tumorigenesis. Inactivation of NF-κB in myeloid cells through ablation of IKKβ, the protein kinase required for its activation, inhibits production of inflammatory mediators, including cytokines such as IL-6 and TNF-α, and prevents IEC proliferation during CAC induction. As a result, tumor load is reduced due to a decrease in tumor frequency and size (Greten et al., 2004). One of the NF-κB-dependent tumor growth factors released by myeloid cells could be IL-6, a multifunctional cytokine important for immune responses, cell survival, apoptosis, and proliferation (Kishimoto, 2005). IL-6 binds to soluble or membrane-bound IL-6 receptor (IL-6Rα) polypeptides that signal by interacting with the membrane-associated gp130 subunit, whose engagement triggers activation of Janus kinases (JAKs), and the downstream effectors Stat3, Shp2-Ras, and phosphatidylinositol 3-kinase (PI3K)-Akt (Kishimoto, 2005). IL-6 is also critical for T cell survival and differentiation and therefore has a central pathogenic role in T cell-dependent autoimmune disorders, including IBD (Atreya et al., 2000; Strober et al., 2007). By regulating the differentiation and survival of pathogenic T helper (Th) cells, IL-6 can perpetuate chronic inflammation and ensure the continuous production of cytokines and growth factors required for malignant cell survival and growth. IL-6 also has an important role in tissue homeostasis and regeneration (Dann et al., 2008; Tebbutt et al., 2002), suggesting that it may have direct prosurvival and protumorigenic effects. Several studies have demonstrated a correlation between circulating or local IL-6 levels and the clinical activity of IBD (Atreya and Neurath, 2005). IL-6 protein and mRNA are also often upregulated in serum and tumor samples of humans and mice suffering from breast, prostate, lung, liver, and colon cancer (Heikkila et al., 2008). IL-6 enhances the proliferation of human colon carcinoma cells in vitro, and interference with IL-6 signaling during late stages of CAC development slows down tumor growth (Becker et al., 2004, 2005). However, it has not been determined whether IL-6 is also involved in tumor promotion and proliferation of premalignant IECs during early stages of CAC. Furthermore, during late stages of CAC development in a model with disrupted TGF-β signaling in T cells, IL-6 was found to be produced mainly by T cells (Becker et al., 2004), whereas genetic experiments that we conducted have revealed that IL-6 is produced mainly by myeloid cells during early stages of CAC (Greten et al., 2004). The exact signaling pathways through which IL-6 promotes tumor development and growth have not been established.

Here we show that IL-6, which is produced in an NF-κB-dependent manner in innate immune cells within the lamina propria in response to intestinal injury, regulates the survival and proliferation of IECs and their preneoplastic derivatives during acute colonic inflammation and CAC induction. The cytoprotective and protumorigenic effects of IL-6 are mainly due to

Stat3 activation. Ablation of Stat3 in IECs effectively inhibits CAC induction and growth, demonstrating the critical oncogenic function of this cytokine-activated transcription factor.

RESULTS

Ablation of IL-6 Reduces CAC Tumorigenesis

IKKβ-dependent NF-κB activation in myeloid cells controls production of cytokines and growth factors that stimulate neoplastic growth in mice subjected to CAC induction (Greten et al., 2004). One of these factors may be the inflammatory cytokine IL-6, whose expression during colitis induction is diminished by inactivation of IKKβ in myeloid cells. As inhibition of IL-6 signaling slows down the growth of adenomas during late-stage CAC tumorigenesis (Becker et al., 2004), we sought to examine the impact of complete IL-6 deficiency on CAC development and determine whether it acts as a tumor promoter. We therefore injected wild-type (WT) and $Il6^{-/-}$ mice with the procarcinogen azoxymethane (AOM) followed by three rounds of dextran sodium sulfate (DSS) exposure to elicit colitis. As expected, DSS exposure increased colonic IL-6 production (see Figure S1 available online). The IL-6 deficiency decreased tumor numbers in mice treated with either high or low doses of DSS (Figures 1A and 1B).

Notably, the absence of IL-6 had a stronger effect when mice were treated with a lower dose of DSS. Tumor size was also reduced (Figure 1C), and $Il6^{-/-}$ mice exhibited a higher frequency of smaller adenomas than WT mice (Figure 1D). Correspondingly, average tumor load, a sum of the diameters of all tumors in a given mouse (Neufert et al., 2007) was significantly lower in $Il6^{-/-}$ mice (Figure 1E). All of the tumors analyzed were adenomas, and no carcinomas were noted. IL-6 status did not exert a significant effect on the proportion of low- or high-grade dysplastic adenomas (data not shown). Taken together, these data indicate that IL-6 is important for both tumor development and tumor growth in CAC.

IL-6 Regulates Survival and Proliferation of IECs

Differences in tumor multiplicity and load may be explained by altered proliferation and/or death of tumor progenitors. Decreased tumor multiplicity in $Il6^{-/-}$ mice suggested that IL-6 may be involved in early tumor promotion, which in this model is linked to inflammation. To determine the role of IL-6 in inflammation, we treated WT and $Il6^{-/-}$ mice with DSS to induce acute colitis. Upon DSS treatment, $Il6^{-/-}$ mice exhibited more severe colitis with greater body weight loss than WT mice (data not shown), shortening of the colon and loss of crypt structure, ulceration, and infiltration of inflammatory cells (Figures 2A–2C), similar to previous reports (Dann et al., 2008; Tebbutt et al., 2002).

DSS-exposed $Il6^{-/-}$ mice exhibited elevated IEC apoptosis (Figure 2D). To examine whether IL-6 might regulate proliferation of IECs in the inflamed colon, we injected naive and DSS-treated mice with 5-bromo-2-deoxyuridine (BrdU), which incorporates into newly synthesized DNA, and sacrificed the animals 3 hr later. Staining with BrdU or Ki-67-specific antibodies did not reveal any significant differences in basal crypt proliferation rates between naive WT and $Il6^{-/-}$ mice (data not shown). However, IEC proliferation within crypts of $Il6^{-/-}$ mice was slightly but
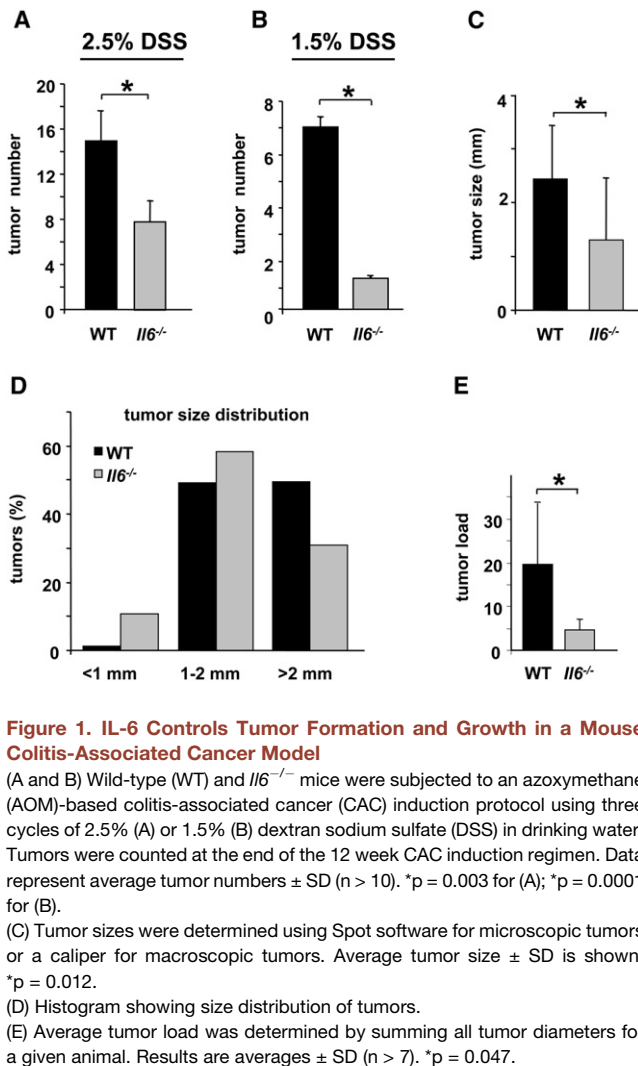
Figure 1. IL-6 Controls Tumor Formation and Growth in a Mouse Colitis-Associated Cancer Model

