IFNγ-dependent, spontaneous development of colorectal carcinomas in SOCS1-deficient mice

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Approximately 20% of human cancers are estimated to develop from chronic inflammation. Recently, the NF-κB pathway was shown to play an essential role in promoting inflammation-associated cancer, but the role of the JAK/STAT pathway, another important signaling pathway of proinflammatory cytokines, remains to be investigated. Suppressor of cytokine signaling-1 (SOCS1) acts as an important physiological regulator of cytokine responses, and silencing of the *SOCS1* gene by DNA methylation has been found in several human cancers. Here, we demonstrated that SOCS1-deficient mice (SOCS1$^{-/-}$Tg mice), in which SOCS1 expression was restored in T and B cells on a SOCS1$^{-/-}$ background, spontaneously developed colorectal carcinomas carrying nuclear β-catenin accumulation and p53 mutations at 6 months of age. However, interferon (IFN)γ$^{-/-}$SOCS1$^{-/-}$ mice and SOCS1$^{-/-}$Tg mice treated with anti-IFNγ antibody did not develop such tumors. STAT3 and NF-κB activation was evident in SOCS1$^{-/-}$Tg mice, but these were not sufficient for tumor development because these are also activated in IFNγ$^{-/-}$SOCS1$^{-/-}$ mice. However, colons of SOCS1$^{-/-}$Tg mice, but not IFNγ$^{-/-}$SOCS1$^{-/-}$ mice, showed hyperactivation of STAT1, which resulted in the induction of carcinogenesis-related enzymes, cyclooxygenase-2 and inducible nitric oxide synthase. These data strongly suggest that SOCS1 is a unique antioncogene which prevents chronic inflammation-mediated carcinogenesis by regulation of the IFNγ/STAT1 pathways.

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Inflammatory bowel diseases (IBDs), such as ulcerative colitis (UC) and Crohn's disease, are well known to increase the risk of developing colorectal cancer. Indeed, IBDs rank among the top three high-risk conditions for colorectal cancer, together with familial adenomatous polyposis and hereditary nonpolyposis colorectal cancer (1). Epidemiological studies have indicated that regular administration of nonsteroidal antiinflammatory drugs lowers mortality from sporadic colorectal cancer and causes regression of adenomas in patients with familial adenomatous polyposis (2). Recently, the NF-κB pathway is shown to be one of the key molecular mechanisms for developing inflammation-related cancer (3, 4). The role of other proinflammatory signal pathways remains unknown.

The JAK/STAT pathway is another major signaling pathway for modulating pro- and antiinflammatory responses. It is also closely correlated with IBDs, since UC and Crohn's disease are associated with a predominance of IFNγ-producing T helper (Th)1 cells and IL-4 producing Th2 cells, respectively (5). Suppressor of cytokine signaling-1 (SOCS1) is an intracellular protein that inhibits JAK-mediated cytokine signaling by binding to JAKs (6). SOCS1 has been shown to be an important physiological negative regulator of various cytokines including IFNγ and IL-4. SOCS1 also modulates toll-like receptor (TLR) signaling in macrophages (7). SOCS1-deficient mice (SOCS1$^{-/-}$) die neonatally because of multiorgan inflammation (6). We also

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reported that SOCS1$^{-/-}$TCR$\alpha^{-/-}$ mice develop very severe colitis within 9 wk of age which resembles human UC (8). Development of this colitis was dependent on both IFNγ and IL-4. Thus, SOCS1 is an important negative regulator of inflammation by limiting cytokine and TLR signaling.

