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Essential Roles of IL-6 *Trans*-Signaling in Colonic Epithelial Cells, Induced by the IL-6/Soluble–IL-6 Receptor Derived from Lamina Propria Macrophages, on the Development of Colitis-Associated Premalignant Cancer in a Murine Model

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Activation of the IL-6/Stat3 via IL-6 *trans*-signaling plays an important role in the pathogenesis of inflammatory bowel disease. Colitis-associated cancer (CAC) is a large bowel cancer and occurs with long-standing inflammatory bowel disease. The role of the IL-6/Stat3 in the development of CAC has not been fully understood. We investigate whether IL-6 *trans*-signaling contributes to the development of CAC using a mouse colitis-associated premalignant cancer (CApC) model. Chronic colitis (CC) was induced in BALB/c mice using dextran sodium sulfate. CApC was induced by dextran sodium sulfate treatment to CC-affected mice. IL-6 expression was determined by quantitative RT-PCR and immunofluorescence staining in colon. Phospho-Stat3 expression was examined by Western blotting and immunofluorescence analysis. The expression of IL-6 receptors (i.e., the IL-6R α-chain and gp130) and tumor necrosis factor-α converting enzyme in the colon was examined by laser-capture microdissection and immunofluorescence staining. Soluble IL-6Rα (sIL-6Rα) was examined by Western blotting of epithelial cell-depleted colonic tissues. We also investigated whether a soluble gp130-Fc fusion protein could prevent CApC. IL-6 expression was increased in the colon of CC- and CApC-affected mice and was restricted to lamina propria-macrophages. The expression of IL-6Rα and tumor necrosis factor-α converting enzyme was increased in the lamina propria CD11b-macrophages of CC-affected mice. sIL-6Rα expression was also increased in these tissues. Reduced levels of IL-6Rα generation were observed in the colonic epithelial cells of CC- and CApC-affected mice and were associated with the increased expression of gp130 and phospho-Stat3. Treatment with soluble gp130Fc significantly reduced the CApC. IL-6 *trans*-signaling in epithelial cells induced by macrophage-derived IL-6/sIL-6Rα plays a crucial role in the development of CAC. *The Journal of Immunology*, 2010, 184: 1543–1551.

The inflammatory cytokine IL-6 has multiple functions (1, 2). It exerts its biological action by binding to two types of membrane receptors, namely IL-6Rα and the gp130 molecule (3, 4). IL-6 binds to IL-6Rα on the cell membrane of target cells and this complex in turn associates with gp130 and induces signal transduction via phosphorylation of Stat3. IL-6Rα is expressed by specific cells, such as neutrophils, monocytes/macrophages, hepatocytes, and in certain lymphocyte phenotypes, whereas gp130 is widely expressed on the cell membrane of various cell types (5, 6). There is an increase in the serum levels of soluble IL-6Rα (sIL-6Rα) during inflammation (7). sIL-6Rα is produced by the proteolytic cleavage of membrane IL-6Rα or by the differential splicing of IL-6Rα mRNA (8). sIL-6Rα also binds to IL-6, forming the IL-6/sIL-6Rα complex that can interact with membrane gp130 and induce IL-6 signal transduction, termed IL-6 *trans*-signaling (9, 10). The importance of this signaling pathway in conditions with chronic inflammation, such as asthma, experimental colitis, and rheumatoid arthritis, has been well documented (11–13). We have previously reported that IL-6/Stat3 signaling via IL-6 *trans*-signaling plays a crucial role in the development of ileitis in SAMP1/Yit mice and that neutralization of this signaling pathway helps to prevent ileitis (14, 15).

Colorectal cancer is one of the most common fatal malignancies in the world. Recent studies have suggested a clear relationship between the onset of malignancy and inflammation (16, 17). Inflammatory bowel disease is known to be associated with a high risk of developing colon cancer (18). A model of azoxymethane (AOM)-induced colon cancer indicated that IL-6 *trans*-signaling is important for the development of colon cancer (19). However, there are many unresolved issues regarding the mechanism by which IL-6 *trans*-signaling modulates tumor development in colitis-associated cancer (CAC), which is associated with colonic inflammation. In this study, by using a murine model of colitis-associated premalignant cancer (CApC), we elucidated the critical roles played by IL-6/IL-6Rα derived from lamina propria mononuclear cells (LPMCs), in IL-6 *trans*-signaling in colonic epithelial cells during the development of CApC. We also characterized the involvement of the *trans*-signaling pathway in the development of CApC and examined whether specific inhibition of the *trans*-signaling pathway could the prevent CAC.

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Abbreviations used in this paper: AID, activation-induced cytidine deaminase; AOM, azoxymethane; CAC, colitis-associated cancer; CApC, colitis-associated premalignant cancer; CC, chronic colitis; COX-II, cyclooxygenase II; DSS, dextran sodium sulfate; FCM, flow cytometric; iNOS, inducible NO synthase; LMD, laser-capture microdissection; LP, lamina propria; LPMC, lamina propria mononuclear cells; RT-PCR, real-time PCR; sIL-6Rα, soluble IL-6Rα; TACE, tumor necrosis factor-α converting enzyme.

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Materials and Methods

Mice

Six-week-old female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan).

Induction of CApC

CApC was induced in BALB/c mice ($n = 20$) by the method described by Okayasu et al. with minor a modification (20). In brief, the mice were subjected to nine cycles of treatment with 4–5% dextran sodium sulfate (DSS; MP Biomedicals, Illkirch, France) in drinking water for 7 d and with normal drinking water for 7 d. The mice were sacrificed 3 wk after the final administration cycle, and their tissues were examined under a stereomicroscope to determine whether CApC had been induced. The CApC tissues were fixed and stained with H&E and observed under a microscope.

Immunohistochemistry

Frozen sections were prepared from normal mice and those exhibiting chronic colitis (CC) and CApC. The sections were stained with mAbs or polyclonal Abs raised against CD4 (BD Biosciences Immunocytometry System, San Jose, CA), CD11b (BD Bioscience), IL-6 (BioLegend, San Diego, CA), IL-6Rα (R&D Systems, Minneapolis, MN), gp130 (R&D Systems), inducible NO synthase (iNOS; Santa Cruz Biotechnology, Santa Cruz, CA), cyclooxygenase II (COX-II; Santa Cruz), tumor necrosis factor-α converting enzyme (TACE; Santa Cruz), activation-induced cytidine deaminase (AID; ProSci, Poway, CA), Ki-67 (DakoCytomation, Glostrup, Denmark), or p53 (Vision BioSystem, Newcastle, U.K.). Nuclear staining was performed using TO-PRO-3 iodide (Invitrogen, Carlsbad, CA), and the sections were observed under a confocal laser microscope (LSM-500; Carl Zeiss, Oberkochen, Germany).

