

STAT2 Contributes to Promotion of Colorectal and Skin Carcinogenesis

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Abstract

Signal transducer and activator of transcription 2 (STAT2) is an essential transcription factor in the type I IFN (IFN- α/β) signal transduction pathway and known for its role in mediating antiviral immunity and cell growth inhibition. Unlike other members of the STAT family, IFNs are the only cytokines known to date that can activate STAT2. Given the inflammatory and antiproliferative dual nature of IFNs, we hypothesized that STAT2 prevents inflammation-induced colorectal and skin carcinogenesis by altering the inflammatory immune response. Contrary to our hypothesis, deletion of STAT2 inhibited azoxymethane/dextran sodium sulfate-induced colorectal carcinogenesis as measured by prolonged survival, lower adenoma incidence, smaller polyps, and less chronic inflammation. STAT2 deficiency also inhibited 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate-induced skin carcinogenesis as indicated by reduced papilloma multiplicity. A potential mechanism by which STAT2 promotes carcinogenesis is through activation of proinflammatory mediators. Deletion of STAT2 decreased azoxymethane/dextran sodium sulfate-induced expression and release of proinflammatory mediators, such as interleukin-6 and CCL2, and decreased interleukin-6 release from skin carcinoma cells, which then decreased STAT3 activation. Our findings identify STAT2 as a novel contributor to colorectal and skin carcinogenesis that may act to increase the gene expression and secretion of proinflammatory mediators, which in turn activate the oncogenic STAT3 signaling pathway. *Cancer Prev Res*; 3(4); 495–504. ©2010 AACR.

Introduction

Local chronic inflammation is believed to be a contributing component in the malignant transformation of the intestinal and skin epithelium (1). Patients suffering from chronic inflammatory bowel disease, such as ulcerative colitis and Crohn's disease, are at a higher risk of developing colorectal cancer (2). However, the molecular pathogenesis of colorectal cancer is poorly

understood. Local chronic inflammation can alter the homeostasis of the intestinal microenvironment via persistent recruitment of immune cells and by prolonging the activation of NF- κ B and interleukin-1 (IL-1) receptor signaling pathways (3–6). Inflammatory cytokines, such as tumor necrosis factor- α , IL-1, IL-6, and IFN- γ , create a microenvironment that promotes colorectal and skin carcinogenesis by enhancing cell proliferation and angiogenesis (7–12).

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The Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is an additional signal transduction pathway important in regulating proinflammatory and anti-inflammatory responses. Activation of this pathway is closely linked to inflammatory bowel disease. Among the cytokines that can activate the JAK/STAT pathway, IFN- γ plays an indispensable role in experimental inflammatory bowel disease and spontaneous development of colorectal carcinomas in mice (10, 13). In contrast, IFN- α/β is protective in dextran sodium sulfate (DSS)-induced colitis (14) and when administered to ulcerative colitis patients (15).

STAT2 is a necessary transcription factor in the IFN- α/β signaling pathway (16). Unlike other members of the STAT family that are activated by multiple growth factors and cytokines, thus far, only IFN- α/β activates STAT2 (17). IFN- α/β exerts antiproliferative, immunomodulatory, and antiviral effects via a complex consisting of STAT1, STAT2, and IRF-9, which enters the nucleus and binds in the

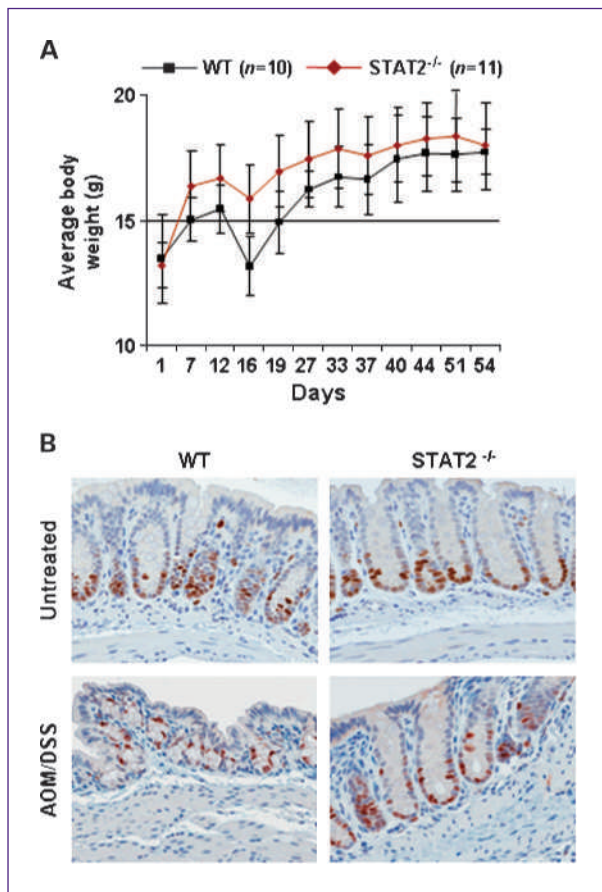


Fig. 1. STAT2 deletion attenuates sensitivity to AOM/DSS-induced colorectal carcinogenesis. Age-matched WT mice ($n = 10$) and STAT2^{-/-} mice ($n = 11$) were i.p. injected with AOM followed by three cycles of 3% DSS in drinking water for 5 d followed by 16 d of normal drinking water and weighed two to three times weekly. A, attenuated weight loss (an indicator of colon inflammation) after first DSS cycle in STAT2^{-/-} mice. B, less crypt destruction and inflammatory infiltrate in STAT2^{-/-} mice after the first DSS cycle. Colon sections were stained for Ki67-positive cells within well-oriented crypts (stained brown) and counterstained with hematoxylin (blue) to visualize the whole tissue. At least two slides from each mouse (two mice per group) were analyzed.

promoter region of IFN-stimulated genes (18). Studies using STAT2-deficient cell lines and mice showed that STAT2 is necessary for the antiviral, apoptotic, and cell growth-inhibitory effects of IFN- α/β (19–21). Yet, the role of STAT2 in inflammation and tumorigenesis is largely unknown. STAT2^{-/-} mice expressing an IFN- α transgene in the central nervous system died prematurely with neuroblastomas and had excessive production of the proinflammatory cytokine IFN- γ (22). Lymphocytes from colons of ulcerative colitis and Crohn's disease patients had decreased STAT2 protein levels (23). Thus, we hypothesized that STAT2 prevents carcinogenesis by modulating the inflammatory immune response.