SOCS1 has also been suggested to function as an antioncogene. Mutations and deletions of the *SOCS1* gene have been found in several lymphomas (9). Yoshikawa et al. reported that aberrant methylation in the CpG island of SOCS1 was correlated with transcriptional silencing of the *SOCS1* gene in hepatocellular carcinoma (10). Moreover, restoration of SOCS1 suppressed both the growth rate and the anchorage-independent growth of the cells in which SOCS1 was methylation silenced. In addition, *SOCS1* methylation has also been reported in various types of human cancers, including colorectal cancer (11, 12). Experimentally, Rottapel et al. and our group showed that SOCS1-deficient fibroblasts were more sensitive to both spontaneous and oncogenes (v-ABL, p210 BCR-ABL, 70Z/3 CBL, and papilloma virus E7)-induced transformation than wild-type fibroblasts (13, 14). Furthermore, we demonstrated that carcinogen-induced hepatocellular carcinoma development was enhanced in SOCS1$^{+/-}$ mice, indicating that SOCS1 functions as an antioncogene in vivo (15). Interestingly, we found that *SOCS1* gene silencing by DNA methylation is frequently observed in the pretransformed liver infected with human hepatitis C virus (15). *SOCS1* gene methylation was well correlated with the severity of liver fibrosis, suggesting that reduction of *SOCS1* gene expression promotes liver inflammation. These findings suggest that SOCS1 is a unique antioncogne that prevents inflammation-associated carcinogenesis. However, the precise molecular function of SOCS1 in cancer development is unknown.

