Targeting STAT3 in Adoptively Transferred T Cells Promotes Their In Vivo Expansion and Antitumor Effects

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Abstract

Adoptive cell therapy with engineered T cells to improve natural immune response and antitumor functions has shown promise for treating cancer. However, the requirement for extensive ex vivo manipulation of T cells and the immunosuppressive effects of the tumor microenvironment limit this therapeutic modality. In the present study, we investigated the possibility to circumvent these limitations by engineering Stat3-deficient CD8+ T cells or by targeting Stat3 in the tumor microenvironment. We show that ablating Stat3 in CD8+ T cells prior to their transfer allows their efficient tumor infiltration and robust proliferation, resulting in increased tumor antigen-specific T-cell activity and tumor growth inhibition. For potential clinical translation, we combined adoptive T-cell therapy with a Food and Drug Administration–approved tyrosine kinase inhibitor, sunitinib, in renal cell carcinoma and melanoma tumor models. Sunitinib inhibited Stat3 in dendritic cells and T cells and reduced conversion of transferred FoxP3+ T cells to tumor-associated regulatory T cells while increasing transferred CD8+ T-cell infiltration and activation at the tumor site, leading to inhibition of primary tumor growth. These data show that adoptively transferred T cells can be expanded and activated in vivo either by engineering Stat3-silenced T cells or by targeting Stat3 systemically with small-molecule inhibitors. Cancer Res; 70(23); 9599–610. ©2010 AACR.

Introduction

Adoptive T-cell therapy has shown promise for cancer therapy. Despite several recent advances, however, this treatment modality still faces a number of challenges. One major challenge is that ex vivo expanded, antigen-specific T cells must proliferate and preserve their effector functions and homing abilities over many weeks prior to infusion into patients and then remain active after infusion in order to generate therapeutic effects (1, 2). Even when T cells are engineered and expanded for optimal tumor specificity and homing, the tumor microenvironment plays a major role in determining the success of immune-based therapy (3, 4). T lymphocyte populations within a tumor are heterogeneous, and infiltrating T cells have been associated with either improved or poor prognosis, depending on the type of T-cell population (5, 6). Antitumor immune responses driven by effector T cells are limited by their susceptibility to the immunosuppressive tumor microenvironment. The immunosuppressive effects are largely generated by cytokines and other tumor-produced factors and by immune cells within the tumor microenvironment such as myeloid derived suppressor cells (MDSC) and regulatory T cells (Tregs; refs. 7, 8). In addition, tumors can also express ligands, such as PD-L1, for turning off T-cell antitumor effects (9).

Signal transducer and activator of transcription 3 (Stat3) acts as a point of convergence for several oncogenic signaling pathways and is persistently activated in numerous tumors as well as in various immune cells within the tumor microenvironment (4, 10, 11). By virtue of its ability to upregulate expression of multiple factors that are upstream of Stat3, the activity of Stat3 can be propagated from tumor cells to diverse immune cells, and vice versa, creating a cross talk between cancer cells and surrounding stroma (4, 11). Moreover, Stat3-regulated factors, such as vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), interleukin-10 (IL-10), and interleukin-23 (IL-23), among many others, promote tumor growth, angiogenesis, and invasion (12–14). Stat3 signaling in both tumor cells and the tumor-associated immune cells plays an important role in promoting MDSC and Tregs (4, 15). In addition to promoting expression of immunosuppressive molecules, Stat3 negatively regulates expression of immunostimulatory factors in both tumor cells and myeloid cells, resulting in a microenvironment strongly...
reducing immune recognition and response against tumors. Our previous studies indicate that blocking Stat3 signaling within the myeloid compartment enhances antitumor immune responses through interruption of the immunosuppressive network that inhibits normal function of both adaptive and innate immunity (16, 17). However, whether Stat3 signaling within CD8+ T cells is inhibitory to their antitumor effector functions remains unknown.