We used STAT2^{-/-} mice in two-stage models of inflammation-induced colorectal and skin carcinogenesis and showed

that, contrary to our hypothesis, STAT2 was required for promotion of colorectal and skin carcinogenesis. A potential mechanism is via a STAT2-dependent increase in the gene expression and secretion of proinflammatory mediators such as IL-6, which then activate the oncogenic STAT3 signaling pathway.

Materials and Methods

Animals

STAT2-deficient (STAT2^{-/-}) mice were generated and kindly provided by Dr. Christian Schindler (Columbia University, New York, NY; ref. 24). Strain-matched wild-type (WT) mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick (Frederick, MD). All mice were on a 129SvJ genetic background. Mice were bred in our own animal facility and housed under specific *Helicobacter* pathogen-free environment. These studies were done in accordance to the National Cancer Institute-Frederick Animal Care and Use Committee guidelines.

Colorectal carcinogenesis model

To examine the effect of STAT2 on tumor incidence and multiplicity, 10 WT and 11 STAT2^{-/-} female mice were i.p. injected at 6 wk of age with the carcinogen azoxymethane (AOM) at 10 mg/kg body weight. One week later, the mice were started on the first of three 21-d DSS cycles, consisting of receiving for 5 d the tumor promoter DSS at 3% in the drinking water followed by 16 d of receiving regular water. Body weights of mice were measured weekly. Mice showing signs of morbidity were sacrificed. The remaining mice were sacrificed 16 wk after AOM induction and 58 d after removal from DSS in the water. Colons were removed and flushed with PBS buffer and cut longitudinally. The entire colons from all 21 mice were examined by experienced histotechnicians using a dissecting microscope, and all visible lesions were counted and measured, fixed in 10% neutral-buffered formalin, and embedded in paraffin. A board-certified pathologist examined H&E-stained tissue sections containing the largest and the smallest proliferative lesion of each mouse (32% of the 129 colon lesions) to confirm the specificity of macroscopic count and to evaluate tumor progression according to Boivin et al. (25).

To examine the early stages of colorectal carcinogenesis, the same protocol was used except mice were sacrificed after 5 d within the first DSS cycle. Age-matched WT and STAT2^{-/-} female mice not subjected to the colorectal carcinogenesis protocol were used as normal controls. Colon tissue sections were either paraffin embedded for immunohistochemistry, snap frozen for RNA analysis, or used for protein analysis or colon tissue culture.

Immunohistochemistry of colon tissue

The effect of STAT2 on proliferation and cell crypt structure in early stages of colorectal carcinogenesis was measured by detection of Ki67 antigen in colon tissue sections of two mice per group. The effect of STAT2 on STAT3 activation was

measured by detection of Tyr⁷⁰⁵-phosphorylated STAT3 in adenoma-containing colonic tissue collected from WT and STAT2^{-/-} mice 16 wk after AOM induction. Embedded colon sections were deparaffinized and rehydrated, and antigen was retrieved in a pressure cooker for 4 min in 0.01 mol/L citric acid (pH 6.0). Sections were then cooled for 25 min followed by pretreatment with 1.5% hydrogen peroxide dissolved in methanol for 10 min. After blocking with 10% normal goat serum, sections were incubated with anti-Ki67 antibody (1:10,000; Vector Laboratories) or with anti-phospho-STAT3-Y705 (1:50; Cell Signaling) for 1 h at room temperature. After incubation with biotin-conjugated secondary antibody and streptavidin-horseradish peroxidase, signals of Ki67-stained and phospho-STAT3-stained nuclei were detected by using Vector Elite kit (Vector Laboratories).

RNA expression analysis of colon tissue

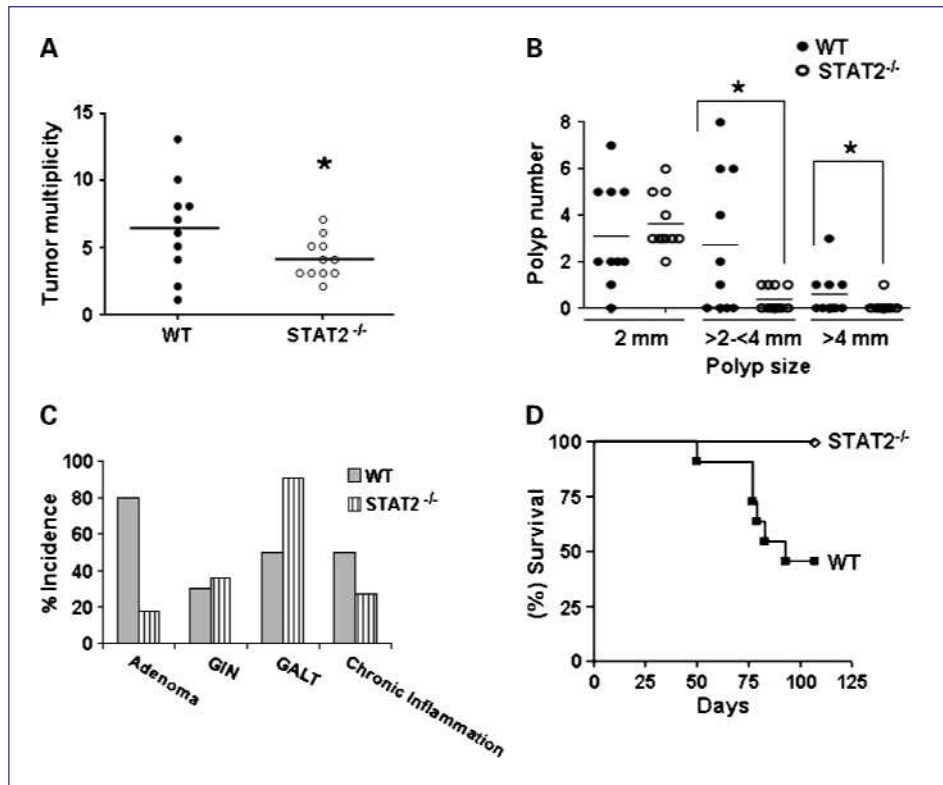
RNA of colon tissue sections was quantified from four individual mice per AOM/DSS-treated group and two individual mice per untreated group. RNA was isolated from colon tissue section using a combination of the Mini-Beadbeater (BioSpec) to disrupt and homogenize samples and the RNeasy Mini kit (Qiagen). All samples were DNase treated during the on-column RNA isolation steps to eliminate genomic DNA contamination. The RNA Cleanup kit (Qiagen) was used to purify RNA samples if needed. Total RNA (2 µg) was reverse transcribed using the Super-

