Tumor STAT1 Transcription Factor Activity Enhances Breast Tumor Growth and Immune Suppression Mediated by Myeloid-derivedSuppressor Cells∗

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Background: The role of STAT1 in promoting tumor progression is not well understood.

Results: STAT1 activity is elevated in human and mouse breast cancers, and STAT1 promotes breast cancer progression.

Conclusion: Tumor STAT1 transcription factor activity enhances breast tumor growth and immune suppression mediated by MDSCs.

Significance: STAT1 activity in breast cancer cells is responsible for shaping an immunosuppressive tumor microenvironment, and inhibiting STAT1 activity is a promising immune therapeutic approach.

Previous studies had implicated the IFN-γ transcription factor signal transducer and activator of transcription 1 (STAT1) as a tumor suppressor. However, accumulating evidence has correlated increased STAT1 activation with increased tumor progression in multiple types of cancer, including breast cancer. Indeed, we present evidence that tumor up-regulation of STAT1 activity in human and mouse mammary tumors correlates with increasing disease progression to invasive carcinoma. A microarray analysis comparing low aggressive TM40D and highly aggressive TM40D-MB mouse mammary carcinoma cells revealed significantly higher STAT1 activity in the TM40D-MB cells. Ectopic overexpression of constitutively active STAT1 in TM40D cells promoted mobilization of myeloid-derived suppressor cells (MDSCs) and inhibition of antitumor T cells, resulting in aggressive tumor growth in tumor-transplanted, immunocompetent mice. Conversely, gene knockdown of STAT1 in the metastatic TM40D-MB cells reversed these events and attenuated tumor progression. Importantly, we demonstrate that in human breast cancer, the presence of tumor STAT1 activity and tumor-recruited CD33+ myeloid cells correlates with increasing disease progression from ductal carcinoma in situ to invasive carcinoma. We conclude that STAT1 activity in breast cancer cells is responsible for shaping an immunosuppressive tumor microenvironment, and inhibiting STAT1 activity is a promising immune therapeutic approach.

Cancer breast remains the most common malignant tumor in women (1). It is well documented that tumors evolve strategies to evade detection and eradication by the immune system (2, 3). In addition, more recent studies suggest that the immune system is conducive to collaborate with the tumor by orchestrating cancer-associated inflammation and suppressing antitumor immunity (4, 5). Several lines of evidence implicate tumor-derived factors accumulating over time in promoting tumor tolerance and immune suppression, thereby driving aggressive cancer progression (6, 7). In order to investigate tumor-derived factors that promote breast cancer progression, we have developed a syngeneic orthotopic transplantation mouse model of breast cancer progression (8). In this model, the TM40D-MB tumor cells, originally derived from the TM40D tumor cells, developed mammary tumors that are more aggressive and metastatic when they were implanted to the mammary fat pads of BALB/c mice. A microarray comparison between TM40D and TM40D-MB tumor cell lines revealed gene expression alterations in the TM40D-MB cells related to immune control of tumor progression (9, 10). Interestingly, many IFN-γ-activated genes were found to be up-regulated, including a 4-fold expression increase of STAT1 in TM40D-MB cells.

STAT1 has been classically defined as a T helper 1 proimmune and antitumor transcription factor, based on its canonical role in IFN-γ signaling and on studies using STAT1−/− tumor cells and mouse models (11, 12). Based on this, it was widely believed that STAT1 acts as a tumor suppressor (13). However, STAT1 has been found to be up-regulated in several late stage human cancers, including breast cancer (14–17). STAT1 has been shown to regulate DNA repair pathways, and is a predictive marker for breast cancer chemotherapy and radiotherapy resistance (18–20). The multipotent effects of STAT1 on the immune system include regulating numerous genes involved in promoting chronic inflammatory disease, and inhibiting STAT1 signaling for the treatment of autoimmune disease is an active area of research (21).

In this study, we demonstrate that constitutively active STAT1 is significantly overexpressed in human biopsies of invasive carcinoma, as compared with ductal carcinoma in situ (DCIS)2 specimens. In addition, we present the novel finding

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2 The abbreviations used are: DCIS, ductal carcinoma in situ; MDSC, myeloid-derived suppressor cell; Dox, doxycycline; Treg, CD4+ Foxp3+ regulatory T cell.
that in human breast tumor biopsies, increasing tumor progression from DCIS to invasive carcinoma correlates with increased tumor recruitment of CD33+ myeloid cells that have been described by others as myeloid-derived suppressor cells (MDSCs), immune cells that are well established suppressors of antitumor immunity (22, 23). Using our syngeneic orthotopic transplantation mouse model of late stage mammary carcinoma, we show that STAT1 overexpression promotes aggressive tumor growth, whereas gene knockdown of STAT1 significantly delays tumor progression. Further, tumor expression of STAT1 directly recruits suppressive CD11b+Gr1+ cells, which have characteristics of granulocytic MDSCs, to the tumor microenvironment. We demonstrate that STAT1 induces expression of proinflammatory TNF-α as well as TGFβ and IL-13, factors known to promote suppressive immune cell function (24). MDSCs are known to potently suppress adaptive T cell-mediated antitumor immunity (25). In our study, we show that STAT1 overexpression in TM40D tumors alters their immune profile from a high infiltration of CD4+ and CD8+ T cells to a low infiltration of these cells, whereas knockdown of STAT1 in the TM40D-MB tumors reverses this phenotype. Based on these findings, we propose that inhibition of STAT1 in breast cancer will prevent the homing of suppressive immune cells to the tumor microenvironment and enable immune mediated tumor rejection.

