Cytokine Induction of Tumor Necrosis Factor Receptor 2 Is Mediated by STAT3 in Colon Cancer Cells

Kathryn E. Hamilton, James G. Simmons, Shengli Ding, Laurianne Van Landeghem, and P. Kay Lund

Abstract
The IL-6/STAT3 and TNFα/NFκB pathways are emerging as critical mediators of inflammation-associated colon cancer. TNF receptor (TNFR) 2 expression is increased in inflammatory bowel diseases, the azoxymethane/dextran sodium sulfate (AOM/DSS) model of colitis-associated cancer, and by combined interleukin (IL) 6 and TNFα. The molecular mechanisms that regulate TNFR2 remain undefined. This study used colon cancer cell lines to test the hypothesis that IL-6 and TNFα induce TNFR2 via STAT3 and/or NFκB. Basal and IL-6 + TNFα-induced TNFR2 were decreased by pharmacologic STAT3 inhibition. NFκB inhibition had little effect on IL-6 + TNFα-induced TNFR2, but did inhibit induction of endogenous IL-6 and TNFR2 in cells treated with TNFα alone. Chromatin immunoprecipitation (ChIP) revealed cooperative effects of IL-6 + TNFα to induce STAT3 binding to a −1,578 STAT response element in the TNFR2 promoter but no effect on NFκB binding to consensus sites. Constitutively active STAT3 was sufficient to induce TNFR2 expression. Overexpression of SOCS3, a cytokine-inducible STAT3 inhibitor, which reduces tumorigenesis in preclinical models of colitis-associated cancer, decreased cytokine-induced TNFR2 expression and STAT3 binding to the −1,578 STAT response element. SOCS3 overexpression also decreased proliferation of colon cancer cells and dramatically decreased anchorage-independent growth of colon cancer cells, even cells overexpressing TNFR2. Collectively, these studies show that IL-6- and TNFα-induced TNFR2 expression in colon cancer cells is mediated primarily by STAT3 and provide evidence that TNFR2 may contribute to the tumor-promoting roles of STAT3. Mol Cancer Res; 9(12): 1718–31. ©2011 AACR.

Introduction
Patients with inflammatory bowel diseases (IBD), such as Crohn’s disease and ulcerative colitis, have an increased lifetime risk of developing inflammation-associated colorectal cancer (CRC; refs. 1–3). Chronic increases in proliferation of intestinal epithelial cells (IEC) driven by proinflammatory factors have been shown to promote tumorigenesis. The IL-6/STAT3 (4–9) and TNFα/NFκB (10–12) pathways are both major mediators of inflammation-associated CRC, and recent studies show that TNFR2 blockade decreases intestinal tumor formation in mice (13, 14).

TNFα signals through 2 receptors, TNFR1 and TNFR2. TNFR1 exerts proapoptotic functions due to its intracellular death domain (15). TNFR2, which lacks a death domain, has been linked to increased proliferation of IEC in animal models of colitis and colon cancer cells (16, 17). This supports a concept that TNFR2 may mediate protumorigenic effects of TNFα. The role of TNFR2 in inflammation-associated cancer is a topic of increasing interest, as recent studies indicate that TNFR2 is upregulated in IBD and in the azoxymethane/dextran sulfate model of inflammation-associated cancer (14, 16, 18). In vitro studies have shown that TNFR2 is induced in colon cancer cells by TNFα and IL-6 combined but neither cytokine alone (16). Other studies have shown TNFR2 induction by IFNγ (19). These findings suggest that the STAT pathways activated by IL-6 or IFNγ and/or NFκB pathways typically activated by TNFα may interact to induce TNFR2 expression. In support of this possibility, the human TNFR2 promoter contains 2 consensus STAT-binding sites as well as 2 consensus NFκB-binding sites (20). The present study tested the hypothesis that IL-6 and TNFα interact to induce TNFR2 expression by activation of STAT3, NFκB, or both STAT3 and NFκB.

Suppressors of cytokine signaling (SOCS) proteins are negative feedback inhibitors of the JAK/STAT pathway (21). IEC-specific SOCS3 gene deletion increased tumor load in the AOM/DSS model of colitis-associated CRC (22). This effect was associated with enhanced activation of both STAT3 and NFκB (22). In vitro, SOCS3 overexpression reduced proliferation of colon cancer cell lines and inhibited both IL-6-induced STAT3 activation and TNFα-induced NFκB activation (22). Furthermore, SOCS3 genes are silenced by promoter hypermethylation in various human
cancers, including CRC (23–26). Together these data provide strong evidence that SOCS3 normally acts as a suppressor of inflammation-associated CRC. The current study tested the hypothesis that SOCS3 overexpression limits the cytokine induction of TNFR2 and/or growth-promoting effects of TNFR2 in colon cancer cells.

Our studies reveal a novel pathway where IL-6 and TNFα cooperatively induce TNFR2 in colon cancer cells by interactions at multiple levels. TNFα, acting through NFκB, induces endogenous IL-6, but combined effects of IL-6 and TNFα to induce TNFR2 gene expression depend on STAT3 activation. We also provide direct evidence that TNFR2 promotes growth of colon cancer cells. Overexpression of the known STAT3 inhibitor SOCS3 decreases TNFR2 expression, decreases STAT3 binding to the TNFR2 promoter, and potently inhibits proliferation and anchorage-independent growth of colon cancer cells.

Materials and Methods

Cell culture and cytokine treatments

The 2 colon cancer cell lines primarily used in this study were SW480 and COLO205 cells, both of which express low levels of endogenous SOCS3. SW480 cells were used for the majority of studies because our prior studies showed that these cells are responsive to both IL-6 and TNFα, which robustly activate STAT3 and NFκB, respectively (22). COLO205 cells were used as an independent cell line to confirm cytokine induction of TNFR2. Because COLO205 cells grow well in soft agar, they were also used to address effects of TNFR2 and SOCS3 on anchorage-independent growth. SW480, COLO205, and Caco2 cells were obtained from the American Type Culture Collection. Human intestinal epithelial cell line (HIEC) was generously provided from the American Type Culture Collection.