Western blotting analysis

Colonic tissues were homogenized using lysis buffer (15). The homogenate was centrifuged, and the supernatant was subjected to Western blotting by using anti–phospho-Stat1, anti–phospho-Stat3, anti–phospho-SHP-2, anti–phospho-NFκB, anti–phospho-p38MAPK, anti–β-actin, anti-Stat3 (all obtained from Cell Signaling Technology, Danvers, MA), or anti-TACE (Santa Cruz) Abs. Chemiluminescence was visualized by using an ECL Plus kit (GE Healthcare, Buckinghamshire, U.K.). The images were analyzed by using an LAS-3000 imaging system (Fujifilm, Tokyo, Japan). To analyze the expression of the sIL-6R in the colonic mucosa, colonic epithelial cells were removed from the tissue samples by incubating the latter with HBSS containing 2 mM EDTA. The tissue samples were then subjected to Western blotting by using an anti–IL-6Rα Ab (Santa Cruz), and chemiluminescence was visualized. sIL-6Rα was detected as a protein of ~50 kDa (21).

Flow cytometry

Colonic LPMCs were prepared as described previously (14). LPMCs were treated with FITC-CD4/PE-IL-6Rα/PE-Cy5-gp130 or FITC-F4/80/PE-IL-6Rα/PE-Cy5-gp130, and flow cytometric (FCM) analysis was performed using the Epics Altra system (Beckmann-Coulter, Fullerton, CA). For the analysis of active TACE expression on LPMCs, LPMCs that were isolated from normal and CC-affected mice were treated with an FITC-F4/80/anti-TACE Abs and then incubated with PE-conjugated anti-rabbit IgG.

Laser-capture microdissection and quantitative real-time RT-PCR

Colonic tissue samples were snap frozen in liquid nitrogen. Cryosections were prepared and quickly air dried. Colonic epithelial tissues on the sections were immediately collected onto AdhesiveCaps (PALM, Microlaser Technologies, Bernried, Germany) by using a laser-capture microdissection (LMD) system (PALM MB-III, Microlaser Technologies). Total RNA was purified from the LMD-isolated epithelial tissues, colonic LPMCs, or whole colonic tissues and purified by using an RNA Micro kit (Qiagen, Hilden, Germany). Quality control analysis of the isolated RNA was performed by using the Bioanalyzer RNA 6000 Pico Assay. In addition, a one-step real-time PCR (RT-PCR) was performed on an ABI-7500 RT-PCR system (Applied Biosystems, Foster City, CA), using specific primers for IL-6, TNF-α, SOCS3, IL-10, gp130, IL-6Rα, TACE, or GAPDH (all obtained from Qiagen). In some experiments, total RNA was isolated from whole colonic tissues by using the TRIzol reagent (Invitrogen). Quantitative RT-PCR was performed using the Perfect Real-Time PCR system (Takara Bio, Shiga, Japan). All expression data were calculated relative to the levels of the *GAPDH* housekeeping gene. To analyze the levels of IL-6 mRNA in the lamina propria (LP) macrophages isolated from the mice with CC or CApC, total RNA was purified from F4/80$^+$ LP macrophages using MACS (purity >90%; Miltenyi Biotec, Gladbath, Germany), that were prepared from CC- and CApC-affected BALB/c mice, and then the levels of IL-6 mRNA were quantified as described above.

Determination of the tissue levels of IL-6 and sIL-6Rα

Colonic tissue homogenates were prepared from normal, CC- or CApC-affected BALB/c mice by using lysis buffer (15). After centrifugation, the supernatant was collected, and the tissue levels of IL-6 and sIL-6Rα were assayed by using ELISA kit from R&D Systems.

sIL-6Rα production in LP macrophage

F4/80$^+$ LP macrophages were isolated using MACS (Miltenyi Biotec). In brief, LPMCs, isolated from CC-affected BALB/c mice, were treated with biotinylated F4/80 (BioLegend). Finally, F4/80$^+$ LP macrophages were purified using streptoavidin microbeads (Miltenyi Biotec). Heat-killed commensal bacteria were prepared by the following method. Cecal contents were prepared from BALB/c mice and suspended in sterile distilled water. The suspensions were centrifuged at $50\times g$ to remove the intestinal debris. The supernatants were centrifuged at $10{,}000\times g$ for 30 min and the pellets were washed twice with ice-cold distilled water. The suspension was boiled at 100°C for 30 min and lyophilized. The lyophilized sample was used as the source of heat-killed commensal bacteria. F4/80$^+$ LP macrophages were stimulated with the heat-killed commensal bacteria (5 μg/ml) in the presence or absence of 2 nM TNF-α processing inhibitor-1 (Biomol International, LP, Plymouth Meeting, PA). After 6, 12, 24, and 48 h culture, the culture supernatants were collected and the amounts of sIL-6Rα were determined with sIL-6Rα specific ELISA (R&D Systems).

Soluble gp130Fc treatment

The mice were i.p. administered soluble gp130Fc (sgp130Fc; 500 or 50 μg/mouse) or a vehicle ($n = 10$ per group) on the first day of each cycle during DSS treatment cycles 6–9, at 14-d intervals. The mice were sacrificed, and the incidence of CApC was compared between the vehicle- and gp130Fc-treated mice. Western blot analysis was performed to detect phosphorylated transcription factors in the colonic tissue samples, according to the method described previously.

Statistical analysis

Each experimental group consisted of 10–20 animals. The data are presented as the mean ± SD. Two-tailed Student t test was used to evaluate the statistical significance. The p values of < 0.05 were considered to be statistically significant. The experiments were repeated 2–4 times.