**EXPERIMENTAL PROCEDURES**

**Creation of STAT1-modulated Cell Lines**—The low metastatic TM40D cells were engineered to express a constitutively activated STAT1 (TM40D-STAT1C). The STAT1C gene contains a disulfide linkage mutation that dimerizes STAT1, enabling constitutive tyrosine autophosphorylation and activation (a gift from Dr. John Crispino, Northwestern University). STAT1C cDNA was cloned into a retroviral vector under the control of a tetracycline (doxycycline)-inducible promoter (RevTetOn, Clontech) and used to transduce TM40D cells. Empty vector was also transduced in these cells as a control (TM40D-TetOn). Constitutive expression of phosphorylated STAT1 protein is induced by treating cells in culture with (1 μg/ml) doxycycline (Dox; Research Products International) for 48 h in DMEM with Tet-compatible FBS (Clontech). To knock down protein expression of STAT1 in the TM40D-MB cells, a retrovirus expressing an shRNA inhibitor of STAT1 (pSM2; Open Biosystems) was used to transduce TM40D cells. Empty vector was also transduced in these cells as a control (TM40D-MB-pSM2). For the human tumors, a single set of paraffin-fixed sections containing human DCIS samples and invasive breast sections from patients with various breast cancer metastases were obtained from Dr. Song Zhao (University of Wisconsin, Madison, WI). Slides were stained with primary anti-Gr1, anti-CD11b, anti-CD4, anti-CD8α, and anti-CD45R. To exclude dead cells from analysis, cells were stained with LIVE/DEAD fixable violet blue (Invitrogen). Cells were sorted on a FACS Canto II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Immunohistochemistry and Immunofluorescence**—For each tumor group, 5-μm-thick sections of formalin-fixed paraffin-embedded mammary tumors (3 mice/group) at maximum tumor size were deparaffinized and rehydrated in graded alcohol. Antigen retrieval was done using 1× Target retrieval solution (DAKO) and incubated with 1% bovine serum albumin for 30 min at room temperature. Sections were then incubated with biotinylated anti-mouse Gr1 (BD Biosciences) or anti-mouse F4/80 (Abcam) primary antibodies overnight at 4 °C. For the mouse tumors, slides were washed with PBS and incubated for 15 min with 4’,6-diamidino-2-phenylindole (Sigma) and then washed with PBS and mounted with antifade mounting medium. For the human tumors, a single set of paraffin-fixed sections containing human DCIS samples and invasive breast tumors from patients with various breast cancer metastases were obtained from Dr. Song Zhao (University of Wisconsin, Madison, WI). Slides were stained with primary anti-phospho-STAT1 (Tyr701; Cell Signaling), and 3,3’-diaminobenzidine staining was performed using the ABC staining kit (Pierce) according to the manufacturer’s recommendations.

**Fluorescence-activated Cell Sorting (FACS) Analysis**—At maximum tumor size, spleen and tumor were excised and homogenized to obtain single cell suspensions, and erythrocytes were lysed as described previously (10). To test for immune cell recruitment in spleen and tumor, 2 × 10^6 cells from each sample were preincubated with anti-CD16/CD42 (2.4G2; eBioscience) to avoid nonspecific binding of antibodies to FcγR (28). Cells were stained with the following fluorophore-conjugated anti-mouse monoclonal antibodies (BD Biosciences): anti-Gr1, anti-CD11b, anti-CD4, anti-CD8α, and anti-CD45R. To exclude dead cells from analysis, cells were stained with LIVE/DEAD fixable violet blue (Invitrogen). Cells were sorted on a FACS Canto II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Arginase Assay**—Tumor extracts were prepared from TM40D-MB tumors by homogenizing tumors in PBS and harvesting the supernatant. Purified Gr1+ cells from TM40D-MB tumors and CD11b+ cells from naive bone marrow were obtained by labeling cells with either anti-Gr1-biotin or anti-CD11b-biotin (BD Biosciences) and separating cells using Magnetic-activated cell sorting streptavidin-conjugated beads and LS columns (Miltenyi Biotec). Supernatants from tumor extracts after intracardiac injection of TM40D cells as described previously (9). For all tumor experiments, mice were injected bilaterally into the fourth mammary fat pads with 1 × 10^6 tumor cells. Tumor volume measurements were taken every 3 days, and tumor volume was calculated using the formula, length × width^2/2. Mice were euthanized when tumors reached 2.0 cm.