Sunitinib is an orally bioavailable oxindole small-molecule tyrosine kinase inhibitor of vascular endothelial growth factor receptor (VEGFR)-1, VEGFR-2, VEGFR-3, platelet-derived growth factor receptor α (PDGFR-α), PDGFR-β, and stem cell factor (18). Growth inhibition of multiple implanted solid tumors and eradication of larger, established tumors has been shown in mouse xenograft models (19). Sunitinib therapy has shown improved survival for patients with metastatic renal cell carcinoma (RCC) and has become a front-line therapy for the disease (20). Sunitinib has also shown antitumor efficacy in multiple tumor types, indicating its multifaceted role in tumor growth inhibition (21). Recent studies have evaluated the role of sunitinib in modulating immune cells within the tumor microenvironment. Sunitinib has been shown to inhibit MDSC and Tregs in RCC patients (22, 23) and in mouse tumor models (24, 25). In addition, sunitinib can inhibit Stat3, leading to increased tumor apoptosis and decreased MDSC and Tregs in tumor-bearing mice (26).

Although the role of Stat3 in both tumor cells, myeloid cells and Tregs, in promoting the immunosuppressive tumor microenvironment is well known, whether Stat3 signaling within CD8+ T cells affects T-cell effector functions remains unclear. Therefore, we investigated the feasibility of targeting Stat3 in T cells to promote tumor infiltration and expansion of transferred T cells and stimulation of T-cell functions. Our results showed that these criteria can be met by either transferring Stat3-deficient CD8+ naïve T cells or by systemic sunitinib treatment to inhibit Stat3 signaling in conjunction with T-cell transfer. These findings serve as a platform to genetically modify T cells to block Stat3 prior to transfer and/or to use effective direct or indirect Stat3 inhibitors to modulate the tumor microenvironment to improve adoptive T-cell immunotherapy.

Materials and Methods

Cell lines

The B16 cell line was obtained originally from American Type Culture Collection. B16OVA cell line was kindly provided by Dr. J. Mule from Moffitt Cancer Center. The Renca murine cell line was obtained from Dr. A. Chang (University of Michigan Medical Center). These cell lines were maintained in RPMI supplemented with 5% to 10% fetal bovine serum and 100 U/mL of penicillin/streptomycin. Intracellular staining and flow cytometry of B16 cells were used to check expression of melanoma-specific HMB-45 antigen (data not shown). The expression of exogenous OVA antigen and B16 cell–specific endogenous TRP2 and p15E antigens was confirmed by ELISPOT performed within the last 6 months. The ability of these cells to form melanoma in C57BL/6 mice and to elicit OVA-specific response was monitored.

Animals

Stat3+/- mice were provided by S. Akira (Laboratory of Host Defense, World Premiere International Immunology Frontier Research Center and Research Institute for Microbial Diseases, Osaka University) and K. Takeda (Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University). C57BL/6 and BALB/c mice were purchased from the National Cancer Institute. CD4Cre mice were purchased from Taconic, OVA TCR (OT-I), GFP+, Rag1-/- and Rag2-/- transgenic mice were purchased from the Jackson Laboratory. FoxP3-GFP knock-in mice were provided by D. Zeng (Departments of Diabetes Research & Hematopoietic Cell Transplantation, Beckman Research Institute, City of Hope National Medical Center). Mouse care and experimental procedures were conducted under pathogen-free conditions in accordance with established institutional guidance and approved protocols from the Institutional Animal Care and Use Committee of Beckman Research Institute at City of Hope National Medical Center.

In vivo experiments and T-cell adoptive transfer

B16 or B16OVA melanoma cells (1 x 10^6) were injected subcutaneously into Rag1-/- or wild-type mice. Renca tumor cells (2.5 x 10^5) were injected subcutaneously into Rag2-/- mice. Mice received adoptive transfer of (8–10) x 10^6 CD8+ T cells 1 day prior or at days 5 or 6 after tumor challenge. CD8+ cells were isolated from spleens and lymph nodes of donor animals by magnetic beads enrichment using positive selection EasySep kits (STEMCELL Technologies) or MACS cell separation system (Miltenyi Biotec). T cells were transferred by retro-orbital injection. In experiments for in vivo Tregs conversion, Rag1-/- mice received 1 x 10^8 CD4+GFP- cells isolated from FoxP3-GFP knock-in C57BL/6 mice.