(A and B) Wild-type (WT) and $Il6^{-/-}$ mice were subjected to an azoxymethane (AOM)-based colitis-associated cancer (CAC) induction protocol using three cycles of 2.5% (A) or 1.5% (B) dextran sodium sulfate (DSS) in drinking water. Tumors were counted at the end of the 12 week CAC induction regimen. Data represent average tumor numbers ± SD (n > 10). *p = 0.003 for (A); *p = 0.0001 for (B).

(C) Tumor sizes were determined using Spot software for microscopic tumors or a caliper for macroscopic tumors. Average tumor size ± SD is shown; *p = 0.012.

(D) Histogram showing size distribution of tumors.

(E) Average tumor load was determined by summing all tumor diameters for a given animal. Results are averages ± SD (n > 7). *p = 0.047.

significantly decreased after DSS exposure relative to WT mice (Figure 2E). These results were confirmed by immunohistochemical determination of Ki-67-expressing cells in the crypts (Figure 2F) and immunoblot analysis for proliferating cell nuclear antigen (PCNA), whose levels were generally higher in WT colonic lysates (Figure 2G). Therefore, IL-6 enhances the proliferation of IECs and increases their resistance to apoptosis with potential effects on tissue regeneration. Presumably, IL-6 exerts the same effects on premalignant IECs in mice treated with AOM + DSS.

IL-6 Is Produced by Multiple Cell Types during CAC Tumorigenesis

To gain further insights into the cellular source of IL-6 during tumorigenesis, we generated reciprocal bone marrow chimeras by introducing WT bone marrow into lethally irradiated $Il6^{-/-}$ (WT→$Il6^{-/-}$) or WT (WT→WT) hosts and $Il6^{-/-}$ bone marrow into $Il6^{-/-}$ ($Il6^{-/-}$→$Il6^{-/-}$) or WT ($Il6^{-/-}$→WT) mice. The extent of repopulation was determined by flow cytometric analysis of the congenic markers Ly5.1 and Ly5.2 and was routinely 93%–98% for CD3$^+$ T cells and 90%–95% for CD11b$^+$ myeloid cells

(data not shown). The chimeric mice were subjected to the standard CAC induction protocol, and tumor multiplicity and load were evaluated. Tumor numbers were markedly reduced when IL-6 was inactivated in hematopoietic cells (Figure 3A). IL-6 deficiency in bone marrow-derived cells also decreased tumor size and load (Figures 3B and 3C). We observed an increase in tumorigenicity in $Il6^{-/-}$→WT mice in comparison with $Il6^{-/-}$→$Il6^{-/-}$ mice, but the effect was not statistically significant (Figure 3A).

We have previously found a critical contribution of myeloid cells to IL-6 production during the initial stage of DSS-induced colitis (Greten et al., 2004). However, others who have conducted their analyses during late stages of CAC tumor growth have suggested that T cells are the major IL-6 producers, at least in the model where TGF-β signaling is inactivated in T cells (Becker et al., 2004). Another report has suggested that intestinal dendritic cells are responsible for IL-6 production triggered by proinflammatory stimuli (Denning et al., 2007). To determine the cellular source of IL-6 during the colitis phase and in already developed CAC, we performed immunohistochemical staining with anti-IL-6 antibody. We found strong IL-6 expression in infiltrating immune cells and weak but detectable expression in epithelial cells both in the mucosa of DSS-challenged mice and in CAC adenomas (Figure 3D). To further delineate cellular sources of IL-6, we isolated lamina propria cells from colons of CAC-bearing mice and from CAC adenomas using a protocol that increases the yield of myeloid cells, which otherwise are largely lost during the isolation procedure (Denning et al., 2007). Intracellular cytokine staining and flow cytometry revealed that lamina propria and tumor-infiltrating CD11c$^+$ (dendritic cells) and CD11b$^+$ (macrophages) cells were the major IL-6 producers during CAC growth, followed by CD3$^+$ (T cells) cells (Figures 3E and 3F). Analysis of IL-6 mRNA in cells sorted by fluorescence-activated cell sorting (FACS) from lamina propria and CAC adenomas confirmed that dendritic cells (DCs) followed by macrophages are the major IL-6 producers (Figure 3G). Myeloid cells were less abundant in the adenoma leukocyte population than T cells (Figure 3H), but their overall contribution to IL-6 production was greater (Figure 3F and 3G).

IL-6 Is Important for Tumor Growth and Proliferation

We analyzed cell proliferation in WT and IL-6-deficient tumors. PCNA nuclear staining was modestly decreased in $Il6^{-/-}$ adenomas (Figure 4A), as was expression of PCNA mRNA (Figure 4B). Analysis of Ki-67 expression revealed a small but significant difference in proliferation rates between WT and IL-6-deficient tumors (Figures 4C and 4D). Expression of cyclin D was also lower in $Il6^{-/-}$ tumors (Figure 4C), indicating decreased growth capacity for the tumors in the absence of IL-6. Cyclin D2 mRNA expression was also decreased in total RNA prepared from $Il6^{-/-}$ colon tumors of CAC-bearing mice (Figure S4).