Flow cytometric analysis of surface TNFR2 expression

Cell surface expression of TNFR2 was assessed using flow cytometry as previously described (16). SW480 cells were trypsinized, washed with serum-free RPMI-1640 media (Gibco), and incubated in medium alone or medium plus IL-6 and TNFα (50 ng/mL) for 10 hours at 37°C with rotation in 15 mL conical tubes. Cells were then resuspended in wash buffer [PBS supplemented with 1% bovine serum albumin and 1 mg/mL DNase (Roche)], and Fc-blocked with 1 μg human IgG (R&D Systems) for 15 minutes. Cells were incubated with fluorescein-conjugated anti-TNFα (R&D Systems) or isotype control (BD Pharmingen) for 45 minutes. Following antibody incubation, cells were washed and resuspended in 2% paraformaldehyde. Flow cytometric analysis of surface TNFR2 expression was then conducted using a CyAn flow cytometer (Beckman-Coulter-Dako). Effect of cytokine treatment on TNFR2 surface expression was measured on the basis of fluorescence intensity.

Semiquantitative real-time PCR analyses

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. Reverse-transcription was carried out using AMV-RT (Promega). PCR and analyses were completed on the Rotorgene 2000 (Qiagen) using Invitrogen Platinum qPCR Supermix-UDG and the following TaqMan primer–probe sets (Applied Biosystems): human TNFR2 Hs00961755_m1, human TNFR1 Hs00533568_g1, human IL-6 (Hs00985639_m1), human ICAM-1 (positive control as NFκB-induced gene). Hydroxymethylbilane synthase (human HMBS) Hs00609297_m1 was used as an invariant control. Non–reverse-transcribed samples were used as negative controls. Gene expression was calculated using the \( R = 2^{-\Delta\Delta C_T} \) method, where changes in C values for the genes of interest were normalized to HMBS. In all cases, gene expression for particular treatment groups was expressed as fold change versus mean values for no treatment control. Real-time PCR reactions were carried out in triplicate and replicated in at least 3 independent experiments.

ELISA for TNFR2

Soluble TNFR2 levels in cell supernatants were measured using Quantikine ELISA system (R&D System) according to manufacturer’s instructions. Samples were normalized to total protein as measured by BCA protein assay (Pierce).

Chromatin immunoprecipitation to assess STAT3 and NFκB binding to the TNFR2 promoter

For chromatin immunoprecipitation (ChIP), SW480 cells were serum deprived overnight followed by treatment...
with IL-6, TNFα, or both cytokines (50 ng/mL) for 5 to 60 minutes. After treatment, cells were cross-linked with 1% formaldehyde for 10 minutes. Subsequent steps were conducted as specified in the ChIP-IT Express (Active Motif) user manual. Briefly, cross-linked cells were lysed and sonicated, followed by overnight immunoprecipitation with anti-STAT3 (SC-483x; Santa Cruz Biotechnology) and anti-p65 NFκB (SC-372) or negative control IgG antibody (Active Motif). Eluted, reverse cross-linked protein:DNA complexes were digested with proteinase K for 1 hour. Digested samples were then purified using QIAquick columns according to manufacturer’s instructions (QIAquick PCR Purification Kit, Qiagen). PCR amplification was subsequently carried out using primers specific to STAT3 and NFκB-binding elements within the TNFR2 promoter. Oligomers used to amplify these binding sites were purchased from Sigma, and sequences are shown in Table 1. Densitometry was conducted to quantify the PCR-amplified transcription factor–binding sites.

Western blot for activated STAT3 in nuclear extracts

Western blot analyses were conducted on nuclear extracts from SW480 cells to determine whether IL-6 combined with TNFα enhanced levels of activated or total nuclear STAT3. Nuclear extracts were prepared as previously described (31). Briefly, SW480 cells were grown to confluence, serum-deprived overnight, and treated with IL-6 and TNFα (50 ng/mL) for 30 minutes. Cells were then pelleted in lysis buffer containing 10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol (DTT), and 2 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonylfluoride (PMSF). Nuclei were obtained by adding 10% NP40 and centrifuging for 5 minutes at 15,000 × g. Pellets were then resuspended in buffer containing 20 mmol/L HEPES, pH 7.9, 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and 1 mmol/L PMSF to release nuclear proteins. The nuclear suspension was centrifuged at 15,000 × g for 5 minutes, and supernatants containing nuclear extracts were then subjected to immunoprecipitation and immunoblot with the following antibodies: anti-phospho-tyrosine STAT (total); rabbit polyclonal pTyr705 (#9131; Cell Signaling); anti-STAT3 (total): rabbit polyclonal SC-7179 (Santa Cruz Biotechnologies). Coomassie-stained protein gels verified equivalent amounts of nuclear protein in samples used for immunoprecipitation.

Constitutive activation of STAT3

Constitutively active STAT3 (CA-STAT3) adenovirus was kindly provided by Dr. Christian Jobin (University of North Carolina, Chapel Hill, NC). This vector is constitutively activated due to C661A and C663N mutations and has been functionally characterized in prior studies (32, 33). SW480 cells were treated with CA-STAT3 at a multiplicity of infection (MOI) of 50 or with IL-6 and TNFα for 10 hours prior to mRNA extraction for evaluation of TNFR2 expression.

TNFR2 and SOCS3 expression constructs

Cells treated with TNFR2 or SOCS3 expression constructs were used to directly evaluate the proliferative and growth-promoting effects of TNFR2 overexpression and test whether SOCS3 could inhibit TNFR2 expression or its growth-promoting effects. Retroviral expression vector pQCXIP was obtained from BD Biosciences Clontech. pQCXIP containing c-myc–tagged human TNFR2 was kindly provided by Dr. Daniella Männel, University of Regensburg, Germany. HEK293 cells were cotransfected with retroviral vectors and packaging vector as previously described (34) using jetPei (Polyplus Transfection) according to manufacturer’s instructions. Media containing TNFR2 retrovirus was collected from transfected HEK293 cells and used to treat SW480 or COLO205 cells for 24 to 48 hours. TNFR2 overexpression was confirmed by Western immunoblot (data not shown). Plasmid pBIG2i expressing human SOCS3 and empty vector were kindly provided by Drs. Richard Furlanetto and Peter Nisley. These plasmids were used to generate adenovirus expressing human SOCS3 or empty vector control as previously described (22). Adenoviruses were used at an MOI of 100 unless otherwise noted. Cells were treated with adenovirus for 24 to 48 hours in complete media and switched to serum-free media overnight prior to cytokine stimulation. Adenovirus-mediated overexpression of SOCS3 was confirmed by Northern blot analysis and qRT-PCR (data not shown).