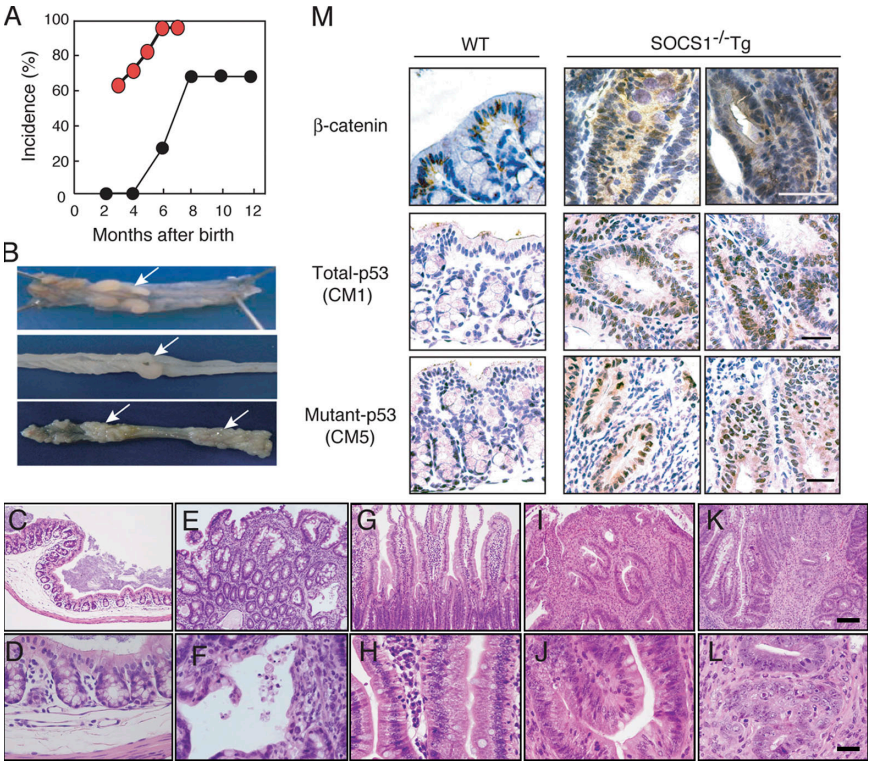

Figure 1. Colorectal tumors in SOCS1$^{-/-}$Tg mice. (A) Percentage of histologically determined colitis (red circle) and tumor (black circle) incidence in SOCS1$^{-/-}$Tg mice. (B) Macroscopic view of colon tumors in SOCS1$^{-/-}$Tg mice. Arrows indicate tumors. (C–L) HE-stained sections of colitis and grades of dysplasia and neoplasia in SOCS1$^{-/-}$Tg mice. The top and bottom panels in each row show medium- and high-magnification views of the mucosa, respectively. (C and D) Histology of a wild-type control mouse. (E and F) Colitis, indefinite for dysplasia in SOCS1$^{-/-}$Tg mice. (G and H) Mildly active cryptitis with neutrophilic infiltration and goblet cell depletion are evident. The nuclei are mildly swollen but uniform in size and shape. Epithelial maturation toward the surface is preserved. (G and H) Low-grade dysplasia with villous configuration in SOCS1$^{-/-}$Tg mice. The crypts are uniformly lined with tall epithelial cells containing mildly elongated and hyperchromatic nuclei. (I and J) Colitis with high-grade dysplasia in SOCS1$^{-/-}$Tg mice. Eroded and inflamed mucosa with cryptoabscesses can be seen. In addition, the tubuli show an irregular arrangement and budding. The nuclei are elongated, hyperchromatic, and pseudostratified. (K and L) High-grade dysplasia and intramucosal carcinoma in SOCS1$^{-/-}$Tg mice. The desmoplastic stroma has assumed early invasive growth. Bars: (C, E, G, I, K) 200 μm; (D, F, H, J, I) 50 μm. (M) Immunohistochemical staining for β-catenin, total p53 (CM1), and mutant p53 (CM5) in colon tumors from SOCS1$^{-/-}$Tg mice and WT littermates. Bars, 50 μm.

RESULTS AND DISCUSSION

SOCS1$^{-/-}$Tg mice spontaneously develop colon cancer

SOCS1$^{-/-}$Tg mice, in which exogenous SOCS1 is only expressed in T and B cells, survived for more than 1 yr (16). However, typical colitis, including hyperplasia of the crypt epithelium, the loss of goblet cells, crypt abscess formation, and mixed inflammatory cellular infiltration in the lamina propria mucosa, were observed in SOCS1$^{-/-}$Tg mice after 3 mo of age (Fig. 1 A). In addition, we discovered frequent development of colon tumors in SOCS1$^{-/-}$Tg mice after 6 mo of age. The frequency of colon tumors in these mice increased as the mice became older (Fig. 1 A). Most of the tumors in SOCS1$^{-/-}$Tg mice occurred in the proximal parts of the colon, similar to human colitis-associated colorectal cancers (1) (Fig. 1 B). Histologically, these colon tumors were developed from dysplastic epitherial cells at inflammation sites (Fig.1, C–L). Regenerative mucosa and low- to moderate-grade dysplasia, which are found at high frequency in human UC, were also detected in the colon of SOCS1$^{-/-}$Tg mice (Fig. 1, E–J). β-catenin gene mutations and accumulation of this protein in the nucleus are very important events in colorectal carcinogenesis (17). As expected, strong β-catenin expression was seen in the nucleus and cytoplasm of adenocarcinoma cells in the immunohistochemical staining (Fig. 1 M). Furthermore, p53 staining using two rabbit polyclonal antibodies (CM1 specific for both wild-type and mutant p53 proteins and CM5 specific for the mutant p53 protein) revealed the accumulation of mutant p53 proteins in the nuclei of tumor cells (Fig. 1 M). These results suggest that SOCS1 deficiency is related to colon tumor initiation nd/or promotion.

Colitis and colon tumor development is dependent on IFNγ but not TNFα in SOCS1$^{-/-}$Tg mice

As shown in Fig. 1 A, colitis was ahead of the development of colon tumors. Although IFNγ$^{-/-}$SOCS1$^{-/-}$ mice survived for more than 1 yr, they did not develop strong colitis and any colon tumors, suggesting that IFNγ plays an essential role in tumorigenesis. Therefore, we examined the effect of the depletion of IFNγ by anti-IFNγ antibody treatment. We also compared the effect of anti-TNFα antibody because TNFα has been suggested to play an important role in hepatocellular carcinoma developed in Mdr2-deficient mice (4). As shown in Fig. 2 (A and B), anti-IFNγ antibody, but not anti-TNFα antibody, blocked colitis as well as colon tumor development