Immunoblot analysis of total colon lysates of WT and $Il6^{-/-}$ CAC-bearing mice revealed marked downregulation of Stat3 phosphorylation, as well as significant decreases in proliferation (PCNA) and inflammation (COX2 and MMP9) markers in $Il6^{-/-}$ mice (data not shown). Therefore, IL-6 is important for tumor growth and expression of prosurvival and proproliferative genes during CAC tumorigenesis. However, since WT mice have higher tumor loads, it is difficult to conclude whether the changes in protein expression are a direct reflection of IL-6 deficiency or
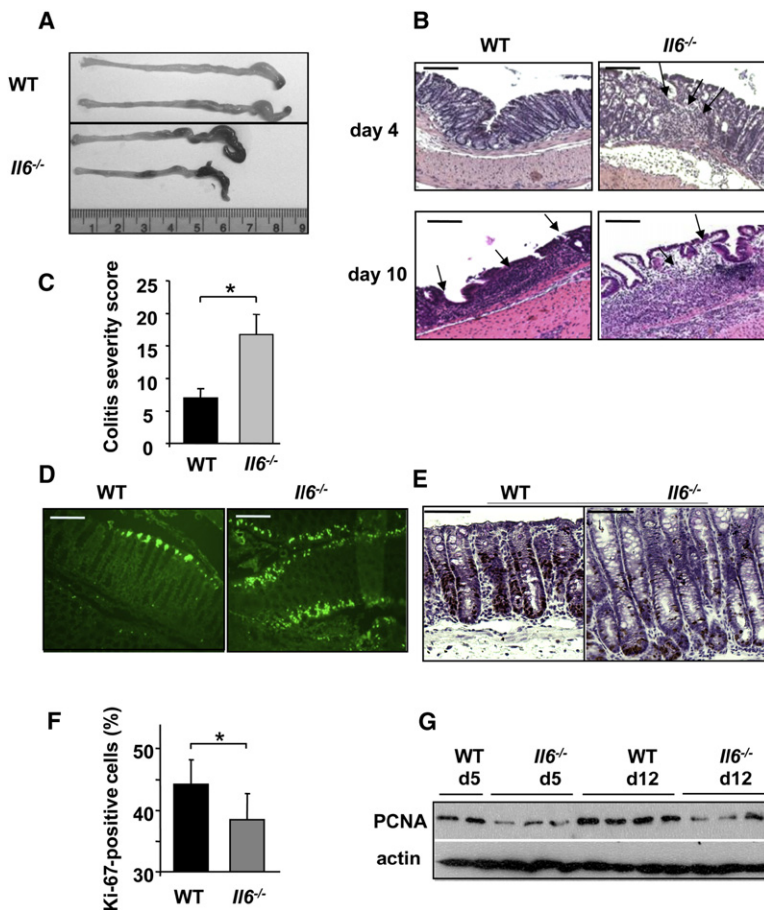
A

WT

Il6^{-/-}

C

D

WT   Il6^{-/-}

F

B

WT   Il6^{-/-}

day 4

day 10

E

WT   Il6^{-/-}

G

	WT d5	Il6^{-/-} d5	WT d12	Il6^{-/-} d12

PCNA

actin

Figure 2. IL-6 Is Required for Maintenance of Mucosal Integrity

(A) WT and Il6^{-/-} mice exhibit colon shortening after 7 days of 2.5% DSS exposure.

(B) Mucosal histology was examined in WT and Il6^{-/-} mice 4 or 10 days after initiation of 2.5% DSS treatment by hematoxylin and eosin (H&E) staining of paraffin-embedded sections. Scale bars = 50 μm.

(C) Colitis severity score after 3% DSS exposure was determined on day 10. Results are averages ± SD (n = 5). *p ≤ 0.05.

(D) Apoptosis in colons of 3% DSS-treated mice was evaluated by TUNEL staining on day 4 after DSS administration. Scale bars = 50 μm.

(E) The extent of intestinal epithelial cell (IEC) proliferation in colons of DSS-treated mice was determined by BrdU labeling and immunohistochemistry. Scale bars = 50 μm.

(F) The percentage of Ki-67-positive cells among all crypt cells in colons of DSS-treated mice was enumerated. Results are averages ± SD (n = 6). *p = 0.001.

(G) Lysates of distal colons prepared on the indicated days after initiation of 3% DSS treatment were analyzed for PCNA expression by immunoblotting.

kinase (S6K), a target for PI3K/mTOR signaling, and ERK, a target for Shp2-Ras signaling, was not significantly altered by the absence of IL-6, Stat3 activation was considerably reduced (Figure 5A). IL-6 signaling has also been reported to downmodulate TGF-β/BMP signaling by inducing expression of its inhibitor Smad7 (Jenkins et al., 2005). However, we did not observe increased amounts of phospho-Smad2/3 in nuclei of IECs of Il6^{-/-} mice (Figure S5).

To determine the contribution of Stat3 in IECs to CAC tumorigenesis, we generated mice with a conditional Stat3 deletion in enterocytes (Stat3[ΔIEC] mice) by crossing Stat3[F/F] mice (Takeda et al., 1999) with villin-Cre mice (Madison et al., 2002). Stat3[ΔIEC] mice, which were phenotypically normal, were almost completely devoid of Stat3 protein in IECs (Figures 5B and 5C) but retained Stat3 expression in lamina propria cells (Figure 5C). A considerable reduction in total and phosphorylated Stat3 was also seen in crude colonic lysates of Stat3[ΔIEC] mice (Figure 5D).

Stat3[ΔIEC] mice developed more severe DSS-induced colitis than WT counterparts, with pronounced colonic ulceration and body weight loss (Figures 5E and 5F). Expression of Hsp70 (Hsp72) and Bcl-X[L] was significantly downregulated in colonic lysates and IECs of Stat3[ΔIEC] mice (Figures 5G and 5H). This phenotype of Stat3[ΔIEC] mice was similar to that of Il6^{-/-} mice, although Stat3[ΔIEC] mice developed more severe colitis and displayed more pronounced epithelial injury. Stat3[ΔIEC] mice also exhibited decreased IEC proliferation after DSS exposure (Figure 6A). Most importantly, hardly any adenomas were found in AOM + DSS-treated Stat3[ΔIEC] mice (Figure 6B), an effect also somewhat greater than the one caused by ablation of IL-6. In addition, Stat3-deficient adenomas were smaller than WT counterparts (Figures 6C and 6D). Immunohistochemical staining confirmed absence of Stat3 in Stat3[ΔIEC] cancer cells, indicating that the tumors did not originate from progenitor cells that escaped Cre-mediated deletion (data not shown). DSS-induced apoptosis of IECs was increased in Stat3[ΔIEC] mice in

are partially due to reduced tumor load. To circumvent this difficulty, we examined the consequences of IL-6 deficiency during colitis induction and confirmed a considerable reduction in phosphorylated Stat3 in IECs from Il6^{-/-} mice (Figure 4E). We also analyzed nuclear accumulation of phosphorylated Stat3 in acute DSS-induced colitis and in CAC adenomas and found that ablation of IL-6 resulted in weaker phospho-Stat3 nuclear staining, while the frequency of phospho-Stat3-positive cells was not different between WT and Il6^{-/-} mice (Figure S3). Therefore, IL-6 (together with other cytokines) is an important Stat3 activator in IECs during acute DSS-induced colitis and tumor growth. We also noted downregulation in IECs from Il6^{-/-} mice of the antiapoptotic and cytoprotective proteins Hsp70 (Hsp72) (data not shown) and Bcl-X[L] (Figure 5H), which were suggested to mediate IEC survival (Rakoff-Nahoum et al., 2004) and whose genes contain STAT-binding sites (Madamanchi et al., 2001). We also analyzed expression of the tissue-protective factors Tff3, which has been implicated in IL-6-mediated intestinal protection during colitis (Tebbutt et al., 2002), and RegIIIγ and found a small reduction in the absence of IL-6 (Figure 4F).

