Inhibitory Roles of Signal Transducer and Activator of Transcription 3 in Antitumor Immunity during Carcinogen-Induced Lung Tumorigenesis

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Abstract
Stat3 mediates a complex spectrum of cellular responses, including inflammation, cell proliferation, and apoptosis. Although evidence exists in support of a positive role for Stat3 in cancer, its role has remained somewhat controversial because of insufficient study of how its genetic deletion may affect carcinogenesis in various tissues. In this study, we show using epithelium-specific knockout mice (Stat3–/–) that Stat3 blunts rather than supports antitumor immunity in carcinogen-induced lung tumorigenesis. Although Stat3–/– mice did not show any lung defects in terms of proliferation, apoptosis, or angiogenesis, they exhibited reduced urethane-induced tumorigenesis and increased antitumor immunity and natural killer (NK) cell immunity. Comparative microarray analysis revealed an increase in Stat3–/– tumors in proinflammatory chemokine production and a decrease in MHC class I antigen expression associated with NK cell recognition. Consistent with these findings, human non–small cell lung cancer (NSCLC) cells in which Stat3 was silenced displayed an enhancement of proinflammatory chemokine production, reduced expression of MHC class I antigen, and increased susceptibility to NK cell–mediated cytotoxicity. In addition, supernatants from Stat3-silenced NSCLC cells promoted monocyte migration. Collectively, our findings argue that Stat3 exerts an inhibitory effect on antitumor NK cell immunity in the setting of carcinogen-induced tumorigenesis. Cancer Res; 72(12); 2990-9. ©2012 AACR.

Introduction
The importance of inflammatory mediators and cells in the tumor microenvironment has recently become the focus of much attention. In some types of cancer, inflammatory conditions initiate or promote oncogenic transformation, whereas in other types of cancer, genetic and epigenetic changes in malignant cells conversely generate an inflammatory microenvironment that further supports tumor progression (1). In addition, cumulative experimental findings indicate that the immune system controls tumor incidence and growth (2). For instance, in colon and ovarian cancers, the presence of infiltrating CTLs or natural killer (NK) cells is associated with a better prognosis (3, 4). In addition, innate immune cells, such as macrophages, NK cells and dendritic cells, can destroy tumor cells when appropriately activated. T cells from the adaptive arm of the immune system can also attack tumors when activated in a type 1 helper T (Th1) immunologic milieu (5, 6). By contrast, tumor–associated macrophages have been shown to promote cancers, partly through their ability to secrete angiogenic, metastatic, and growth factors (1). Another related myeloid lineage, myeloid-derived suppressor cells, which are increased in cancers, not only suppress antitumor immunity but also directly contribute to tumor growth (7). Therefore, it remains controversial how inflammation and immunity influence tumorigenesis.

Stat3 mediates signals for interleukin-6 (IL-6), IL-11, oncostatin M, leukemia inhibitory factor, and ciliary neurotropic factor, and activates the transcription of various target genes. Although Stat3 was originally identified as an inducible DNA-binding protein that binds an IL-6–responsive element within the promoter of hepatic acute phase protein genes, Stat3 is expressed in a variety of cells and tissues and exerts pleiotropic
functions. Because a Stat3 deletion leads to embryonic lethality in mice (8), the physiologic and pathologic significance of Stat3 in adult organs has been investigated using conditional knock-out animals generated using the Cre/loxP recombination system (9). These studies revealed that Stat3 exerts a variety of biologic functions, including cell growth, antiapoptosis, and cell motility, depending on the cell types and stimuli. With regard to the role of Stat3 in promoting or inhibiting cancer, Stat3 has been implicated in the survival and proliferation of various cancer cells by upregulating the expression of various genes, including Bcl-xl (10), Mcl-1 (11), cyclin D1 (12), and c-Myc (13). In addition to these cell autonomous effects, Stat3 has been shown to have cancer-promoting properties through "bystander" effects, such as stimulating angiogenesis and regulating inflammatory and immune responses in tumor microenvironments (14). On the basis of these in vitro studies with cancer cell lines, in vivo tumorigenesis models using tissue-specific knockout mice have revealed that Stat3 promotes tumorigenesis in the colon (15, 16), stomach (17), and pancreas (18, 19) by enhancing tumor-associated inflammation and tumor proliferation. In fact, the association between inflammation and tumorigenesis is very strong in these organs. However, no firm causal relationships with inflammation or infection have been established for lung tumorigenesis (20–22). In addition, the role of Stat3 in carcinogen-induced lung tumorigenesis has not been examined.