Table 1. Oligomers used in ChIP assays

<table>
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<tr>
<th>Site within TNFR2 promoter</th>
<th>Oligomers</th>
<th>Product size, bp</th>
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<tbody>
<tr>
<td>STAT (−1,578)</td>
<td>F-5'-CTGCAGTGAGCTATGGGTGA-3'</td>
<td>223</td>
</tr>
<tr>
<td></td>
<td>R-5'-GAGGTTGGGTCCGAAGTGAC-3'</td>
<td></td>
</tr>
<tr>
<td>STAT (−364)</td>
<td>F-5'-CTGCAGTGAGCTATGGGTGA-3'</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>R-5'-GGGTTGGGATCTTTTGGGA-3'</td>
<td></td>
</tr>
<tr>
<td>NFkB (−1,890)</td>
<td>F-5'-TTGAAATGGTCCCCAGGATG-3'</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td>R-5'-CTAGTTGTCGCCACACACAG-3'</td>
<td></td>
</tr>
<tr>
<td>NFkB (−1,517)</td>
<td>F-5'-AAAGGCTTTGTCGTCATCCCCAG-3'</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td>R-5'-GGCTGTCTGAAAGAGTAG-3'</td>
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**Table 2. Genotyping oligomers for VC-SOCS3^{+/−} mice**

<table>
<thead>
<tr>
<th>Target</th>
<th>Oligomers</th>
<th>Product size, bp</th>
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<tr>
<td>VC transgene</td>
<td>F′-CGTGGAGACAGCAAGTGGACGGC-3′</td>
<td>324</td>
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<tr>
<td></td>
<td>R′-CCCTGACACCTCGACGGTCT-3′</td>
<td></td>
</tr>
<tr>
<td>Wild-type or pLox-modified SOCS3 alleles</td>
<td>F′-GGATTTTCTTGGCGCTTCTCTA-3′</td>
<td>402 or 534</td>
</tr>
<tr>
<td></td>
<td>R′-TGATTACTGCTTGGAGGCTGAA-3′</td>
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</tr>
</tbody>
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**TNFR2 or SOCS3 knockdown**

SW480 cells were grown to approximately 50% to 70% confluence in RPMI-1640 medium (Gibco, with 10% FBS, plus antibiotics). Cells were trypsinized and counted. A total of 5 × 10^4 cells per condition were transected using Nucleofector Technology according to manufacturer’s instructions (Kit V, Lonza) or by using Lipofectamine 2000 (Invitrolog) in Opti-MEM (Gibco) with scramble control, TNFR1, or TNFR2 siRNAs (Applied Biosystems) according to manufacturer’s instructions. Additional TNFR2 knockdown experiments were carried out using the Santa Cruz plasmid sc-36689-SH. Knockdown was mediated SOCS3 knockdown using siRNAs (sense 5′-CCAAAGACCCUGCGCAUCCAdTdT-3′, antisense 5′-UGGAUGCGCAGGUUCUUGGdTdT-3′; Dharmacon) and methods previously described for prostate cancer cells (35).

**Analysis of cell proliferation and anchorage-independent growth**

Assays of [3H]-thymidine incorporation into DNA were used to assess S-phase of the cell cycle as one measure or proliferation and were conducted as previously described (36). Briefly, SW480 cells were plated in 24-well plates at a density of 1 × 10^4 cells per well and treated with TNFR2 and/or SOCS3 expression constructs for 24 hours. Medium was then supplemented with 2 μCi/mL [3H]-thymidine overnight, and thymidine incorporation into DNA was measured using scintillation counting. Values are expressed as fold change compared with empty vector control. As an independent measure of effects of TNFR2 knockdown on cell growth, WST-1 assays (Roche), which measure numbers of viable cells, were conducted according to manufacturer’s instructions. Briefly, cells were transfected with control or TNFR2 siRNAs in 96-well plates, then switched to media supplemented with 0.3% agar followed by plating in 6-well culture dishes coated with 5% agar. Cells were treated with expression constructs at days 1, 7, and 14. At day 21, viable cells were stained with MTT for 4 hours and colonies were quantified using NIH ImageJ software (37).

**Evaluation of TNFR2 in colon of AOM/DSS-treated mice with IEC-specific disruption of SOCS3 genes**

C57BL6 mice homozygous for pLox-modified SOCS3 alleles were crossed with mice hemizygous for the villin-Cre transgene (Jackson Laboratories) as previously described (22). Study mice are denoted as VC-SOCS3^{+/-} and WT-SOCS3^{+/-} controls with pLox-modified, but intact SOCS3 alleles. Genotyping primers are listed in Table 2. All procedures were conducted with Institutional Animal Care and Use Committee approval and in accordance with the NIH guidelines for use of live animals. VC-SOCS3^{+/-} and WT-SOCS3^{+/-} were treated with AOM/DSS as previously described (22). Briefly, 8- to 10-week-old mice were given an intraperitoneal injection of 10 mg/kg AOM. After 7 days, mice were given 3 cycles of 2.5% DSS in the drinking water for 5 days, allowing 14 days of recovery between each DSS treatment. Animals were euthanized 80 days after initial AOM injection. Colon samples were collected and fixed in 10% zinc-buffered formalin for immunohistochemical staining.