**Tumor STAT1 Promotes Breast Cancer Progression**
Tumor STAT1 Promotes Breast Cancer Progression

and lysates from Gr1+ and CD11b+ cells were quantified for total protein by a BCA protein assay (Thermo Scientific), according to the manufacturer’s directions. A total of 10 μg of protein from each sample was assayed for arginase activity using the Quantichrom arginase assay kit (BioAssay Systems) according to the manufacturer’s instructions. Arginase activity (units/liter of sample) was calculated as follows.

\[
\text{Activity} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{water}}} \times [\text{urea standard}] \times 50 \times 10^4 / (40 \times t) \quad (\text{Eq. 1})
\]

Migration Assay—CD11b+ cells were separated from total mononuclear cells (MNCs) isolated from naive mouse spleenocytes using biotinylated anti-CD11b (BD Biosciences), streptavidin magnetic MACS beads, and MACS LS columns (Miltenyi Biotec). The purity of CD11b+ cells isolated was verified using FACS. For the migration assay, the upper chamber of a 5-μm uncoated 96-well ChemoTx system (Neuro Probe) was used and was seeded with 22.5 μl of isolated CD11b+ cells or MNCs, resuspended in serum-free RPMI at 106 cells/ml concentration, and seed in triplicate. The bottom well was loaded in triplicate with either 29 μl of serum-free DMEM (negative control), conditioned medium obtained from TM40D-TetOn or TM40D-STAT1C cells treated for 48 h with Dox (1 μg/ml), or conditioned DMEM from TM40D-MB-pSM2 or TM40D-MB-shSTAT1 cells after 48 h of culture. For studies targeting TNFα as a regulator of CD11b+ cell migration, either 10 μg/ml anti-TNFα antibody (eBioscience) or vehicle was included in TM40D or TM40D-STAT1C conditioned medium. After a 4-h incubation at 37 °C, cells that migrated into the lower chamber were fixed with 1% paraformaldehyde, imaged by microscope with an attached camera (Leica), and quantified using ImageJ software.

T Cell Proliferation Assay—T cells were separated from naive splenocytes using biotinylated anti-T cell (BD Biosciences), streptavidin magnetic MACS beads, and MACS LS columns (Miltenyi Biotec). The purity of CD11b+ cells isolated was verified using FACS. For the migration assay, the upper chamber of a 5-μm uncoated 96-well ChemoTx system (Neuro Probe) was used and was seeded with 22.5 μl of isolated CD11b+ cells or MNCs, resuspended in serum-free RPMI at 106 cells/ml concentration, and seed in triplicate. The bottom well was loaded in tripli culate with either 29 μl of serum-free DMEM (negative control), conditioned medium obtained from TM40D-TetOn or TM40D-STAT1C cells treated for 48 h with Dox (1 μg/ml), or conditioned DMEM from TM40D-MB-pSM2 or TM40D-MB-shSTAT1 cells after 48 h of culture. For studies targeting TNFα as a regulator of CD11b+ cell migration, either 10 μg/ml anti-TNFα antibody (eBioscience) or vehicle was included in TM40D or TM40D-STAT1C conditioned medium. After a 4-h incubation at 37 °C, cells that migrated into the lower chamber were fixed with 1% paraformaldehyde, imaged by microscope with an attached camera (Leica), and quantified using ImageJ software.

Real-time RT-PCR Analysis—Total mRNA was isolated using the RNeasy kit (Qiagen) from cultured TM40D-MB-pSM2 and TM40D-MB-shSTAT1 cells as well as TM40D-TetOn and TM40D-STAT1C cells cultured for 48 h with Dox (1 μg/ml). RNA was reverse-transcribed into cDNA using the Superscript II kit (Invitrogen). Real-time RT-PCR was performed on cDNA using Power SYBR Green master mix and the 7900 HT real-time PCR machine (Applied Biosystems), according to the manufacturer’s recommendations. Primer sets for the cytokines tested are as follows, with GAPDH as the endogenous control: mouse IL-13, 5′-ATCTACAGGACCAGCGGATA-

TTGC-3′ (sense) and 5′-CTGATGTGAGAAAGAAATGAGTCC-3′ (antisense); mouse TGFβ1, 5′-CGCCATCTATGAGAAAAAC-3′ (sense) and 5′-GTAACGCCAGGAAT-TGT-3′ (antisense); mouse TNFα, 5′-TCCAGGTCTTCTTCAAGGGA-3′ (sense) and 5′-GGTGAGGACGCTAGTCCGG-3′ (antisense); mouse IL-1β, 5′-ATGGCAACTTTGCCGAACTCAACT-3′ (sense) and 5′-CAGGAGGATTAGATTCTTCTTTT-3′ (antisense); mouse IL-6, 5′-AGGATCCACTCCCAACACGCT-3′ (sense) and 5′-CAAAGTGCATCATCGTTGTTCATAC-3′ (antisense).

Statistical Analysis—To determine statistical significance, Student’s paired t tests, χ2 tests, or log rank tests were used. A value of p < 0.05 was considered statistically significant. Data were analyzed using Excel for Mac 2008 (Microsoft) and Prism 5 (GraphPad).

RESULTS

Tumor Expression of Constitutive STAT1 Correlates with Disease Progression in Human Breast Cancer—Several studies have now correlated tumor up-regulation of STAT1 with advanced breast cancer (15, 17, 29). Previous work by the Brown group had demonstrated increased tumor STAT1 immunostaining, especially at the tumor-stromal borders, in human breast tumor specimens as compared with normal breast tissue (30). We sought to validate and extend these findings in human biopsy samples scored as either DCIS or as invasive carcinoma. Importantly, we wanted to compare the level of phospho-STAT1 (Tyr701) staining because previous studies had only examined non-phosphorylated and thus potentially inactive STAT1. Our results of the immunohistochemical staining on the invasive tumor sections showed significant staining of phospho-STAT1 (in brown) in tumor cells (counterstained in purple) throughout the tumor sections, with very little staining in the DCIS tumors (n = 10 for each group, p = 0.0053) (Fig. 1, A and B). These results provide conclusive evidence that tumor STAT1 activation correlates with increasing disease progression in human breast cancer.