In this study, we used urethane-induced lung tumorigenesis models and found that tumorigenesis was significantly reduced in Stat3−/− mice, although these mutant mice did not show any abnormalities in proliferation, apoptosis, or angiogenesis. Interestingly, tumor-associated antitumor inflammation increased in Stat3−/− mice, suggesting that Stat3 signaling is important in evading antitumor immunity during lung tumorigenesis. Of note, microarray analysis identified 2 significant Stat3-mediated pathways. One pathway involves the upregulation of proinflammatory chemokine production by tumor cells in Stat3−/− mice. The other pathway leads to the suppression of NK cell–related MHC class I expression in tumor cells in Stat3−/− mice, which may allow the tumor cells to stimulate NK cell–mediated immunosurveillance. Collectively, our studies with a carcinogen-induced tumorigenesis model revealed a novel role for Stat3 in inhibiting antitumor immunity during lung tumorigenesis.

Materials and Methods

Animal experiments

Stat3−/− mice were generated as previously described (23, 24). Briefly, the human surfactant protein (hSP)-C promoter was used to express the reverse tetracycline transactivator (rtTA). In the presence of doxycycline, rtTA binds to the (tetO)2 promoter, activating transcription of the Cre recombinase. LoxP sites were inserted into introns 20 and 21 of the Stat3 gene (Stat3fl/fl), in which exon 21 was deleted after recombination to produce the Stat3fl/fl locus. Triple transgenic (Stat3−/−) and nondeleted littermate (Stat3−/−/−) control mice were used for the experiments. Six-week-old mice were intraperitoneally injected with urethane (1 mg/g body weight; Sigma).

After 4 to 6 months, the lung tumors were scanned by micro-CT (GE eXplore Locus micro-CT scanner; GE Healthcare). Images were reconstructed and the total lung tumor volume per mouse (TV) was quantified using MicroView analysis software (GE Healthcare). After CT scanning, the mice were sacrificed for each experiment. All protocols were approved by the Institutional Animal Care and Use Committee at Osaka University.

Bronchoalveolar lavage

Mice (N = 14 for Stat3fl/fl group and N = 8 for Stat3−/− group) were sacrificed with a lethal intraperitoneal phenobarbital injection 6 months after urethane treatment. The trachea was cannulated and the lungs were lavaged 3 times with 1 mL of saline. The volumes of recovered bronchoalveolar lavage (BAL) fluid from mice in both groups were similar. After centrifugation, BAL cells were counted using a hemocytometer to determine total BAL cell count. Differential cell counts based on cell morphology were carried out after cytospin followed by May–Giemsa staining to classify the infiltrating cells as monocytes/macrophages, lymphocytes, neutrophils, or eosinophils. Data were analyzed using JMP version 9.0.2 (SAS Institute). The normality of distribution was examined by visually inspecting Q-Q plots. Two groups (Stat3fl/fl vs. Stat3−/−) were compared using the ANOVA model, which included the following factors: the mouse groups, TV, and the interaction between the groups and TV. A 2-sided P value less than or equal to 0.05 was considered significant. A nonparametric analysis was also conducted to confirm the robustness of the ANOVA analysis results.

