SOCS3 Deficiency in Myeloid Cells Promotes Tumor Development: Involvement of STAT3 Activation and Myeloid-Derived Suppressor Cells

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Abstract

Suppressor of cytokine signaling (SOCS) proteins are negative regulators of the JAK/STAT pathway and generally function as tumor suppressors. The absence of SOCS3 in particular leads to heightened activation of the STAT3 transcription factor, which has a striking ability to promote tumor survival while suppressing antitumor immunity. We report for the first time that genetic deletion of SOCS3, specifically in myeloid cells, significantly enhances tumor growth, which correlates with elevated levels of myeloid-derived suppressor cells (MDSC) in the tumor microenvironment, and diminishes CD8+ T-cell infiltration in tumors. The importance of MDSCs in promoting tumor growth is documented by reduced tumor growth upon depletion of MDSCs. Furthermore, SOCS3-deficient bone-marrow-derived cells exhibit heightened STAT3 activation and preferentially differentiate into the Gr-1+CD11b+Ly6G+ MDSC phenotype. Importantly, we identify G-CSF as a critical factor secreted by the tumor microenvironment that promotes development of MDSCs via a STAT3-dependent pathway. Abrogation of tumor-derived G-CSF reduces the proliferation and accumulation of Gr-1+CD11b+ MDSCs and inhibits tumor growth. These findings highlight the critical function of SOCS3 as a negative regulator of MDSC development and function via inhibition of STAT3 activation. Cancer Immunol Res; 3(7); 727–40. ©2015 AACR.

Introduction

The JAK/STAT signaling pathway is utilized by numerous cytokines and is critical for induction of innate and adaptive immunity, and ultimately suppressing inflammatory and immune responses (1). Of the seven STAT proteins, STAT3 has been implicated in inducing and maintaining an immunosuppressive tumor microenvironment (2, 3). The persistent activation of STAT3 mediates tumor-promoting inflammation, tumor survival and invasion, and suppression of antitumor immunity (3). Hyperactivation of STAT3 is implicated in tumor progression and poor patient prognosis in a large number of cancers, including breast, prostate, melanoma, pancreatic, and brain tumors (3). Activating mutations in STAT3 are rare; thus, STAT3 hyperactivation is usually caused by an overabundance of cytokines such as IL6 and/or dysregulation of endogenous negative regulators, most notably suppressor of cytokine signaling (SOCS) proteins (4–6). There are eight SOCS proteins: SOCS1–7 and CIS, which inhibit the duration of cytokine-induced JAK/STAT signaling. The predominant function of SOCS3 is inhibition of STAT3 activation by inhibiting JAK kinase activity (5, 7). As such, loss of SOCS3 expression leads to hyperactivation of JAKs and downstream STAT3 and expression of STAT3-mediated genes.

SOCS3 is tightly linked to cancer cell proliferation, as well as cancer-associated inflammation (8). Yet, the role of SOCS3 in various types of cancer is controversial; there are reports of either increased or reduced SOCS3 expression in breast and prostate cancer (9–12). In other cancers, including gastric cancer, hepatocellular carcinoma, head and neck squamous cell carcinoma, and colon cancer, SOCS3 functions as a tumor suppressor (8). The loss of SOCS3 expression by hypermethylation of the SOCS3 promoter is generally associated with poor clinical outcome, metastasis, and aggressive phenotype (9). In preclinical models, conditional knockdown of SOCS3 results in accelerated tumorigenesis, which is associated with hyperactivation of various signaling pathways, including STAT3 (8).

The inflammatory milieu within the microenvironment of cancers supports tumor cell survival and angiogenesis. In tumor models and human cancers, innate leukocytes are predominantly of myeloid origin and are composed of tumor-associated macrophages, dendritic cells (DC), and myeloid-derived suppressor cells (MDSC; refs. 13, 14). MDSCs, characterized by expression of CD11b and Gr-1, are a heterogeneous population of activated immature myeloid cells found within tumors that exert immunosuppressive properties (13–15). MDSCs have the capacity to suppress the cytotoxic activities of natural killer (NK) and NKT cells and adaptive immune responses elicited by CD4+ and CD8+ T cells (15, 16). Under normal conditions, Gr-1+CD11b+ cells are maintained at very low levels, but in patients with tumors, those cells can constitute up to 50% of total CD45+ hematopoietic cells in the tumor mass (17). The number of MDSCs in tumors is negatively associated with overall...
survival and treatment efficacy in patients with colorectal, pancreatic, and prostate cancer (18). In a tumor-promoting environment, MDSCs expand and migrate from the bone marrow into the blood, spleen, and tumors induced by numerous cytokines and soluble mediators, including macrophage colony-stimulating factor (M-CSF), G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, IL-1, TNFα, and S100A8/S100A9 (14, 19–23). The expansion and functional activation of MDSCs involve numerous transcription factors, with STAT3 being the most crucial (24).

We recently demonstrated that deletion of SOCS3 in myeloid cells (neutrophils, DCs, monocytes/macrophages) leads to heightened activation of STAT3, and enhanced expression of proinflammatory genes, including IL-1, TNFα, IL-6, and inducible nitric oxide synthase (iNOS; refs. 25, 26). To investigate the function of myeloid SOCS3 in tumor growth, the TRAMP model of prostate cancer was examined in SOCS3-floxed (SOCS3\textsuperscript{fl/fl}) and SOCS3 myeloid-specific deletion (SOCS3\textsuperscript{MycKO}) mice. Our results demonstrate that prostate tumor growth is significantly enhanced in SOCS3\textsuperscript{MycKO} mice and is associated with elevated levels of Gr-1\textsuperscript{+}CD11b\textsuperscript{+} MDSCs in tumors of SOCS3-deficient mice. We identify G-CSF as a critical factor secreted by the tumor microenvironment that promotes MDSC expansion via a STAT3/SOCS3-dependent pathway. Abrogation of tumor-derived G-CSF inhibits tumor growth by reducing the accumulation of MDSCs. Our results highlight the important role of myeloid SOCS3 in regulating the development of MDSCs and demonstrate that in the absence of SOCS3, an immunosuppressive milieu is established in the tumor microenvironment that promotes tumor growth.