Array RT² First Strand kit (SABiosciences). The resulting cDNAs were analyzed by quantitative PCR using the Mouse Signal Transduction and the Stress and Toxicity RT² Profiler PCR Arrays (SABiosciences). PCR was done on Bio-Rad iQ5 cyclor according to the manufacturer's instructions.

Protein analysis of colon tissue

Colon cell lysates were prepared from three individual mice per group. Colons were flushed in cold PBS supplemented with 30 units/mL penicillin and 30 µg/mL streptomycin (Invitrogen), cut longitudinally, and then cut into pieces of 200 to 300 mg. Individual colon pieces were placed in a 12-well flat tissue culture plate and covered with 2 mL of serum-free RPMI 1640 supplemented with high doses of penicillin and streptomycin. Colon tissue was incubated at 37°C for 24 h and then digested for 2 h at 37°C in a shaking bath in a solution of 1× PBS containing 10% FCS, 15 mmol/L HEPES, 5 mmol/L EDTA, antibiotics, collagenase (50×), and DNase (100×). Collected cells were filtered, washed, and lysed in ice-cold lysis buffer previously described (20). After centrifugation at 4°C for 10 min, lysate was collected and proteins were resolved by SDS-PAGE (Invitrogen). Proteins were transferred to a polyvinylidene difluoride membrane, which was blocked for 30 min with Blocker casein in TBS (Pierce) and then blotted with phospho-STAT1-Y701 (BD Biosciences Pharmingen), cyclooxygenase-2 (Cayman Chemical), IRF-1 (M-20) and STAT2 (Santa Cruz Biotechnology),

Fig. 2. STAT2 deletion decreases colon tumor development and progression and improves long-term survival in response to AOM/DSS-induced colorectal carcinogenesis. Age-matched WT mice (*n* = 10) and STAT2^{-/-} mice (*n* = 11) were i.p. injected with AOM followed by three cycles of DSS. Number of colon lesions was counted using a dissecting microscope in all 21 mice. A, decreased number of polyps in STAT2^{-/-} mice. B, decreased size of tumors in STAT2^{-/-} mice. C, decreased tumor progression in STAT2^{-/-} mice. Histology of tumor sections of two representative tumors from each mouse was done. GIN, gastrointestinal intraepithelial neoplasia. D, improved long-term survival in STAT2^{-/-} mice. *, *P* ≤ 0.05.