Immunohistochemistry

Lungs were inflation fixed (25 cmH2O) with 4% paraformaldehyde/PBS for 1 minute, immersed in the same fixative for 18 hours at 4°C and then processed according to standard methods for paraffin-embedded blocks. Immunohistochemistry (IHC) was carried out on 5-μm thick sections. The following primary antibodies were used: Clara cell secretory protein (CCSP; 1:1,500, rabbit polyclonal; Seven Hills Bioreagents), prosurfactant protein (SP)-C (1:1,000, rabbit polyclonal; Seven Hills Bioreagents), phospho-Stat3 (Tyr705; rabbit polyclonal; Cell Signaling Technology), VEGF receptor (VEGFR)-2 (1:300, rabbit IgG; Cell Signaling Technology), phospho-histone-3 (PH-3; 1:500, rabbit polyclonal; Santa Cruz Biotech), cleaved caspase-3 (1:1,600, rabbit polyclonal; Cell Signaling Technology), and Bcl-XL (1:300, rabbit polyclonal; Cell Signaling).

Cell lines

A549 was obtained from the Japanese Collection of Research Bioresources. KHYG-1 was obtained from Health Science Research Resource Bank. HCC827 and H3255 were kindly gifted from Pasi A. Jänne (Dana-Farber Cancer Institute/Department of Medical Oncology, Boston, MA). HFL-1 was obtained from Riken BioResource Center. NCI-H292, 3T3, H441, and THP-1 were purchased from the American Type Culture Collection.

Western blot analysis and ELISA

Lung tissue was snap-frozen in liquid nitrogen. Frozen tissue was broken into pieces using a mortar and lysed in 5 mL of...
T-PER Tissue Extraction Reagent (Pierce). After centrifuging at 1,000 × g for 5 minutes, the supernatants were stored at −80°C. Western blot analysis was done using 20 μg of protein and the following primary antibodies: phospho-histone-3 (PF-3; 1:500, rabbit polyclonal; Santa Cruz Biotech), VEGFR-2 (1:250, rabbit IgG; Cell Signaling Technology), phospho-Stat3 (1:1,000, rabbit polyclonal; Cell Signaling Technology), mouse MHC class I (ER-HR52; 1:200, rat monoclonal IgG2a; Santa Cruz), human MHC class I (W6/32; 1:400, mouse monoclonal IgG2a; Santa Cruz), and actin [1:1,000, horseradish peroxidase (HRP)-conjugated goat polyclonal; Santa Cruz]. A goat anti-rabbit IgG (H+L)-HRP conjugated (BioRad) or HRP-goat anti-rat IgG (H+L; Invitrogen) antibody was used as the secondary antibody. The levels of IFN-γ, TNF-α, and IL-6 in the supernatants were determined using quantitative murine sandwich ELISA kits (R&D Systems Inc.) and normalized to the total lung tumor volume.

Microarray analysis

Lung tumors were microdissected and pooled. RNA was extracted with RNeasy Plus (Takara Bio) and purified with an RNeasy Mini Kit (Qiagen). The RNA quality and quantity were analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies) and NanoDrop ND-1000 (Thermo Fisher Scientific), respectively. Gene expression was analyzed using GeneChips mouse genome 430 2.0 array (Affymetrix) according to the manufacturer’s protocol. Arrays were scanned on a GeneChip scanner 3000 (Affymetrix).

Construction of protein–protein interaction networks and functional analysis of the differentially expressed genes by characterization of enriched biologic associations

Protein–protein interactions (PPI) for genes that showed increased or decreased expression in the microarray studies were retrieved from the BioGRID (25) and iRefIndex 8.0 (26) databases using the TargetMine data warehouse (27). Bioelectric pathway data from KEGG (28) were used to assign functional annotations to the genes in the upregulated and downregulated networks. The overexpression of specific KEGG pathway associations within each network was estimated by carrying out the hypergeometric test within TargetMine. The inferred P values were further adjusted for multiple test corrections to control for the false discovery rate using the Benjamini and Hochberg method (29), and the pathway annotations were considered significant if P value was less than or equal to 0.05.