Materials and Methods

Mice

Six- to 8-week-old mice were used. C57Bl/6 and transgenic OT-1 mice [specific to ovalbumin (OVA) peptide\textsubscript{257-264} (27)] were bred at the University of Alabama at Birmingham (UAB). SOCS3 conditional knockout (SOCS3\textsuperscript{MycKO}) mice were generated by breeding of SOCS3\textsuperscript{fl/fl} mice (28), with mice expressing Cre recombinase under the control of the LysM promoter, in which the conditional SOCS3 allele is excised in myeloid cells (25). All experiments were approved by the Institutional Animal Care and Use Committee of UAB.

Cell lines and primary cells

Murine epithelial prostate cancer cells TRAMP-C1 and TRAMP-C2 were cultured in DMEM medium with 10% FBS (Sigma). Conditioned media (CM) were generated by incubation of TRAMP-C1 or C2 cells for 48 hours. Primary bone marrow cells were flushed from the femur and tibia of mice (25, 26) and cultured under conditions to generate MDCSs, including TRAMP CM, 10 ng/mL of murine GM-CSF, or 20 ng/mL of murine G-CSF for 3 to 4 days.

Peptides, antibodies, cytokines, and lentiviral vector

OVA\textsubscript{257-264} (SIINFEKL) was obtained from AnaSpec. Antibodies (Ab) against phospho-STAT3 (Tyr705) and STAT3 were from Cell Signaling Technology, and Ab against GAPDH was from Abcam. Neutralizing Ab to G-CSF (MAB414) and isotype control (IgG1) were from R&D Systems. Neutralizing Ab to Gr-1 (RB6-8C5) and isotype control (IgG2b) were from BioXcell. Recombinant murine G-CSF and GM-CSF were from R&D Systems. Lentiviral expression of SOCS3 was generated as described (29).

Tumor models and Ab treatment

Subcutaneous TRAMP tumors, prostate-containing tumor, and normal prostate tissue were minced into fragments and incubated in collagenase solution in the presence of DNase I (1 mg/mL) at 37°C for 1 hour. Dissociated cells were passed through a 100-μm cell strainer. Spleens or bone marrow extracted from mouse femurs were homogenized and passed through a 100-μm cell strainer. Cells were resuspended and stained with direct labeled Abs against: CD45 (30–F11), CD11b (M1/70), Gr-1 (RB6-8C5), Ly6G (1A8), and Ly6C (HK1.4) (BioLegend); and B220 (RA3–6B2), F4/80 (BM8), and CD11c (N418) (eBioscience). For T-cell analysis, Abs from BioLegend were used: CD3 (145–2C11), CD4 (GK1.5), CD8 (53–6.7), IFNγ (XMG1.2), and Foxp3 (MF-14). Mouse G-CSF R/CD114 Ab was from R&D Systems. For intracellular staining, Abs against phospho-STAT3 (Y705) and phospho-STAT5 (Y694) (BioLegend) were used. Staining was performed as previously described (25, 31). All samples were analyzed on an LSRIIB FACS instrument and were further analyzed with FlowJo software.

Isolation of Gr-1\textsuperscript{+}CD11b\textsuperscript{+} cells and functional T-cell suppression assay

Single-cell suspensions were prepared from tumors or spleens by digestion with Collagenase/Dispase and DNase for 1 hour at 37°C. Gr-1\textsuperscript{+}CD11b\textsuperscript{+} myeloid cells were sorted by FACSaria into cultures of 10 × 10^6 cells/mL and incubated with carboxyfluorescein succinimidyl ester (CFSE; 0.5 μmol/L) at room temperature for 10 minutes. Suppression of T cells was evaluated in a coculture system with OT-1 splenocytes (1 × 10^5 per well), with increasing ratios of Gr-1\textsuperscript{+}CD11b\textsuperscript{+} cells in the presence of OVA peptide (2 μg/mL). OT-1 CD8\textsuperscript{+} T cells were evaluated on day 3 by flow cytometry for CFSE dilution. The percentage of dividing cells was determined by drawing a gate outside a reference CFSE peak from unstimulated CFSE\textsuperscript{+} cells.

Nitric oxide detection, IFNγ production, and arginase activity assay

Nitric oxide production was evaluated in supernatants from cocultured Gr-1\textsuperscript{+}CD11b\textsuperscript{+} cells and OT-1 T cells using the Griess Reagent System [Promega (25)]. The same culture supernatants were evaluated for IFNγ production using an IFNγ ELISA kit [BioLegend (25)]. Arginase activity was measured in cell lysates.
using the Quantichrom Arginase Assay Kit (BioAssay Systems) following the manufacturer’s instructions.

**Cytology of Gr-1⁺CD11b⁺ cells**

CD45⁺Gr-1⁺CD11b⁺ cells were sorted from spleens or tumors of TRAMP-C1 tumor–bearing mice using FACSaria. Cytospin preparations were stained with Diff-Quick modified Giemsa reagent (Polysciences) according to the manufacturer’s protocols.

**In vivo cell proliferation assay**

Naïve or TRAMP-C1 tumor–bearing mice were injected i.p. with 100 μg of 5-ethyl-2’-deoxyuridine (EdU; Invitrogen). After 20 hours, mice were sacrificed, and single-cell suspensions were prepared from bone marrow, spleens, and tumors. Cells were stained with Alexa Fluor 647 Azide according to the Click-iT EdU Flow Cytometry Assay Kits (Invitrogen; ref. 31).

**Histopathology and immunofluorescence**

Slides were stained with nuclear dye (hematoxylin), and then stained with counterstain (eosin). Immunofluorescence was performed with Abs to CD45 (1:50) or Gr-1 (1:100). Sections were fixed in 3% formaldehyde, blocked with 10% donkey serum for 30 minutes, labeled with primary antibody overnight at 4°C, and then incubated with Alexa Fluor 568 secondary antibody (Invitrogen; 1:200) along with DAPI (Invitrogen; 1:1,000) for 1 hour at room temperature. Remaining steps were performed as described (25, 26).

**RNA isolation, RT-PCR, and gene expression assays**

Total RNA was isolated from untreated or cytokine-stimulated MDSCs (26). RNA (500 ng) was used to reverse transcribe into cDNA and subjected to qRT-PCR. The abundance of mRNA was normalized to that of 18S or HPRT (hypoxanthine-guanine phosphoribosyl transferase), and the data were analyzed using the comparative Ct method to obtain relative quantitation values (26).