Immunostaining for TNFR2 was conducted on parafin-embedded sections of distal colon tumor and nontumor tissues. Only VC-SOCS3^{+/-} had histologically detectable tumors in these experiments. After deparaffinizing and hydrating, sections were pretreated with 0.05 mol/L Tris Triton epitope retrieval solution at room temperature for 30 minutes. Endogenous peroxidase activity was blocked with 3% H2O2 for 10 minutes, followed by blocking in 5% normal goat serum (NGS)/TT buffer for 1 hour. The sections were incubated with TNFR2 antibody (NB1-03130, Novus Biologicals) at a dilution of 1:250 in 5% NGS/TT buffer overnight at 4°C in a humidity chamber. After washing 3 times in 0.05 mol/L Tris buffer, the sections were incubated with biotinylated goat anti-rabbit IgG (11-065-114, Jackson ImmunoResearch) for 1 hour at room temperature. ABC (Vector Labs) was applied for 1 hour, followed by adding 3 3′—diaminobenzidine (DAB; Zymed) for 10 to 14 minutes and the reaction was stopped in dH2O. Counterstaining was conducted with hematoxylin for 25 seconds and coverslips were mounted in DPX mount (VWR International Ltd.). Images were taken by Imager A2 microscope (Zeiss).
Statistics

Values are expressed as mean ± SE. Comparisons between cell treatments were analyzed using Student t test for comparisons between 2 treatments or one-way ANOVA for comparisons of multiple treatments followed by post hoc, pairwise comparisons using the Fisher protected least significant difference (PLSD) test. A value of \( P < 0.05 \) was considered statistically significant for all experiments.

Results

IL-6 and TNFα induce TNFR2 in COLO205 and SW480 cells

Prior studies in colon cancer cells indicate that TNFR2 expression is increased by combined IL-6 or TNFα treatment, with only modest effects due to either cytokine alone (16). Figure 1A and C confirm elevation of TNFR2 mRNA levels by combined IL-6 and TNFα in SW480 and COLO205 cells. TNFR1 mRNA levels were not significantly altered with cytokine treatment. Evaluation of TNFR2 protein by ELISA and flow cytometry (Fig. 1C–E) verified these data. In SW480 cells, treatment with TNFα alone induced TNFR2 to levels similar to those observed with combined IL-6 and TNFα. Because TNFα has been reported to induce IL-6 in cancer cell lines from other organs (38–42), we tested whether TNFα upregulated endogenous IL-6 mRNA levels. As shown in Fig. 1F, TNFα treatment and TNFα combined with IL-6 significantly increased endogenous IL-6 mRNA. This result suggests that autocrine or paracrine actions of TNFα-induced IL-6 may contribute to the ability of TNFα to induce TNFR2.

A predominant role of STAT3 in mediating basal and cytokine-induced TNFR2 expression

To assess the functional role of STAT3 or NFκB in regulating TNFR2 expression, we examined the effects of a STAT3 (cucurbitacin) or NFκB (Bay 11-7082) inhibitor on basal and IL-6 + TNFα-induced TNFR2 mRNA (Fig. 2A). On the basis of published and pilot studies, we used

Figure 1. Increased TNFR2 mRNA and protein levels in colon cancer cell lines treated with IL-6 and TNFα. Histograms (A–D) show levels of TNFR2 mRNA or soluble TNFR2 in SW480 and COLO205 cells treated with 50 ng/mL IL-6 plus 50 ng/mL TNFα for 10 hours. TNFR2 mRNA was normalized to HMBS. sTNFR2 was measured by ELISA on cell supernatants and values were normalized to total protein. All values are expressed as fold change (mean ± SE) versus mean levels in untreated controls. (*, \( P \leq 0.05 \) compared with no treatment controls). TNFR2 mRNA was significantly increased by IL-6 and TNFα treatment in both cell lines (A and C). Consistent with findings for TNFR2 mRNA, protein levels of soluble TNFR2 were significantly increased with IL-6 and TNFα treatment (B and D). E, representative histogram of surface TNFR2 expression as measured by flow cytometry. Cells treated with IL-6 and TNFα exhibit an increase in surface TNFR2 expression. Negative controls include isotype and no antibody (Ab) controls for each condition. F, histograms show IL-6 mRNA levels in SW480 cells. Note that TNFα alone or TNFα + IL-6 elicit similar, significant increases as IL-6 mRNA (*, \( P \leq 0.05 \) compared with no treatment). For all experiments, \( n \geq 3 \) independent experiments were carried out in duplicate. FITC, fluorescein isothiocyanate.
maximally effective doses of 20 μmol/L cucurbitacin or 5 μmol/L Bay 11-7082. Cucurbitacin significantly inhibited basal TNFR2 mRNA and TNFR2 mRNA induction by combined IL-6 and TNFα. In contrast, Bay 11-7082 had no significant effect on basal or IL-6 + TNFα-induced TNFR2 mRNA. Furthermore, effects of combined inhibition of STAT3 and NFκB on basal or IL-6 + TNFα-induced TNFR2 were not significantly different than the inhibitory effect of cucurbitacin alone. These data suggest a predominant role of STAT3 compared with NFκB pathways in induction of TNFR2 by combined IL-6 and TNFα. Confirmatory studies were conducted in cells treated with IL-6 or TNFα alone plus STAT or NFκB inhibitors. IL-6 alone did not induce TNFR2 mRNA above basal levels, but cucurbitacin, and not Bay 11-7082, reduced TNFR2 mRNA in IL-6–treated cells.

