Microenvironment and Immunology

Immunologic Consequences of Signal Transducers and Activators of Transcription 3 Activation in Human Squamous Cell Carcinoma

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Abstract

Paracrine cross-talk between tumor cells and immune cells within the tumor microenvironment underlies local mechanisms of immune evasion. Signal transducer and activator of transcription 3 (STAT3), which is constitutively activated in diverse cancer types, is a key regulator of cytokine and chemokine expression in murine tumors, resulting in suppression of both innate and adaptive antitumor immunity. However, the immunologic effects of STAT3 activation in human cancers have not been studied in detail. To investigate how STAT3 activity in human head and neck squamous cell carcinoma (HNSCC) might alter the tumor microenvironment to enable immune escape, we used small interfering RNA and small-molecule inhibitors to suppress STAT3 activity. STAT3 inhibition in multiple primary and established human squamous carcinoma lines resulted in enhanced expression and secretion of both proinflammatory cytokines and chemokines. Although conditioned medium containing supernatants from human HNSCC inhibited lipopolysaccharide-induced dendritic cell activation in vitro, supernatants from STAT3-silenced tumor cells reversed this immune evasion mechanism. Moreover, supernatants from STAT3-silenced tumor cells were able to stimulate the migratory behavior of lymphocytes from human peripheral blood in vitro. These results show the importance of STAT3 activation in regulating the immunomodulatory mediators by human tumors and further validate STAT3 as a promising target for therapeutic intervention. Cancer Res; 70(16); 6467–76. ©2010 AACR.

Introduction

Human solid malignancies, notably, head and neck squamous cell carcinoma, as well as glioblastoma multiforme, melanoma, prostate, and breast cancer display constitutive activation of signal transducers and activators of transcription 3 (STAT3) that regulates multiple genes associated with angiogenesis, apoptosis, cell cycle progression, and inflammation (1–3). Subsequently, the characterization of the pleiotropic role of STAT3 in tumorigenesis has prompted the development of drugs to disrupt STAT3 signaling (4–7). Interestingly, in preclinical studies, STAT3 targeting in tumor cells elicited a “bystander” antitumor effect that was attributed to infiltration of immune cells in the tumor microenvironment (8, 9). STAT3 can serve as a negative regulator of chronic inflammatory responses in vivo but is also critical for the generation of Th17 cell response, characterized by the production of interleukin (IL)-17A (10–12). STAT3 null mice in the myeloid compartment induced inflammatory bowel disease, and its macrophages were abnormally activated, corroborating its in vivo role in mediating an immunologic “brake” against certain destructive inflammatory responses (13, 14). In this vein, IL-6-dependent suppression of dendritic cell (DC) maturation was found to be STAT3 dependent (15). On the other hand, STAT3-driven Th17 responses can induce inflammation, which in one case has recently been shown to be procarcinogenic (16).

In the context of immunologic responses to established tumors in mice, STAT3 has been noted to orchestrate the immune components of the tumor microenvironment (1, 16–18). In the B16 model, STAT3 activity inhibited the expression of multiple Th1 cytokines that can potentially induce DC maturation, resulting in immune evasion response in vivo (17, 19). In a follow-up study, Kortylewski and colleagues (20) showed that genetic ablation of STAT3 in the myeloid hematopoietic compartment also elicited an antitumor immune response characterized by activation of natural killer cells, neutrophils, and T cells within the tumor microenvironment. These studies, together with the finding that STAT3 suppression in DCs can break tumor antigen–specific T-cell anergy (21), showed that tumor cells can use STAT3 signaling as an important mechanism to suppress the antitumor potential of the immune cells that infiltrate their microenvironment. From these murine studies, there

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are now over 30 downstream STAT3-dependent factors that can potentially immunomodulate the tumor microenvironment (18). Given the complexity of the tumor microenvironment, one valid hypothesis is that these STAT3-dependent factors may act combinatorially to induce the antitumor phenotypes.