Results

Characterization of CApC induced by DSS treatment

The development of CApC could be macroscopically observed in >60–80% of mice after the nine DSS treatment cycles (Fig. 1*A*, 1*B*). Histologically, we observed the proliferation of gland epithelial cells, resulting in the formation of a polypoid mass (Fig. 1*C*). The glands had branched and irregular tubules develop, and a marked depletion of goblet cells was observed (Fig. 1*D*). Observation under high-power magnification revealed the nuclei with distinct nucleoli were elongated and stratified (Fig. 1*E*). In several cases (~20% of mice), the cancers consisted of glands that invaded into the muscularis mucosae or into the submucosa (Fig. 1*F*, arrow). The invasive cancers were sometimes associated with the overlying ulceration (data not shown). However, we could not observe any metastasis of a cancer into other organs such as the liver, lung, and brain (data not shown). It should be noted that nuclear expression of p53 was usually induced in the epithelial cells in CApC-affected mice (Fig. 2). Normal BALB/c and CC-affected mice did not express p53 Ag in the nucleus. The p53 Ag was often colocalized with the Ki-67 cell proliferative marker in the nuclei of epithelial cells from CApC-affected mice (Fig. 2, arrow). Therefore, we defined the colonic lesion of our model as the changes associated with the pathway to malignancy, such as nuclear p53, dysplasia, and glandular structures that appear to be invasive into the submucosa were evident. Immunofluorescence analysis indicated the presence of inflammatory infiltrates, CD4$^+$

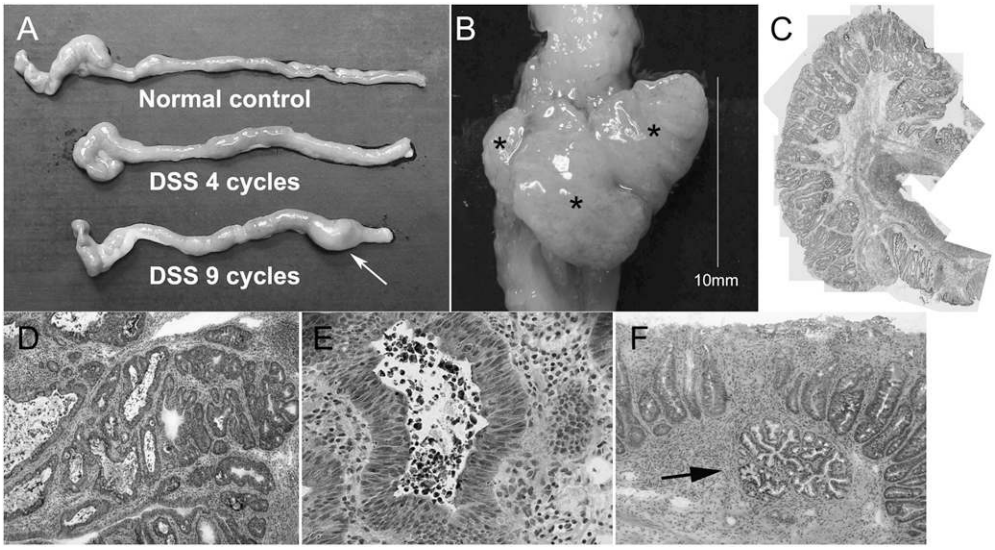

FIGURE 1. *A*, Characterization of a murine model of DSS-induced CApC. Stereomicroscopic observation of CApC in BALB/c mice. The arrow indicates colorectal cancer. *B*, CApC under high-power magnification. The asterisk indicates the large nodular mass in *A*. *C–F*, Histology of CApC. H&E staining, original magnifications ×40 (*C*), ×100 (*D*), ×400 (*E*), and ×40 (*F*).

and CD11b$^+$ cells, in the LP of the mice with CApC as well as those with CC (Fig. 2). It has previously been reported that AID, an enzyme involved in class switching, hypermutation, and tumorigenesis, plays an important role in *Helicobacter pylori*-induced gastric carcinogenesis (22–25). We observed the induction of AID expression in the colonic epithelial cells and LPMCs of the CC- and CApC-affected mice (Fig. 2).

Expression of phosphorylated signal-transduction molecules and cytokine profiles in the mucosa of CApC-affected mice

We previously observed the increased expression of phospho-Stat3 in the colonic mucosa of CC-affected mice when compared with normal control mice (26). In the current study, we compared the expression of phosphorylated signal-transduction molecules in the colonic mucosa of CC- and CApC-affected mice. The expression levels of phospho-Stat3, phospho-SHP2, and phospho-NFκB, but not those of phospho-Stat1 and p38MAPK, were markedly increased in the colonic mucosa of the CApC-affected mice when compared with the CC-affected mice (Fig. 3*A*). We examined the cytokine profiles in the colonic mucosa and observed the prominent expression of IL-6 mRNA in the mucosa of the CApC-affected mice. SOCS3 mRNA expression was also elevated in the mucosa of these mice, and we also observed a weak induction of TNF-α and IL-10 mRNA (Fig. 3*B*). Confocal microscopic analysis revealed that IL-6–positive LPMCs were abundant in the LP and submucosal colonic regions of CApC-affected mice. Double immunofluorescence analysis clearly revealed that almost all IL-6–positive cells coexpressed CD11b (Fig. 3*C*). These IL-6–positive LPMCs were rare in normal control mice and were gradually increased in CC- and CApC-affected mice. Supporting these results, IL-6 was progressively augmented in the tissues of CC- and CApC-affected mice (Fig. 3*D*). However, we observed comparable cellular levels of IL-6 in CD11b$^+$ macrophages in the CApC- and CC-affected mice. There was a marked phosphorylation of Stat3 proteins in the nuclei of colonic epithelial cells in the mucosa of CC- and CApC-affected mice that accompanied the infiltration of IL-6–producing cells (Fig. 4). There was increased phosphorylation of Stat3 proteins in the nuclei of epithelial cells from CApC-affected mice than in CC-affected mice. In addition, the localization of iNOS overlapped with phospho-Stat3 in the epithelial cells and LPMCs. In contrast, COX-II proteins were constitutively expressed in the colonic epithelial cells and LP of normal mice. Interestingly, despite the elevation of SOCS3 mRNA during the development of CApC, the expression of the mature SOCS3 protein decreased in the colonic epithelial cells in the mucosa of CApC-affected mice.