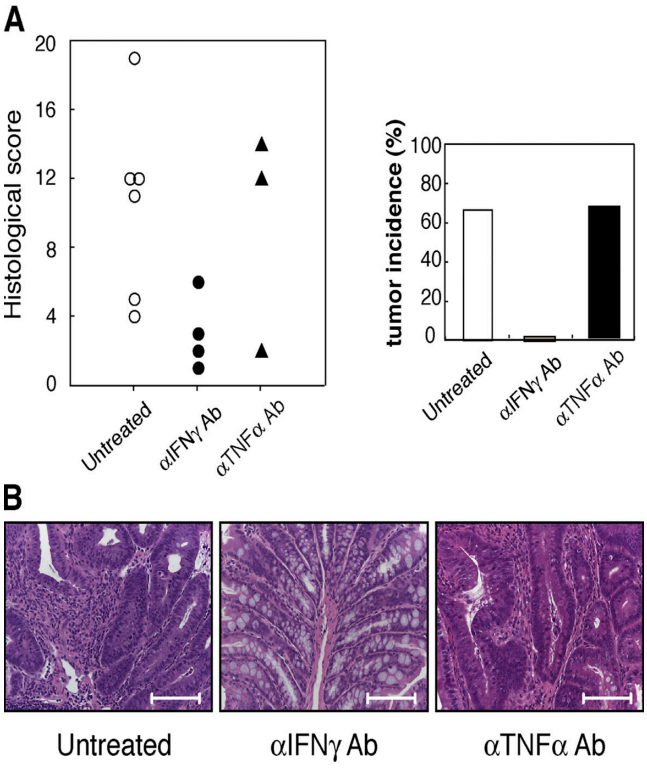

Figure 2. Anti-IFNγ mAb but not anti-TNFα mAb treatment ameliorated the colitis and colon tumor development in SOCS1$^{-/-}$Tg mice. SOCS1$^{-/-}$Tg mice at 2 mo of age were treated either with control IgG, anti-IFNγ, or anti-TNFα mAb for 4 mo. The colitis score (A) and tumor incidence (B) are shown. (C) The representative HE staining of the colonic sections from the SOCS1$^{-/-}$Tg mice after antibody treatment. Bars, 100 μm.

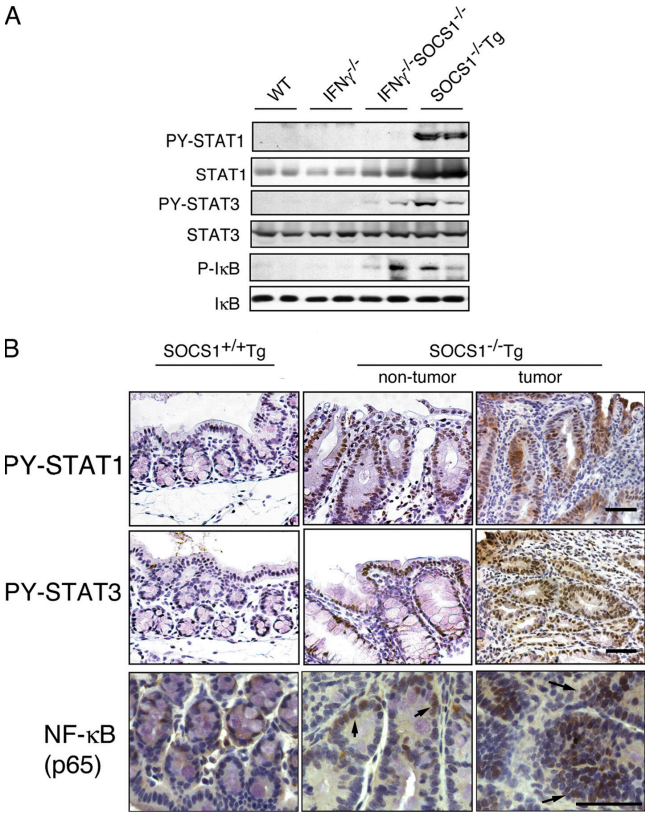

Figure 3. Activation of STAT1, STAT3, and NF-κB in SOCS1$^{-/-}$Tg colons. (A) Western blot analysis of whole colon samples from indicated mice were performed with the indicated antibodies. The data are representative of three independent experiments each experiment using two mice per group. (B) Immunostaining for phospho-STAT1, phospho-STAT3, and NF-κB (p65) in the colons of SOCS1$^{+/+}$Tg and SOCS1$^{-/-}$Tg mice. Arrows indicate nuclear accumulation of p65. Bars, 50 μm.