Sunitinib treatment

Sunitinib (SU11248; Sutent) was purchased from LC Laboratories or supplied by Pfizer Inc. Tumor-bearing mice received sunitinib treatment at a dose of 30 to 40 mg/kg dissolved in 0.5% solution of carboxymethyl cellulose, administered orally daily for up to 14 days.

Flow cytometry

Cell suspensions from lymph nodes and tumor tissues were prepared as described previously (3) and stained with different combinations of fluorochrome-coupled antibodies to CD4, CD8, CD25, FoxP3, CD11c, CD11b, Gr1, phospho-Tyr705-Stat3 (BD Biosciences). Fluorescence data were collected on Accuri C6 (Accuri Cytometers) or CyAn (Dako Cytomation, Inc.) and analyzed using CFlow software (Accuri Cytometers) or FlowJo software (Tree Star). For in vitro and in vivo experiments for Tregs conversion, CD4+GFP+ cells were sorted using MoFlo MLS cell sorter (Dako Cytomation, Inc.).

Immunofluorescence

Tumor tissue sections were fixated in 2% paraformaldehyde and permeabilized in methanol. After blocking in PBS containing 10% goat serum and 2.5% mouse serum, slides were...
incubated overnight with antibodies: rat anti-mouse CD4 (1:20; BD Biosciences) and rabbit anti-FoxP3 (1:500; Abcam). Next day, the slides were washed and secondary antibodies were applied for 1 hour (goat anti-rabbit, Alexa Fluor 488 labeled and goat anti-rat, Alexa Fluor 555, both 1:200; Molecular Probes).

**Intravitral multiphoton microscopy**

Rag1<sup>−/−</sup> mice bearing B16<sup>OVA</sup> tumors received adoptive transfer of OVA-specific Stat3<sup>+/+</sup> and Stat3<sup>−/−</sup> CD8<sup>+</sup> cells (1.5 × 10<sup>7</sup> of each) previously labeled with cell trackers (CMAC) or OrangeCMTMR reagents (Molecular Probes) according to the manufacturer’s instructions. After 48 or 72 hours, mice were anaesthetized with isoflurane/oxygen, followed by intravenous injection (via retro-orbital route) with dextran-fluoresceine (100 μg; Molecular Probes). Fifteen minutes later, tumor tissues of mice were surgically exposed for intravitral multiphoton microscopy (IVMMP) and imaged with an Ultima Multiphoton Microscopy System (Prairie Technologies). For recording fluoresceine and rhodamine emission signals, λ = 860-nm excitation wavelength was used. Extracellular matrix (ECM) was recorded using second harmonic generation (2HG) using λ<sub>exc</sub> = 890 nm. Band-pass filters optimized for and rhodamine (BP λ = 570–620 nm), coumarin, and 2HG (BP λ = 460/50 nm) were used for detection.

**Enzyme-linked immunosorbent spot assay**

To detect IFN-γ or granzyme B enzyme-linked immunosorbent spot (ELISPOT) assay was conducted according to the manufacturer’s protocols (Diaclone and R&D Systems, respectively). Briefly, 5 × 10<sup>4</sup> cells isolated from tumor draining lymph nodes were cultured over night in the presence or absence of peptides (SYMFCEL for B16<sup>OVA</sup> experiments and p15E for B16 experiments, both at concentration of 10 μg/mL). Peptide-specific IFN-γ and granzyme B–positive spots were detected following the manufacturer’s protocol and manually counted under the binocular.