Analyses of the effects of cucurbitacin and Bay 11-7082 on TNFR2 mRNA in cells treated with TNFα alone revealed that either the NFκB or the STAT3 inhibitors significantly attenuated TNFα induction of TNFR2, but combined inhibitors did not have a more dramatic effect than either inhibitor alone. These results are consistent with our findings that TNFα induces IL-6 and suggests that NFκB likely mediates TNFα induction of IL-6 in SW480 cells. To directly test this, we measured IL-6 mRNA levels in cells treated with TNFα or TNFα + IL-6 in the presence of Bay 11-7082 or DMSO control. Treatment with Bay 11-7082 dramatically decreased IL-6 mRNA in TNFα– or TNFα + IL-6-treated cells, confirming that TNFα induction of IL-6 in SW480 cells is mediated through NFκB (Fig. 2B). Because it has been established that cytokines induce ICAM-1 through NFκB–dependent mechanisms, we also

Figure 2. Effects of STAT3 or NFκB inhibitors and constitutively activated STAT3 on TNFR2, and effects of NFκB inhibitor on IL-6 and ICAM. A, histograms show levels of TNFR2 mRNA in SW480 cells treated with vehicle, IL-6 + TNFα, IL-6 alone, or TNFα alone, for 10 hours in the absence (−) or presence (+) of the STAT3 inhibitor cucurbitacin (20 μmol/L) or the NFκB inhibitor Bay 11-7082 (5 μmol/L). Values are expressed as mean fold change versus basal values in the absence of inhibitor; *, P ≤ 0.05 compared with basal; **, P ≤ 0.05 for effect of STAT or NFκB inhibitor; NS, not significant. Note the dramatic inhibitory effect of cucurbitacin on both basal and IL-6 + TNFα-induced TNFR2 compared with Bay 11-7082. Note that IL-6 alone did not induce TNFR2 but cucurbitacin reduced TNFR2 mRNA in IL-6–treated cells. TNFα alone induced TNFR2, and this was inhibited by Bay 11-7082 or cucurbitacin, with no greater inhibition when both STAT3 and NFκB inhibitors were combined. B, IL-6 mRNA levels were measured in SW480 cells treated with TNFα or IL-6 + TNFα in the absence (−) or presence (+) of Bay 11-7082 (5 μmol/L). Note that Bay 11-7082 completely abolished TNFα or IL-6 + TNFα–mediated increases in IL-6 mRNA levels. *, P ≤ 0.05 compared with no treatment; **, P ≤ 0.05 for effect of NFκB inhibitor. For all experiments, n ≥ 3 independent experiments were carried out in duplicate. C, ICAM mRNA induction in SW480 cells treated with IL-6 + TNFα in the absence (−) or presence (+) of Bay 11-7082 (5 μmol/L). Bay 11-7082 completely abolished IL-6 + TNFα–mediated increases in ICAM mRNA levels. *, P ≤ 0.05 compared with no treatment; **, P ≤ 0.05 for effect of NFκB inhibitor. D, SW480 cells were treated with adenovirus to overexpress constitutively active STAT3 (CA-STAT3) for 10 hours followed by mRNA collection. Expression of CA-STAT3 significantly increased TNFR2 mRNA levels to the same level as that found with IL-6 and TNFα treatment. *, P ≤ 0.05 compared with empty vector.


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verified the efficacy of Bay 11-7082 by showing that IL-6 and TNFα induced a 4.4 ± 0.6-fold increase in ICAM-1 mRNA, and treatment with Bay 11-7082 potently inhibited this effect (Fig. 2C). Thus, the modest effects of NFκB versus STAT3 inhibitor on TNFR2 expression in cells treated with IL-6 and TNFα were not due to a lack of effective Bay 11-7082 dosing. Together these findings suggest a predominant role of STAT3 in mediating cytokine-induced TNFR2 expression.

To confirm a role for STAT3 in TNFR2 expression, we treated SW480 cells with a constitutively active STAT3 (CA-STAT3) adenovirus. Expression of CA-STAT3 in the absence of cytokine treatment induced TNFR2 mRNA levels to the same degree as observed with combined IL-6 and TNFα treatment (Fig. 2D). Together, the data with STAT3 inhibitor and constitutively activated STAT3 suggest that STAT3 is necessary and sufficient for basal and IL-6/TNFα-induced TNFR2 expression.

**IL-6 and TNFα induce STAT3, but not NFκB binding, to the TNFR2 promoter**

We verified a predominant role of STAT3 in the control of TNFR2 expression by ChIP assays that allowed us to directly assess the effect of cytokines on STAT3 and NFκB binding to putative binding elements in the TNFR2 promoter (Fig. 3A). Combined IL-6 and TNFα treatment induced binding of STAT3 to both of the putative STAT-binding sites, with maximal binding at 30 and 60 minutes for −364 and −1,578 sites, respectively (Fig. 3B). Densitometry revealed that IL-6 and TNFα induced 2.0 ± 0.4-fold and 3.8 ± 1.6-fold and increases in STAT3 binding to the −364 and −1,578 sites, respectively. There was a small degree of basal NFκB binding, but this was not enhanced by IL-6 and TNFα treatment.

To delineate the individual contribution of IL-6 and TNFα on STAT3 binding to the TNFR2 promoter, we treated cells with either cytokine alone or in combination

![Image](mcr.aacrjournals.org)
and conducted ChIP for STAT3. Treatment with IL-6 and TNFα alone for 30 minutes modestly induced STAT3 binding to the −1,578 STAT3–binding site, but both cytokines combined induced dramatic increases in STAT3 binding to the −1,578 binding site (Fig. 3C). Putative STAT3–binding site −364 differed in that IL-6 alone, but not TNFα, induced STAT3 binding and combined cytokines had similar effects as IL-6 alone. Thus, the cooperative effects of IL-6 and TNFα to activate STAT3 appear selective for the −1,578 STAT3–binding site.

Because combined IL-6 and TNFα led to a dramatic increase in STAT3 binding to the −1,578 STAT3–binding site in the TNFR2 promoter, we assessed whether the 2 cytokines in combination enhanced tyrosine phosphorylation of STAT3 relative to IL-6 or TNFα alone. Western blot analysis on nuclear extracts revealed that IL-6 increased phosphorylated and total STAT3 in the nucleus whereas TNFα alone had no effect (Fig. 3D). Both cytokines together did not dramatically augment tyrosine phosphorylation of STAT3 or total nuclear STAT3 as compared with IL-6 alone. Thus, enhanced tyrosine phosphorylation of STAT3 does not appear to account for the combinatorial effects of IL-6 and TNFα on STAT3 binding to the −1,578 STAT3–binding site. It is important to note that the treatment time points used to examine effects of TNFα alone on STAT3 binding by ChIP or STAT3 tyrosine phosphorylation were much shorter than the time points (10 hours) needed for TNFα to induce IL-6. Thus, the Western immunoblot and ChIP data together indicate that IL-6 alone enhances STAT3 activation and nuclear localization, but interaction with TNFα is required to promote IL-6–induced binding of STAT3 to the TNFR2 promoter.