**Immunoblotting**

Cell lysate (30 μg) was separated by electrophoresis on 10% SDS-polyacrylamide gels and probed with specific Abs (26).

**ELISA and multiplex analysis of cytokine expression**

Tumor supernatants, TRAMP tumor cell CM, and serum were collected and analyzed using a C-CSF ELISA kit (R&D Systems). Millipore mouse cytokine/chemokine panel LI (MPXMCYTO-70K; Millipore) was used for detection of cytokines and chemokines. Expression levels of cytokines/chemokines were normalized to total protein levels.

**Statistical analyses**

Graphpad Prism v6.01 was used for the graphs and for statistics. The ANOVA test was performed. All results are shown as mean ± SD. A P value of <0.05 was considered to be statistically significant.

**Results**

**Myeloid-specific SOCS3 loss promotes tumor growth**

We utilized a syngeneic tumor model of murine TRAMP C1 and C2 prostate cancer cells (32) inoculated into the flanks of C57BL/6 SOCS3(-/-) and SOCS3+MycKO mice. Tumor growth was significantly increased in SOCS3+MycKO mice compared with SOCS3(-/-) mice (Fig. 1A). Immunofluorescence staining demonstrates enhanced tumor infiltration of CD45⁺ hematopoietic-derived cells and Gr-1⁺ myeloid cells in SOCS3+MycKO mice at day 45 (Fig. 1B). The Gr-1⁺CD11b⁺ cell population was substantially increased in SOCS3+MycKO mice compared with SOCS3(-/-) mice (Fig. 1C), whereas the percentages of other cells, such as macrophages (F4/80(high), CD11b⁺), DCs (CD11b⁺, CD11c⁺), B cells (B220⁺), and T cells (CD3⁺), were largely unchanged (Fig. 1D). Associated with the increased frequency of Gr-1⁺CD11b⁺ cells in the tumor, there was a significant reduction in infiltrating CD8⁺ T cells in the tumors of SOCS3+MycKO mice (Fig. 1E). The percentage of Gr-1⁺CD11b⁺ cells was elevated in the spleens of SOCS3+MycKO mice compared with that of SOCS3(-/-) mice (Supplementary Fig. S1A), although no changes in CD8⁺ T cells were observed in the spleens (Supplementary Fig. S1B). Similar percentages of CD4⁺Foxp3⁺CD25⁺ regulatory T cells (Treg) were detected in tumors from SOCS3+MycKO and SOCS3(-/-) mice (Supplementary Fig. S1C). To determine if the increased tumor growth observed in SOCS3+MycKO mice is due to enhanced MDSC function, depletion of Gr-1⁺ MDSCs with anti-Gr-1 neutralizing antibody in tumor-bearing mice was conducted. TRAMP-C1 cells were injected into the flanks of SOCS3(-/-) and SOCS3+MycKO mice, and anti–Gr-1 mAb or isotype control was administrated at day 24 (Supplementary Fig. S1D). We observed reduced levels of MDSCs in the spleens of SOCS3+MycKO mice after treatment with neutralizing Gr-1 mAb (Supplementary Fig. S1E). More importantly, tumor growth was significantly reduced after treatment with anti–Gr-1 mAb in SOCS3+MycKO mice (Fig. 1F). These results suggest a critical role for Gr-1⁺ MDSCs in contributing to tumor growth in SOCS3+MycKO mice.

Gr-1⁺CD11b⁺ cells from tumor-bearing mice suppress antigen-specific T-cell responses

Gr-1⁺CD11b⁺ cells from the tumors of TRAMP-C1 mice displayed multilobed nuclei (Fig. 2A). Gr-1 identiﬁes the monocyte and neutrophil markers Ly6C and Ly6G, respectively. MDSCs consist of two major subsets of Ly6G⁻Ly6Chigh granulocytic and Ly6G⁺Ly6Chigh monocytic cells (33). Gr-1⁺CD11b⁺ cells from TRAMP-C1 tumors are a mixture of granulocytic and monocytic cells, with the majority of cells expressing Ly6G (Fig. 2B). Further, higher percentages of both granulocytic and monocytic MDSCs were detected in SOCS3+MycKO mice (Fig. 2B). We next examined the inﬂuence of SOCS3 on various MDSC effector functions, first testing the ability of Gr-1⁺CD11b⁺ cells isolated from tumors to suppress in vitro proliferation of OVA-specific CD8⁺ T cells. The immunosuppressive function of tumor-associated MDSCs isolated from SOCS3+MycKO mice was more potent than MDSCs from SOCS3(-/-) mice (Fig. 2C). MDSCs from the tumors of SOCS3+MycKO mice decreased IFNγ production by T cells more potently (Supplementary Fig. S2A) and produced higher levels of nitrite upon coculture with OT-1 splenocytes (Supplementary Fig. S2B) than MDSCs from SOCS3(-/-) mice. Gene expression analysis of MDSCs isolated from tumors was performed. Loss of SOCS3 increased expression of mediators of immune suppression, including Arginase 1 and S100A8, with a trend of increased iNOS and S100A9 (Fig. 2D). MDSCs from the spleens of SOCS3+MycKO mice inhibited T-cell proliferation more potently (Supplementary Fig. S2C) and had signiﬁcantly increased arginase activity compared with MDSCs from SOCS3(-/-) mice (Supplementary Fig. S2D). Furthermore, Gr-1⁺CD11b⁺ cells isolated from the bone marrow,
Figure 1.
Myeloid-specific SOCS3 loss promotes tumor growth. A, SOCS3fl/fl (n = 25) and SOCS3MyeKO (n = 22) mice were inoculated s.c. with 3.0 × 10⁶ TRAMP-C1 or -C2 cells. Tumor size is indicated as volume (mm³ ± SD) and evaluated up to 45 days. B, H&E staining of TRAMP-C1 tumors from mice at 45 days. Scale bar, 50 μm. C, Immunofluorescence staining of tumor-infiltrating CD45⁺ or Gr-1⁺ cells (n = 3 for SOCS3fl/fl mice, n = 3 for SOCS3MyeKO mice). Scale bar, 45 μm. C, flow cytometry plot of Gr-1⁺CD11b⁺ cells from tumors of TRAMP-C1 mice (top), and quantification of tumor-infiltrating Gr-1⁺CD11b⁺ cells (bottom; n = 7–8 for SOCS3fl/fl mice or SOCS3MyeKO mice). D, SOCS3fl/fl (n = 3) and SOCS3MyeKO (n = 3) mice were inoculated s.c. with 3.0 × 10⁶ TRAMP-C1 cells. After 45 days of inoculation, tumor-infiltrating CD45⁺ cells were gated, and the percentage of the indicated cell subsets was determined by flow cytometry. E, flow cytometry plot of CD3⁺ and CD8⁺ T cells (gated in boxes) from tumors of TRAMP-C1 mice (top), and quantification of tumor-infiltrating CD8⁺ T cells (bottom; n = 8–10 for SOCS3fl/fl mice or SOCS3MyeKO mice). F, SOCS3fl/fl and SOCS3MyeKO mice were inoculated s.c. with 3.0 × 10⁶ TRAMP-C1 cells. At 24 days, mice were randomly assigned to receive anti-Gr-1 or isotype antibodies (100 μg per mouse) every 2 days for a total of 9 treatments (n = 5 for SOCS3fl/fl mice, n = 5 for SOCS3MyeKO mice). Tumor size indicated as volume (mean mm³ ± SD) was evaluated at 41 days. *, P < 0.05; **, P < 0.01.
spleens, and tumors of SOCS3MyeKO mice had elevated STAT3 activation compared with cells from SOCS3fl/fl mice (Fig. 2E).