A Critical Role for Epithelial Stat3 in IL-6-Dependent Tumorigenesis

To decipher the molecular mechanisms that mediate effects of IL-6 on IEC physiology and CAC development, we analyzed total colon lysates of DSS-treated WT and Il6^{-/-} animals for activation of various IL-6 effectors. Whereas the extent of activation of S6
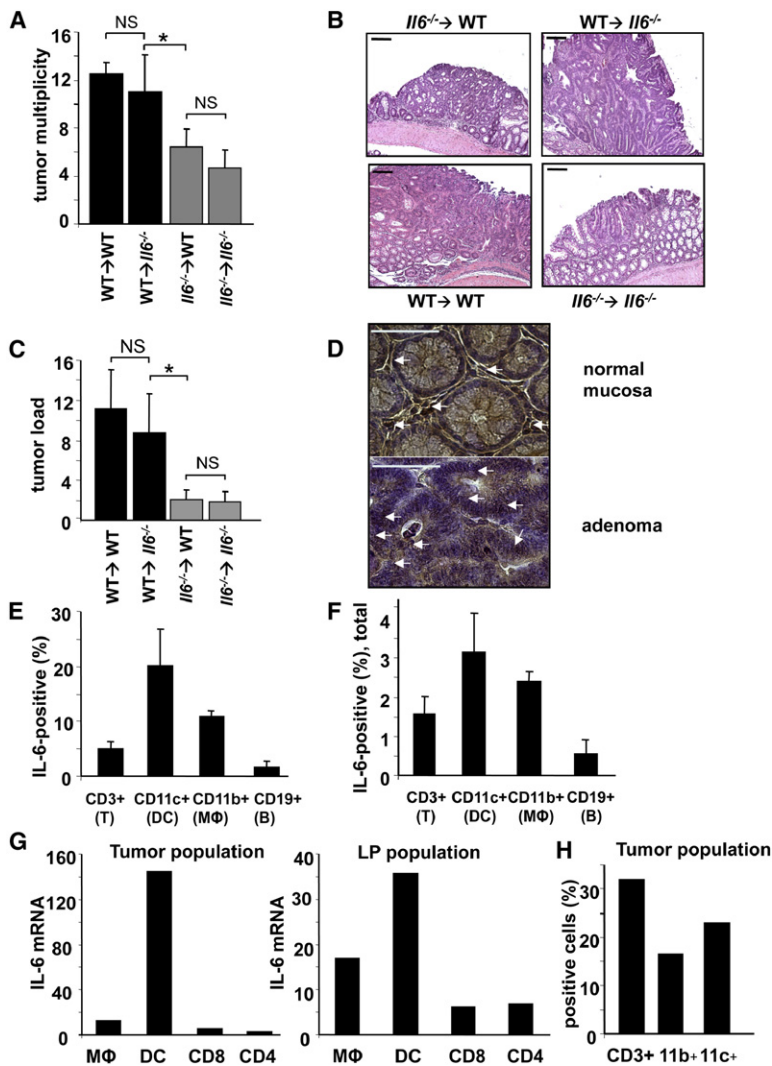
Figure 3. IL-6 Produced by Bone Marrow-Derived Cells Is Required for CAC Tumorigenesis

(A) Tumor multiplicity in radiation chimeras subjected to induction of CAC using 2.5% DSS. Results are averages ± SD (n > 8). *p < 0.01.

(B) Paraffin-embedded sections of adenoma-containing colons stained with H&E. Scale bars = 100 μm.

(C) Average tumor loads in radiation chimeras. Results are averages ± SD (n = 5). NS, not significant; *p < 0.01.

(D) Immunohistochemical analysis of IL-6 expression in DSS-treated colons (normal mucosa) or CAC-bearing colons of WT mice. Scale bars = 50 μm.

(E and F) Intracellular IL-6 cytokine staining of PMA + ionomycin-restimulated tumor-infiltrating cells analyzed by flow cytometry. Results are averages ± SD (n = 3).

(E) Percentages of IL-6-expressing cells in each given population (dendritic cells [DCs], macrophages [Mφ], T cells, and B cells).

(F) Percentages of each population positive for IL-6 cells among all tumor-infiltrating cells.

(G) Macrophages (CD45⁺CD11b⁺CD11c⁻), dendritic cells (CD45⁺CD11b⁺CD11c⁺), and T cells (CD45⁺CD8α⁺ or CD45⁺CD4⁺) isolated by fluorescence-activated cell sorting were analyzed for IL-6 mRNA by qRT-PCR. Tumor population = tumor-infiltrating cells from pooled tumors; LP population = lamina propria cells from colons from which the tumors were excised.

(H) Percentage of macrophages (CD11b⁺), T cells (CD3⁺), and dendritic cells (CD11c⁺) in total lamina propria cells isolated from pooled CAC tumors from WT mice.

IL-6 or hyper-IL-6 also enhanced Stat3 phosphorylation in colon (Figure 7G) and, importantly, resulted in elevated serum levels of IL-6 (Figure S6), in agreement with previously published data (Peters et al., 1998). Thus, IL-6 signaling can affect both tumor multiplicity and size if activated during early stages of CAC induction and therefore has an impact on tumor formation and growth.