However, despite these findings in murine models, there have been no studies to determine whether STAT3 plays a parallel role in human cancer. As an initial approach to address this question, we explored the immunologic consequences of STAT3 blockade in human cell lines and mouse tumor xenografts. Specifically, we investigated the role of tumor cell STAT3 signaling in the regulation of proinflammatory cytokine expression, human DC activation, and human leukocytic migration.

Materials and Methods

Cell lines

Human Cal27, HN11, and Fadu cell lines were purchased from the American Type Culture Collection. HN6, HN22, HN28, and HN29 were derived from tumor specimens and were kindly provided by Dr. David Sidransky (Johns Hopkins School of Medicine, Baltimore, MD).

Small interfering RNA transfection and lentiviral transduction

Transient transfections of tumor cells with STAT3 small interfering RNA (siRNA) oligonucleotide (Santa Cruz) were conducted using Lipofectamine 2000 (Invitrogen Life Technologies). For control samples, cells were transfected with scrambled siRNA oligonucleotide or Lipofectamine alone. The cells were transfected with 15 nmol/L of STAT3 or scrambled siRNA and cultured for 2 days.

Mission TRC-Hs (Sigma) clone sets of sequence-verified short hairpin RNA lentiviral plasmids were obtained from the JHU High Throughput Biology Center. The sequences of B7 and B8 shRNAs are reported in Supplementary Table S1. VSV-G-pseudotyped virus was produced by the Johns Hopkins Neurosurgery Vector Core by cotransfecting 293T cells with an shRNA-transducing vector and two packaging vectors: psPAX2 and PMD2.G.

Annexin V staining

Cells were stained with 5 μL of phycoerythrin Annexin V according to the manufacturer’s protocol (BD Pharmingen) and analyzed by flow cytometry within 1 hour.

Quantitative real-time PCR

Total RNA was extracted and DNase I digested using the RNeasy kit according to the manufacturer’s protocol (Qiagen). One microgram of RNA was used as template for the reverse transcription reaction (SuperScript III; Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed using a iCycler MyiQ detection system (Bio-Rad). The primer sequences are listed in the Supplementary Table S2. To analyze the relative changes in gene expression of the treatment group versus the nontreated control, the $2^{-\Delta\Delta Ct}$ method was used (22).

ELISA

Human IP-10, IL-6, IL-8, and vascular endothelial growth factor (VEGF)-A were measured by ELISA (Quantikine HS; R&D Systems) in the culture supernatants of siRNA-treated and nontreated cell lines according to the manufacturer’s instructions. The lower detection limit was 1.0 pg/mL.

Electrophoresis mobility shift assay

Nuclear extracts were prepared, and 5 to 10 μg of crude extracts were incubated with the $^{32}$P-labeled high-affinity SIE probe derived from the c-fos gene promoter that binds STAT1 and STAT3 as previously described (23). Protein-DNA complexes were resolved on 5% nondenaturing polyacrylamide gels and analyzed by autoradiography using Kodak film. Supershift binding reaction was performed using polyclonal rabbit antibody specific for human STAT3 (Santa Cruz).

DC maturation assay

Human DCs were prepared from Buffy coat layers purchased from Baxter Healthcare Corporation. CD14+ monocytes were isolated from peripheral blood mononuclear cells (PBMC) by positive selection using a MACS system (Miltenyi Biotech), according to the manufacturer’s protocol, and were cultured for 6 days in 10% FCS RPMI 1640 supplemented with 1,000 U/mL granulocyte macrophage colony-stimulating factor (R&D Systems) and 500 U/mL IL-4 (Peprotech). Subsequently, the immature DCs were incubated with 100 ng/mL of LPS from Escherichia coli 026: B6 (Sigma) for 48 hours. Immature DCs and mature DCs were labeled with FITC-conjugated IgG specific for HLA-DR (BD Bioscience), phycoerythrin-conjugated IgG specific for CD86 (eBioscience), and Allophycocyanin-conjugated IgG monoclonal antibody specific for CD11c (BD Bioscience) for 20 minutes at 4°C.