Orthotopic prostate tumor growth is enhanced in SOCS3MyeKO mice

We next investigated the functional importance of SOCS3 in myeloid cells in the prostate microenvironment. TRAMP-C2 cells represent more advanced prostate cancer than TRAMP-C1 cells, due to enhanced tumor growth and elevated expression of Ras and Myc (34). TRAMP-C2 cells were injected intraprostatically into SOCS3fl/fl and SOCS3MyeKO mice (30), and prostate weight was compared between the two groups. Orthotopic growth of TRAMP-C2 cells was significantly increased in SOCS3MyeKO mice (Fig. 3A). Hematoxylin and eosin (H&E) staining of TRAMP-C2 tumors in SOCS3fl/fl and SOCS3MyeKO mice showed poorly differentiated tumors compared with control prostate (Fig. 3B). Prostate tumors from SOCS3MyeKO mice have higher numbers of infiltrating CD45+CD11b+ cells (Fig. 3B). Orthotopic TRAMP-C2 tumors in SOCS3MyeKO mice exhibited increased Gr-1+CD11b+ cell infiltration, compared with SOCS3fl/fl mice (Fig. 3C), and a significant reduction in the percentage of infiltrating CD8+ T cells (Fig. 3D). Significantly higher levels of S100A8 and S100A9, inflammation- and cancer-promoting factors expressed by Gr-1+CD11b+ MDSCs (35), were detected in prostate tumors from SOCS3MyeKO mice compared with those from SOCS3fl/fl mice (Fig. 3E).

Figure 2.

Gr-1+CD11b+ cells from SOCS3MyeKO tumor-bearing mice suppress antigen-specific T-cell responses. A, Diff-Quick stain of cytospin preparation of CD45+Gr-1+CD11b+ cells from TRAMP-C1 tumors at 42 days. B, Flow cytometric analysis of myeloid cells in tumors formed by TRAMP-C1 cells at 42 days. Numbers indicate the percentage of CD45+CD11b+Ly6G+ or CD45+CD11b+Ly6C+ cells. Data are representative of 3 experiments. C, CD45+Gr-1+CD11b+ cells were isolated from TRAMP-C1 tumors by flow cytometry at 42 days, and antigen-specific suppression of CD8+ T cells was evaluated in a coculture assay. Splenocytes from OT-1 transgenic mice were cultured in the presence of increasing ratios of Gr-1+CD11b+ cells and stimulated with OVA-derived peptide. Proliferation of OVA-specific CD8+ T cells was evaluated by flow cytometric analysis of CFSE dilution after 72 hours of coculture. Data are mean ± SD and representative of five experiments. D, gene expression of CD45+Gr-1+CD11b+ cells from tumors of TRAMP-C1 mice by qRT-PCR at day 42. Data are representative of three experiments. E, SOCS3fl/fl (n = 3) and SOCS3MyeKO (n = 3) mice were inoculated s.c. with 3.0 × 10^6 TRAMP-C1 cells. Thirty-one days after inoculation, intracellular staining for p-STAT3 was performed in Gr-1+CD11b+ cells from bone marrow, spleens, and tumors. Numbers indicate the quantification of mean fluorescence intensity. *, P < 0.05.
Loss of SOCS3 promotes proliferation of Gr-1\(^+\)CD11b\(^+\) cells

TRAMP tumor-bearing mice developed splenomegaly compared with naïve mice, and SOCS3\(^{MyeKO}\) mice showed a statistically significant increase in spleen size and weight compared with SOCS3\(^{fl/fl}\) mice (Fig. 4A). Perturbed myelopoiesis and spleen hematopoiesis (36) may account for the accumulation of Gr-1\(^+\)CD11b\(^+\) cells in tumors and increased spleen size. EdU cell proliferation assays were performed to address the role of SOCS3 on myeloid cell proliferation. We observed no differences in myelopoiesis (Supplementary Fig. S3A), spleen size (Supplementary Fig. S3B), and bone marrow myeloid cell proliferation (Supplementary Fig. S3C) between naïve SOCS3\(^{fl/fl}\) and SOCS3\(^{MyeKO}\) mice. Gr-1\(^-\)CD11b\(^+\) cells from the spleens of naïve SOCS3\(^{fl/fl}\) mice had approximately 6.4% proliferating cells, and both TRAMP-C1 tumor-bearing SOCS3\(^{fl/fl}\) and SOCS3\(^{MyeKO}\) mice showed increased proliferating cells compared with naïve mice (Fig. 4B). In addition, the percentage of proliferating Gr-1\(^-\)CD11b\(^+\) cells from the spleens of SOCS3\(^{MyeKO}\) mice was higher than that of SOCS3\(^{fl/fl}\) mice. Similarly, Gr-1\(^{high}\)CD11b\(^+\) cells and Gr-1\(^{int}\)CD11b\(^+\) cells from the bone marrow of TRAMP-C1 tumor-bearing SOCS3\(^{MyeKO}\) mice exhibited an enhanced proliferation rate (Fig. 4C). TRAMP tumor development was associated with a 2- to 3-fold increase in circulating white blood cells (WBC) in SOCS3\(^{MyeKO}\) mice (Fig. 4D), due to a 3- to 5-fold increase in granulocytes (Fig. 4E). This suggests that factors secreted by the tumor may increase the mobility of bone marrow progenitor cells to enter the bloodstream.