IL-6 and TNF-α Cross-Regulation and the Role of TNF-α in CAC

TNF-α is another NF-κB-regulated cytokine that is critical for IBD development in humans and mice (Kollias et al., 1999). CAC development and growth have been found to be attenuated in TNF receptor 1 (TNFR1) knockout mice or in mice treated with a soluble TNFR2 decoy (Enbrel) (Popivanova et al., 2008). However, the methods used in this previous study did not distinguish between the involvement of TNF-α and lymphotoxin-α, which also signals through TNFR1 and is neutralized by Enbrel. We therefore examined whether monoclonal antibodies to mouse TNF-α can decrease CAC development. Indeed, treatment with anti-TNF-α antibodies at late stages of CAC reduced the number of macroscopically detectable tumors and decreased average tumor load in CAC-bearing mice (Figures S7A and S7B). TNF-α blockade also inhibited expression of IL-6 mRNA in colonic lysates from CAC-bearing mice (Figure S7C), which may partially explain how TNF-α controls tumor growth.
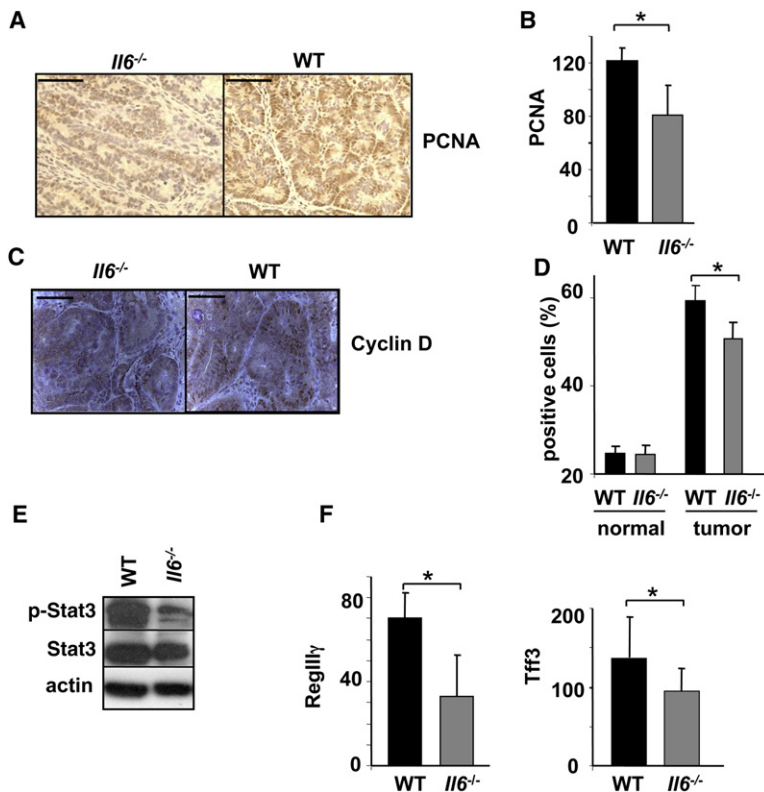
comparison with WT counterparts or even *Il6⁻/⁻* mice (Figure 6E). Therefore, epithelial Stat3 is required for transduction of tumor-promoting signals from IL-6 and other cytokines and is important to maintain survival and regenerative capacity of IECs.

IL-6 Is Continuously Required for Stimulation of CAC Tumor Growth

WT mice were treated with recombinant IL-6 and the so-called hyper-IL-6 recombinant protein, which triggers IL-6 *trans*-signaling (Fischer et al., 1997; Mitsuyama et al., 2006). Hyper-IL-6 treatment during colitis or at late stages of CAC growth increases T cell survival and tumor burden without affecting tumor multiplicity (Becker et al., 2004). We confirmed that continuous treatment with either recombinant IL-6 or hyper-IL-6 during early or late stages of CAC resulted in a significant increase in tumor size (Figures 7A–7E). As reported previously, effects on tumor multiplicity during late CAC development were marginal (Figure 7C). However, when IL-6 or hyper-IL-6 was administered during early CAC induction, they did enhance tumor multiplicity (Figures 7D–7F). Treatment with recombinant

Figure 4. IL-6 Regulates Cell Proliferation and Expression of Genes Involved in Proliferation, Survival, and Inflammation

(A) Colons of adenoma-bearing mice were stained with anti-PCNA antibody. Scale bars = 50 μm.

(B) Lysates of distal colons of adenoma-bearing mice were prepared, and PCNA mRNA expression was analyzed by qRT-PCR. Results are averages ± SD (n = 3). *p < 0.05.

(C) Sections of adenoma-bearing colons were stained with antibodies to cyclin D. Scale bars = 50 μm.

(D) Percentages of Ki-67-positive cells within colonic crypts. Results are averages ± SD (n = 6). *p = 0.005.

(E) IECs from mice treated with 2.5% DSS were purified on day 10. Expression of the indicated proteins was analyzed by immunoblotting after gel separation.

(F) RegIIIγ and Tff3 mRNA expression was analyzed by qRT-PCR. Results are averages ± SD (n = 3). *p < 0.05 (left); *p = 0.05 (right).

However, we also found that IL-6 can affect TNF-α expression. Blockade of IL-6 *trans*-signaling slightly decreased TNF-α mRNA expression in colon, while administration of hyper-IL-6 resulted in a modest increase in TNF-α mRNA (Figure S7D). Thus, the two NF-κB-dependent cytokines IL-6 and TNF-α cross-regulate each other during CAC development to enhance chronic inflammation and tumorigenesis.

DISCUSSION

Although cell-autonomous events such as proliferation and death evasion control tumor development, the tumor microenvironment also makes a major contribution and influences the physiology of malignant cells (Radisky and Bissell, 2004). Nearly all tumors contain inflammatory and immune cells, such as dendritic cells, macrophages, and lymphocytes, which produce cytokines and other factors that promote tumor growth and survival (Balkwill et al., 2005; Coussens and Werb, 2002; Lin and Karin, 2007). The most obvious tumor-promoting role of immune cells is manifested in inflammation-associated cancers, where tumors arise and grow at sites of chronic inflammation. In previous work, we found that inhibition of NF-κB activation in myeloid cells, exerted through the cell-type-specific ablation of IKKβ, inhibits the proliferation of premalignant IECs in a mouse model of CAC (Greten et al., 2004). Based on these results, we concluded that myeloid cells in the lamina propria of mice subjected to CAC induction produce cytokines that stimulate the proliferation of adjacent premalignant IECs, which harbor activating β-catenin mutations (Greten et al., 2004). The results described above suggest that one of these cytokines is IL-6.

Using genetic and pharmacological tools, we demonstrated that IL-6 is an important regulator of CAC development and growth. However, IL-6 did not have significant effect on early tumor promotion in the CAC model since its absence did not alter the distribution of high- and low-grade dysplastic adenomas. As reported by other groups, it is plausible that in other cancer models, IL-6 may be an important player in tumor progression and invasiveness (Poutahidis et al., 2007).

The major protumorigenic IL-6 effector is the transcription factor Stat3, whose ablation in IECs also results in decreased tumor multiplicity and growth. Although Stat3 has been known as an oncogenic transcription factor (Bromberg et al., 1999; Sriuranpong et al., 2003; Wang et al., 2004; Yu et al., 2007), it has only recently been proven to be critical for tumor initiation and growth in vivo in a model of skin cancer (Chan et al., 2004; Kataoka et al., 2008). Here, we demonstrate that specific Stat3 ablation in intestinal epithelial cells interferes with tumor formation and tumor growth in a mouse model of CAC.