DC maturation inhibition experiments were performed with CD14+ monocytes in standard DC medium supplemented with tumor cell supernatants (50%). Tumor cell supernatant was added to the culture on day 0, 2, 4, and 6, at which point lipopolysaccharide (LPS) was added. On day 8, cultures were stained and analyzed by flow cytometry.

Migration assay

Functional ability to induce lymphocytic chemotaxis was assessed with the ChemoTx system (3-μm pore, 5.7-mm site, 300 μL 96-well, Neuro Probe) according to the manufacturer’s protocol. Tumor cell supernatant, serum-free media (negative control), or 100% FCS (positive control) were placed in the lower wells. In 11 wells, the supernatant was replaced by a serial dilution of PBMC to serve as a standard curve for the CyQuant cell proliferation assay. PBMCs from normal donors were placed on top of each filter site. Cell numbers were quantified with the CyQuant NF Cell Proliferation Assay (Invitrogen). The fluorescence was measured...
with the SpectraMax Gemini XS Fluorometer (Molecular Devices) with a 485/538-nm filter set. The fluorescence readout was correlated to cell numbers by the PBMC standard curve.

Small molecule inhibitor
Statcic (5) was purchased from Calbiochem and diluted in DMSO following the manufacturer’s protocol (final DMSO concentration of 1%). Cell lines were treated with Statcic at 1, 10, and 20 μmol/L concentration, or DMSO. Cells were harvested after 24, 48 and 72 hours. Annexin V staining was performed to test for apoptosis.

Statistical analysis
We used paired t test to calculate two-tailed P value to estimate statistical significance of differences between two treatment groups. Statistically significant P values were P < 0.01 (*) and P < 0.05 (*). Data were analyzed using Excel software.

Results

Suppression of STAT3 alters the proinflammatory cytokine and chemokine profile of human HNSCC tumor cell lines
To study the immunologic consequences of STAT3 activation of human tumor cells, we focused on commercially available HNSCC cell lines Cal27, HN11, and Fadu, as well as four other human HNSCC primary culture cell lines (HN6, HN22, HN28, and HN29), which are known to express high levels of constitutively active phospho-STAT3 (p-STAT3; ref. 24). Previously, the immunomodulatory role of STAT3 was evaluated by transient transfection of STAT3 antisense oligonucleotides that had modest transfection efficiency (19). Therefore, we chose to optimize the blockade of STAT3 signaling by using siRNA knockdown to specifically suppress its expression. We optimized the transfection conditions to assure >60% STAT3 mRNA suppression in all the cell lines by using a Lipofectamine system. We also transduced two of the HNSCC cell lines, HN11 and Cal27, with STAT3 siRNA lentivirus to obtain stably transduced cell lines. We observed minimal change in apoptosis (10%) as confirmed by Annexin V staining that was performed at the end of each siRNA transfection and infection (data not shown). As shown in Fig. 1A, quantitative qRT-PCR on STAT3 siRNA–transfected cell lines detected a statistically significant suppression of STAT3 mRNA compared with nontreated cells or cells transfected with scrambled siRNA used as controls. Comparable results were noted for HN11 and Cal27 cell lines transduced with STAT3 siRNA lentivirus (Fig. 1B). To show that STAT3 siRNA suppression resulted in decreased p-STAT3 activation in the nuclei, electrophoresis mobility shift assay (EMSA) was performed. As shown in Fig. 1C, there was a significant reduction of DNA-binding p-STAT3 in the siRNA-treated cell lines compared with the controls.