Knockdown experiment with siRNA

Cells were transfected with either siRNA against human Stat3 or control cocktail RNAs (B-Bridge International), using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocol. After a 48-hour incubation, the cells were replated in serum-containing medium (5 × 10^4/mL) and cultured for another 30 hours. Protein and RNA expression analysis was done by Western blot and TaqMan real-time reverse transcriptase PCR (Applied Biosystems), respectively. The concentrations of CCL5 and CXCL10 in the culture supernatants were determined using quantitative murine sandwich ELISA kit (R&D).

In vitro migration assay

The migration of monocytes (THP-1 cells) was measured by a modified Boyden chamber migration assay. THP-1 cells were loaded into Transwells inserts (5-μm porous membrane; Corning). The culture supernatants of non–small cell lung cancer (NSCLC) cell lines transfected with Stat3 siRNA or control siRNA were placed in the lower chamber. Cells on the upper side of the membrane were removed after 4 hours and the number of migratory cells was determined by Guava ViaCount assays (Millipore).

Cytotoxic assay

Susceptibility to NK cell–mediated cytotoxicity was measured by a conventional 4-hour ^51^Cr-release assay as previously described (30). Briefly, the target cells, an NSCLC cell line (H3255 and H441) that was transfected with Stat3 or control siRNA, were labeled with ^51^Cr and mixed with effector cells, KHYG-1 cells, at different effector/target (E/T) ratios. After a 4-hour incubation, the supernatant was collected for liquid scintillation counting. Spontaneous ^51^Cr-release was measured in target cells incubated without effector cells, and the maximal release was measured by treating the target cells with 5% Triton X-100. The percent specific lysis was determined using the following formula: % specific lysis = 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release).

Statistical analysis

All statistical analyses were done using JMP version 9.0.2 (SAS). Data are expressed as the means ± SE. The differences between the 2 groups were evaluated using 2-sided Student t test or ANOVA. P values less than 0.05 were considered statistically significant.

Results

Urethane-induced lung tumorigenesis was inhibited in Stat3^fl/fl^ mice

To investigate the pathologic role of Stat3 in lung tumorigenesis, we first examined urethane-induced tumorigenesis in Stat3^fl/fl^ mice. Stat3^fl/fl^ mice did not show any abnormalities in terms of lung size, lung morphology, or respiratory functions before urethane administration (23, 24). At 4 months after urethane injection, we examined the size of the lung tumors by micro-CT (Fig. 1A). Lung tumors had developed in 90.3% (28 of 31) of the Stat3^fl/fl^ mice and 76.9% (10 of 13) of the Stat3^lox/lox^ mice. The total lung tumor volume per mouse (TV) was significantly decreased in Stat3^fl/fl^ mice (average 2.9 mm^3^ per mouse) compared with Stat3^lox/lox^ mice (average 6.5 mm^3^ per mouse; P = 0.037; Fig. 1B). A histologic examination revealed that tumors in both mice displayed the same morphologic characteristics of lung adenomatous tumor, except that Stat3^fl/fl^ mice never acquired large tumors compared with Stat3^lox/lox^ mice (Fig. 1C, Supplementary Fig. S1). IHC for CCSP, a Clara cell marker, and pro-SP-C, an alveolar type II...
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Figure 1. Carcinogen-induced lung tumorigenesis was impaired in Stat3<sup>fl/fl</sup> mice. A, representative micro-CT images of Stat3<sup>lox/lox</sup> (a) and Stat3<sup>fl/fl</sup> (b) mice showing lung tumors (arrowheads). Mice were intraperitoneally injected with urethane (1 mg/g body weight) and tumor formation was visualized after 4 months. B, each tumor volume was quantified and the total tumor volume was determined. The total lung tumor volume per each mouse was significantly decreased in Stat3<sup>fl/fl</sup> mice, N = 31 for Stat3<sup>lox/lox</sup> group and N = 13 for Stat3<sup>fl/fl</sup> group. *P < 0.05. C, representative hematoxylin and eosin stain (H&E)-stained sections of tumors in Stat3<sup>fl/fl</sup> (b) compared with Stat3<sup>lox/lox</sup> (a) mice. Immunohistochemical analysis of CCSP (c and d), pro-SP-C (e and f), and phospho-Stat3 (g and h) and in lung tumors from Stat3<sup>lox/lox</sup> (c, e, and g) and Stat3<sup>fl/fl</sup> (d, f, and h) mice.