Tumor Recruitment of CD33+ Myeloid Cells Correlates with Invasive Disease Progression in Human Breast Cancer—Several recent studies have demonstrated a strong correlation between tumor progression and cancer-associated inflammation (24, 31). One mechanism for how inflammation promotes tumor progression is through the ability to recruit MDSCs to sites of inflammation (32). MDSCs are a heterogeneous population of immature myeloid cells, collectively identified by their surface expression of Gr1+ and CD11b+ in mice (CD33+ and CD11b+ in humans) (33). MDSCs collectively function to suppress anti-tumor immunity through mechanisms involving arginase 1 (ARG1)-mediated arginine depletion, inducible nitric-oxide synthase production of NO, and generation of reactive oxygen species (22, 23). In multiple animal models of cancer, MDSC accumulation in the spleen and tumor sites positively correlated with tumor progression. Previous work by Montero and colleagues (36) had correlated expansion of CD33+ MDSCs in circulating blood with increasing clinical stage of human breast cancer, and inhibiting MDSCs has been shown to improve clinical outcomes (34–37). However, to date, no group has directly demonstrated CD33+ MDSC infiltration in human biopsy sam-
Tumor STAT1 Promotes Breast Cancer Progression

FIGURE 1. Tumor STAT1 expression and MDSC recruitment in human breast tumors correlates with disease progression. A and B, immunohistochemical staining of phospho-STAT1 in human DCIS and invasive tumor tissue sections. A, representative images of DCIS and invasive carcinoma. Nuclear staining with hematoxylin is indicated in purple, and cells staining positive for phospho-STAT1 (Tyr701; Cell Signaling) using 3,3′-diaminobenzidine are in brown (×20 magnification). The arrows indicate phospho-STAT1-positive cells. B, histogram quantifying the total number of phospho-STAT1+/total hematoxylin+ tumor cells. Data are mean ± S.E., 5 slides/tumor group, compiled from two independent experiments (a total of 10 samples for each group). **, p ≤ 0.01, Student’s t test. C and D, immunofluorescence analysis of the expression of CD33+ MDSCs in human DCIS and invasive tumor tissue sections. Black scale bar, 50 μm. C, representative images of DCIS and invasive carcinoma. Nuclear staining with DAPI is indicated in blue, and cells staining positive for anti-CD33+ (BD Biosciences) are in red. The arrows indicate CD33+ cells. D, histogram quantifying CD33+ staining in tumors. Total numbers of defined red CD33+ cells were counted per field and averaged from five randomly selected fields at the same magnification (×10) for each slide. White scale bar, 50 μm. Data are mean ± S.E. (error bars), 10 tumor slides/group, compiled from two independent experiments. ***, p ≤ 0.001, Student’s t test.

STAT1 Promotes Tumor Progression in a Syngeneic Orthotopic Transplantation Model of Breast Cancer—In several human studies of cancer, gene microarrays comparing early and late stage tumors revealed significant up-regulation of many proinflammatory genes, including STAT1 (14, 38). This was similarly demonstrated in our syngeneic orthotopic transplantation model of metastatic breast cancer. In this model, we found that TM40D-MB cells had a 4-fold expression increase of STAT1 compared with TM40D tumor cells through a gene microarray analysis (10). In order to directly study the mechanism of STAT1 modulation on tumor progression using our mouse model, mouse mammary carcinoma cell lines were created with varying STAT1 protein expression levels. We first generated a TM40D cell line expressing a constitutively activated STAT1 under the control of a tetracycline (doxycycline)-inducible promoter (TM40D-STAT1C). Protein levels of STAT1 as assessed by immunoblot were significantly higher in Dox-induced TM40D-STAT1C cells than in TM40D-TetOn (vector control) cells (Fig. 2, A and B). Importantly, Dox-treated TM40D-STAT1C cells induced high expression of active tyrosine-phosphorylated STAT1, similar to TM40D-TetOn upon IFN-γ stimulation.

To study the effects of STAT1 down-regulation in the highly metastatic TM40D-MB cells, a retrovirus expressing an shRNA inhibitor of STAT1 was used to transduce TM40D-MB cells (TM40D-MB-shSTAT1). This resulted in significantly decreased protein levels of STAT1, as compared with TM40D-MB-pSM2 (vector control) cells (Fig. 2, A and B). Importantly, basal levels of phospho-STAT1 were inhibited in the TM40D-MB-shSTAT1 cells and diminished even upon IFN-γ stimulation.