To evaluate the immunologic consequences of STAT3 blockade in these human HNSCC lines, we initially tested the mRNA expression of several cytokines and chemokines by qRT-PCR. We noticed a pattern of expression similar to the murine models. In particular, we found that IFNγ-inducible protein 10 (IP10), IL-8, tumor necrosis factor α (TNFα), and IL-6 mRNAs were elevated in all seven HNSCC cell lines transfected with STAT3 siRNA (Supplementary Fig. S1; Fig. 2). Interestingly, in contrast to observations in murine tumor systems, STAT3 suppression did not consistently result in upregulation of RANTES or IFNγ mRNAs in all of the human HNSCC tumor cell lines tested. We next investigated whether these STAT3-mediated changes in cytokine/chemokine transcription correlated with protein expression. Using ELISA assays, we showed that STAT3 suppression in tumor cell lines resulted in significantly higher secretion of IP-10, IL-8, and IL-6 in the culture supernatant of each of the STAT3 siRNA–treated cell lines, compared with controls (Fig. 3). Because STAT3 is known to directly control VEGF transcription in mice (25), we also tested for VEGF secretion and we noticed a statistically significant reduction in the samples treated with STAT3 siRNA (Fig. 3). qRT-PCR using VEGF primers in the STAT3-suppressed tumor cells also showed reduced expression of VEGF mRNA (Supplementary Fig. S2), correlating with the reduction of VEGF protein. Therefore, we hypothesized that constitutive expression of STAT3 in human tumor cells that is responsible for inhibiting the production of inflammatory mediators in the tumor microenvironment may induce the tumor-infiltrated immune cells to differentiate along an immunosuppressive phenotype. Moreover, given the differential expression patterns of paracrine factors among the HNSCC cell lines tested, we initially focused on the combinatorial effects of STAT3-dependent cytokines and chemokines in the tumor microenvironment.

STAT3 suppression in the tumor cell can affect DC maturation
One mechanism by which cancer cells can potentially modulate the immune response is to regulate the expression of DC maturation inhibitory factors, such as VEGF and IL-10, into the tumor microenvironment. In the B16 murine melanoma model, STAT3 overexpression prevented efficient maturation of murine bone marrow–derived DCs (19). To test whether an analogous phenomenon is operative in the cross-talk between human tumor cells and human DCs, we exposed human immature monocyte-derived DCs to conditioned medium (CM) containing supernatants from STAT3 siRNA–treated or nontreated HNSCC cell lines. In the presence of LPS, human DCs are activated, as defined by an augmented coexpression of MHC class II and CD86 (Fig. 4, top row, right). The majority of DCs cultured in the presence of conditioned media containing supernatant of HNSCC cell lines were shown to remain immature after LPS stimulation (Fig. 4, middle and lower rows, left). However, LPS-induced maturation of DCs in the presence of CM containing STAT3 siRNA–treated cell line supernatants revealed that the percentage of MHC class IIhigh and CD86high DCs was restored to that level observed by standard LPS stimulation (Fig. 4, middle rows,
right). Comparable results were noted using HN11 cell lines transduced with lentivirus containing B7 and B8 STAT3 shRNA (Fig. 4, bottom row). This indicated that blocking STAT3 signaling in human tumor cell lines may result in an enhanced LPS-induced activation of DC in vitro. DC maturation assays were also performed without LPS as control experiments. The percentage of MHC class II$^{\text{high}}$ and CD86$^{\text{high}}$ were slightly elevated without LPS induction when incubated with CM from STAT3-suppressed cell lines as shown in Supplementary Fig. S3. However, the differential effect on DC maturation without LPS was minimal compared with the significant effect on the DC maturation assay in the presence of LPS. Given that the DC maturation is sensitive to variable such as temperature, time, and even mechanical manipulation, the quantitative effect of STAT3 signaling in the HNSCC cells to suppress LPS-induced DC maturation underscores how STAT3 can reverse a potent immunostimulant to render the DC immature to potentially
produce an immunosuppressive phenotype in the tumor microenvironment.

VEGF, whose expression is controlled by STAT3, has been shown as a potential mediator that can suppress DC maturation (1). By ELISA (Fig. 3) and qPCR (Supplementary Fig. S2) we found that the level of VEGF mRNA and secreted protein is statistically diminished in the supernatant from STAT3 siRNA–treated HNSCC cell lines. Preliminary experiments were performed to directly test whether VEGF by itself can suppress LPS-induced DC maturation. Several concentrations of human recombinant VEGF were titrated back into cultured medium obtained from HNSCC cell lines with suppressed STAT3 siRNA, but even concentrations as high as 20 ng/mL did not inhibit DC maturation to the level noted with cultured medium from nontreated HNSCC cell lines (Supplementary Fig. S4).