IL-6-deficient mice developed fewer and smaller adenomas than WT mice. Also, fewer myeloid cells (macrophages and neutrophils) were recruited to IL-6-deficient colons after DSS administration (Figure S2), consistent with a role for IL-6 and IL-6-like signaling in leukocyte migration (Romano et al., 1997; Sander et al., 2008). However, it should be noted that in acute DSS-induced colitis, leukocyte recruitment not only depends on direct IL-6-dependent signals but also is regulated by the degree of injury. We also found fewer IL-17A-producing T helper (Th17) cells in colons of *Il6*$^{-/-}$ mice subjected to CAC (Figure S2), which is in line with the role of IL-6 in Th17 lineage differentiation (Bettelli et al., 2006; Veldhoen et al., 2006). We do not rule out indirect effects of IL-6 on CAC development due to its impact on immune cells; however, IEC-specific ablation of the IL-6 target Stat3 phenocopies IL-6 ablation as far as CAC induction and growth are concerned. However, IKKβ ablation in myeloid cells, which affects IL-6 induction, does not compromise IEC survival (Greten et al., 2004). Thus, in addition to control of IL-6 expression, NF-κB may also be involved in production of damage-inducing cytokines, whose effect becomes more
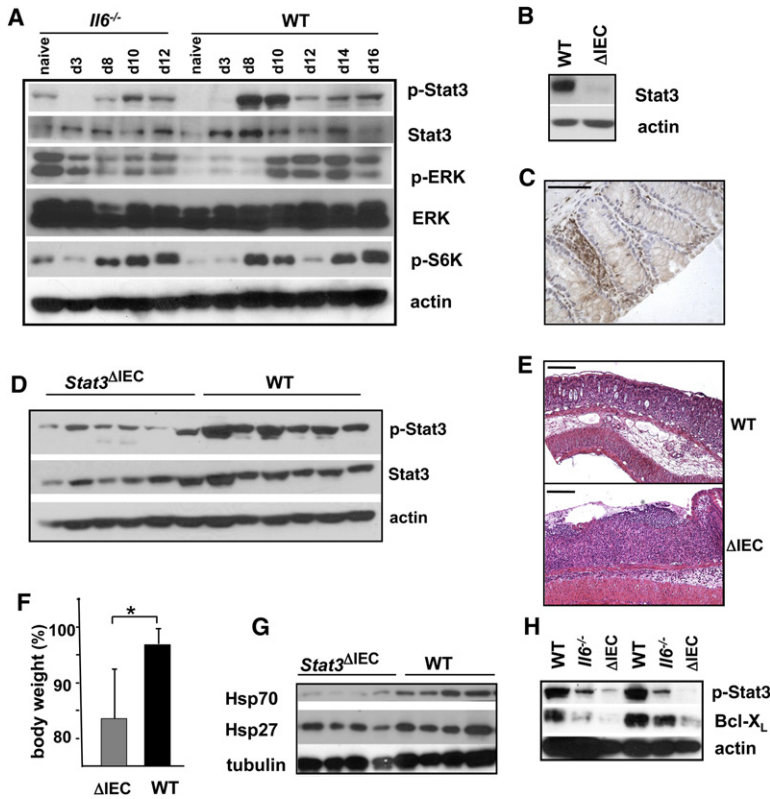
Figure 5. Stat3 Maintains Mucosal Integrity during Acute Colitis

(A) Mice were exposed to 2.5% DSS. Colonic lysates were prepared at the indicated time points and analyzed for expression and phosphorylation of the indicated proteins.

(B) Deletion of Stat3 in IECs of Stat3$^{\Delta IEC}$ mice was examined by immunoblotting of IEC lysates.

(C) Deletion of Stat3 in colons of Stat3$^{\Delta IEC}$ mice was analyzed by immunohistochemistry with anti-Stat3 antibody. Scale bar = 50 μm.

(D) Total colon lysates of WT and Stat3$^{\Delta IEC}$ mice were analyzed for Stat3 expression and phosphorylation by immunoblot analysis.

(E) Increased susceptibility of Stat3$^{\Delta IEC}$ mice to DSS-induced colitis. Colons of WT and Stat3$^{\Delta IEC}$ mice were analyzed by sectioning and H&E staining 10 days after initiation of 2.5% DSS exposure. Scale bars = 100 μm.

(F) Body weights of the indicated mice were measured 10 days after initiation of 2.5% DSS exposure. Results show percentage of body weight on day 0 and are averages ± SD (n = 6). *p < 0.05.

(G and H) Total colon lysates (G) or IEC lysates (H) from mice on day 10 after 2.5% DSS administration were analyzed for expression of the indicated proteins by immunoblotting.

pronounced in the absence of IL-6. Candidates for such cytokines are TNF-α and Fas ligand (FasL), which do not induce apoptosis in normal IECs and whose proapoptotic effect can be partially blocked by IL-6 or by activation of Stat3 (Dann et al., 2008; Yamaoka et al., 2008).

Our current results affirm our earlier conclusions that during early CAC induction, when it acts as a tumor promoter, IL-6 is produced mainly by myeloid cells. Nonetheless, we also detected IL-6 production by other immune cell types, such as T cells, and by IECs. Correspondingly, mice lacking IL-6 in bone marrow-derived cells exhibited fewer tumors, with a substantial reduction in overall CAC tumor load. While the role of immune cell IL-6 seems greater in cancers associated with underlying chronic inflammation, IL-6 produced by epithelial and cancer cells may still contribute to tumorigenesis in models without an obvious inflammatory component (Ancrile et al., 2007; Gao et al., 2007; Grivennikov and Karin, 2008; Hodge et al., 2005; Sansone et al., 2007).

By binding to its gp130-associated receptor, IL-6 activates three separate signaling pathways, namely Shp2-Ras-ERK, JAK1/2-Stat3, and PI3K-Akt-mTOR (Kishimoto, 2005). Our results suggest that among these, Stat3 is a critical IL-6 effector in CAC induction. Stat3 is highly phosphorylated not only during DSS-induced colitis in mice but also in the mucosa and lamina propria of human IBD patients (Fu, 2006). Stat3 has been found to be activated in various adenomas and carcinomas, although the mechanisms of its activation are obscure (Klampfer, 2008; Kusaba et al., 2005). Stat3 induces expression of genes important for proliferation (such as cyclin D and PCNA) and suppression of apoptosis (Bcl-X$_L$, Bcl-2, and Mcl-1)

(Becker et al., 2005; Klampfer, 2008). Stat3 signaling in IECs also controls expression of the inducible form of the cytoprotective chaperone Hsp70 (Hsp72), known to be encoded by a Stat3 target gene (Madamanchi et al., 2001), and Bcl-X$_L$ but has an insignificant effect on the small heat-shock protein Hsp27, also thought to protect the intestinal mucosa from damage (Rakoff-Nahoum et al., 2004). Hsp27 was previously shown to be controlled by p38 MAPK signaling (Sakurai et al., 2008). Other cytoprotective factors regulated by IL-6 in IECs are the intestinal trefoil factor (ITF)/Tff3 and RegIIIγ, which are important for intestinal protection during DSS-induced colitis (Tebbutt et al., 2002). However, Tff3 expression is presumably regulated by the Stat3 pathway (Tebbutt et al., 2002). The remaining Stat3 activation seen in IECs of Il6$^{-/-}$ mice and the more severe manifestations of DSS-induced colitis and decreased tumorigenesis seen in Stat3$^{\Delta IEC}$ mice imply that IL-6 is not the only Stat3 activator. This suggests the possible involvement of other cytokines that play cytoprotective and proliferative roles, in particular EGF, IL-22 (Sugimoto et al., 2008; Zheng et al., 2008), IL-11 (Ernst et al., 2008; see also Bollrath et al. [2009] in this issue of Cancer Cell), and others.