Next, we cultured bone marrow cells from SOCS3\(^{fl/fl}\) and SOCS3\(^{MyeKO}\) mice in the absence or presence of TRAMP CM for 4 days, and total numbers of Gr-1\(^-\)CD11b\(^+\) cells were determined by flow cytometry. The absolute number of Gr-1\(^-\)CD11b\(^+\) cells generated from SOCS3\(^{MyeKO}\) mice was approximately 2-fold higher than SOCS3\(^{fl/fl}\) mice (Supplementary Fig. S3D). Cell-cycle analysis was performed, and results indicate increased numbers of SOCS3\(^{fl/fl}\) bone marrow cells in S and M phase compared with that of SOCS3\(^{fl/fl}\) bone marrow cells (Supplementary Fig. S3E). These results indicate that factors from the tumor microenvironment contribute to the differentiation and proliferation of Gr-1\(^-\)CD11b\(^+\) cells, and that SOCS3 plays a critical role in regulating myeloid cell proliferative responses to these factors.

Absence of SOCS3 enhances STAT3 activation and bone marrow differentiation into Gr-1\(^-\)CD11b\(^+\) MDSCs

We utilized CM from TRAMP cells to assess functional effects on MDSCs. TRAMP CM, but not control medium, promoted bone marrow cells to differentiate into Gr-1\(^-\)CD11b\(^+\) cells, with higher
percentages detected in SOCS3MyeKO mice (Fig. 5A). In addition, Gr-1\(^+\)CD11b\(^+\) cells from SOCS3MyeKO mice incubated in TRAMP CM exhibited a greater capacity to suppress antigen-specific T-cell proliferation (Fig. 5B) and IFN\(\gamma\) production (Fig. 5C) than did cells from SOCS3\(^{fl/fl}\) mice. Gr-1\(^+\)CD11b\(^+\) cells lacking SOCS3 also produced elevated levels of nitrite (Fig. 5D) and had increased arginase activity (Fig. 5E). Cells from SOCS3MyeKO mice showed enhanced and prolonged STAT3 activation in the presence of TRAMP CM compared with that in SOCS3\(^{fl/fl}\) mice (Fig. 5F). Loss of SOCS3 increased expression of arginase 1, iNOS, S100A8, S100A9, and IDO1 (37) by bone marrow–derived Gr-1\(^+\)CD11b\(^+\) cells incubated with TRAMP CM (Fig. 5G). TRAMP CM–cultured Gr-1\(^+\)CD11b\(^+\) cells had low expression of MHC class II, with SOCS3MyeKO cells expressing less MHC class II compared with that expressed by SOCS3\(^{fl/fl}\) cells (Fig. 5H), suggesting that loss of SOCS3 maintains MDSCs in an undifferentiated state. The majority of Gr-1\(^+\)CD11b\(^+\) cells generated in the presence of TRAMP CM express Ly6G (Fig. 5I). These results demonstrate that prostate tumor cells secrete factors that drive the generation of Gr-1\(^+\)CD11b\(^+\) cells from hematopoietic progenitor cells of the bone marrow, and this effect is amplified in the absence of SOCS3.

G-CSF induces the differentiation of bone marrow precursors into functional MDSCs in a STAT3-dependent manner

We next examined potential prostate tumor–derived soluble factors that may contribute to the generation of Gr-1\(^+\)CD11b\(^+\) cells. Expression of G-CSF in the serum of na\(\€/C3\), P < 0.05; na\(\€/C3/C3\), P < 0.01.
Figure 5.
Absence of SOCS3 enhances STAT3 activation and bone marrow differentiation into Gr-1⁺CD11b⁺ MDSCs. 

A, bone marrow cells from SOCS3^fl/fl^ and SOCS3^MyeKO^ mice were cultured in TRAMP-C1 CM (100% volume), and Gr-1⁺CD11b⁺ cells were analyzed by flow cytometry at day 4. Numbers are expressed as percentage of total CD45⁺ cells.