in SOCS1$^{-/-}$Tg mice. Failure of the suppression of colitis by anti-TNFα antibody was also observed in SOCS1$^{-/-}$TCRα$^{-/-}$ mice (8), suggesting that colitis developed by SOCS1 deficiency does not depend on TNFα. Colitis found in SOCS1$^{-/-}$Tg mice resembled that of SOCS1$^{-/-}$TCRα$^{-/-}$ mice (8), but much less severe than that of SOCS1$^{-/-}$TCRα$^{-/-}$ mice, and all SOCS1$^{-/-}$TCRα$^{-/-}$ mice died by 20 wk of age. Thus, chronic inflammation for certain period seems to be necessary for the development of tumors.

Hyperactivation of STAT1, STAT3, and NF-κB in colons of SOCS1-deficeint mice

It has been demonstrated that transcription factors STAT1, STAT3, and NF-κB were activated in the colon of human IBD and in mouse colitis models. Therefore, we examined the activation status of these transcription factors in SOCS1$^{-/-}$Tg mice by Western blotting (Fig. 3 A). NF-κB activation was assessed by the phosphorylation of inhibitor of NF-κB (IκB). Low levels of activation of STAT1, STAT3, and NF-κB were detectable in WT and IFNγ$^{-/-}$ mice. On the other hand, constitutive STAT3 activation and IκB phosphorylation was seen in the colons of both SOCS1$^{-/-}$Tg and IFNγ$^{-/-}$SOCS1$^{-/-}$ mice (Fig. 3 A). These data suggest that SOCS1 may regulate STAT3 and NF-κB activations in colon cells even in the absence of IFNγ. However, strong STAT1 activation was observed in the colon of SOCS1$^{-/-}$Tg mice, but not in IFNγ$^{-/-}$SOCS1$^{-/-}$ mice. Thus, constitutive activation of STAT1 signaling appears to depend on IFNγ and to be necessary for tumor development.

Next, we examined in which cells these transcription factors are activated by the immunohistochemical staining (Fig. 3 B). Immunoreactivities for phosphorylated STAT1 and STAT3 were found in the nuclei of both normal epithelial cells and tumor cells in SOCS1$^{-/-}$Tg colon. Low levels