Stat3 inhibitors reduce growth and promote apoptosis in colon cancer cell lines (Rivat et al., 2004). Conversely, mice lacking SOCS3, a negative regulator of receptor-mediated activation of Stat3 (Suzuki et al., 2001), in IECs show enhanced CAC development (Rigby et al., 2007). Similarly, gp130 mutations that prevent SOCS3 binding lead to Stat3 hyperactivation and spontaneous gastrointestinal tumorigenesis (Ernst et al., 2008; Jenkins et al., 2005; Judd et al., 2004; Bollrath et al., 2009).
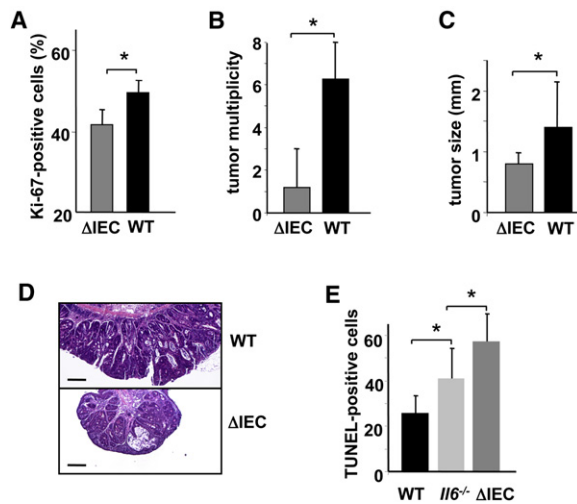
Figure 6. Stat3 Is Critical for CAC Tumorigenesis

(A) Percentage of Ki-67-positive cells in WT and $Stat3^{\Delta IEC}$ colonic crypts 10 days after initiation of DSS exposure. Results are averages ± SD (n = 3). *p = 0.03.

(B) Tumor multiplicity in WT and $Stat3^{\Delta IEC}$ mice subjected to induction of CAC. Results are averages ± SD (n = 8). *p = 0.004.

(C) Tumor size in WT and $Stat3^{\Delta IEC}$ mice. Results are averages ± SD (n = 8). *p = 0.012.

(D) Paraffin-embedded sections of adenoma-containing colons of WT and $Stat3^{\Delta IEC}$ mice were stained with H&E. Scale bars = 100 μm.

(E) Apoptosis in colons of DSS-treated mice was evaluated on day 4 after DSS administration by TUNEL staining. Number of TUNEL-positive cells per microscope field was determined. Results are averages ± SD (n = 3). *p = 0.05.

Besides its importance during early tumor promotion, IL-6 signaling also affects tumor growth during late stages of CAC (Becker et al., 2004). Enhanced IL-6 *trans*-signaling or both classical and *trans*-signaling during late CAC development accelerate tumor growth without affecting tumor multiplicity (Figures 7A–7C). Interestingly, IL-6 signaling during that stage increases TNF-α production, and interference with TNF-α signaling curtails tumor growth and reduces IL-6 production. Thus, the beneficial effect of TNF-α inhibition may be partially due to reduced IL-6 expression. While TNF-α can directly induce IL-6 production by NF-κB-, NF-IL-6-, and AP-1-dependent mechanisms (Legrand-Poels et al., 2000), it also can facilitate the recruitment and survival of proinflammatory immune cells capable of IL-6 production (Kollias et al., 1999). Conversely, IL-6 can directly induce TNF-α transcription and sustains chronic inflammation, in particular by ensuring the continuous presence of TNF-α-producing cells (Atreya et al., 2000). Such cross-regulation is not unique to IL-6 and TNF-α since, for example, IL-1 can also induce IL-6 production (Legrand-Poels et al., 2000) and have an indirect impact on immune cell recruitment (Dinarello, 1994). Indeed, IL-1α released by necrotic hepatocytes induces IL-6 synthesis (Sakurai et al., 2008), whereas IL-1β is involved in CAC development (Garlanda et al., 2007; Xiao et al., 2007) and is also a well-established IL-6 inducer in immune cells and even in IECs (Parikh et al., 1997).

In summary, our results establish a role of IL-6 and Stat3 signaling in IECs during inflammation-associated colon carcino-

genesis and create a rationale for the use of IL-6 blockers and Stat3 inhibitors in the treatment and prevention of CAC.

EXPERIMENTAL PROCEDURES

Animals and Tumor Induction

$Il6^{-/-}$ ($Il6^{tm1Kopf}/Il6^{tm1Kopf}$), *villin*-Cre (B6.SJL-Tg(*vil*-Cre)997Gum/J), and Ly5.1 and Ly5.2 congenic C57BL/6 mice were obtained from The Jackson Laboratory. $Stat3^{F/F}$ mice (Takeda et al., 1999) were obtained from C. Drake with permission from S. Akira. Cre-negative mice were used as WT controls. All mice were maintained in filter-topped cages on autoclaved food and water at UCSD according to NIH guidelines, and all experiments were performed in accordance with UCSD and NIH guidelines and regulations.

CAC was induced as described previously (Greten et al., 2004). Briefly, on day 1, mice were injected intraperitoneally (i.p.) with 12.5 mg/kg azoxymethane (AOM; National Cancer Institute) and maintained on regular diet and water for 5 days. After 5 days, mice received water with 2.5% (unless stated otherwise) dextran sulfate sodium (DSS; MP Biomedicals, molecular weight 35,000–50,000 kDa) for 5 days. After this, mice were maintained on regular water for 14 days and subjected to two more DSS treatment cycles. On day 100, mice were injected i.p with 100 mg/kg 5-bromo-2-deoxyuridine (BrdU; Sigma) and sacrificed 3 hr later. Macroscopic tumors were counted and measured with a caliper. One half of the distal colon was taken as a tissue sample and snap frozen in liquid nitrogen or maintained in RNA stabilization solution (RNAlater, Ambion). The other half was fixed in 10% neutral buffered formalin for 24 hr and transferred to 70% ethanol for subsequent paraffin embedding and histological analysis. The clinical course of disease was followed daily by measurement of body weight and monitoring for signs of rectal bleeding or diarrhea.

IL-6 and TNF-α Agonists and Antagonists

Purified hyper-IL-6, recombinant IL-6, and sgp130Fc fusion protein have been described previously (Fischer et al., 1997). Monoclonal antibody to mouse TNF-α (C258D) and isotype control were generously provided by Centocor Inc. These reagents were diluted in sterile PBS and injected i.p.