epithelial cell marker, revealed that the tumor cells were CCSP-negative and pro-SP-C-positive, indicating that they developed from alveolar type II epithelial cells (Fig. 1C). IHC for phospho-Stat3 confirmed that the Stat3 expressed in tumor cells was activated in Stat3<sup>fl/fl</sup> mice (Fig. 1C). H&E for phospho-Stat3 confirmed that the Stat3 expressed in tumor cells was activated in Stat3<sup>lox/lox</sup> mice but not in Stat3<sup>fl/fl</sup> mice, indicating that the tumors in Stat3<sup>fl/fl</sup> mice did not originate from progenitor cells that escaped Cre-mediated deletion (Fig. 1C). In addition, we confirmed that the expression and function of Stat3 was intact in myeloid cells and lymphocytes (Supplementary Fig. S2). These findings indicated that a Stat3 deletion in airway epithelial cells inhibited tumor formation in a urethane-induced lung tumorigenesis model.

Proliferation, apoptosis, and angiogenesis in lung tumors in Stat3<sup>fl/fl</sup> mice

Previous studies reported that Stat3 is involved in mitosis, survival, and angiogenesis during tumorigenesis. Thus, we examined the possibility that a Stat3 deletion in airway epithelial cells results in impaired mitosis, survival, and angiogenesis in urethane-induced lung tumors. First, we assessed proliferation by IHC and Western blot analysis using phospho-histone-3 (PH-3) as a marker, which was not different between Stat3<sup>fl/fl</sup> mice and Stat3<sup>lox/lox</sup> mice (Fig. 2A and B). Next, we examined apoptosis by immunohistochemically examining cleaved caspase-3. There were few positive cells in both Stat3<sup>fl/fl</sup> mice and Stat3<sup>lox/lox</sup> mice, suggesting that apoptosis plays a minimal role during urethane-induced tumorigenesis (data not shown). In addition, we examined the expression of Bcl-xL, an antiapoptotic protein, by IHC and Western blot analysis and found that there were no apparent differences between Stat3<sup>fl/fl</sup> mice and Stat3<sup>lox/lox</sup> mice (Fig. 2A and B). With regard to vascular development, immunohistochemical analysis for VEGFR-2 showed that the expression of VEGFR-2 was specifically stained in the vascular endothelial cells but not in tumor cells, in which there were no difference between Stat3<sup>fl/fl</sup> mice and Stat3<sup>lox/lox</sup> mice (Fig. 2A). In addition, we did not see significant differences in the levels of VEGFR-2 and VEGF in the lung homogenates between Stat3<sup>fl/fl</sup> mice and Stat3<sup>lox/lox</sup> mice (Fig. 2B and C). These findings unexpectedly showed that Stat3 in tumor cells has a minimal effect on mitosis, apoptosis, and angiogenesis in this model.

Tumor-associated inflammation was augmented in Stat3<sup>fl/fl</sup> mice

It is well known that inflammation in the tumor microenvironment acts to either promote or inhibit tumors depending on the tissue type (1). Thus, we next examined the effects of an epithelial cell–specific Stat3 deletion on tumor-associated inflammation in the lung and surprisingly found significantly more inflammatory cells surrounding tumors in Stat3<sup>fl/fl</sup> mice compared with Stat3<sup>lox/lox</sup> mice (Fig. 3A). Therefore, we carried out BAL to count inflammatory leukocytes in the lung. The number of inflammatory cells, including macrophages, lymphocytes, and neutrophils, recovered in the BAL fluid correlated with the TV, suggesting that inflammation correlated with tumor growth. Indeed, the ratios of inflammatory cell numbers to TV were significantly higher in Stat3<sup>fl/fl</sup> mice compared with Stat3<sup>lox/lox</sup> mice (Fig. 3B). The relative concentrations of antitumor inflammatory mediators, such as IFN-γ, TNF-α, and IL-6, to TV were also increased in the lungs of Stat3<sup>fl/fl</sup> mice compared with Stat3<sup>lox/lox</sup> mice (Fig. 3C). Taken together...
with the fact that tumor growth was suppressed in Stat3^Δ/Δ mice, these data implied that a Stat3 deletion in tumor cells promotes antitumor inflammatory responses in urethane-induced tumorigenesis.