B, bone marrow–derived cells incubated with TRAMP-C1 CM for 4 days were cocultured with CFSE (0.5 μmol/L)-labeled OT-1 splenocytes in the presence of OVA-derived peptide. Proliferation of CD8⁺ T cells was evaluated by flow cytometric analysis of CFSE dilution after 48 hours of coculture. Data are mean ± SD and representative of three experiments. (Continued on the following page.)
tumor-bearing mice was examined, and elevated levels of G-CSF were detected from mice with orthotopic or s.c. TRAMP tumors (Supplementary Fig. S4A). Prostate tissues from orthotopic tumor-bearing mice and flank tumor tissue were evaluated for G-CSF concentrations. Higher amounts of G-CSF were detected in TRAMP tumor-bearing mice compared with that in tissue from naïve mice (Fig. 6A), although the levels of G-CSF were not different between tumor-bearing SOCS3<sup>MyeKO</sup> and SOCS3<sup>fl/fl</sup> mice. CM from TRAMP cells also contained G-CSF (Fig. 6A). The expression of G-CSF was validated at the mRNA level (Supplementary Fig. S4B). Enhanced and prolonged activation of STAT3 was observed in G-CSF–treated bone marrow cells from SOCS3<sup>MyeKO</sup> mice (Fig. 6B), which was validated by flow cytometry (Fig. 6C). G-CSF also activated STAT5 in Gr-1<sup>+</sup>CD11b<sup>+</sup> cells, but SOCS3 deficiency did not affect this response (Fig. 6C). SOCS3 mRNA expression was induced by G-CSF treatment of cells from SOCS3<sup>fl/fl</sup> mice, but not in cells from SOCS3<sup>MyeKO</sup> mice (Fig. 6D). Inclusion of G-CSF in bone marrow cultures promoted differentiation into Gr-1<sup>+</sup>CD11b<sup>+</sup> cells, with SOCS3<sup>MyeKO</sup> cells having higher levels (Fig. 6E, left). SOCS3 overexpression was utilized to validate the negative regulatory role of SOCS3 in G-CSF promotion of MDSCs (29). Overexpression of SOCS3 in bone marrow cells from SOCS3<sup>fl/fl</sup> and SOCS3<sup>MyeKO</sup> mice diminished the generation of Gr-1<sup>+</sup>CD11b<sup>+</sup> cells in response to G-CSF (Fig. 6E, right). To substantiate the involvement of STAT3 in MDSC differentiation, JAK and STAT3 inhibitors were tested. A significant reduction in Gr-1<sup>+</sup>CD11b<sup>+</sup> cells in the presence of the pan-JAK inhibitor P6 (38) or the STAT3 inhibitor Statin (39) was observed (Fig. 6F). These results indicate that SOCS3 negatively regulates G-CSF–induced generation of Gr-1<sup>+</sup>CD11b<sup>+</sup> cells by inhibiting JAK/STAT3 activation. In the presence of G-CSF, loss of SOCS3 increased expression of arginase 1, iNOS, S100A8, S100A9, and IDO1 (Fig. 6G). Furthermore, Gr-1<sup>+</sup>CD11b<sup>+</sup> cells from SOCS3<sup>MyeKO</sup> mice incubated in G-CSF exhibited a greater capacity to suppress T-cell proliferation (Fig. 6H), and had increased arginase activity compared with Gr-1<sup>+</sup>CD11b<sup>+</sup> cells from SOCS3<sup>fl/fl</sup> mice (Fig. 6I). Thus, we identify G-CSF as an important factor in the development of Gr-1<sup>+</sup>CD11b<sup>+</sup> MDSCs via STAT3 activation, which is regulated by SOCS3.

G-CSF neutralization limits Gr-1<sup>+</sup>CD11b<sup>+</sup> cell proliferation and tumor growth

Bone marrow cells isolated from SOCS3<sup>fl/fl</sup> and SOCS3<sup>MyeKO</sup> mice were cultured in the presence of TRAMP CM or G-CSF. Flow cytometric analysis demonstrates that addition of anti–G-CSF Ab, but not isotype IgG, inhibits generation of Gr-1<sup>+</sup>CD11b<sup>+</sup> cells (Supplementary Fig. S5A). Neutralization of G-CSF partially inhibited bone marrow cell proliferation in the presence of TRAMP CM and abolished proliferation induced by G-CSF (Fig. 7A).

The contribution of G-CSF to tumor growth was next assessed. TRAMP-C1 cells were injected into the flanks of SOCS3<sup>fl/fl</sup> and SOCS3<sup>MyeKO</sup> mice, and anti–G-CSF mAb or isotype control was administrated at day 22 (Fig. 7B). In both SOCS3<sup>fl/fl</sup> and SOCS3<sup>MyeKO</sup> mice, tumor volume (Fig. 7B), weight (Fig. 7C), and serum G-CSF levels (Supplementary Fig. S5B) were significantly reduced after treatment with anti–G-CSF mAb. Circulating WBC (Supplementary Fig. S5C) and granulocytes (Supplementary Fig. S5D) were also decreased after treatment with neutralizing G-CSF mAb in SOCS3<sup>MyeKO</sup> mice. Moreover, infiltration of MDSCs in tumors was significantly reduced after treatment with neutralizing G-CSF mAb (Fig. 7D), as were levels of MDSCs in the spleens of SOCS3<sup>MyeKO</sup> mice, but not in SOCS3<sup>fl/fl</sup> mice (Fig. 7E). These data highlight the importance of myeloid SOCS3 in regulating prostate tumor growth in response to G-CSF. To exclude a direct action of anti–G-CSF Ab on TRAMP-C1 tumor cells, G-CSF receptor (G-CSFR) expression was assessed. We observed high levels of G-CSFR expression on spleen or tumor Gr-1<sup>+</sup> cells compared with TRAMP-C1 tumor cells and nonmyeloid CD11b<sup>+</sup> cells from tumor-bearing mice (Supplementary Fig. S3E).

Discussion

We demonstrate that SOCS3 expression in myeloid cells is an important determinant of tumor growth, indicating a critical influence of the tumor microenvironment in cancer progression. The loss of SOCS3 in myeloid cells promotes the differentiation of bone marrow–derived progenitor cells into Gr-1<sup>+</sup>CD11b<sup>+</sup> MDSCs and enhances the immunosuppressive functions of these cells. Importantly, STAT3 activation is essential for this process. Tumor-derived G-CSF is crucial for the mobilization and recruitment of Gr-1<sup>+</sup>CD11b<sup>+</sup> MDSCs to tumors, where they decrease the presence of CD8<sup>+</sup>T cells. Neutralization of G-CSF is effective in limiting the differentiation and functionality of MDSCs, which in vivo manifests as restricted tumor growth. These findings establish a circuitry between MDSCs, tumor cells, and G-CSF in the tumor microenvironment; G-CSF secreted by tumor cells promotes the recruitment and differentiation of MDSCs, which is dependent on STAT3 activation in MDSCs. In the absence of SOCS3, this response and circuitry is amplified (Fig. 7F). These results support a role for SOCS3 in repressing MDSC differentiation, which ultimately relieves immunosuppression in the prostate tumor microenvironment.