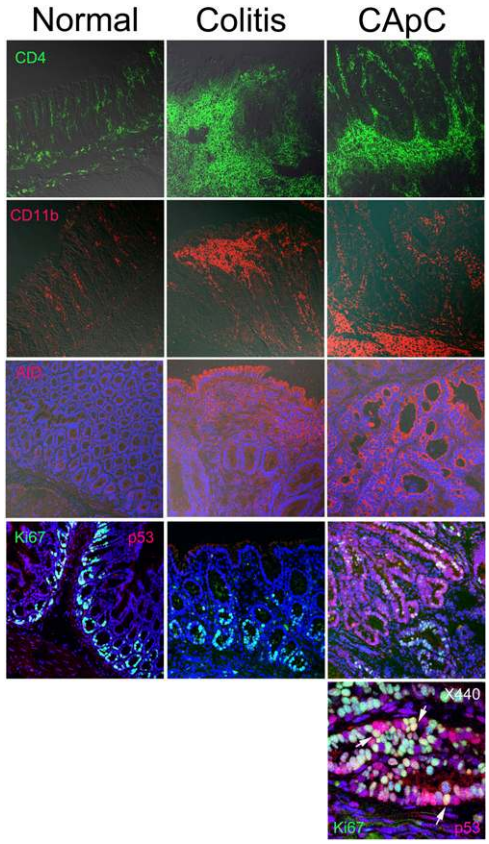

FIGURE 2. Immunofluorescence analysis of CD4, CD11b, AID, Ki-67, and p53 expression during the development of CApC. In AID, Ki-67, and p53 staining, the nuclei were counterstained with TO-PRO-3 dye (blue). The arrows indicate the colocalization of Ki-67 and p53 Ags in the nuclei of cancer epithelial cells. The results of four independent experiments are shown (original magnifications ×100 and ×440).

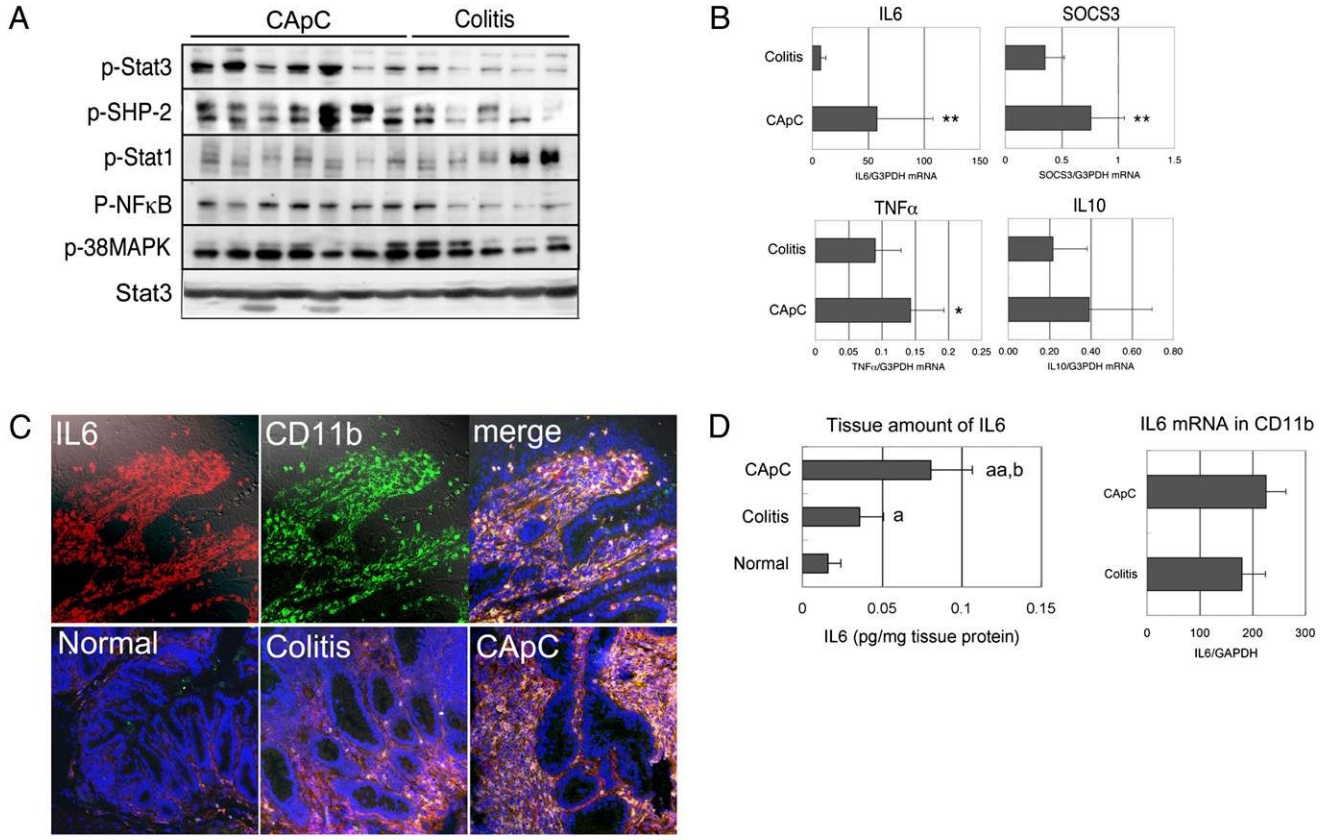

FIGURE 3. *A*, Expression of phosphorylated transcription factors in the mucosa of CApC-affected mice. Colonic tissue homogenates were subjected to Western blotting with polyclonal Abs raised against phospho-Stat3, phospho–SHP-2, phospho-Stat1, phospho-NFκB, phospho-p38MAPK, and Stat3. The images were analyzed with the LAS-3000 image analyzer. *B*, Cytokine mRNA profiles in the CApC tissues. Total RNA was isolated from colon tissues obtained from CC- or CApC-affected mice. Quantitative RT-PCR was performed using specific primer sets. The data are represented as the mean ± SD ($n = 10$). $*p < 0.05$; $**p < 0.01$. The expression data were calculated relative to the levels of the *GAPDH* housekeeping gene. *C*, Immunofluorescence analysis of the IL-6 and CD11b Ags in normal, CC, and CApC tissues. Cryostat sections were incubated with Cy3-labeled mAbs against IL-6 and FITC-labeled mAbs against CD11b (original magnification ×200). *D*, Quantification of tissue and cellular IL-6. The IL-6 levels in mucosal homogenates purified from normal, CC-, and CApC-affected mice were determined using an ELISA specific for IL-6. Quantitative RT-PCR was performed using specific primer sets (IL-6 and GAPDH). $CD11b^+$ LP macrophages were prepared from CC- or CApC-affected mice by MACS. *a*, $p < 0.05$ versus normal; *aa*, $p < 0.01$ versus normal; and *b*, $p < 0.05$ versus colitis. The data are presented as the mean ± SD from three independent experiments.