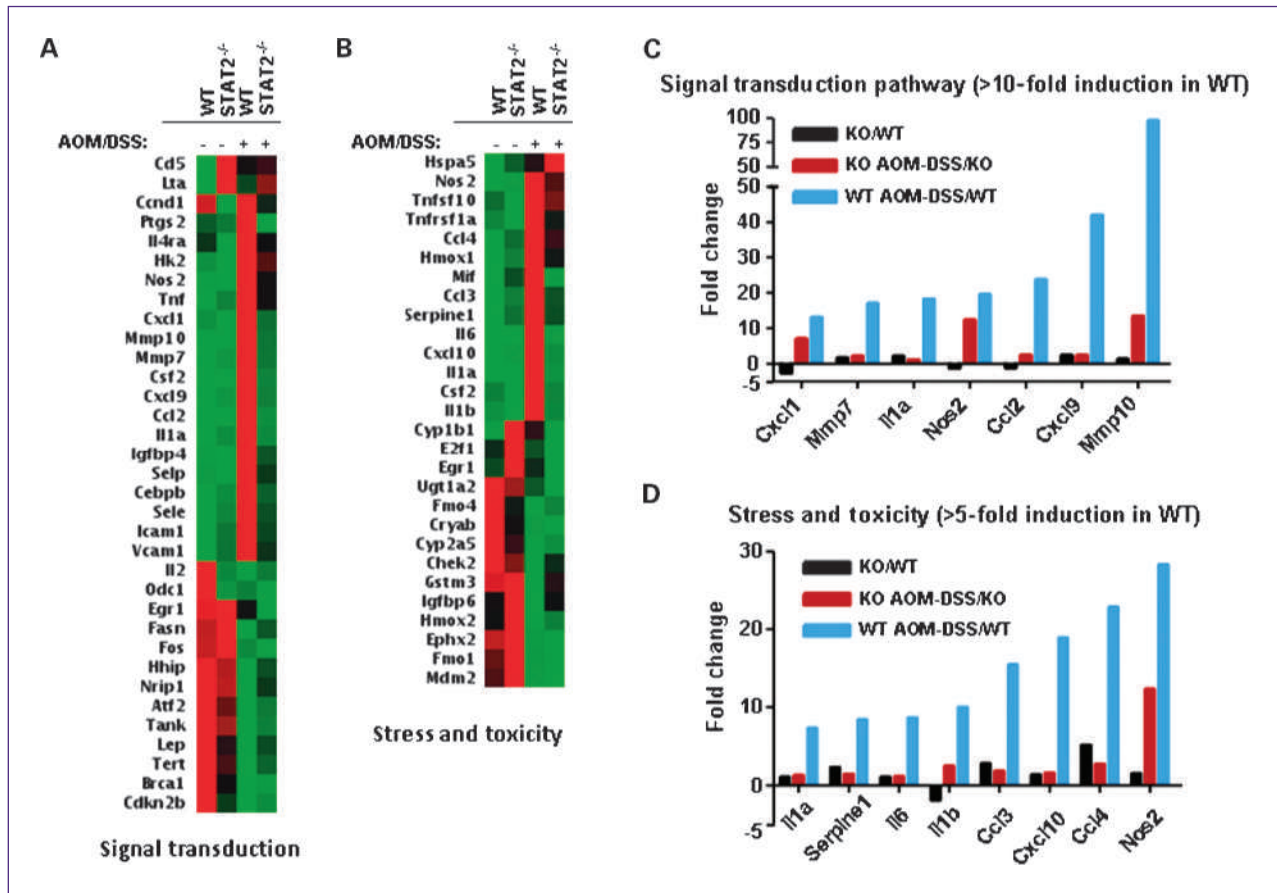


Fig. 3. STAT2 deletion attenuates the proinflammatory gene response to AOM/DSS. RNA was isolated from colons of AOM/DSS-treated mice ($n = 4$) at the end of the first DSS treatment. Untreated mice ($n = 2$) served as controls. Reverse transcription-PCR focused pathway arrays, signal transduction (A and C) and stress and toxicity (B and D), were used for evaluating changes in gene expression of a subset of genes. Cluster maps (A and B) depict genes that varied significantly ($P < 0.05$) between one or more groups of mice. Green depicts lower expression and red depicts higher expression. Graphs (C and D) show a subset of genes that were induced >10 -fold (C) and >5 -fold (D) in the WT mice treated with AOM/DSS compared with the untreated WT mice (blue bars). Black bars show the difference between STAT2^{-/-} [knockout (KO)] and WT untreated mice. Red bars show the effects of AOM/DSS on the KO mice relative to the untreated KO mice.

and actin (Abcam). Following incubation with horseradish peroxidase-conjugated secondary antibody, membranes were washed and developed by chemiluminescence using the ECL Western blotting system (Pierce). Total protein concentrations were measured by a standard Bio-Rad Bradford protein assay.

Cytokine production of colon tissue culture

Cytokine release in early stages of colorectal carcinogenesis was quantified in mouse colons. Colons were flushed in cold PBS supplemented with 30 units/mL penicillin and 30 μ g/mL streptomycin (Invitrogen), cut longitudinally, and then cut into pieces of 200 to 300 mg. Individual colon pieces were placed in a 12-well flat tissue culture plate and covered with 2 mL of serum-free RPMI 1640 supplemented with penicillin and streptomycin. Culture plates were incubated at 37°C for 24 h. Supernatants were collected, centrifuged at 14,000 rpm at 4°C for 5 min, and stored at -80°C. Cytokine and chemokine concentrations

in the supernatant were measured using the mouse inflammation cytometric bead array kit (BD Biosciences) according to the manufacturer's instructions.

Skin carcinogenesis model

The biological implication of STAT2 in papilloma incidence and multiplicity was assessed by applying 102 μ g of 7,12-dimethylbenz(a)anthracene (DMBA) in 0.2 mL acetone to the shaved dorsal skin area of WT and STAT2^{-/-} female mice ($n = 10$ per group) at 7 wk of age. Starting 2 wk after initiation, 6 μ g of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in acetone were applied to the same skin area twice weekly for 20 wk. Mice were visually examined weekly for skin papilloma and squamous cell carcinoma number and size. Papillomas were counted when lesions were palpable. Only tumors that reached at least 2 mm in diameter and had been present for at least 2 wk were included in the analysis. Mice with papillomas that had progressed to carcinoma

were euthanized. Carcinomas were harvested to establish tumor cell lines.