**Effects of TNFR2 overexpression or knockdown on proliferation and cell number**

Prior in vivo studies suggest that TNFR2-null mice show reduced crypt proliferation during intestinal inflammation (16). To directly test the effects of TNFR2 on colon cancer cell proliferation, we overexpressed TNFR2 in SW480 and COLO205 cells and measured [3H]-thymidine incorporation into DNA. TNFR2 overexpression significantly enhanced [3H]-thymidine incorporation in both cell lines (Fig. 4A). In complementary experiments, we knocked down TNFR1 or TNFR2 using siRNA (Fig. 4B) and measured [3H]-thymidine incorporation over 24 hours. The maximal knockdown achieved after testing multiple TNFR2-targeted siRNAs was a 60% reduction in TNFR2 mRNA levels (Fig. 4B). However, this was a specific effect because expression of TNFR1 mRNA was unaffected by the TNFR2 siRNA. Similarly, TNFR1 siRNA had no effect on TNFR2 mRNA but inhibited TNFR1 expression by 80%. TNFR2 knockdown resulted in a modest but significant decrease (14% ± 2.5%) in [3H]-thymidine incorporation in SW480 cells (Fig. 4C). WST-1 assays, which measure number of viable cells rather than just S-phase, revealed that knockdown of TNFR1 significantly increased cell number, whereas knockdown of TNFR2 reduced cell number up to 40% compared with control siRNA and up to

**Figure 4.** Effects of TNFR2 overexpression or siRNA-mediated knockdown on colon cancer cell proliferation. A, histogram of [3H]-thymidine incorporation into DNA as a measure of COLO205 or SW480 cell proliferation after 24-hour overexpression of TNFR2. TNFR2 overexpression significantly increased cell proliferation. *, P ≤ 0.05 compared with empty vector. B, SW480 cells were treated with control, TNFR1, or TNFR2 siRNA for 24 hours. Histogram shows fold change in expression of TNFR1 or TNFR2 mRNA. TNFR2 siRNA led to a 60% decrease in TNFR2 mRNA, with no effect on TNFR1 mRNA. *, P ≤ 0.05 compared with other treatments. C, untreated SW480 cells transfected with TNFR2 siRNA exhibit a modest, but significant (†, P ≤ 0.05) decrease in [3H]-thymidine incorporation into DNA. D, cytokine-treated SW480 cells with TNFR2 knockdown exhibited a significant decrease in cell number measured by WST-1 assay, whereas TNFR1 knockdown significantly increased cell number. *, P ≤ 0.05 compared with control siRNA; †, P ≤ 0.05 compared with cells treated with TNFR1 siRNA. n = 3.
70% compared with cells with knockdown of TNFR1 (in which TNFR2 is the predominant TNFR; Fig. 4D). We have observed similar effects of TNFR2 knockdown in COLO205, Caco2, and HIECs (Supplementary Tables S1–S3). The more dramatic effect of TNFR2 knockdown on cell number than [3H]-thymidine incorporation suggests that TNFR2 knockdown likely impacts cell survival and/or other phases of cell cycle than S-phase.

**SOCS3 inhibits cytokine-induced TNFR2 expression, STAT3 binding to the −1578 consensus site, and proliferation and anchorage-independent growth of colon cancer cells**

Negative regulation of STAT3 by SOCS3 is well established (43–45). To test whether SOCS3 inhibits TNFR2 expression, we treated COLO205 and SW480 cells with SOCS3 adenovirus or empty vector control and examined TNFR2 mRNA. As anticipated, SOCS3 overexpression significantly inhibited cytokine-induced TNFR2 in both cell lines (Fig. 5A). ChIP assay also revealed that SOCS3 overexpression dramatically inhibited cytokine-induced STAT3 binding to the −1,578 site but had variable and nonsignificant effects on STAT3 binding to the −364 site (Fig. 5B).

We have previously reported that SOCS3 overexpression reduces proliferation of SW480 cells (22). We used these cells to test whether SOCS3 could prevent the increase in proliferation resulting from TNFR2 overexpression. SOCS3 overexpression reduced [3H]-thymidine incorporation in SW480 cells transfected with empty vector or TNFR2 expression construct (Fig. 5C). However, the magnitude of the increase in [3H]-thymidine incorporation resulting from TNFR2 overexpression was similar in SOCS3 overexpressing and control cells suggesting that SOCS3 overexpression cannot reverse TNFR2-associated increases in DNA synthesis.

SOCS3 overexpression had more potent effects on anchorage-independent growth of COLO205 cells. COLO205 cells overexpressing TNFR2 showed a small but significant increase in colony formation when compared with empty vector controls. SOCS3 overexpression dramatically decreased (>70%) colony formation in both empty vector and TNFR2 overexpressing cells (Fig. 5D and E).

**Increased TNFR2 immunostaining in colon tumors of mice with IEC-specific deletion of SOCS3**

Previously published work showed a dramatic increase in AOM/DSS-induced tumor number and size in mice with villin-cre (VC)-mediated SOCS3 gene disruption in IEC (VC-SOCS3fl/fl) compared with controls with floxed but intact SOCS3 genes (WT-SOCS3fl/fl). We therefore assessed TNFR2 expression in colon of these AOM/DSS-treated mice. In normal colon tissue, TNFR2 immunostaining was weak in both WT-SOCS3fl/fl and VC-SOCS3fl/fl mice (Fig. 6A–F). Only VC-SOCS3fl/fl mice showed histologically detectable tumors, and these tumors showed dramatic increases in TNFR2 immunostaining (Fig. 6G–I). Thus, the increase in tumor load in VC-SOCS3fl/fl mice is associated with increased expression of TNFR2 within the tumors.