Next we wanted to directly assess the effects of STAT1-modulated tumor cells on tumor progression using our syngeneic mouse model of breast cancer. For these experiments, TM40D-STAT1C and TM40D-TetOn tumor cells were transplanted into the mammary fat pads of BALB/c mice and monitored for tumor progression, with Dox administered to the drinking water for the duration of the experiment to induce high levels of STAT1C expression. TM40D-STAT1C tumor cells exhibited highly aggressive tumor growth as compared with control tumor cells, with tumors becoming palpable significantly earlier (day 8, p = 0.011) and reaching maximum tumor size earlier than the TM40D-TetOn cells (Fig. 2C). However, when TM40D-TetOn and TM40D-STAT1C were grown in cell culture in vitro, they grew at a similar rate (data not shown). These results strongly suggest that STAT1 acts as the tumor promoter in the mouse model in vivo.
The TM40D-MB mouse mammary tumor cell line possesses different growth kinetics compared with the TM40D tumor cell strain, which could be attributed to the changed expression of nearly 100 genes (10). When implanted to the mammary fat pads, TM40D-MB tumor cells grow more slowly than TM40D tumor cells. To validate the tumor promoting activity of STAT1 in TM40D-MB cells, we compared the growth kinetics of STAT1 knockdown TM40D-MB-shSTAT1 cells with the TM40D-MB-pSM2 (vector control) cells. We used the Kaplan-Meier survival curve to assess differences between TM40D-MB-shSTAT1 and TM40D-MB-pSM2 (vector control) cells. Mice were administered Dox (2 mg/ml) in drinking water and monitored for tumor growth by caliper measurement, and mice were euthanized at maximum tumor size (2 cm). For day 8 (the earliest significant difference detected), asterisks indicate \( p = 0.011 \), Student's t test. Results represent a total of 10 animals in each tumor group, compiled from two independent experiments. D. mice were implanted with either high STAT1-expressing cells transduced with vector (TM40D-MB-pSM2) or TM40D-MB shSTAT1 knockdown cells (TM40D-MB-shSTAT1). Mice were monitored for tumor growth, and maximum tumor size was scored as end point for survival. \( * \), \( p = 0.02 \), log rank test. Results represent a total of 10 animals in each tumor group, compiled from two independent experiments.

STAT1 Promotes Systemic CD11b\(^+\) Gr1\(^+\) Cell Expansion and Primary Tumor Recruitment—Next we sought to determine whether tumor STAT1 activity might also promote MDSC recruitment in our mouse model. At the experimental end point, cells isolated from spleens and hind leg bones were analyzed by FACS for the murine MDSC cell surface markers Gr1 and CD11b (25). Overexpression of STAT1C in the TM40D tumor cells was found to significantly increase spleen and bone densities of MDSCs as compared with TM40D-TetOn \( (p = 0.05 \) and \( p = 0.0001 \), respectively) (Fig. 3, A–C). Conversely, knockdown of STAT1 in the TM40D-MB-shSTAT1 cells significantly decreased spleen and bone densities of MDSCs \( (p = 0.004 \) and \( p = 0.003 \), respectively). In the tumor microenvironment, these findings were validated and extended by immunohistochemistry. In the TM40D-TetOn tumor group, localization of Gr1\(^+\) cells was confined to the borders of the tumor (Fig. 3D). In contrast, Gr1\(^+\) cells in the TM40D-MB-pSM2 tumors had significantly infiltrated into the tumor. The TM40D-STAT1C tumors, with highly overexpressed STAT1, had significantly higher densities of MDSCs...
FIGURE 3. STAT1 promotes systemic Gr1+ CD11b+ MDSC expansion and primary tumor recruitment. Spleens and hind leg bones were harvested from mice at maximum tumor size. Shown are representative pictures (A) and a histogram (B) quantifying the frequency of live splenocytes analyzed by flow cytometry (BD FACS Canto II) positive for expression of MDSC surface markers Gr1-PerCP-Cy5.5 and CD11b-PE. Data are mean ± S.D. (error bars), 5 mice/tumor group. C, histogram quantifying the frequency of live Gr1+ CD11b+ bone marrow cells analyzed by FACS. Data are mean ± S.D., 5 mice/tumor group. Data are representative of two independent experiments. D and E, formalin-fixed sections of tumors from three mice in each tumor group were prepared as described. Slides were stained with biotinylated anti-mouse Gr1 (BD Pharmingen) and counterstained with hematoxylin (eBioscience). For each tumor group, 15 slides/tumor were counted and quantified using ImageJ. D, representative pictures of Gr1+ cells (in red) for each tumor group. Black scale bar, 50 μm. E, histogram quantifying the total number of Gr1+ cell counts for each tumor group. Data are mean ± S.E., 3 mice/tumor group. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; Student’s t test.
both in the periphery and within the tumor, as compared with TM40D-TetOn control cells ($p = 0.0028$) (Fig. 3, D and E). In contrast, knockdown of STAT1 in the TM40D-MB-shSTAT1 tumors resulted in significantly less Gr1$^+$ staining as compared with TM40D-MB-pSM2 control cells ($p = 0.02$), and staining was localized in the tumor vasculature as opposed to infiltrating into the tumor (Fig. 3, D and E). Thus, in agreement with our human tumor data, tumor STAT1 overexpression correlates with an increased frequency of Gr1$^+$ CD11b$^+$ MDSCs in both primary tumor and systemic sites.