IL-6Rα and gp130 mRNA expression in the colonic epithelial cells and LPMCs

We quantified the expression levels of IL-6Rα and gp130 mRNA in LMD-isolated colonic epithelial cells and LPMCs from normal, cholitic, and CApC-affected mucosal tissues. Quantitative RT-PCR indicated that the downregulation of IL-6Rα mRNA in the epithelial cells of the colitic and CApC-affected mucosa when compared with the normal mucosa (Fig. 5*A*). The gp130 and TACE mRNA levels were augmented in the colonic epithelial cells in CApC-affected mice. The protein levels of IL-6Rα and gp130 were comparable with their mRNA levels in the colonic epithelial cells. In brief, the expression of IL-6Rα decreased, whereas gp130 levels increased in the colonic epithelial cells during the development of CApC (Fig. 5*B*). In contrast, the mRNA expression levels of these molecules in the LPMCs behave differently. The expression levels of both IL-6Rα and gp130 mRNA increased markedly in the LPMCs of the CC-affected mice (Fig. 6*A*). The expression of TACE mRNA in the LPMCs also increased at this stage. FCM analysis clearly revealed that the expression of the IL-6Rα was markedly augmented in $F4/80^+$ LP macrophages isolated from the mice with ongoing CC (Fig. 6*B*). In the $CD4^+$ LP-T cells, induction of the IL-6Rα was marginal, whereas there was a significant induction of gp130. The expression of the TACE protein was also increased in the LPMCs during the development of CApC, which was consistent with the upregulation of IL-6Rα biosynthesis in LP macrophages. In fact, FCM analysis clearly revealed that TACE was induced on the cell surface of $F4/80^+$ LP macrophages in the CC-affected mice (Fig. 6*B*). We then compared the expression of sIL-6Rα in the colonic mucosa between the normal and CC-affected mice by Western blotting and sIL-6Rα–specific ELISA. The epithelial cell-depleted colonic tissues of normal mice expressed the membrane-bound form of IL-6Rα. In contrast, the tissues from CC-affected mice expressed the soluble form of IL-6Rα (≈50 kDa) as the major components instead of the membrane-bound form (≈80 kDa) (Fig. 6*C*). The results of sIL-6Rα–specific ELISA clearly indicated that there were large mounts of sIL-6Rα in the tissue isolated from mice with ongoing CC when compared with the normal control animals. $F4/80^+$ LP macrophages purified from the mice with ongoing CC actively cleaved sIL-6Rα into the culture supernatant after stimulation with heat-killed commensal bacteria (Fig. 6*D*); however, TACE inhibitor markedly inhibited this cleavage.

Effect of sgp130Fc on the development of CApC

The sgp130Fc is a dimerized fusion protein of sgp130 that specifically suppresses the activation of gp130 via the IL-6/sIL-6Rα complex. Becker et al. reported that sgp130Fc prevented AOM-induced colon carcinogenesis (19). As shown in Fig. 7*A*, the expression levels of phospho-Stat3 proteins in the colonic mucosa were lower in the

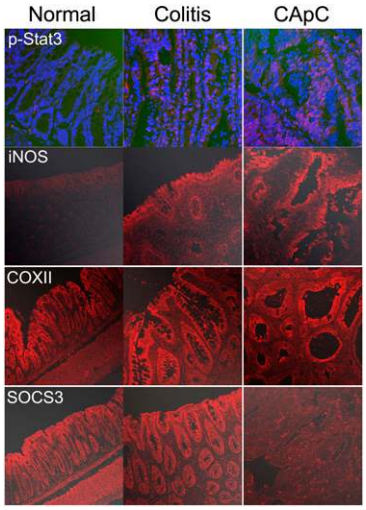

FIGURE 4. Immunofluorescence analysis of phospho-Stat3, iNOS, COX-II, and SOCS3 proteins in the colonic mucosa of normal, CC- and CApC-affected mice. Original magnification ×200 (iNOS, COX-II, and SOCS3) and ×400 (phospho-Stat3). The same results were observed in three independent experiments.

sgp130Fc-treated BALB/c mice than in the vehicle-treated mice. Furthermore, for both doses of sgp130Fc, the incidence and number of tumors were significantly lower in the sgp130Fc-treated mice than in the vehicle-treated mice (Fig. 7*B*). Histologically, we observed mild CC in the sgp130Fc-treated group. We observed that the numbers of Ki-67–positive epithelial cells tended to decrease in the mice treated with sgp130Fc when compared with those treated with vehicle (data not shown). It should be noted that the suppressive effect of sgp130Fc was substantial even in the mice that were treated with the low dose of sgp130Fc.

Discussion

Colonic carcinogenesis represents a model to the connection between chronic inflammation and the etiology of cancer. In this study, we identified the specific roles played by mucosal macrophage-derived IL-6/sIL-6Rα, which induces IL-6 *trans*-signaling in colonic epithelial cells, in the development of inflammation-induced colon cancer. Moreover, by using a specific IL-6 *trans*-signaling inhibitor, we examined whether the inhibition of this signaling pathway is useful for prevention of CAC using a murine model of CApC. We observed that LP macrophage-derived IL-6/sIL-6Rα plays a specific role in the development of CApC. The protein and mRNA expression levels of the membrane-bound form of IL-6Rα were augmented in F4/80$^+$ LP macrophages during the development of CApC. Furthermore, the induction of TACE mRNA in the LPMCs was associated with increased IL-6Rα biosynthesis. Moreover, the tissue expression of sIL-6Rα increased remarkably at this time. In contrast, the protein and mRNA levels of membrane-bound IL-6Rα in the epithelial cells decreased in CC- and CApC-affected mice. Thus, the biosynthesis of membrane-bound IL-6Rα is suppressed in colonic epithelial cells during inflammation-based colon carcinogenesis. In contrast, the protein and mRNA levels of membrane-bound gp130 were selectively augmented in the mucosal epithelial cells of the CApC-affected mice. FCM analysis clearly revealed that the expression of gp130 but not IL-6Rα was augmented in CD4$^+$ lymphocytes. On the basis of these results, we speculate that the IL-6/sIL-6Rα complex derived from LP macrophages targeted the IL-6 *trans*-signaling in the colonic epithelial cells, thus inducing colonic carcinogenesis.