**Genome wide mRNA expression profiling revealed the upregulation of proinflammatory chemokines and downregulation of NK-related MHC class I in Stat3^Δ/Δ mice**

To understand the mechanisms underlying the antitumor inflammatory responses evoked in the absence of Stat3 in lung tumor cells, we carried out a microarray analysis using RNA extracted from the tumors. A comparison of gene expression between Stat3^Δ/Δ and Stat3^flox/flox^ tumors revealed that 298 and 338 genes were upregulated and downregulated, respectively, in Stat3^Δ/Δ mice compared with Stat3^flox/flox^ mice (Supplementary Tables S1 and S2).

Because most genes function in association with other genes and gene products, to further understand the biologic significance of the different gene expression levels, we retrieved the PPIs for the upregulated and downregulated genes and inferred PPI networks using TargetMine. Next, we investigated the upregulated and downregulated gene expression cum PPI networks (comprising 396 and 490 genes, respectively) for the enrichment of specific KEGG pathway associations (Supplementary Tables S3 and S4). We observed that deleting Stat3 in tumor cells led to a significant increase in genes associated with chemokine production (CCL2, CCL9, CCL12, CCL17, and CCL21c), which were mapped to the enriched KEGG pathway, "chemokine signaling pathway" (P = 1.53 × 10^{-4}) and function in inflammatory immune responses (Fig. 4A). Also, there was a significant decrease in genes involved in NK cell–related MHC class I expression (H2-D1, H2-K1, and H60a), which were mapped to the enriched KEGG pathway, "NK cell–mediated cytotoxicity" (P = 8.46 × 10^{-6}) and are associated with the innate immune system (Fig. 4B). In this context, we hypothesized that a Stat3 deletion promotes chemokine production and inhibits of MHC class I expression in tumor cells, leading to the upregulation of antitumor inflammatory responses.

**Stat3 blockade increased chemokine expression in lung epithelial cell lines**

To investigate whether Stat3 activation in tumor cells negatively regulates the expression of inflammatory mediators, we suppressed Stat3 expression in NSCLC cells using siRNA transfection. As reported previously, all NSCLC cells showed various degrees of constitutively activated Stat3 (Fig. 5A). Introducing Stat3 siRNA significantly reduced Stat3 expression and upregulated the expression of chemokines, including CCL5 (RANTES) and CXCL10 (IP-10; Fig. 5B and C). To confirm that the inflammatory factors that were produced by Stat3-suppressed NSCLC cell lines affect immunity, we carried out monocyte migration assays using cell culture supernatants from Stat3 siRNA- or control siRNA-transfected cells. As shown...
in Fig. 5D, cellular migration was considerably promoted in the presence of culture supernatants from Stat3 siRNA–transfected NSCLC cells. These findings support the hypothesis that inhibition of Stat3 signaling in tumor cells promotes the production of chemotactic factors that enhance antitumor immunity.

Stat3 increased MHC class I expression in tumor cells and inactivated NK cells

NK cells attack malignant cells with impaired MHC class I expression (2). To confirm the results of the microarray analysis, which showed that a Stat3 deletion results in the downregulation of MHC class I in tumor cells, we examined the expression of MHC class I in tumor-bearing lung tissues from Stat3^D/D^ mice. Western blot analysis using an antibody against mouse MHC class I revealed that MHC class I expression was decreased in tumor-bearing lung tissues from Stat3^D/D^ mice compared with Stat3^flox/flox^ mice (Fig. 6A). siRNA-mediated knockdown of Stat3 also decreased MHC class I expression in human lung cancer cell lines (Fig. 6B). We finally tested whether Stat3 activation affects NK cell cytotoxic activities against tumor cells using ^31Cr-release assays with NSCLC cells (H3255 and H441) that were transfected with Stat3 siRNA. Stat3 knockdown increased the susceptibility of NSCLC cells to NK cell–mediated cytotoxicity (Fig. 6C). These data indicated that Stat3 deletion in tumor cells downregulates the expression of the MHC class I to stimulate NK cell–mediated antitumor immunity.