Persistent STAT3 activation is associated with poor prognosis in many cancer types, including patients with prostate cancer (40). Loss of the androgen receptor leads to the development of...
prostate cancer stem cells, which requires STAT3 activation (41). Stem-like cells from patients with prostate cancer secrete high levels of IL6 and exhibit hyperactivation of STAT3 (42). STAT3 activation in cells of the hematopoietic lineage is also associated with creating a tumor microenvironment conducive to tumor growth. This is especially true for MDSCs, which rely on STAT3 for differentiation and functionality. Ablation of STAT3 in multiple lineages of immune cells (neutrophils, NK cells, DCs) enhanced their antitumor activity (39). Although MDSCs were not directly examined, heightened activation of STAT3 in MDSCs, as shown by our study, may contribute to tumor immune tolerance. MDSCs lacking SOCS3 with STAT3 hyperactivation have potent immunosuppressive capabilities, such as inhibition of antigen-specific Cd8 T-cell proliferation and IFNγ production, and enhanced nitrite production and arginase activity. Also, SOCS3-deficient MDSCs expressed MHC class II at lower levels than SOCS3 wild-type MDSCs, suggestive of an immature suppressive phenotype. Circulating MDSCs from patients with prostate cancer displayed reduced expression of HLA-DR compared with that in age-matched controls, and had more potent ability to suppress T-cell proliferation (43). It will be informative to examine SOCS3 expression and STAT3 activation status in prostate tumor cells, prostate stem cells, and prostate tumor–associated MDSCs to determine the functional impact of SOCS3/STAT3 in patients with prostate cancer. Nonetheless, our findings clearly demonstrate the importance of SOCS3 in restricting MDSC-mediated antitumor immunity.

Using TRAMP C1/C2 cell lines in immune competent mice, elevated levels of MDSCs were detected in the tumors of SOCS3MyeKO mice compared with those in SOCS3+/fl mice, in both flank and orthotopic models. This was associated with a decrease of Cd8+ T cells in the tumors, which supports the notion that MDSCs interfere with T-cell activation and proliferation. MDSCs also selectively expand Foxp3+/CD25+ Tregs, which promote tumor growth by a variety of mechanisms (44). In our studies, Foxp3+ Tregs were present at comparable levels in tumors from both SOCS3MyeKO and SOCS3+/fl mice; thus, Tregs may not have a prominent role in the TRAMP prostate cancer model (45).

G-CSF plays a crucial role in hematopoiesis by stimulating the proliferation, differentiation, and survival of myeloid progenitor cells, particularly cells within the granulocytic lineage (28). Jak1, Jak2, and Tyk2 are recruited to the G-CSF receptor upon stimulation with G-CSF, and then, in turn, activate STAT1, STAT3, and STAT5, of which STAT3 is the most important. SOCS3 is induced by G-CSF in myeloid cells and serves as a negative regulator of G-CSF–induced cellular responses by binding to the G-CSF receptor (28, 46). G-CSF is one of a number of soluble mediators that promote the expansion and migration of MDSCs from the bone marrow to tumors. Although the levels of G-CSF were comparable in SOCS3+/Myc5 and SOCS3−/Myc5 tumor–bearing mice, SOCS3MyeKO bone marrow–derived cells are hyper-responsive to G-CSF, as documented by increased duration and intensity of G-CSF–induced STAT3 phosphorylation, which led to MDSC differentiation at a greater percentage than cells from SOCS3+/fl mice. In addition, G-CSF–induced MDSCs from SOCS3MyeKO tumor–bearing mice exhibited a greater capacity to suppress antigen-specific T-cell proliferation. Our in vivo findings demonstrate that treatment of tumor-bearing mice with neutralizing G-CSF antibody reduced circulating granulocytic cells as well as infiltration of MDSCs in tumors. These findings highlight the critical role of tumor-derived G-CSF in MDSC development and the importance of SOCS3 as an essential negative regulator of this process. SOCS3 expression in MDSCs negatively regulates the expression of soluble mediators, such as S100A8 and S100A9, and products of iNOS and arginase 1 that support an immunosuppressive milieu in tumors. In the absence of SOCS3, MDSCs are hyper-responsive to tumor-produced cytokines, such as G-CSF, and aberrantly activate STAT3, which in turn contributes to chronic cancer-related inflammation and suppression of antitumor immune responses.

Clinical information regarding SOCS3 expression in prostate cancer is inconclusive at this time. Pierconti and colleagues (9) demonstrated that methylation of the SOCS3 promoter was significantly associated with intermediate- to high-grade Gleason score and with an unfavorable outcome. In benign prostate hyperplasia and normal controls, the SOCS3 promoter was unmethylated. Analysis of the Oncomine database shows that SOCS3 mRNA expression is significantly lower in prostate carcinoma compared with prostate cancer precursor (Luo dataset), but the Tomlins dataset shows the opposite results (https://www.oncomine.org/resource/main.html#a%3A985;d%3A46%;3Bds%3AgeneOverex%3Bd%3Aprede%3Bscr%3Adatasets%3Bss%3Aanalysis%3Ab2C150003%2C150004%3Bf%3A33306%3Bg%3A9021%3Bgt%3Aboxplot%3Bp%3A200000762%3Bepv%3A150001.151078%2C2937%2C3508%3Bet%5B2%5D%3Bp%3A3.483%3Bc3%2C3601%2C2C150003%2C150004%3Bb%3Adatastes%3Bs%3Aanalysis%3Bv%3A18). Data from ebiPortal [Prostate Adenocarcinoma, Memorial Sloan Kettering Cancer Center (MSKCC)] indicate that