Becker et al. suggested the importance of epithelial cells in the generation of IL-6 *trans*-signaling in AOM-induced colon carcinogenesis (19). In their paper, colonic epithelial cells but not mucosal macrophages in a model of AOM-induced colon carcinoma expressed considerable levels of IL-6Rα mRNA and the augmented expression of the TACE protein. Moreover, the expression of TACE protein on the colonic epithelium was more abundant in Apc$^{Min/+}$ mutants than in control mice (21). Therefore, treatment with mutagenic agents targeting colonic epithelial cells or APC gene mutations in these epithelial cells may induce sIL-6Rα biosynthesis after activation by TACE; this in turn induces IL-6 *trans*-signaling and accelerates colon carcinogenesis. However, the current study revealed that in inflammation-based colon carcinogenesis, the production of IL-6/sIL-6Rα in mucosal macrophages might be more important than

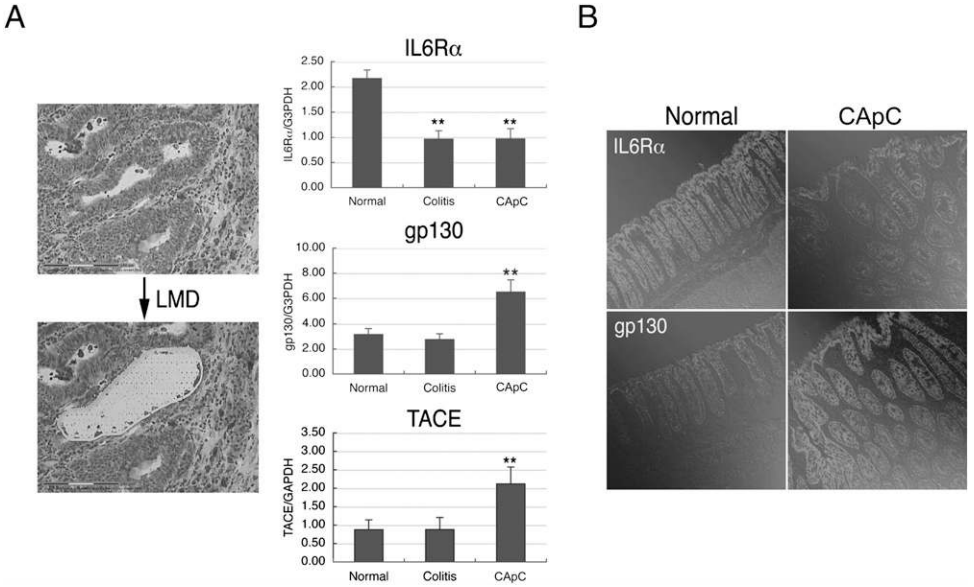

FIGURE 5. *A*, Biosynthesis of IL-6Rα and gp130 in colonic epithelial cells during the development of CApC. Analysis of the mRNA expression levels of IL-6Rα, gp130, and TACE in colonic epithelial cells during the pathogenesis of CApC. Total RNA isolated from colonic epithelial cells using LMD. A one-step RT-PCR was performed using specific primers for IL-6Rα, gp130, TACE, or GAPDH. The data are represented as the mean ± SD (n = 6). Data were calculated relative to the levels of *GAPDH* housekeeping gene. *B*, Immunofluorescence analysis of the IL-6Rα and gp130 proteins in the colonic mucosa of CApC (CAC)-affected mice (original magnification ×200).