Discussion

In this study, we investigated the role of Stat3 in carcinogen-induced lung tumorigenesis. Although Stat3 has been implicated in tumor growth, survival, and angiogenesis, we unexpectedly found that proliferation, apoptosis, and angiogenesis were not altered in Stat3^D/D^ lung tumors. Instead, we found that tumorigenesis was significantly reduced in Stat3^D/D^ mice, whereas tumor-associated inflammation was increased. We further carried out genome-wide mRNA profiling and found that Stat3 knockdown increased the expression of proinflammatory chemokines and decreased the expression of MHC class I. Thus, we provide evidence that Stat3 negatively regulates antitumor immunity during lung tumorigenesis.

Inhibitory roles of Stat3 in antitumor immunity during carcinogen-induced tumorigenesis

The role of Stat3 in inflammation-associated tumorigenesis has been extensively investigated in several organs, including the colon (15, 16), stomach (17), and pancreas (18, 19). These studies indicated that Stat3 activation increases cancer-associated inflammation to promote proliferation and inhibit tumor cell apoptosis. In addition, it was reported that overexpressing a constitutively active Stat3 in airway epithelial cells in the lung promoted lung inflammation and tumor formation (31). However, the roles of Stat3 in antitumor immunity during lung tumorigenesis have not been determined. In this study, we...
examined a carcinogen-induced lung tumorigenesis model and found that Stat3 plays a novel role in negatively regulating antitumor immunity. Indeed, NSCLC is not strongly associated with inflammation compared with cancers in other organs (20–22). In our studies, tumor-associated inflammation increased in Stat3
d/D mice, which exhibited reduced tumorigenesis. According to the increased inflammation in Stat3
D/D lungs, antitumor inflammatory mediators such as TNF-α and IFN-γ were elevated in the lungs of Stat3
D/D mice compared with Stat3
fl/ox mice, although we carefully evaluated their local concentrations. By contrast, we did not observe any differences in proliferation, apoptosis, or angiogenesis between Stat3
D/D and Stat3
fl/ox tumors. Although previous studies indicate that Stat3 has mitotic and antiapoptotic roles in cancer cell lines (10–13), we did not observe these Stat3-related effects in our model. Stat3 might inhibit antitumor immunity in the early phase of tumorigenesis to alter the tumor microenvironment and promote proliferation or survival in the late phase of tumorigenesis. Taken together, our findings indicate that the inflammation observed in the lungs of Stat3
D/D mice had anticancer effects. Further studies are required to determine how Stat3 differentially regulates antitumor immunity in different organs.

Negative regulation of chemokine production by Stat3

Here, we showed that Stat3 negatively regulates chemokine genes, including CCL5 and CXCL10, which play important roles in cancer-associated inflammation by attracting various subpopulations of immune effector cells (32, 33). Consistent with this finding, Wang and colleagues reported that constitutively active Stat3 inhibits the production of multiple proinflammatory cytokines and chemokines in mouse cancer cell lines (34). Mice with a selective disruption of Stat3 in keratinocytes were reported to have defective wound healing associated with increased inflammatory infiltrates at the wound sites (35). Thus, Stat3-mediated inhibition of proinflammatory factors affects not only the tumor microenvironment but also the site of physiologic wound healing.