**Short-term SOCS3 knockdown is not sufficient to upregulate TNFR2**

We used Caco2 colon cancer cells, which are known to have higher basal SOCS3 expression than SW480 and COLO205 cells and show cytokine-mediated upregulation of SOCS3 (ref. 30 and unpublished data), to test whether SOCS3 knockdown was sufficient to increase basal or cytokine-stimulated TNFR2 expression. Caco2 cells were transfected with control or SOCS3 siRNA followed by cytokine treatment for 24 hours. As shown in Supplementary Fig. S1A, SOCS3 siRNA treatment reduced the basal and cytokine-induced levels of SOCS3 mRNA, but we were unable to completely abolish the increase in SOCS3 mRNA resulting from cytokine treatment. SOCS3 siRNA did not enhance but rather reduced TNFR2 expression in these cells (Supplementary Fig. S1B). Thus, while SOCS3 overexpression is sufficient to inhibit basal and TNFR2 overexpression, short-term knockdown of SOCS3 expression was itself not sufficient to increase basal or cytokine-stimulated TNFR2 expression.

**Discussion**

The etiology of inflammation-associated CRC is based strongly on the model that chronically upregulated cytokines drive excessive proliferation of IECs, tumor initiation, and progression. TNFR2 has recently emerged as a proprolifera-
tive factor that is upregulated in IBD and in the AOM/DSS model of IBD-associated cancer (14, 16). Mechanisms regulating TNFR2 expression in IBD or CRC are not fully defined, although prior studies suggest that combined effects of IL-6 and TNFα promote TNFR2 expression (16). The current study provides novel evidence that IL-6 and TNFα act predominantly through STAT3 and a consensus STAT3-binding site in the TNFR2 promoter to induce TNFR2 in CRC. We also show that SOCS3 overexpression inhibits cytokine induction of TNFR2 and STAT3 binding to this STAT3 consensus site and can dramatically decrease anchorage-independent growth of colon cancer cells, even those overexpressing TNFR2.

Mizoguchi and colleagues provided the first evidence that TNFR2 was upregulated during acute DSS-colitis and that this was preceded by IL-6/STAT3 activation. They also showed that mice with global TNFR2 gene disruption exhibited decreased IEC proliferation in the T-cell receptor α (TCRα)-null model of colitis (16). Furthermore, TCRα mice with disruption of both IL-6 alleles showed reduced colitis severity and decreased TNFR2 expression compared with TCRα mice with intact IL-6 (16). While these studies suggested an association between IL-6/STAT3 and TNFR2, the ability of STAT3 to directly regulate TNFR2 expression has not been previously reported. Prior in vitro studies indicated that both IL-6 and TNFα were required to induce TNFR2 in CRC cells, suggesting that TNFR2 induction requires a specific microenvironment of multiple cytokines,
as found in IBD or IBD-associated CRC. The current study confirmed induction of TNFR2 mRNA and protein by combined IL-6 and TNFα in 2 different colon cancer cell lines. Importantly, we provide novel evidence for a model of IL-6 and TNFα interaction in regulating TNFR2 in colon cancer cells (Fig. 7). Our studies show a predominant role of STAT3 in TNFR2 induction by IL-6 and TNFα, which involves cooperative effects of IL-6 and TNFα to induce
STAT3 binding to a consensus element within the TNFR2 promoter. Our studies also reveal that TNFα acts through NFκB to induce endogenous IL-6 and promote TNFR2 induction via autocrine effects of IL-6 (Fig. 7).

The TNFR2 promoter contains 2 putative STAT-binding sequences and NFκB-binding sequences (20), but functional effects of these binding elements have not been reported. Because TNFα typically activates NFκB and IL-6 typically activates STAT3, we hypothesized that IL-6 and TNFα induction of TNFR2 would be mediated by activation of both of these transcription factors. We provide several independent pieces of evidence to indicate that STAT3, rather than NFκB, is the predominant mediator of TNFR2 induction by IL-6 and TNFα. Specifically, STAT3 inhibition reduced basal TNFR2 expression and completely reversed the induction of TNFR2 by IL-6 and TNFα. In contrast, NFκB inhibition had no effect on basal TNFR2 expression and only nonsignificantly reduced induction of TNFR2 by combined IL-6 and TNFα. This was despite data verifying that the NFκB inhibitor potently and completely reversed cytokine induction of ICAM-1 mRNA, whose expression is known to be dependent on NFκB. Importantly, combined STAT3 and NFκB inhibitors did not further reduce basal or cytokine-induced TNFR2 expression compared with STAT3 inhibitor alone. Constitutively activated STAT3 was able to induce TNFR2 to a similar extent as IL-6 and TNFα. Together these findings indicate a predominant role of STAT3 in mediating TNFR2 induction and show that STAT3 activation alone is sufficient to mimic cytokine effects on TNFR2 expression.

ChIP assays also confirmed that IL-6 and TNFα-induced STAT3 binding to two putative STAT3-binding sites on TNFR2 promoter but had no effect on NFκB binding. This was despite the fact that TNFα is known to induce phosphorylation of NFκB in this same cell system (22). It is also notable that combined IL-6 and TNFα more potently induced STAT3 binding to the −1,578 binding site in the TNFR2 promoter than the −364 site. Interestingly, the −1,578 STAT3-binding site also showed dramatic cooperative effects of IL-6 and TNFα to induce STAT3 binding, whereas either cytokine alone only modestly induced STAT3 binding to this site. These combinatorial effects of IL-6 and TNFα to dramatically enhance STAT3 binding to the −1,578 site were not associated with effects of combined IL-6 and TNFα to increase tyrosine phosphorylation or nuclear levels of STAT3. Collectively, these observations provide compelling evidence that IL-6 and TNFα interact to promote maximal STAT3 binding to the TNFR2 promoter and TNFR2 induction, and that this cooperative effect appears to occur primarily at the −1,578 STAT-binding site. The specific mechanisms by which TNFα promotes STAT3

![Figure 6. Increased TNFR2 immunostaining in colon tumors from mice with VC-mediated SOCS3 gene deletion in IEC cells.](image-url)