Cytokine production and detection of active STAT3 in skin carcinoma cell lines

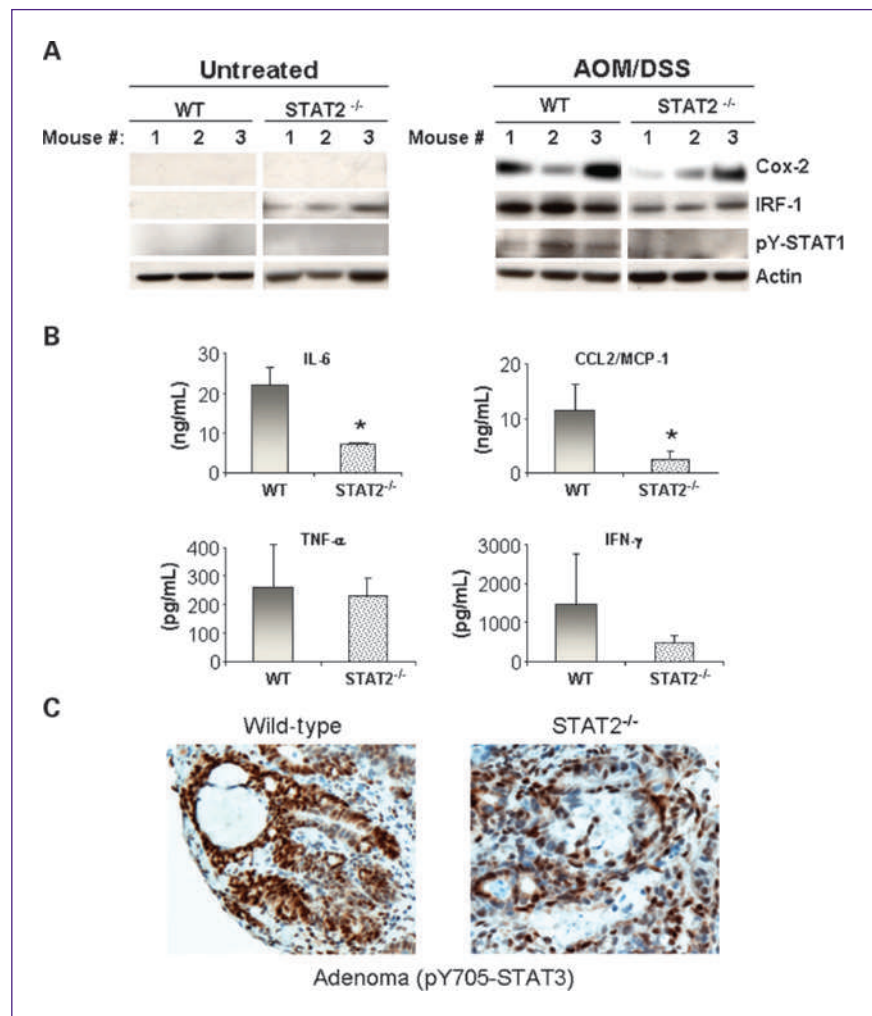
Skin carcinoma cell lines were established from carcinomas induced on WT (LD791) and STAT2^{-/-} (AG1279) mice by adapting a protocol used for establishing melanoma cell lines (26). Briefly, freshly excised tumors were minced into small pieces and allowed to adhere to collagen-coated plates in MEL 2% medium (80% MCDB 131 medium, 20% L-15 medium, 2% fetal bovine serum, 5 µg/mL bovine insulin, 15 µg/mL bovine pituitary extract, 5 ng/mL epidermal growth factor and 1.68 mmol/L, 10 units/mL penicillin, 10 µg/mL streptomycin, and 2.2 g of GlutaMax). Once monolayers were obtained, cells were trypsinized and passaged onto regular plastic dishes with 10% FCS containing DMEM supplemented with antibiotics and GlutaMax. The AG1279 skin tumor cell line was later stably reconstituted with human STAT2. A flag-tagged human STAT2 construct in pCDNA3 mammalian

expression vector whose expression is under the control of a cytomegalovirus promoter was kindly provided by C. Horvath (Northwestern University, Evanston, IL). Cells were transfected using Lipofectamine 2000 (Invitrogen) in combination with either 5 µg of a pCDNA3 empty vector (negative control) or pCDNA3 encoding STAT2. Following an overnight incubation, cells were placed under selection with 10 µg/mL puromycin (Sigma). STAT2 and tyrosine-phosphorylated STAT3 expression of the tumor cell lines was assessed by Western blot analysis as previously described. Additionally, cells were treated or not with exogenous murine IL-6 (PeproTech, Inc.) and analyzed for phospho-STAT3-Y705. Actin was used to monitor for equal protein loading. IL-6 and CCL2/MCP-1 concentrations were measured using the mouse inflammation cytometric bead array kit according to the manufacturer's instructions. The measurements were repeated twice with three replications each.

Statistical analysis

Student's *t* test was used to compare body weight, RNA, and protein data of two groups, and generalized least

Fig. 4. STAT2 deletion attenuates activation of the IFN- α/β signaling pathway and secretion of IL-6 and CCL2/MCP-1 in response to AOM/DSS. A, colons were harvested and whole-cell extracts were prepared from AOM/DSS-treated and untreated individual mice ($n = 3$) after 24-h incubation in medium and resolved by SDS-PAGE analysis. Cyclooxygenase-2 (Cox-2), IRF-1, and phospho-STAT1-Y701 were detected by Western blot analysis. Actin was used to monitor for equal loading of protein. B, same as in A, except supernatants of overnight incubated colons ($n = 4$) were collected. Detection of inflammatory cytokines was measured by multiplex mouse inflammatory flow cytometric analysis. Values are presented as mean \pm SE taken from individual mice. This experiment was done twice, four mice per group. *, $P < 0.05$. TNF- α , tumor necrosis factor- α . C, sections with adenomatous tissue of representative colons from WT mice (left) and STAT2^{-/-} mice (right) were stained for phospho-STAT3-Y705. Immunostaining was scored on a 0 to 3 scale (3 being the highest intensity). WT adenomas were scored 2 to 3, whereas STAT2^{-/-} were scored 1 to 2.



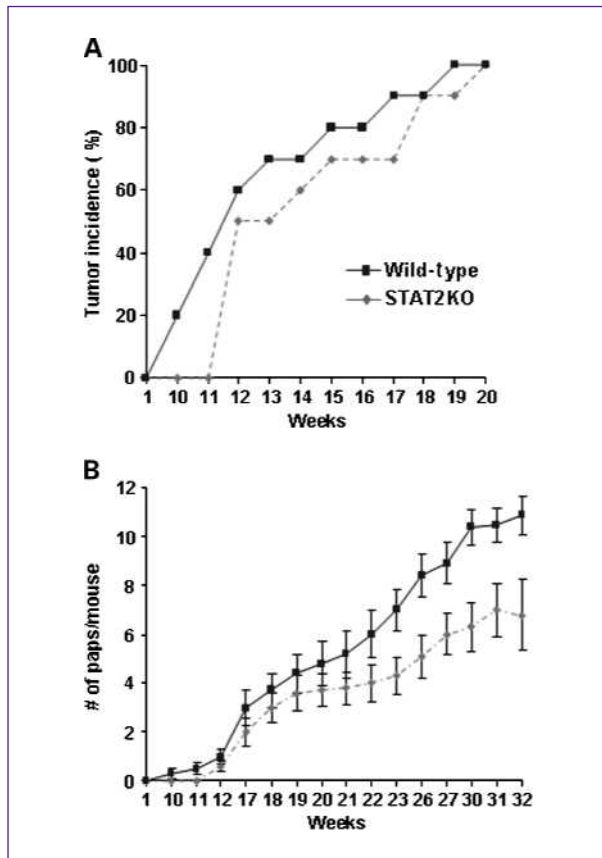


Fig. 5. STAT2 deletion attenuates DMBA/TPA-induced skin carcinogenesis. Age-matched WT mice ($n = 10$) and STAT2^{-/-} mice ($n = 10$) were initiated with a single dose of DMBA (week 1) followed by tumor promoter TPA treatment for 20 wk, twice a week. X axis, number of weeks after the initial DMBA application. A, tumor incidence is the number of mice with palpable papillomas. B, number of papillomas per mouse scored weekly. Significant statistical differences were detected at week 11 in tumor incidence and from week 23 and forward for papilloma multiplicity ($P < 0.05$). Results represent mean \pm SE.