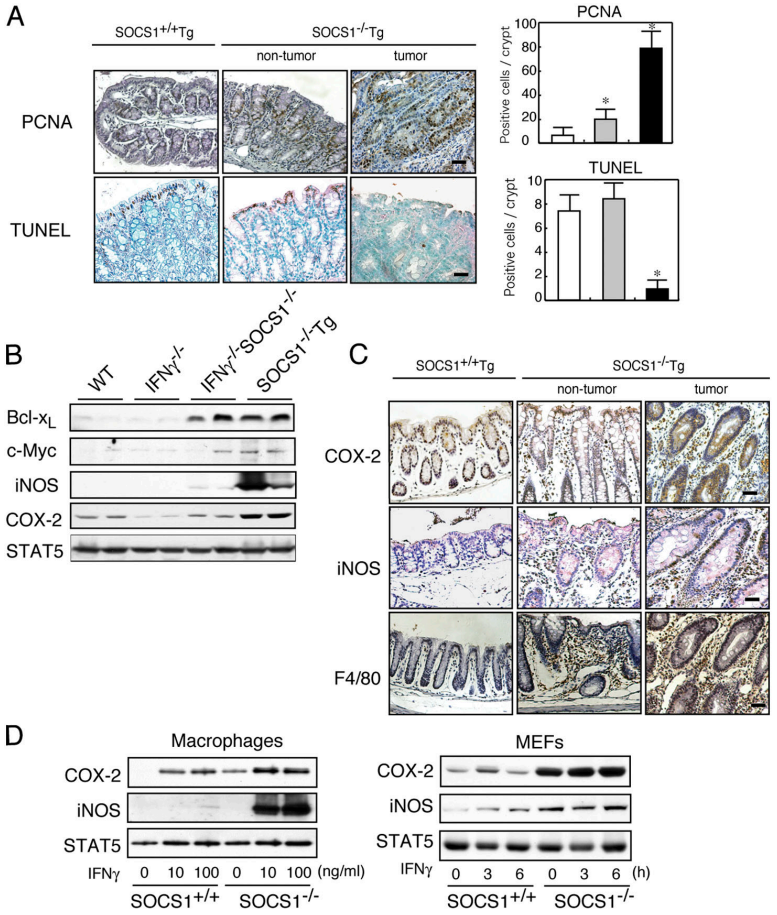

Figure 4. Expression of tumorigenic factors in SOCS1-deficient colons. (A) Immunostaining for PCNA and TUNEL staining in the colons of SOCS1$^{+/+}$Tg and SOCS1$^{-/-}$Tg mice at 6 mo of age. Bars, 50 μm. The average numbers of PCNA-positive cells and TUNEL-positive cells per one side of the colonic crypts are indicated in the bar graph. Error bars represent ± SE. *, $P < 0.05$ compared with SOCS1$^{+/+}$Tg mice. White bars, SOCS1$^{+/+}$Tg; gray bars, SOCS1$^{-/-}$Tg nontumor; black bars, SOCS1$^{-/-}$Tg tumor. (B) Western blot analysis of the Bcl-x_L, c-Myc, iNOS, COX-2, and STAT5 levels in whole colonic extracts from indicated mice. (C) Immunostaining for iNOS, COX-2, and F4/80. Bars, 50 μm. (D) Western blot analysis of the COX-2 and iNOS expression in response to IFNγ in the macrophages from SOCS1$^{+/+}$Tg and SOCS1$^{-/-}$Tg mice and MEFs from WT and SOCS1$^{-/-}$ mice.

of STAT activation were detected in infiltrated mononuclear cells. Nuclear accumulation of p65 subunit of NF-κB was observed in epitherial cells in nontumor regions and in tumor cells in SOCS1$^{-/-}$Tg mice but not in WT mice (Fig. 3 B). High levels of extracellular signal-regulated kinase activation were observed in both normal and tumor epitherial cells, but not in mononuclar cells in SOCS1$^{-/-}$Tg mice (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20060436/DC1).

To define the molecular basis of STAT3 and NF-κB activation in SOCS1-deficient colons, we analyzed the mRNA expression levels of IL-1β, IL-6, and TNFα. Although IL-1β and IL-6 were not elevated in SOCS1$^{-/-}$ colons, TNFα expression was significantly higher ($P < 0.05$) in both IFNγ$^{-/-}$SOCS1$^{-/-}$ and SOCS1$^{-/-}$Tg mice than in their control IFNγ$^{-/-}$ and SOCS1$^{+/+}$Tg mice, respectively (Figs. S1 and S2, available at http://www.jem.org/cgi/content/full/jem.20060436/DC1). TNFα expression was observed in infiltrated mononuclear cells in both tumor and nontumor regions in SOCS1$^{-/-}$Tg mice (Fig. S1). This may account for constitutive activation of NF-κB signaling observed in SOCS1$^{-/-}$Tg colons (Fig. 3, A and B). However, because IL-6 was not up-regulated in SOCS1-deficient colons, the cytokines responsible for the STAT3 hyperactivation remain unclear.

Factors that are involved in tumorigenesis in SOCS1-deficient colons

To address the proliferative states of the colonic crypts of SOCS1$^{+/+}$Tg and SOCS1$^{-/-}$Tg mice, we performed PCNA staining in these mice at 6 mo of age. The numbers of proliferating cells stained with anti-PCNA antibody were increased not only in the tumor areas but also in nontumor areas of SOCS1$^{-/-}$Tg mice compared with SOCS1$^{+/+}$Tg mice (Fig. 4 A). These data demonstrate that proliferation of the intestinal epithelium are augmented in SOCS1$^{-/-}$Tg mice, even in nontumor areas. To examine the state of epithelial apoptosis, the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed. TUNEL-positive cells were seen in the surface epithelium of the colon in both SOCS1$^{+/+}$Tg and SOCS1$^{-/-}$Tg mice, and there was no substantial difference in the number of apoptotic cells between these mice. On the other hand, TUNEL-positive cells were significantly decreased ($P < 0.05$) in the tumor areas of the colon in SOCS1$^{-/-}$Tg mice (Fig. 4 A). These results indicate that the apoptotic balance is disrupted at sites showing tumorigenic transformation of the intestinal epithelium in SOCS1$^{-/-}$Tg mice, but normal in nontumor areas.