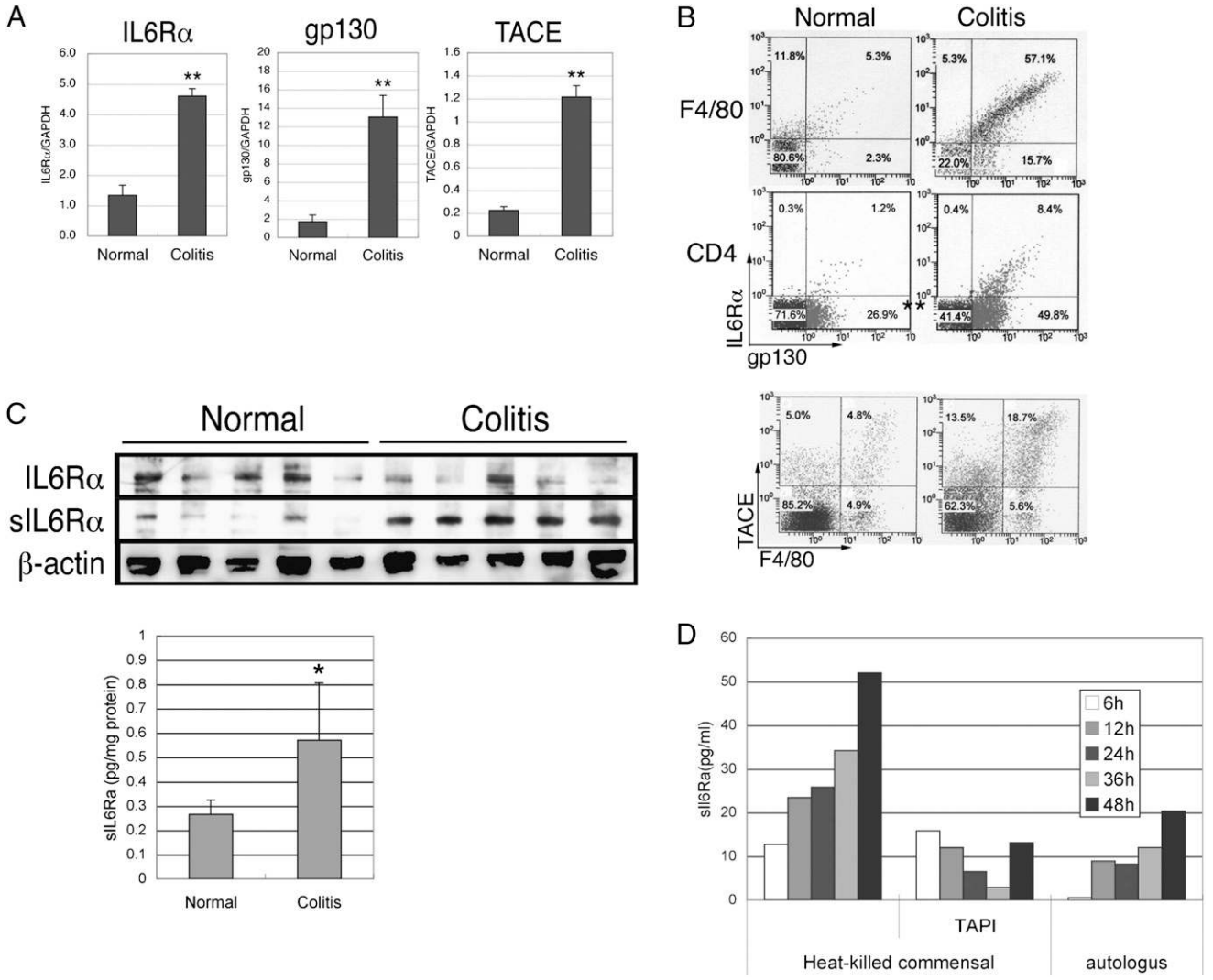

FIGURE 6. *A*, mRNA expression of IL-6Rα, gp130, and TACE in the colonic LPMCs of CC-affected mice. Total RNA was purified from LPMCs using an RNA Micro kit. A one-step RT-PCR was performed using specific primers for IL-6Rα, gp130, TACE, or GAPDH. Data are represented as the mean ± SD ($n = 6$). The expression data were calculated relative to the levels of *GAPDH* housekeeping gene. *B*, FCM analysis of IL-6Rα and gp130 expression in the LPMCs, F4/80$^+$ macrophages, and CD4$^+$ lymphocytes from normal and CC-affected mice. LPMCs were stained with FITC-F4/80/PE-IL-6Rα/Cy5-gp130, FITC-CD4/ PE-IL-6Rα/Cy5-gp130, or FITC-F4/80/PE-TACE Abs. FCM analysis clearly revealed the augmented expression of gp130 and membrane-bound IL-6Rα in F4/80$^+$ LP macrophages. The expression of TACE on F4/80$^+$ LP macrophages was markedly increased in the mice with ongoing CC. *C*, The expression of membrane-bound IL-6Rα or sIL-6Rα in the colonic mucosa. After the dissociation of colonic epithelial cells, the resulting colonic tissues were subjected to Western blotting using anti–IL-6Rα. The images revealed the membrane-bound form of IL-6Rα as a protein of ~80 kDa and the soluble form of IL-6Rα as 50 kDa. Colonic tissue homogenates were prepared from normal or CC-affected mice. After centrifugation, the supernatant was collected and the amounts of sIL-6Rα were assayed using the sIL-6Rα–specific ELISA. $*p < 0.05$. *D*, sIL-6Rα cleavage in LP macrophage. F4/80$^+$ LP macrophages were prepared from CC-affected mice using MACS. F4/80$^+$ LP macrophages were stimulated with heat-killed commensal bacteria in the presence or absence of 2 nM TNF-α processing inhibitor-1. After 6, 12, 24, and 48 h culture, the culture supernatants were collected and the amounts of sIL-6Rα were examined with the sIL-6Rα–specific ELISA.

in the colonic epithelial cells. In other words, although IL-6 *trans*-signaling is known to be involved in large-bowel cancer, the inductive sites of IL-6/sIL-6Rα, which stimulates IL-6 *trans*-signaling, may differ between sporadic colon cancer and inflammation-based colon cancer. In support of this hypothesis, we demonstrated that treatment with sgp130Fc suppressed the development of CApC. Our results were well correlated with the reported effects of sgp130Fc on AOM-induced colon carcinogenesis (19). It should be noted that the effects of sgp130Fc were more prominent when administered in a low dose (50 μg/mouse) than when administered in a high dose (500 μg/mouse). DSS is a toxic chemical agent for intestinal epithelial cells and it inhibits epithelial restitution (27). Growth factors such as epithelial growth factor and TGF-α accelerate epithelial repair in DSS-induced acute colitis (28, 29). Dauer et al. reported that Stat3 positively regulates common genes involved in wound healing and those in tumor growth (30), therefore complete inhibition of IL-6 *trans*-signaling may limit epithelial repair. This may be a possible explanation for why low-dose administration of sgp130Fc clearly inhibited tumor development.

The mechanisms by which IL-6/Stat3 induced colon carcinogenesis remain unknown. Transcriptional factor Stat3 targeted genes that are upregulated during tumorigenesis in several organs, including those encoding Bcl-X_L, survivin, cyclin D1, and *c-myc* (31). We observed the increased expression of SOCS3 mRNA and abolition of mature SOCS3 protein expression in the colonic epithelial cells of CApC-affected mice. The SOCS gene family is involved in the negative regulation of the Jak/Stat pathway that is induced by cytokine signaling (32). Among the SOCS family genes, SOCS3