Figure 6. G-CSF induces the differentiation of bone marrow precursors into functional MDSCs in a STAT3-dependent manner. A, supernatant of prostate tumors obtained from mice with intraprostactic injection of TRAMP-C2 cells (at day 30) and flank tumors from TRAMP-C1 or -C2 tumor–bearing mice (at day 42), as well as TRAMP-C1 or -C2 tumor cell CM, were analyzed for G-CSF levels by ELISA. Data are mean ± SD and representative of 3 experiments. B, bone marrow–derived cells were treated with G-CSF (10 ng/mL) for the indicated times, and immunoblotting was performed with the indicated antibodies. Data are representative of 4 experiments. C, bone marrow–derived cells were incubated in the absence or presence of G-CSF (10 ng/mL) for 2 hours, and then intracellular staining for p-STAT3 or p-STAT5 was performed. Data are representative of two experiments. D, SOCS3 mRNA expression from cultured bone marrow–derived cells treated with G-CSF (10 ng/mL) for the indicated times was evaluated by qRT-PCR. Data are mean ± SD and representative of two experiments. E, bone marrow cells from SOCS3+/fl and SOCS3MyeKO mice were cultured in G-CSF (20 ng/mL) with Lentiviruses expressing GFP, or Lentiviruses expressing SOCS3. Gr-1+ CD11b+ cells were analyzed by flow cytometry at day 4. Data are representative of five experiments. F, bone marrow cells from SOCS3+/fl and SOCS3MyeKO mice were cultured in G-CSF (20 ng/mL) Vehicle (DMSO), P6 (1 μmol/L), or Statick (1 μmol/L) were added to the cultures at days 0 and 2, and Gr-1+ CD11b+ cells were analyzed by flow cytometry at day 4. Data are representative of two experiments. G, bone marrow cells from SOCS3+/fl and SOCS3MyeKO mice were treated with G-CSF (10 ng/mL) for 4 hours, and expression of indicated genes was evaluated by qRT-PCR. Data are mean ± SD and representative of two experiments. H, bone marrow cells cultured for 4 days with G-CSF (20 ng/mL) were cocultured with 0.5 μmol/L CFSE-labeled OT-1 splenocytes in the presence of OVA peptide. Proliferation of CD8+ T cells was evaluated by flow cytometric analysis of CFSE dilution after 48 hours of coculture. Numbers of the ratio of OT-1 splenocytes/MDSCs. Data are representative of three experiments. I, Gr-1+ CD11b cells were sorted from cultured bone marrow–derived cells, and then treated with G-CSF (10 ng/mL) for 4 hours. Arginase activity was measured in lysates from sorted cells. Assay was performed in triplicate, and data are the mean ± SD. **, P < 0.05; ***, P < 0.01.

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Figure 7.
G-CSF neutralization limits Gr-1\(^{+}\)CD11b\(^{+}\) cell proliferation in vitro and tumor growth in vivo. A, bone marrow cells from SOCS3\(^{fl/fl}\) and SOCS3\(^{MeyKO}\) mice were labeled with CFSE, and then cultured in the presence of TRAMP CM or G-CSF (10 ng/mL) with the indicated antibodies. Proliferation of Gr-1\(^{+}\)CD11b\(^{+}\) cells was evaluated by flow cytometric analysis of CFSE dilution after 4 days. Numbers are expressed as percentage of proliferating cells of total Gr-1\(^{+}\)CD11b\(^{+}\) cells (red, SOCS3\(^{fl/fl}\); black, SOCS3\(^{MeyKO}\)). B, SOCS3\(^{fl/fl}\) and SOCS3\(^{MeyKO}\) mice were inoculated s.c with 3.0 \times 10^{6} TRAMP-C1 cells. Mice were randomly assigned to receive anti-G-CSF or isotype antibodies (10 \(\mu\)g per mouse). Black arrows, Ab administration (n = 6 for SOCS3\(^{fl/fl}\) mice, n = 6 for SOCS3\(^{MeyKO}\) mice). Tumor size indicated as volume (mean mm\(^3\)/SD) was evaluated up to 39 days. C, SOCS3\(^{fl/fl}\) and SOCS3\(^{MeyKO}\) mice were inoculated s.c. with 3.0 \times 10^{6} TRAMP-C1 cells. After 39 days, flank tumors were collected and weighed. Data are expressed as mean/SD. D and E, quantification of flow cytometric analyses of tumor-infiltrating (D) and spleen (E) Gr-1\(^{+}\)CD11b\(^{+}\) cells (n = 3 per group). *, P < 0.05 and **, P < 0.01. F, schematic model. Tumor cell-secreted G-CSF induces activation of the JAK/STAT3 pathway in myeloid cells, which leads to induction of tumor-promoting genes and to SOCS3 expression. SOCS3 is a feedback inhibitor of G-CSF–induced STAT3 activation. In the absence of SOCS3, MDSCs are hyper-responsive to tumor-produced G-CSF, aberrantly activate STAT3, and express enhanced levels of arginase 1, iNOS, and S100A8/9. Elevated levels of MDSCs suppress antitumor immune responses in the tumor microenvironment, in part by inhibiting activity of CD8\(^{+}\) T cells, and eventually promote tumor growth.
patients with prostate cancer with SOCS3 mRNA overexpression trend toward a longer disease-free time than patients with "normal" levels of SOCS3 mRNA (http://www.ncbi.nlm.nih.gov/index.do?caner_study_list=prad_mskcc&cancer_study_id=prad_mskcc&genetic_profile_ids_PROFILE_MUTATION_EXTENDED= prad_mskcc_mutations&genetic_profile_ids_PROFILE_COPY_NUMBER_ALTERATION=prad_mskcc_cna&genetic_profile_ids_PROFILE_DATA=prad_mskcc_mrna_zbynorm2& SCORE_THRESHOLD=2.0&data_priority=0&case_set_id=prad_mskcc_complete&case_ids=&gene_list=SOCS3%0D%0A&clinical_param_selection=null&tab_index=tab_visualize&Action=Submit). Thus, there is clear dysregulation of SOCS3 gene expression in patients with prostate cancer, but the functional and clinical relevance is still under investigation. Nonetheless, it is a clear example of mechanisms, including SOCS3 dysregulation and abundant IL-6 protein production, that contribute to hyperactivation of the JAK/STAT pathway in animal models of prostate cancer and in patients with prostate cancer (4, 42, 47). Inhibitors of IL-6, JAKs, and STAT3 are already proven beneficial in animal models (42). Furthermore, peptides that mimic the SOCS kinase inhibitory domain region, which is responsible for binding to JAKs and suppressing downstream STAT3 activation (48), may prove beneficial in prostate cancer.

These studies not only highlight the significance of the STAT3/SOCS3 pathway in regulating the differentiation and function of MDSCs in cancer, but also identify this intricate protein network as important therapeutic targets to eliminate MDSC-mediated immunosuppression. This is especially important in light of the recent finding that MDSCs are responsible for resistance to immune checkpoint inhibitor, and that elimination of MDSCs led to cures of experimental, metastatic tumors (49).

References


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No potential conflicts of interest were disclosed.

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SOCS3 Deficiency in Myeloid Cells Promotes Tumor Development: Involvement of STAT3 Activation and Myeloid-Derived Suppressor Cells

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