Then, we investigated the target genes of activated STATs and NF-κB. STAT3 has been proposed to participate in oncogenesis through up-regulation of genes such as apoptosis inhibitors (Bcl-x_L, Mcl) and cell cycle regulators (c-Myc, cyclins D1/D2) (18). Greten et al. also demonstrated that IKKβ acts by suppressing the mitochondrial apoptosis pathway through induction of the NF-κB target gene Bcl-x_L in a DSS/AOM colon cancer model (3). Up-regulation of Bcl-x_L and cell cycle regulators during tumor promotion appears to be important in colorectal cancer development (19). As expected, the expression levels of Bcl-x_L and c-Myc were up-regulated in SOCS1$^{-/-}$Tg mice (Fig. 4 B).

However, regardless of the up-regulations of Bcl-x_L and c-Myc (Fig. 4 B), IFNγ$^{-/-}$SOCS1$^{-/-}$ mice did not develop any colon tumors, suggesting that IFNγ/STAT1-specific molecules may be essential for tumor initiation. We examined inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, which are induced by IFNγ (20, 21) are thought to be crucial for inflammation-mediated colon carcinogenesis (22, 23). The expression levels of iNOS and COX-2 were considerably higher in SOCS1$^{-/-}$Tg colons than in IFNγ$^{-/-}$SOCS1$^{-/-}$ colons, and these levels were in parallel with the STAT1 phosphorylation (Fig. 4, B and C). Immunohistochemical staining revealed that COX-2 was strongly expressed in macrophages in nontumor areas of SOCS1$^{-/-}$Tg mice compared with SOCS1$^{+/+}$Tg mice (Fig. 4 C). In the tumor areas of SOCS1$^{-/-}$Tg mice, COX-2 was detected in both tumor cells and macrophages. iNOS was also strongly detected in macrophages in both nontumor and tumor areas, but weakly detected in tumor cells (Fig. 4 C). Therefore, activated macrophages may be the main producer of iNOS in the colon of SOCS1$^{-/-}$Tg mice. The numbers of macrophages infiltrated the colon were much higher in SOCS1$^{-/-}$Tg mice than in SOCS1$^{+/+}$Tg mice (Fig. 4 C). These aberrantly activated and increased macrophages may have an important progressive role for the colon tumors in SOCS1$^{-/-}$Tg mice.

We then investigated iNOS and COX-2 expression in response to IFNγ in peritoneal macrophages and mouse embryonic fibroblasts (MEFs). As expected, SOCS1-deficient macrophages and MEFs produced COX-2 and iNOS more extensively than SOCS1$^{+/+}$ cells (Fig. 4 D). These data demonstrate the importance of SOCS1 in IFNγ signal regulation not only for epithelial cells but also for the stromal cells, including macrophages and fibroblasts in tumor formation.

In this study, we have shown that SOCS1 is one of the candidate tumor suppressor genes for inflammation-associated colon cancer. SOCS1 deficiency enhanced STAT3, NF-κB, and STAT1 activations which induced apoptosis inhibitors, cell cycle regulators, COX-2, and iNOS (Fig. 5). COX-2 expression in tumor-infiltrating macrophages is an early event in colon carcinogenesis, and inhibition of COX-2 activity represents an effective chemopreventive strategy (24). In addition, there is a positive correlation between iNOS activity and G:C to A:T mutations at 5-methylcytosine sites in *p53* gene in human colon tumors (25). Thus, high expression of iNOS may be one reason for high frequency of p53 mutation in SOCS1$^{-/-}$Tg colon tumors. The importance of altered p53 expression in the development of colitis-related colonic neoplasms in human has been reported (26, 27). The features of the colon cancers in SOCS1$^{-/-}$Tg mice are similar to those of human colitis-associated colon cancer. These unique features of SOCS1$^{-/-}$Tg mice will provide