**Suppressive Function of MDSCs via Arginase 1 and STAT1-mediated Recruitment of MDSC Myeloid Precursor Cells**—Because MDSCs have been shown to suppress antitumor immunity through expression of ARG1, we assayed STAT1-induced tumors with high MDSC frequencies for arginase activity. Tumor extracts from TM40D-MB tumors were found to have high levels of arginase activity, and even higher arginase activity could directly promote recruitment of MDSC myeloid cells. Specifically, CD11b$^+$ cells isolated from TM40D-MB tumors were cultured with anti-CD3 activated T cells of naive mouse splenocytes at CD11b$^+/\text{T cell}$ ratios of 10:1, 1:10, 0:1, and 0:1 without anti-CD3 activation (Fig. 4B; data of ratios of 5:1 and 1:1 not shown due to similar effects as 10:1). The tumor-derived CD11b$^+$ cells effectively suppress CD4$^+$ T cell proliferation at all concentrations compared with T cells not cultured with CD11b$^+$ cells. There were no observed differences in CD8$^+$ T cell proliferation or differences in T cell death between any groups (data not shown). These data indicate that the suppressive nature of the CD11b$^+$ cells parallels the suppressive characteristics of MDSCs.

Next we wanted to assess whether STAT1-induced factors could directly promote recruitment of MDSC myeloid precursor cells using an *in vitro* migration assay (39). For these experiments, CD11b$^+$ myeloid cells were isolated from naive splenocytes, and both the CD11b$^+$ and CD11b-depleted mononuclear cells fractions were assessed for the rate of migration into cell-free supernatants harvested from cultured STAT1-modulated tumor cells. Frequencies of migrated CD11b$^+$ myeloid cells...
were found significantly elevated in the TM40D-STAT1C group as compared with the TM40D-TetOn group ($p = 0.004$) (Fig. 4C). Migration of CD11b$^+$ cells was highest in the TM40D-MB-pSM2 group and was found to be significantly lower in the TM40D-MB-shSTAT1 knockdown group ($p = 0.03$). These data show that modulation of STAT1 expression in these tumor cells has a direct effect on their ability to recruit CD11b$^+$ myeloid cells, the precursors to suppressive MDSCs. Interestingly, no significant differences were found in the ability of CD11b-depleted MNCs to migrate across the membrane (Fig. 4D). Because the CD11b$^-$ MNC fraction contains mainly lymphocytes, there was clearly a preference for myeloid cell recruitment (CD11b$^+$) over lymphocytes.

**STAT1 Induces Tumor Cell Expression of Proinflammatory and Immunosuppressive Cytokines**—Chronic overexpression of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and CCL2 (MCP-1), are associated with metastatic tumor progression in multiple tumor types (40). One mechanism for how these cytokines may promote tumor progression is through their ability to recruit MDSCs to sites of inflammation (32). Because STAT1 signaling is known to be proinflammatory, we sought to identify potential STAT1-regulated factors that may be directly recruiting MDSCs as well as potentially enhancing suppressive MDSC function (41). For this we performed real-time RT-PCR on a panel of cytokines using mRNA extracted from cultured TM40D-MB-pSM2 and TM40D-MB-shSTAT1 cells as well as TM40D-TetOn and TM40D-STAT1C cells that were cultured for 48 h under Dox induction. Among the proinflammatory cytokines IL-1β, IL-6, and TNF-α, only expression of TNF-α was found to be significantly increased upon STAT1 induction of TM40D cells as compared with TM40D-TetOn ($p = 0.009$) (Fig. 5, A and B). Similarly, expression of TNF-α in the STAT1 knockdown TM40D-MB-shSTAT1 cells was significantly decreased as compared with the TM40D-MB-pSM2 cells ($p = 0.001$). Expressions of the cytokines TGFβ and IL-13 were also both significantly increased in the TM40D-STAT1C cells as compared with TM40D-TetOn ($p = 0.002$; IL-13, $p = 0.002$). In contrast, high levels of these cytokines in the TM40D-MB-pSM2 cells were significantly reduced in the TM40D-MB-shSTAT1 cells (TGFβ, $p = 0.002$; IL-13, $p = 0.001$) (Fig. 5, A and B). Thus, tumor induction of STAT1 induces expression of proinflammatory and MDSC-recruiting TNF-α and additionally up-regulates expression of TGFβ and IL-13, factors known to promote suppressive MDSC function and inhibit antitumor immunity.
To assess the role TNFα has on CD11b+ cell recruitment in tumors overexpressing STAT1C, tumor CD11b+ cell migration was characterized utilizing anti-TNFα-neutralizing antibody in addition to the conditioned tumor cell media (Fig. 5C). Specifically, CD11b+ cells from the TM40D-MB primary tumor were assayed for their rate of migration toward either TM40D or TM40D-STAT1C conditioned medium with or without anti-TNFα antibody. The utilization of the anti-TNFα antibody suppressed CD11b+ cell migration in the TM40D-STAT1C conditioned medium group by 40% compared with the TM40D-STAT1C control group. In contrast, there were no observed differences in CD11b+ cell migration in either TM40D conditioned medium groups. These data suggest that TNFα may be one potential factor driving the STAT1-induced MDSC recruitment observed in aggressive breast cancer.