Antibodies

Fluorescent-labeled antibodies for flow cytometry were from eBioscience. Immunoblot analysis and immunohistochemistry were carried out with the following antibodies: anti-Cyclin D (1/2) (Upstate), anti-Ki-67 (Novocastra Laboratories), anti-PCNA, anti-BrdU (BD Pharmingen), anti-Bcl-X$_L$ (BD Pharmingen and Cell Signaling), anti-COX2 (Cayman), anti-actin (Sigma), anti-p-SHP2, anti-p-Stat3, anti-Stat3, anti-p-ERK, anti-ERK, and anti-p-S6 (Cell Signaling). Anti-Hsp70 was from Santa Cruz Biotechnology or Stressgen, and anti-tubulin antibodies were from Santa Cruz Biotechnology.

Histological Analysis

Colons were examined using 6 μm thick, 200 μm step serial sections stained with hematoxylin and eosin. Using Scion Image for Windows, the greatest width of each tumor was measured and recorded. The extent of inflammation was measured and scored as described previously (Greten et al., 2004). For TUNEL assay, an ApoAlert DNA Fragmentation Assay Kit (BD Clontech) or In Situ Cell Death Kit (Roche) was used according to the manufacturer's recommendations. To determine BrdU incorporation, paraffin sections were stained using a BrdU In-Situ Detection Kit (BD Pharmingen) according to the manufacturer's recommendations. Scion Image for Windows was used to add scale bars.

Immunohistochemistry and Immunoblotting

Paraffin-embedded slides were deparaffinized. Antigen unmasking was carried out by incubation in 80°C water bath in 10 mM sodium citrate buffer with 0.1% Tween 20 overnight. Slides were incubated with primary antibodies in PBS containing 1% BSA and 10% goat serum. Biotinylated secondary anti-rat or anti-rabbit antibodies (BD Pharmingen) were added and incubated at room temperature for 1 hr. Streptavidin-HRP (BD Pharmingen) was added, and after 40 min the sections were stained with DAB substrate and counterstained with hematoxylin.
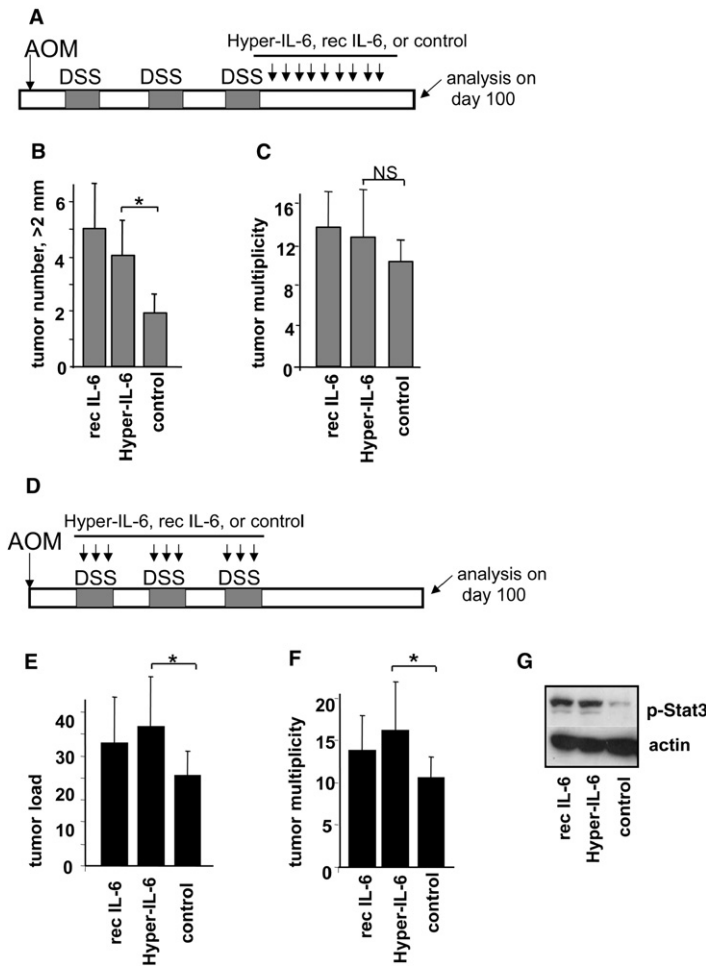
Figure 7. IL-6 Signaling Stimulates Tumor Formation and Growth

(A) Scheme of treatment with IL-6 agonists during late stage of CAC growth. Mice were injected intraperitoneally (i.p.) with 2 μg hyper-IL-6, 5 μg recombinant IL-6 (rec IL-6), or control PBS every 3 days after the last DSS cycle. Tumors were analyzed on day 100 after AOM injection.

(B) Number of tumors > 2 mm. Results are averages ± SD (n = 7). *p < 0.05.

(C) Tumor multiplicity. Results are averages ± SD. NS, not significant.

(D) Scheme of treatment with IL-6 agonists during CAC induction. Mice were injected i.p. with the same amount of IL-6 agonist as in (A) on day 1, 5, and 8 of each DSS cycle. Tumors were analyzed 100 days after AOM injection.

(E) Average tumor load. Results are averages ± SD (n = 6). *p < 0.05.

(F) Tumor multiplicity. Results are averages ± SD (n = 6). *p < 0.05.

(G) Immunoblot analysis of colonic lysates from mice treated with hyper-IL-6, recombinant IL-6, or PBS (control) after exposure to 2.5% DSS for 7 days. Mice were sacrificed on day 10, 30 min after the last treatment.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures and seven figures and can be found with this article online at http://www.cancercell.org/supplemental/S1535-6108(09)00002-6.

Tissue samples were homogenized in standard RIPA buffer, unless stated otherwise, with a cocktail of protease and phosphatase inhibitors. Cytoplasmic and nuclear proteins were prepared using a NE-PER kit (Pierce) according to the manufacturer's instructions. Lysates were separated on a 10%–15% SDS-polyacrylamide gels, and proteins were transferred to PVDF membranes (Whatman). Membranes were blocked in PBST buffer containing 5% skim milk for 1 hr at room temperature, probed with primary antibodies and secondary HRP-conjugated antibodies (GE Healthcare UK), and developed using ECL western blot detection reagent (Pierce).

Flow Cytometry and Cell Sorting

Isolated cells were stained with labeled antibodies in PBS with 2% fetal calf serum and analyzed on a BD FACSCalibur or on an Accuri C6 flow cytometer. For cell sorting, a BD FACSVantage cell sorter was used. Data were analyzed using FlowJo or Accuri CFlow software.

Real-Time PCR Analysis

Total RNA was extracted using TRIzol (Invitrogen) or an RNeasy Plus kit (QIAGEN). After DNase I treatment (Ambion), RNA was reverse transcribed using an iScript kit (Bio-Rad). Real-time PCR was performed using SYBR green (Bio-Rad) on a Bio-Rad iQ5 machine. Expression data were normalized to cyclophilin or GAPDH mRNA expression. Data are presented in arbitrary units and were calculated as $2^{(-\Delta Ct\ cyclophilin\ -\ gene\ of\ interest)}$. Primer sequences are listed in Supplemental Experimental Procedures.

Statistical Analysis

Data are presented as averages ± SD and were analyzed by built-in t test using Microsoft Excel software. p ≤ 0.05 was considered significant.

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