squares means were used to compare data of more than two groups. A logistic rank test was used to compare survival curves. Fisher's exact test was used to compare in all mice, including those that had to be prematurely sacrificed, tumor incidences and first appearance of skin papilloma number. The nonparametric Wilcoxon rank sum test was used to compare tumor multiplicity. For PCR array data analysis, the $\Delta\Delta C_t$ method was used and each gene fold change was calculated as difference in gene expression between naive and AOM/DSS colon samples. Data were normalized against five housekeeping genes that were included in the PCR array by subtracting the average C_t value for five housekeeping genes from the average C_t value of gene of interest (i.e., calculating a $\Delta\Delta C_t$). $\Delta\Delta C_t$ was compared across groups using t tests. Due to the large number of comparisons made in our analyses, differences in measurements of gene expression between groups were considered statistically significant when $P < 0.005$. All tests were two-sided except for tumor multiplicity. Significance was

declared at $P \leq 0.05$, and trends toward significance were declared at $P \leq 0.10$. Unadjusted means and SDs are presented.

Results

STAT2^{-/-} mice are less susceptible to AOM/DSS-induced colorectal carcinogenesis

Deletion of STAT2 protected the mice from the colon irritant DSS. STAT2^{-/-} mice lost less body weight after one DSS cycle (at 16 days, 5% versus 16% in WT mice; $P = 0.0004$; Fig. 1A). We observed in STAT2^{-/-} mice less inflammatory infiltrate after the first DSS cycle, less crypt destruction, and less uncontrolled proliferation with Ki67 staining in cells within well-oriented crypts (Fig. 1B). These changes are characteristic of an attenuated inflammatory response.

STAT2 deletion also decreased tumor multiplicity and size in AOM/DSS-treated mice. STAT2^{-/-} mice developed 1.6-fold fewer tumors than WT mice (6.4 versus 4.1 tumors per mouse; $P = 0.05$; Fig. 2A), and on average, the tumors were smaller (Fig. 2B). Furthermore, a loss of STAT2 inhibited tumor progression. In comparison with WT mice, fewer STAT2^{-/-} mice had histologically confirmed adenomas (18% versus 80%; $P = 0.009$), a similar number of STAT2^{-/-} mice had dysplastic lesions called gastrointestinal intraepithelial neoplasia (36% versus 30%), and more STAT2^{-/-} mice had hyperplastic gut-associated lymphoid tissue (GALT) lesions (91% versus 50%; $P = 0.06$; Fig. 2C; Supplementary Fig. S1). In addition, numerically fewer STAT2^{-/-} mice showed inflammation around the tumors than WT mice (27% versus 50%; Fig. 2C; Supplementary Fig. S1). No adenocarcinoma or flat lesions were observed. We did not observe consistent and distinct morphologic differences between STAT2^{-/-} and WT mice in normal or tumor tissue of the same grade (Supplementary Fig. S1). Consistent with the inhibited tumor development and progression, STAT2 deficiency enhanced survival. STAT2^{-/-} mice showed increased long-term survival at the end of the 16-week study, in which all of these mice ($n = 11$) remained viable, whereas only 40% (4 of 10) of WT mice survived ($P = 0.003$; Fig. 2D). Collectively, these results suggest that STAT2 plays an active role in tumor development and tumor progression.

STAT2^{-/-} mice have an attenuated proinflammatory gene response to AOM/DSS

To discover possible mechanisms for how the STAT2 transcription factor regulates inflammation-induced colorectal carcinogenesis, we did pathway-focused reverse transcription-PCR array analysis using the signal transduction and stress and toxicity arrays with colons from two untreated and four AOM/DSS-treated WT and STAT2^{-/-} mice per group. Ablation of the STAT2 gene was associated with a slight change in gene expression (<3-fold) of only a few genes (8 of 136; Fig. 3A and B). As expected, AOM/DSS treatment induced the expression of multiple genes in the WT mice and deletion of STAT2 attenuated some of

these changes. STAT2 deletion predominantly attenuated gene expression that was induced by AOM/DSS treatment and had less of an effect on gene expression that decreased when compared with WT mice. Closer examination shows that genes whose expressions were attenuated by STAT2 deletion are predominantly in the JAK/STAT and NF- κ B pathway in addition to genes involved in inflammation and angiogenesis (*MMP7* and *MMP10*; Fig. 3C and D). Our data suggest that STAT2 deletion attenuates the activation of inflammatory chemokines (*Ccl2*, *Ccl3*, *Ccl4*, *Cxcl9*, and *Cxcl10*) and cytokines (*IL-1a*, *IL-1b*, and *IL-6*) genes, which showed >5-fold induction in the WT mice. Our data also suggest that STAT2 deletion attenuates the activation of the procarcinogenic JAK/STAT (*Cxcl9*, *MMP10*, and *Nos2*) and NF- κ B (*Cxcl1*, *IL-1a*, and *Nos2*) pathways in response to a carcinogenic stimulus.

STAT2^{-/-} mice show attenuated activation of the IFN- α/β signaling pathway, reduced proinflammatory mediator secretion, and attenuated STAT3 activation in response to AOM/DSS

Consistent with the literature (23), STAT2 deficiency attenuated the DSS-induced increase in STAT1 activity (phosphorylation) and IRF-1 expression. Colon tissue of STAT2^{-/-} mice had, after the first DSS cycle, a smaller induction of cyclooxygenase-2 and IRF-1 and no increase in STAT1 activation compared with colon tissue of WT mice (Fig. 4A). These observations suggest that STAT2 deletion attenuates the activation of the antiproliferative and immunomodulatory IFN- α/β signaling pathway to a carcinogenic stimulus.