**STAT1 Overexpression Alters T Cell Phenotypes in the Tumor Microenvironment**—T cell-mediated adaptive immunity is a well recognized inhibitor of tumor progression (42). MDSCs have been demonstrated to potently inhibit both CD4+ and CD8+ T cell tumor recruitment and their antitumor activity (25). In order to determine the effects of MDSC tumor recruitment on CD4+ and CD8+ T cells in our model, we assessed tumor frequencies of these cells by FACS. We found that tumor densities of CD4+ and CD8+ T cells in the TM40D-TetOn tumor group were significantly higher than in the TM40D-STAT1C group (CD4+, p = 0.001; CD8+, p = 0.007) (Fig. 6, A–C). Comparing the STAT1 knockdown TM40D-MB-shSTAT1 group to the high STAT1-expressing TM40D-MB-pSM2 tumor group, the frequencies of CD4+ and CD8+ T cells were significantly increased in the STAT1 knockdown tumor group (CD4+, p = 0.049; CD8+, p = 0.011) (Fig. 6, A–C). These results identify a previously unrecognized function of tumor STAT1 activity in suppressing infiltration of CD4+ T and CD8+ T cells, thereby disabling potent mediators of adaptive antitumor immunity.

MDSCs are known to recruit and expand another immune suppressor cell, known as CD4+ Foxp3+ regulatory T cells (Tregs) (43). Tregs promote tumor tolerance by inhibiting the function of CD8+ T effector cells of the adaptive immune system (44, 45). Because MDSCs were found to significantly accumulate in STAT1-overexpressed tumors, we investigated whether these tumors also promoted CD4+ Foxp3+ Treg accumulation by immunofluorescence staining. In line with our hypothesis that tumor STAT1 expression promotes immune
suppression, STAT1-overexpressing tumors had significantly reduced frequencies of antitumor CD4⁺ and CD8⁻ T cells, and knockdown of tumor STAT1 expression reduced tumor levels of suppressive Tregs (data not shown).

**DISCUSSION**

IFN-γ/STAT1 activation has been classically associated with antiviral and antitumor immunity (11, 12). Early studies showed that STAT1 activates antiproliferative and proapoptotic genes, thus classifying STAT1 as a tumor suppressor (13). A large body of clinical studies showed the benefit of type I interferon in melanoma and the up-regulation of Stat1 inhibitors in cancer patients’ peripheral blood lymphocytes (46, 47). In addition, several reports showed that loss of STAT1 is associated with breast cancer development, based on data using STAT1⁻/⁻ models (13, 48, 49). However, it is important to point out that in these studies, the immune system in STAT1⁻/⁻ does not function as that in the wild type mice (48, 49). Moreover, accumulating evidence in multiple cancer models has implicated constitutive STAT1 activation as tumor-promoting. Microarray analysis of non-small cell lung carcinoma cells overexpressing constitutive STAT1 revealed significant elevations in proinflammatory gene expression, with no effect on proapoptotic or proliferative genes (38). Comparison microarrays between benign human papilloma virus-negative, benign human papilloma virus-positive, and highly malignant cervical squamous carcinoma cells demonstrated a correlation between increasing STAT1 expression and proliferative gene expression with increasing disease progression (14). STAT1 was found significantly to be overexpressed in a microarray of human melanoma metastases, and knockdown of STAT1 in human melanoma cells significantly reduced their invasive and migratory abilities in vitro and decreased lung metastasis in vivo in a xenograft melanoma mouse model (50). Similarly, STAT1 overexpression in human squamous carcinoma cells was found to induce prosurvival genes and resistance to genotoxic stress (18, 19). In human breast cancer, STAT1 expression was a predictive marker for poor survival as well as chemotherapy and radiotherapy resistance (15, 20).

Based on these data, we hypothesized that STAT1 overexpression in breast cancer might have a tumor-promoting, rather than tumor-suppressing role. Indeed, we demonstrated in human breast cancer biopsies that tumor cell expression of constitutively active STAT1 correlates with increasing disease progression from DCIS to invasive carcinoma. In our syngeneic transplantation mouse model of breast cancer, we discovered a significant up-regulation of STAT1 and other IFN-γ-activated genes in the highly metastatic TM40D-MB tumor cells. We showed that overexpressing constitutive STAT1 in low metastatic TM40D cancer cells promotes aggressive tumor growth, whereas knockdown of STAT1 in highly metastatic TM40D-MB cells significantly delays tumor growth.

We then investigated the mechanism of how STAT1 might promote tumor progression in an immunocompetent model because previous studies demonstrating pro-tumor functions of STAT1 in breast cancer were conducted in cell culture and immunodeficient xenograft models (15, 18–20). In addition to its potential for regulating DNA repair pathway genes (18, 19), STAT1 has been shown to regulate numerous proinflammatory cytokines and chemokines (21, 51–53). Aberrant STAT1 signaling is implicated in several inflammation-driven diseases, such as asthma, inflammatory bowel disease, and rheumatoid arthritis (21). Although acute inflammation is highly beneficial for activating antitumor immunity, chronic inflammation is an established driver of tumorigenesis and is highly correlated with metastatic tumor progression (24, 40). It has been previously established in STAT1⁻/⁻ mouse models that intact IFN-γ/STAT1 signaling is important for early tumor rejection (48, 54, 55). We hypothesized that although acute proinflammatory STAT1 activity is clearly beneficial for preventing early tumorigenesis, activation of STAT1 in late stage cancer may be driving tumor-promoting chronic inflammation.