With regard to the gene regulation by Stat3, Xu Y and colleagues previously reported the effects of a pulmonary epithelial cell–specific deletion of Stat3 using genome-wide mRNA expression profiling, in which they found that chemotaxis-related genes were significantly induced by a Stat3 deletion, and these authors also reported that STAT was the most significantly enriched cis-element in the promoter region of differentially expressed genes, suggesting that Stat3 directly controls the expression of these genes (36).

Decreased MHC class I expression in tumor cells augments antitumor inflammation

We provided evidence that inhibition of Stat3 signals in tumor cells decreased the expression of MHC class I in tumor cells, resulting in enhanced NK cell cytotoxic activities. This raises the question of whether the decreased MHC class I expression in tumor cells affects adaptive antitumor immunity.
mediated by CD8⁺ T cells. Indeed, Schreiber and colleagues reported that, although NK cells are important in the early elimination of tumors, the "dormant" tumor state is controlled by adaptive immunity mediated by CD8⁺ T cells (37). However, it has been reported that MHC class I–low tumor cells still evoke antitumor CD8⁺ T-cell responses because NK cells, activated by MHC class I–low tumor cells, produce IFN-γ that stimulates CD8⁺ T cells (38, 39). Thus, enhanced NK cell responses, together with increased chemokine production by tumor cells, may be primarily responsible for antitumor inflammation during urethane-induced tumors. It is also noteworthy that the efficacy of the antitumor cytotoxic activities of cytotoxic CD8⁺ T cells (CTL) depends not only on the presentation of tumor antigens by the malignant cells but also on their susceptibility to CD8⁺ T-cell–induced lysis. The hypoxic tumor microenvironment confers resistance to this CTL-mediated cytotoxic activity. Interestingly, it was recently reported that Stat3 and hypoxia-inducible factor (HIF)-1α are functionally linked to the alteration of NSCLC susceptibility to CTL-mediated killing under hypoxic conditions (40). In this context, Stat3 activation in tumor cells may affect tumor cell sensitivity to either NK cell– or CD8⁺ T-cell–mediated cell killing.

With regard to the regulation of MHC class I expression by Stat3, there are several possibilities, which affect MHC class I expression during tumorigenesis, such as inflammatory cytokines, oncogenic transcriptional factors, and tumor-associated missing self-phenotypes. Further studies are necessary to clarify the mechanism of how Stat3 regulates the expression of MHC class I molecule.

Involvement of Stat3 in tumorigenesis and its clinical implication

Adenocarcinoma is the most common histologic type of lung cancer, accounting for almost half of all lung cancers. The relevant molecular events frequently observed in lung adenocarcinoma are KRAS mutations, epidermal growth factor receptor mutations, and echinoderm microtubule–associated protein-like 4-anaplastic lymphoma kinase translocations (41). Stat3 is activated and responsible for the oncogenic phenotypes of adenocarcinoma caused by such molecular mutations or alterations (18, 19, 42, 43). Thus, it is possible that Stat3 inhibition not only promotes antitumor immunity but also impairs oncogenic signals in adenocarcinoma. Indeed, several reports suggest that Stat3 activation is relevant to the progression of human NSCLC (44, 45); however, it may be difficult to determine the involvement of Stat3 activation in the in vivo tumorigenesis of NSCLC. We therefore think that our in vivo tumorigenesis model provides a clue to understand the involvement of Stat3 activation in NSCLC tumorigenesis. Collectively, our findings not only indicate a novel role for Stat3 in lung tumorigenesis but also provide a potential therapeutic target for lung cancers.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Figure 6. Stat3 knockdown decreased MHC class I expression on lung tumor cells, resulting in the activation of NK cell-mediated cytotoxicity. A, immunoblot analysis of MHC class I in lung homogenates from Stat3fl/fl mice and Stat3fl/fl mice. B, immunoblot analysis of MHC class I in lysates prepared from a series of NSCLC cell lines that were transfected with control or Stat3 siRNA. C, a cytotoxic assay revealed that Stat3 knockdown increased the susceptibility of an NSCLC cell line (H3255 and H441) to NK cell-mediated cytotoxicity. Results are representative of 3 experiments.
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