In agreement with Fig. 3, STAT2 deletion also attenuated the DSS-induced increase in the secretion of proinflammatory mediators. Supernatant of STAT2^{-/-} mouse colon tissue had, after the first DSS cycle, 2.5-fold lower IL-6 ($P = 0.04$) and 4.5-fold lower CCL2/MCP-1 ($P = 0.01$) concentrations than supernatant of WT mouse colon tissue, whereas no statistical differences in the concentrations of tumor necrosis factor- α and IFN- γ were noted (Fig. 4B). Concentrations of IL-10 and IL-12 were below detection levels (data not shown).

STAT3 is an oncogenic transcription factor activated by IL-6. STAT3 activation was evaluated in adenomas of WT and STAT2^{-/-} mice (Fig. 4C). Constitutive STAT3 activation was detected in adenomatous tissue of WT and STAT2^{-/-} mice, albeit at lower levels in the STAT2^{-/-} mice. The uninvolved normal colonic epithelium of WT and STAT2^{-/-} mice showed undetectable levels of activated STAT3 (data not shown). These data suggest that STAT2 deficiency attenuates and modulates the inflammatory response to a procarcinogenic stimulus via inhibition of STAT3 signaling.

STAT2^{-/-} mice are less sensitive to DMBA/TPA-induced skin carcinogenesis and release less IL-6 from their carcinomas

Similar to our results in the AOM/DSS colorectal model, STAT2 deletion attenuated DMBA/TPA-induced skin carci-

nogenesis. The onset of papilloma in the STAT2^{-/-} group was delayed by 2 weeks compared with the WT group (week 12 versus week 10, respectively; 0% versus 40% tumor incidence in week 11; $P = 0.03$; Fig. 5A). Whereas all of the mice eventually developed papillomas, STAT2^{-/-} mice had, throughout the study, fewer papillomas than the WT mice, with statistically significant differences starting at week 23 (4.3 versus 7 papillomas per mouse; Fig. 5B). The carcinoma incidence and papilloma morphology did not differ between STAT2^{-/-} and WT mice (data not shown).

Reconstitution of STAT2 in an established STAT2^{-/-} skin carcinoma cell line, as shown in Fig. 6A, increased activated STAT3 (Fig. 6B and C) and the secretion of IL-6 (Fig. 6D).

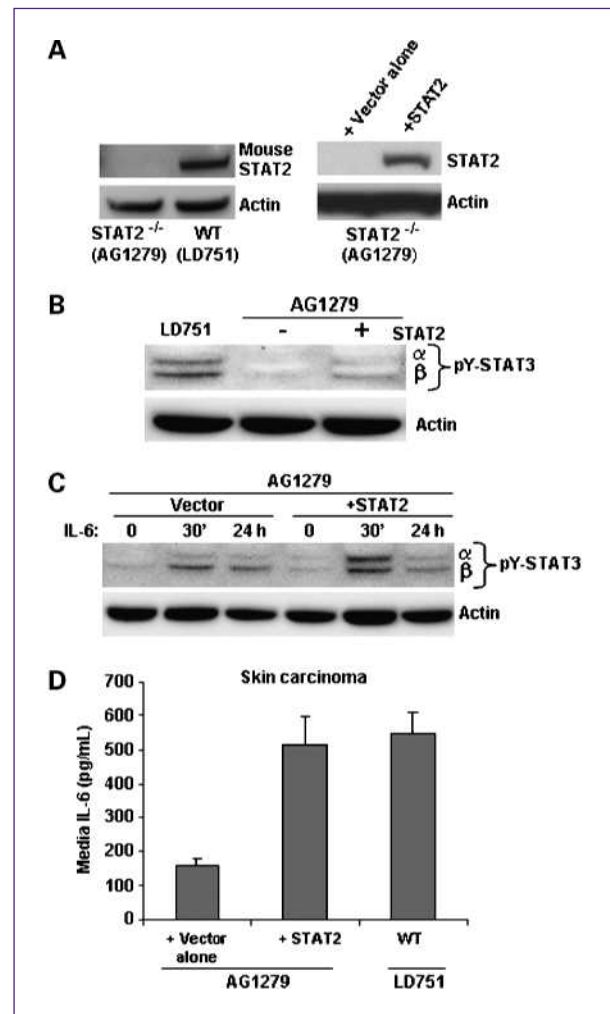


Fig. 6. STAT2-deficient skin carcinoma cells show reduced STAT3 activation and secrete less IL-6. A, STAT2 expression in cell lines from STAT2^{-/-} and WT mice (left) and from STAT2^{-/-} cells transfected with empty vector or STAT2 expression vector (right) was assessed by Western blot analysis. B, cells were examined for phospho-STAT3-Y705. C, cells were treated with IL-6 (100 ng/mL) and analyzed for phospho-STAT3-Y705. Actin was used to monitor for equal loading of protein. D, IL-6 concentrations were measured in supernatants collected from skin carcinoma cell lines. WT, LD751; STAT2^{-/-}, AG1279 and STAT2^{-/-} reconstituted with STAT2.

STAT2^{-/-} carcinoma cells had 3.5-fold lower IL-6 concentrations in the supernatant than STAT2-reconstituted cells, which in contrast had levels similar to those of the WT skin carcinoma (157 versus 551 pg/mL; $P = 0.0002$; 515 pg/mL; Fig. 6D), whereas CCL2/MCP-1 concentrations were not consistently affected (data not shown). Addition of exogenous IL-6 to STAT2^{-/-} cells induced STAT3 tyrosine phosphorylation (Fig. 6C), thus suggesting that activation of STAT3 is not directly STAT2 dependent but is mediated by IL-6. In summary, these observations suggest that STAT2 contributes to tumor development in inflammation-promoted carcinogenesis by increasing gene expression and release of proinflammatory mediators that in turn activate STAT3.