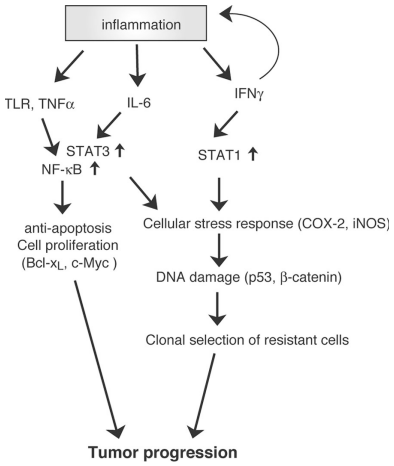

Figure 5. A model for tumor progression caused by proinflammatory cytokines. Aberrantly activated STAT3 and NF-κB signals induce cell proliferation and antiapoptotic factors. Aberrantly activated STAT1 signal induces cellular stress responses and leads to DNA damage, resulting in clonal selection of resistant cells. These signals orchestrate tumor formation in the colon.

novel insights into the pathogenesis of inflammation-associated cancers.

We believe that macrophage activation is important for the development of colitis and tumor. Colitis in SOCS1-deficeint mice was dependent on intestinal flora because we observed almost no colitis in SOCS1$^{-/-}$TCRα$^{-/-}$ mice when antibiotics were included in the drinking water (unpublished data). Therefore, macrophage activation by both TLR and IFNγ must play an important role in the tumor development. This also explains why tumorigenesis is restricted in the colon of SOCS1$^{-/-}$Tg mice.

MATERIALS AND METHODS

Mice. SOCS1$^{-/-}$, IFNγ$^{-/-}$SOCS1$^{-/-}$, and SOCS1$^{-/-}$Tg mice were described previously (15). All experiments using these mice were approved by and performed according to the guidelines of the animal ethics committee of Kyushu University, Fukuoka, Japan.

Histopathological and immunohistochemical studies. Colons were isolated and opened longitudinally to inspect for mucosal tumors. Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Sections (5-μm-thick) were cut and stained with hematoxylin and eosin (HE). The severity of colitis was determined by the histological scoring system as described previously (8). For immunohistochemistry, paraffin-embedded sections were dehydrated and then microwaved in 10 mM citrate buffer (pH 6.0) twice for 5 min each. Then, the sections were incubated with the following antibodies: anti–β-catenin (clone 14; BD Transduction Laboratories; 1:200 dilution), anti-p53 (CM-1 and CM-5; Novocastra Laboratories, Ltd.; 1:1,000 dilution), anti–phospho-STAT1 (Tyr701; Cell Signaling; 1:100 dilution), anti–phospho-STAT3 (Tyr705; Cell Signaling; 1:100 dilution), anti–COX-2 (Cayman Chemical; 1:200 dilution), anti-iNOS (Santa Cruz Biotechnology, Inc.; 1:50 dilution), anti-TNFα (MP6-XT22; 1:100 dilution), anti-PCNA (PC10; DakoCytomation; 1:100 dilution), and anti-F4/80 (CI:A3-1; Serotec; 1:50 dilution). ENVISION+ System-HRP (DakoCytomation) was used for detection. All sections were counterstained with hematoxylin. TUNEL staining was performed using an ApoTag Peroxidase In Situ Apoptosis Detection kit (CHEMICON) according to the manufacturer's instructions.

In vivo monoclonal antibody treatment. For in vivo mAb treatment, rat anti–mouse TNFα mAb (MP6-XT22) and anti–IFNγ mAb (R4.6A2) were used (8). Rat IgG (Zymed Laboratories) was used as the control antibody. The mice were intraperitoneally injected with anti-IFNγ mAb or anti-TNFα mAb or the control antibody (200 μg/mouse) twice a week from the beginning of 2 to 6 mo of age. The mice were killed and then were examined to determine the severity of colitis and tumor development.

Statistical analysis. For statistical analysis, we used Student's *t* test. A 95% confidence limit was taken to be significant and defined as $P < 0.05$.

Online supplemental material. Fig. S1 shows inflammatory cytokine levels determined by real time RT-PCR. Fig. S2 shows localization of TNFα and pERK. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20060436/DC1.

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