STAT3 suppression enhances trafficking of leukocytes in vitro

Given that suppression of STAT3 resulted in the upregulation of potent chemoattractant chemokines such as IP10 (26) or IL-8 (27), we also hypothesized that suppression of STAT3 signaling in the tumor cells may improve immune cell trafficking into the tumor microenvironment, comparable with the B16 model (19). To test this in a human system, we used a standard in vitro chemotaxis assay. We incubated human PBMC in the presence of CM containing supernatant from STAT3 siRNA–treated Cal27 tumor cell line. CM containing

Figure 2. STAT-3 suppression in tumor cells increases the expression of proinflammatory mediators. qRT-PCR analysis showed the upregulation of proinflammatory cytokine and chemokines mRNA by siRNA treatment only, compared with no treatment and to controls in (A) Cal27, (B) HN11, and (C) HN29 cell lines. Histograms represent the means of at least three experiments normalized to GAPDH. Data are presented as fold difference relative to control–nontreated cell lines. *, P < 0.05; **, P < 0.01.
supernatant from cells transfected with scrambled siRNAs was used as controls. We used a 3-mm pore size filter in the chemotaxis plate to allow predominantly lymphocytic migration. We used a fluorescence-based assay to quantify the number of migrated cells. A standard curve with known numbers of PBMC showed the linear range of the assay. We observed a distinct, statistically significant migration of leukocytes only toward the supernatant from Cal27 transfected with STAT3 siRNA (Fig. 5). Further work is in progress to establish if a differential combination of STAT3-dependent chemotactic mediators can stimulate lymphocyte migration.

**Effects of pharmacologic inhibition of STAT3**

Despite advances in siRNA technology, the feasibility of siRNA in cancer therapy is still unknown. Fortunately, because STAT3 has been extensively studied as an oncogenic factor, there are several STAT3-specific small-molecule inhibitors with translational potentials. One such STAT3 inhibitor that is commercially available is Stattic, a cell-permeable vinyl-sulfone compound that acts as an inhibitor of STAT3 cellular function by targeting the STAT3-SH2 domain and preventing its association with upstream kinases (5). First, we titrated the concentration of Stattic needed to specifically reduce the activation, dimerization, and nuclear translocation of STAT3 in human HN11 and Cal27 cell lines. At concentrations between 1 to 10 μmol/L, which is below the reported concentrations at which apoptosis is induced, Stattic resulted in substantial reduction in p-STAT3 and the DNA binding of STAT3 homodimers, whereas binding of STAT1 homodimers to the same DNA probe was not significantly inhibited at those concentrations in all human cell lines tested according to the EMSA (Fig. 6A). We next evaluated the battery of proinflammatory chemokine and cytokine after 48 hours of incubation in the tumor cell lines. We were able to observe a dose-dependent increase in mRNA for RANTES, TNFα, IP-10, IL-8, IFNβ, and IL-6 in both HNSCC cell lines (Fig. 6B). It is informative that the small-molecule STAT3 inhibitor did result in increased mRNA for RANTES and IFNβ, suggesting that the lack of effect on these genes with STAT3 siRNA transfection may have been due to incomplete STAT3 knockdown rather than a fundamental difference between mouse and human tumor lines. These results suggest that targeting STAT3 with a small-molecule inhibitor at doses that may not induce apoptosis may still induce a therapeutic antitumor effect by activating locoregional DCs and by attracting leukocytes into the tumor microenvironment.

**Discussion**

Physiologically, STAT3 transcription factor is a critical negative regulator of tissue wound healing that induces pleiotropic brake on inflammatory cytokines, chemokines, and angiogenic factors to prevent tissue injury during the chronic phase of wound healing (17, 28, 29). It is not surprising that neoplastic epithelial cells have been selected for constitutively active STAT3 as a potent immune evasion mechanism. Previous work from our laboratory in the murine system showed that tumor cells could co-opt the physiologic function of STAT3 to prevent an antitumor host immune response (19, 18).