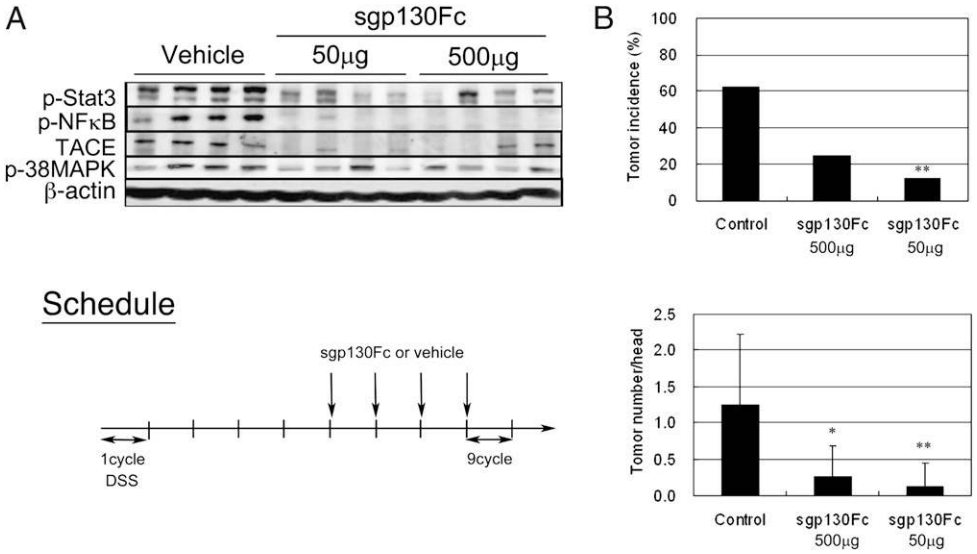

FIGURE 7. Effect of sgp130Fc on inflammation-based colon tumorigenesis. During the induction of CApC, sgp130Fc (500 or 50 μg/mouse) or a vehicle was injected i.p. into BALB/c mice on the first day of each cycle during DSS cycles 6–9 (each group, $n = 10$). *A*, Western blot analysis of phospho-Stat3, phospho-NFκB, phospho-p38MAPK, TACE, and β-actin expression in the colonic tissues of the in sgp130Fc-treated mice. *B*, Incidence or number of CApC tumors in mice treated with sgp130Fc. The data are presented as the mean ± SD ($n = 8–10$).

plays a central role as a negative regulator of Stat3 activation (33). We have previously reported that DSS-induced colitis is mild in mice lacking Stat3 activation but severe in mice that are genetically manipulated to exhibit SOCS3 dysfunction (26). In this study, we observed marked phosphorylation of Stat3 proteins in the mucosa of CApC-affected mice. This may be because of either the excessive induction of IL-6 *trans*-signaling in the colonic epithelial cells or the loss of mature SOCS3 protein expression in the epithelial cells of the CApC-affected mucosa. Consistent with this concept, Rigby et al. reported that disruption of the intestinal epithelial cell-specific *SOCS3* gene accelerates AOM-induced colon tumorigenesis in mice (34). In this study, we did not focus on the mechanisms underlying the loss of mature SOCS3 protein expression in the CApC-affected mucosa; however, posttranslational regulation of the SOCS3 protein in the epithelial cells of the CApC-affected mucosa may be responsible for this phenomenon. Further analysis is required to clarify this important issue.

In this study, the expression of gp130 but not membrane-bound IL-6Rα was augmented in $CD4^+$ lymphocytes. We have previously demonstrated that mucosal $CD4^+$ T cells and intestinal epithelial cells are targets of IL-6 *trans*-signaling during the development of Crohn's disease in a murine model (15). IL-6 *trans*-signaling has recently been reported as the biological consequence of heat responses in T cells, and it plays a critical role in the augmentation of the immune response during fever reactions (35). Atreya et al. reported that the phosphorylation of Stat3 proteins, which is modulated by IL-6 *trans*-signaling, accelerates the induction of antiapoptotic proteins in mucosal T cells (36). It also induces the expression of cell-adhesion molecules, resulting in T cell infiltration into the mucosa (37). Domintzki et al. suggested that IL-6 *trans*-signaling induced by the IL-6/sIL-6Rα fusion protein abrogates the generation of TGF-β–induced FoxP3-positive $CD4^+$–$CD25^+$ peripheral regulatory T cells from $CD4^+$ to $CD25^-$ naive T cells (38). We observed the infiltration of activated $CD4^+$ T cells into the colonic mucosa in CC- and CApC-affected mice. Therefore, the activation of $CD4^+$ T cells by IL-6 *trans*-signaling may be essential for the maintenance of the chronic inflammation that causes CAC.

The induction of TACE in LP macrophages is another important phenomenon that should be investigated further because TACE is a key enzyme involved in the shedding of sIL-Rα, TNF-α, and the ligands of the epithelial growth factor-receptor from the cell membrane (39). We observed TACE-dependent active sIL-6Rα cleavage in $F4/80^+$ LP macrophages that were isolated from the mice with ongoing CC after stimulation with commensal bacteria. TACE mRNA expression is upregulated in human colon carcinoma (40). The importance of TACE activity in many physiological or pathological events has been well established; however, little is known regarding the types of stimuli that modulate its expression (41, 42). Recent evidence has suggested that hypoxia inducible factor-1α binds to the TACE promoter sequence and upregulates its transcription (43). Peyssonnaux et al. reported that LPS induce hypoxia inducible factor-1α mRNA production by macrophages in a TLR4-dependent manner (44). In this study, we observed the activation NFκB in the colonic mucosa during the development of CApC and its inhibition by the sgp130Fc protein. It has been widely reported that enteric bacteria are necessary for the induction of chronic intestinal inflammation in various mouse model (14, 45, 46). The increased presence of mucosal adherent bacteria and the intramucosal translocation of enteric bacteria are usually observed in these models (47). Therefore, we speculate that several species of enteric bacteria may play crucial roles in the regulation of TACE induction in inflammatory macrophages. In our preliminary study, we observed the induction of TACE mRNA in LP macrophages after the stimulation by several enteric bacterial strains. Consistent with this result, Kado et al. reported that the intestinal microflora influence the development of colonic adenocarcinoma in TCR-$\beta^{-/-}$ and $p53^{-/-}$ mice (48). Further research could provide insights into the roles played by commensal bacteria in the pathogenesis of inflammation-based colon carcinogenesis.

Several studies have indicated that IL-6 *trans*-signaling plays an important role in the induction of colon carcinogenesis (19). However, the mechanisms underlying the regulation of IL-6 *trans*-signaling in the colonic mucosa remain unknown. We demonstrated that inflammatory macrophages in the colonic mucosa play essential roles in both the production of IL-6 and the shedding of sIL-6Rα from the cell membrane, thus inducing IL-6 *trans*-signaling in colonic epithelial cells during the development of inflammation-based colon carcinogenesis. Moreover, sgp130Fc, which is a competitive inhibitor of IL-6 *trans*-signaling, suppressed colon carcinogenesis. Therefore, the inhibition of IL-6 *trans*-signaling may be a useful

therapeutic system for the treatment of inflammation-based colon carcinogenesis.

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Disclosures

The authors have no financial conflicts of interests.

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