Discussion

Our study examined the role of STAT2 in inflammation-promoted colorectal and skin carcinogenesis (24). STAT2^{-/-} mice were protected from AOM/DSS-induced tumorigenesis as indicated by the decrease in colon tumor multiplicity, size, progression, and improved long-term survival. During the early stages of colorectal carcinogenesis, lack of STAT2 resulted in less severe weight loss, less crypt destruction, and, most notably, an attenuated increase in gene expression and secretion of proinflammatory mediators such as IL-6. We cannot exclude the possibility that AOM is metabolized differently in the STAT2^{-/-} mice, as this could have contributed to the colon tissue differences seen between the two strains of mice. However, these differences are unlikely because STAT2^{-/-} mice develop tumors, albeit less (our data), whereas WT 129/Sv do not develop tumors with AOM alone (27). Similarly, STAT2^{-/-} mice were protected from DMBA/TPA-induced skin papillomagenesis. During the late stages of skin carcinogenesis, STAT2 may have tumor-promoting effects, as overproduction of IL-6 by skin carcinoma cells that we established in our laboratory seemed to be STAT2 dependent. This is not surprising, as production of IL-6 has been shown previously to be required in skin carcinogenesis (28). Thus, our findings reveal an unexpected and first described role of STAT2 as a potential promoter of skin and colon tumorigenesis that may act to increase gene expression and release of proinflammatory mediators, most notably IL-6.

The use of genetically engineered mouse models of human cancer has provided new methods for studying cancer prevention (29). The creation of mice that are resistant to induced tumorigenesis not only validates the engineered target but also allows the discovery of new molecular targets that are active in the early stages of tumor development. For instance, genetically engineered mice that express TAM67, a dominant-negative inhibitor of activator protein-1, are resistant to chemical, UVB, and virus-induced skin tumorigenesis (30, 31) and oncogene-induced breast cancer (29). For these studies, a few TAM67-regulated genes have been identified and validated as candidate targets for cancer prevention

(32, 33). The observation that STAT2^{-/-} mice are resistant to inflammation-induced colon and skin tumorigenesis will allow us to identify new targets that are active in the early stages of colon and skin cancer, the first of which seems to be the proinflammatory cytokine IL-6.

The range of proinflammatory cytokines and other soluble factors produced by activated innate immune cells can trigger not only tumor promotion but also cell survival (1, 34–36). Activation of the JAK/STAT pathway-induced IFN- γ is thought to drive inflammation and has been linked to colorectal carcinogenesis (10, 13). In a different study, STAT2^{-/-} mice expressing an IFN- α transgene in the central nervous system produced excessive amounts of IFN- γ and developed neuroblastomas (22). In this model, one could postulate that the protective effect of STAT2 deletion was hampered by the high levels of IFN- γ and, therefore, contributed to tumor development. In contrast, IFN- α/β -activated pathways have been shown to elicit a protective effect not only during inflammation-induced colitis but also during the early events of squamous skin carcinogenesis (14, 15, 37). Yet, the mechanism of how this occurs remains largely unknown. One reasonable explanation could be that endogenously secreted IFN- α/β might antagonize stress- and antigen-dependent effector T-cell expansion through a STAT1/STAT2-dependent pathway (24, 38).

The molecular mechanism by which STAT2 deletion attenuates the increase in gene expression of proinflammatory mediators is unclear. Our data suggest that inactivation of the STAT2 signaling pathway confers protection in both early and later stages of colon carcinogenesis. A marked decrease in IL-6 production in the colons of STAT2^{-/-} mice could in part explain the less pronounced inflammatory response seen in these animals. Although IL-6 is needed for the mucosal integrity of the colon, it also promotes the proliferation of premalignant cells and drives tumor formation in the early stages of carcinogenesis via activation of the STAT3 signaling pathway (36). In our study, an accumulation of GALT in areas of less progressed tumors after AOM/DSS treatment is seen when STAT2 is absent. In this case, STAT2 and/or IL-6 may be required for regulation of GALT production. Moreover, lesions that progress to adenoma carry activated STAT3 irrespective of STAT2 expression. One may speculate that the hyperproduction of IL-6 seemingly coming from the inflammatory cells present in GALT is STAT2 dependent and that a strong IL-6-activated STAT3 signaling is what drives progression of the tumors into later stages of carcinogenesis.

Our results depict an autocrine IL-6/STAT3 signaling pathway as an important molecular target of STAT2 in colorectal and skin cells. IL-6 and STAT3 play a critical role in early and late stages of colorectal carcinogenesis (35). STAT3-null mice are resistant to skin carcinogenesis (39). Similar to STAT2^{-/-} mice, IL-6^{-/-} mice show decreased tumor multiplicity and size compared with WT mice after AOM/DSS treatment. However, unlike STAT2^{-/-} mice,

IL-6-deficient mice have more crypt cell destruction and more dramatic weight loss than control mice after DSS treatment, showing a need for basal IL-6 concentrations to maintain mucosal integrity. In our study, the increase in IL-6 gene expression and secretion was attenuated in STAT2^{-/-} mice following a tumor-promoting stimulus. This observation was recapitulated using a skin carcinoma cell line derived from a STAT2^{-/-} mouse in which hyper-IL-6 secretion and constitutive STAT3 activation were seen after STAT2 reconstitution. A recent genomic analysis reported that both IL-6 and STAT2 are downregulated by dietary flavonoids (40). The involvement of STAT2 in the production of proinflammatory mediators (Fig. 3) raises the question of whether STAT2/STAT3 heterodimers form to activate gene transcription. However, a previous study showed that STAT2 does not associate with STAT3 (41).

In conclusion, our results quite unexpectedly link STAT2 to inflammation-associated colorectal and skin carcinogenesis. The model we propose is that STAT2 upregulates IL-6 production, which in turn activates the STAT3 oncogenic pathway to drive tumor promotion. Nonetheless, additional studies in other animal models of cancer are

warranted to define the role of STAT2 in skin and colon carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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