In this report, we showed that STAT3 inhibition in human HNSCC cells could also induce upregulation and secretion of multiple cytokines and chemokines for potential antitumor response in the microenvironment. Both siRNA silencing and pharmacologic small-molecule inhibition of STAT3 were used to substantially reduce STAT3 signaling in the human tumor cells. As shown by this report, HNSCC differentially expressed multiple paracrine factors that can potentially inhibit LPS-induced DC maturation. STAT3 suppression in HNSCC cell lines dramatically reversed this immunosuppressive phenotype in vitro. We also showed that these STAT3-dependent tumor-derived factors...
could stimulate migration of lymphocytes in vitro. The control tumor cells were treated with scrambled siRNA to ensure that variables that can be attributed to transfection or transduction of small RNA sequences did not confound the paracrine effects. Moreover, the two independent methods of STAT3 inhibition inducing comparable effects corroborate our hypothesis that these paracrine cross-talk are STAT3 dependent.

We reasoned that focusing on a single cytokine or chemokine may not reveal the combinatorial effects of multiple factors that can direct cellular behaviors within the tumor microenvironment. To directly examine the various combination of STAT3-induced cytokines and chemokines, we tested the unbiased conditioned media from the STAT3 siRNA–treated tumor cell line culture supernatants to examine the paracrine cross-talk between the human tumor cells and the isolated human immune cells. Although we tested for IP-10, IL-6, IL-8, and VEGF protein levels in the culture supernatants, this report does not address the possibility of the differential expression pattern of other

Figure 4. Inhibition of DC maturation by tumor-derived factors is abrogated upon STAT3 knockdown. Flow cytometry plots show surface expression of HLA-DR versus CD86 of CD11c+–gated DCs. Top row, immature monocyte-derived DCs (middle) and LPS-matured DCs (right). Left, isotype controls on LPS-matured DCs. Middle row, LPS-matured DCs cultured in presence of condition medium from nontreated (left), control (middle), and STAT3 siRNA–treated Cal27 supernatant (right). Bottom row, LPS-matured DCs cultured in presence of condition medium from control lentivirus (LV) vector–transduced HN11 supernatant (left), and STAT3 B7 and B8 shRNA LV vector–transduced HN11 supernatant (middle and left, respectively).
proinflammatory factors that may shape the tumor microenvironment. Our preliminary experiments with VEGF to examine if a single STAT3-dependent factor can mediate some of the antitumor phenotypes of the tumor microenvironment suggest that STAT3 signaling induces transcriptional variations of multiple paracrine factors that work combinatorially to produce an antitumor response. We are currently investigating the presence of other important cytokines, such as IL-12 and IL-23, as well as combinatorial effects of multiple paracrine factors mediating DC activation and lymphocytic chemotaxis in vitro. It would be also very important to study the relationship between corresponding receptors of these proinflammatory mediators and STAT3 signaling suppression in the same tumor cells as well as in the immune cells.

Demonstrating IP10 upregulation consistently and significantly in all the cell lines treated with STAT3 siRNA was an interesting phenomena because it can potentially mediate antitumor activity through two independent mechanisms: by stimulation of leukocytic migration into tumor microenvironment and by inhibition of tumor angiogenesis. It is also well documented in mouse tumor models the synergistic antitumor effects of IP10 in conjunction with IL-12 or with TNFα (30, 31). This "attraction-expansion" hypothesis is based on the idea that the colocalization of proinflammatory and chemotactic mediators could synergistically enhance antitumoral immune response (32). Our results showed concomitant upregulation of various STAT3-dependent immunomodulatory factors that can act as the "recruiters" as well as "activators."

The finding that the blockade of STAT3 in HNCC cancer cell lines could reverse the inhibitory effects of tumor cell supernatant on DC maturation suggested that VEGF may be a critical STAT3-dependent immunomodulator that can interfere with DC differentiation and function in cancer patients. Gabrilovich and colleagues (33) documented that there was a direct association between the elevated numbers of immature DCs in cancer patients and high levels of circulating VEGF. In our in vitro studies, STAT3-dependent VEGF alone did not seem to directly mediate DC maturation. Interestingly, although we focused our initial studies on DC maturation, STAT3-dependent VEGF may induce other hematopoietic cells such as myeloid-derived suppressor cells to shape the tumor microenvironment toward an immunosuppressive phenotype.