It is now well established that inflammation recruits MDSCs (22, 32, 56). In our study, we have presented the first evidence that STAT1 overexpression correlates with a significant tumor infiltration of CD33⁺ myeloid cells in invasive human breast tumors. We similarly demonstrated in our mouse model that tumor STAT1 overexpression directly recruits MDSCs, both in vitro and in vivo. MDSCs are highly suppressive through their production of ARG1 and reactive oxygen species (57). Similarly, we showed that our Gr1⁺ CD11b⁺ cells from STAT1-overexpressing tumors were capable of producing high levels of active arginase 1 and suppressing CD4⁺ T cell proliferation, thereby identifying them as well established suppressors of antitumor immunity.

Because numerous proinflammatory cytokines have been demonstrated to be capable of recruiting MDSCs, we sought to determine whether STAT1-induced cells secreted known MDSC chemotactic factors (41). Using real-time RT-PCR to analyze tumor cell expression of a cytokine panel, we found that whereas expression of chemotactic IL-1β and IL-6 was reduced in STAT1-induced cells, TNF-α was significantly increased. TNF-α is a well established promoter of chronic inflammatory disease and has also been demonstrated to promote tumor progression (58, 59). A recent study demonstrated that STAT1 was capable of directly up-regulating TNF-α, and this resulted in MDSC infiltration and inflammation-mediated cochlear destruction in a rat model of cisplatin-induced ototoxicity (53). Indeed, STAT1 induction of TNF-α is a major MDSC chemottractant in our model as well. In addition, we found that STAT1 induced expression of both TGFβ and IL-13, cytokines well established to drive immune suppression and suppressive MDSC function (25, 43). Thus, tumor STAT1-induced cytokines promote direct MDSC recruitment and may also enhance their suppressive function.

In several human and animal studies, CD4⁺ T helper cells and CD8⁺ cytotoxic T cells have been shown to be potent inhibitors of tumor progression and metastasis (42, 60). As evidence of the ability to suppress this mechanism of antitumor immunity, we found that tumor STAT1 overexpression and MDSC recruitment correlated with a significant decrease in CD4⁺ and CD8⁺ T cell densities in these tumors. Although our data were able to support the known suppressive effects of MDSCs on CD4⁺ T cell proliferation, we could not as readily explain the attenuated CD8⁺ T cell numbers. Similar findings were reported in biopsies from human breast cancer patients,
Tumor STAT1 Promotes Breast Cancer Progression

where the presence of tumor-infiltrating T lymphocytes correlated with improved prognosis and survival as well as a positive response to neoadjuvant chemotherapy (61, 62). The importance of T cells for inhibiting tumor progression in our model was demonstrated in our previous study, which showed a significant tumor growth rate increase when the TM40D-MB cells were implanted into T cell-deficient RAG2 KO mice, as compared with wild type BALB/c mice (10). Thus, tumor-induced STAT1 fosters a highly suppressive tumor microenvironment capable of disabling antitumor T cell immunity, thereby promoting aggressive tumor progression.

MDSCs have been demonstrated to promote recruitment and expansion of suppressive CD4+ Foxp3+ Tregs at the tumor site (43). However, whether Tregs promote or hinder antitumor immunity in human patients remains the subject of controversy (63). In our study, although tumor STAT1 overexpression did not significantly increase tumor frequencies of Tregs, knockdown of STAT1 in the metastatic TM40D-MB cells did significantly reduce tumor Tregs (data not shown). However, because the differences in Treg frequencies between tumor groups were quite modest, it is unclear what their specific contribution is to suppressing antitumor immunity in our model. Additionally, in our study, STAT1 overexpression directly recruited CD11b+ myeloid cells in vitro but not mononuclear lymphocytes, a population including Tregs. This lends further support to the notion that MDSCs, and not Tregs, are the primary drivers of immune suppression in our model. Inhibition of tumor STAT1 activity in late stage cancer and subsequent disabling of MDSC-mediated immune suppression may offer a novel therapeutic strategy to combat advanced disease. Further, therapeutically targeting tumor-induced STAT1 may have other growth-inhibitory benefits, such as inhibiting tumor resistance to genotoxic stress (29). However, in designing such a therapy, it would be important to target it to tumor cells alone because STAT1 activity is necessary for T cell antitumor immunity (64).

In summary, inhibiting breast cancer through bolstering antitumor immunity remains a promising therapeutic direction. We propose a novel mechanism whereby tumor up-regulation of STAT1 recruits and activates suppressive MDSCs to inhibit antitumor T cell function, driving breast cancer progression. Identifying STAT1 as a master-signaling molecule that controls tumor communication with its environment is of key importance. Our data suggest that the impact of STAT1 extends beyond cell proliferation (13). Expression of STAT1 by the tumor enhances the ability of the tumor to harness the host immune system in a pathogenic manner. Thus, inhibition of STAT1 in this context is likely to improve the efficacy of breast cancer immunotherapies.

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Tumor STAT1 Promotes Breast Cancer Progression