Immunohistochemical analysis of TNFR2 in nontumor tissue from WT-SOCS3+/+ (B and E) and VC-SOCS3−/− (C and F) mice compared with negative control, which had no effect on basal TNFR2 expression and only modestly induced STAT3 binding. These combinatorial effects of IL-6 and TNFα to dramatically enhance STAT3 binding to the −1,578 site were not associated with effects of combined IL-6 and TNFα to increase tyrosine phosphorylation or nuclear levels of STAT3. Collectively, these observations provide compelling evidence that IL-6 and TNFα interact to promote maximal STAT3 binding to the TNFR2 promoter and TNFR2 induction, and that this cooperative effect appears to occur primarily at the −1,578 STAT-binding site. The specific mechanisms by which TNFα promotes STAT3
binding to the TNFR2 promoter remain undefined and will require further study. We cannot rule out the possibility that NFκB binds to other regions in the TNFR2 gene than the consensus sites tested, but observations that IL-6 and TNFα did not induce NFκB binding to these NFκB consensus sites and the minimal effects of NFκB inhibitor on basal or IL-6 + TNFα–induced TNFR2 expression support a novel mechanism of TNFα and IL-6 interaction to induce TNFR2 by predominant effects on STAT3.

Recent and increasing evidence implicates TNFR2 as a mediator of colitis-associated cancer. TNFR2 has been shown to increase migration of colon cancer cell lines and is upregulated in mouse models and patients with IBD (16, 17). In addition, disruption of TNFR2 genes was associated with decreased proliferation of crypt epithelial cells in colitis models (16, 17). Recent studies in the AOM/DSS model of inflammation-associated CRC revealed that TNFR2 is preferentially upregulated over TNFR1 and that treatment with the anti-TNFα monoclonal antibody MP6-XT22 reduced the number and size of tumors, although colitis severity was unchanged (14). In a separate study, anti-TNFα antibodies given at late stages of the AOM/DSS model reduced tumor load (9). To our knowledge, a direct effect of TNFR2 on CRC proliferation or transformed phenotype has not been previously shown. Our current study shows in 2 independent colon cancer cell lines that TNFR2 overexpression directly enhances proliferation. In COLO205 cells, TNFR2 overexpression increased anchorage-independent growth. Although effects of TNFR2 overexpression on proliferation and anchorage-independent growth might be considered relatively modest, it is important to emphasize that the colon cancer cell lines used exhibit high rates of basal proliferation and are generally refractory to increases in growth in response to exogenous stimuli. Indeed, we have examined effects of serum deprivation and serum supplementation on these cells and serum, typically a potent growth inducer, does not significantly increase proliferation (unpublished observations). Thus, even the small increases in proliferation and anchorage-independent growth support a direct effect of TNFR2 to promote colon cancer cell proliferation and the concept that TNFR2 is a protumorigenic factor. This is further supported by data showing that siRNA-mediated TNFR2 knockdown modestly decreased proliferation of multiple colon cancer cell lines. A limitation of our study is that the impact of TNFR2 knockdown on cancer cell proliferation was modest. This may reflect the fact that despite testing of multiple siRNAs, the maximum knockdown achieved was 60%, which may reflect the existence of multiple TNFR2 splice variants (34). Future experiments will be required to definitively establish the role of normal levels of endogenously expressed TNFR2 in IEC or colon cancer cell proliferation.

SOCS3 is induced by cytokines, is a known endogenous negative feedback inhibitor of STAT3, and is epigenetically silenced in lung, liver, and squamous cell cancers or cancer cell lines (25, 46–49). Our previous study showed that IEC-specific deletion of SOCS3 led to an increase in tumor load in the AOM/DSS model, supporting the hypothesis that SOCS3 may act as a suppressor of colitis-associated cancer (22). This same study revealed that loss of IEC-SOCS3 resulted in enhanced activation of both STAT3 and NFκB. We report here that SOCS3 overexpression limits TNFR2 expression in colon cancer cell lines and limits STAT3 binding to −1,578 consensus element in the TNFR2 promoter. A predominant effect of SOCS3 on the −1,578 site versus −364 STAT3–binding site further supports a critical role of this region of the TNFR2 promoter in regulation of TNFR2 expression levels. SOCS3 overexpression reduced proliferation of colon cancer cells and dramatically decreased anchorage-independent growth. Also, increased TNFR2 was observed in tumors that develop in AOM/DSS-treated mice with specific SOCS3 gene disruption in IECs. Together, these findings support a role of SOCS3 as a potent inhibitor of colon cancer cell growth and suggest that these effects may, at least in part, involve the ability of SOCS3 to limit cytokine-induced TNFR2 expression. The findings also indicate that loss of SOCS3 in IECs may promote colon tumors by facilitating increases in cytokine-induced TNFR2 expression. The findings also indicate that loss of SOCS3 in IECs may promote colon tumors by facilitating increases in cytokine-induced TNFR2 expression in a setting of chronic intestinal inflammation. However, short-term siRNA-mediated reductions in basal or cytokine-induced SOCS3 mRNA did not enhance TNFR2 expression, indicating that SOCS3 knockdown alone or in the short term is itself not sufficient to cause increased TNFR2 expression.

Anti-TNFα therapies are widely used in the treatment of human IBD (50–54). The effect of anti-TNFα therapy on risk of colitis-associated cancer or other cancers is not well defined and is a topic of intensive investigation (55, 56). Our findings that TNFα cooperates with IL-6 to induce TNFR2, an inducer of colon cancer cell growth, suggests that TNFR2 may prove useful as a biomarker of the potential effects of...
anti-TNFα on colitis-associated cancer risk or could represent a specific target to decrease colon cancer risk in IBD. Indeed, increased circulating TNFR2 was recently reported to be associated with enhanced CRC risk in humans, and those with higher plasma TNFR2 exhibit reduced CRC risk with use of anti-inflammatory drugs (57). Furthermore, the finding that STAT3 is a mediator of TNFR2 induction by combined TNFα and IL-6 adds to the growing evidence for STAT3 as a key mediator of colitis-associated cancer and supports further investigation of STAT3 inhibitors as potential cancer therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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