The role of IL-6 in tumorigenesis has been well documented, and a recent cohort study showed that serum IL-6 may serve as a biomarker for prognosis among head and neck cancer patients (34). In our study, as well as in the murine models, we consistently noted increase level of IL-6 with STAT3 suppression. IL-6 is currently hypothesized as one cytokine that can mediate the STAT3 feed forward loop, which may be dependent on NF-κB signaling (18, 35). However, given the complex relationship between inflammation and tumorigenesis within the tumor microenvironment, the consequences of STAT3-dependent IL-6 variations may be difficult to predict. Because we did not observe any increased growth rate of the tumor cells tested in vitro, this elevation of IL-6 is not correlated with STAT3-independent tumorigenesis. Despite the possible use of IL-6 as a biomarker, however, it may not account for the full combinatorial effects of multiple cytokines and chemokines to direct the development of the tumor. Rather than focusing on a single cytokine, therefore, the focus of our study was to evaluate the role of STAT3 signaling in human tumor cells.

In terms of STAT3 signaling, our laboratory and others have shown that STAT3 signaling in the hematopoietic compartment also plays a significant role in the development of the murine tumor microenvironment (20). Although our study was directed at the STAT3 activity from human carcinoma cells, murine studies have shown that STAT3 signaling in the tumor microenvironment can also induce the expression of IL-23 on tumor-infiltrating macrophages, while inhibiting NF-κB–dependent IL-12/p39 gene expression (36). STAT3 also seems to prolong NF-κB nuclear retention in both tumor cells as well as hematopoietic cells (37). Moreover, STAT3 signaling regulates the level of Th17 cells in vivo, and this proinflammatory environment is procarcinogenic in the colon carcinoma models (12, 16). Cumulatively, STAT3 signaling affects multiple downstream pathways to promote a procarcinogenic “soil” that suppresses an antitumor response.

From a clinical standpoint, therefore, targeting STAT3 signaling in the tumor microenvironment seems to be attractive.

Figure 5. Supernatant from STAT3 siRNA–suppressed tumor cells enhances leukocytes migration. Chemotaxis assay showed enhanced PBMC migration in the presence of CM containing siRNA-treated and control Cal27 culture supernatants. PBMCs were incubated in presence of serum-free medium and 100% serum as negative and positive controls, respectively. Columns, mean (n = 3) for each ELISA; bars, SEM. *, P < 0.05; **, P < 0.01.
The data presented in this report show that the paracrine effects of STAT3 activation in human carcinoma can regulate the human tumor microenvironment analogously to the murine system. Given that STAT3 signaling in tumor cells promotes proliferation and prevents apoptosis, our report provides more evidence that STAT3 targeting may be an avenue of translational research as immunomodulator for clinical trials. As a transcription factor, STAT3 may be difficult to target, but we were able to show that Stattic, a small-molecule inhibitor available commercially, can inhibit STAT3 signaling in the human tumor cells. Interestingly, its initial report used several log-fold higher concentrations for induction of apoptosis (5). Using doses from 1 to 10 μmol/L range, which did not induce apoptosis of the tumor cells in our studies, we showed that Stattic can induce an upregulation of RANTES, TNFα, IP-10, IL-8, IFNβ, and IL-6 to a comparable level as seen with the STAT3 siRNA suppression. These results illustrate the feasibility of pharmacologic STAT3 blockade to induce an immunomodulatory effect.

Currently, several clinical trials that can block STAT3 signaling in various cancer patients are under way (38, 39). Some are upstream regulators of STAT3 such as epidermal growth factor receptor inhibitors and neutralizing antibodies, as well as Src inhibitors. Given the findings in our report, the immunomodulatory effects of STAT3 signaling should be investigated as potential mechanisms of clinical efficacy among patients who are treated with biological agents that work upstream to STAT3 signaling.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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