**In vivo CTL killing assay and proliferation assay**

Splenocytes were harvested and split into 2 populations. Target cell population was pulsed with 2 μg/mL of p15E peptide for 2 hours at 37°C, followed by CFSE<sup>Hi</sup> (10 μmol/L) fluorescent labeling. The control cell population was not pulsed but labeled with CFSE<sup>Lo</sup> (1 μmol/L). Equal numbers of CFSE<sup>Hi</sup> and CFSE<sup>Lo</sup> cells were mixed, followed by adoptive transfer (intravenously) into B16 tumor-bearing animals. Each animal received 2 × 10<sup>7</sup> cells. Cytotoxic T lymphocyte (CTL) cytotoxic effects (decrease of CFSE<sup>Hi</sup> population) in mice that received adoptive transfer of Stat3<sup>+/+</sup> or Stat3<sup>−/−</sup> CD8<sup>+</sup> cells (8 × 10<sup>5</sup>) were analyzed by flow cytometry (Accuri Cytometers). In proliferation assay, tumor-bearing Rag1<sup>−/−</sup> mice received adoptive transfer of OVA-specific Stat3<sup>+/+</sup> or Stat3<sup>−/−</sup> CD8<sup>+</sup> cells (1 × 10<sup>6</sup> of each) labeled with CFSE. CD8<sup>+</sup> cells proliferation was analyzed 72 hours later by flow cytometry (Accuri Cytometers).

**In vitro Tregs conversion**

CD4<sup>+</sup>GFP<sup>+</sup> cells sorted from splenocytes isolated from FoxP3-GFP knock-in C57BL/6 mice were cocultured with Renca tumor cells and irradiated CD11c<sup>+</sup> cells for 72 hours. Presence of CD4<sup>+</sup>GFP<sup>+</sup> cells was checked by flow cytometry.

**T-cells proliferation assay in vitro**

About 1 × 10<sup>5</sup> cells per well of OVA-specific Stat3<sup>+/+</sup> and Stat3<sup>−/−</sup> CD8<sup>+</sup> cells were cocultured with irradiated 5 × 10<sup>5</sup> CD11c<sup>+</sup> cells in the presence of OVA peptide for 72 hours. Eighteen hours before harvesting, cells were incubated in medium containing 1 μCi of [methyl-<sup>3</sup>H]-thymidine (Amersham). Radioactivity was measured as counts per minutes (cpm) on liquid scintilator (Wallac 1450 Microbeta, Perkin Elmer).

**Western blot analysis**

Western blot analysis was conducted as previously described (27).

**Statistics**

An unpaired t test and 2-way ANOVA were used to calculate 2-tailed P values to estimate statistical significance of differences between treatment groups. Statistically significant P values are indicated in figures as follows: ***P < 0.001, **P < 0.01, *P < 0.05. Data were analyzed using GraphPad Prism software.

**Results**

**Ablating Stat3 within CD8<sup>+</sup> cells allows in vivo expansion and activation of infused T cells in tumor-bearing lymphopenic host**

Genetically engineering T cells prior to their infusion into animals or patients has greatly facilitated the advancement of adoptive T-cell transfer therapeutic modality (28). To test whether Stat3 is a molecular target to inhibit in CD8<sup>+</sup> T cells before adoptive transfer, we first generated transgenic mice lacking the Stat3 alleles in T cells. This was achieved by crossing mice containing Stat3 alleles flanked by loxP sites with mice carrying Cre-recombinase transgene under control of the CD4 promoter, which deletes loxP-flanked alleles in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells with very high efficiency. These mice served as donors of naive T cells for adoptive transfer into immunodeficient Rag1<sup>−/−</sup> mice that lack both T and B cells. Some of current adoptive T-cell therapy protocols prior to T-cell infusion introduce chemolympo-depletion alone or combined with radiation (29, 30). Adoptive transfer of Stat3<sup>−/−</sup> CD8<sup>+</sup> T cells before tumor implantation significantly inhibited growth of poorly immunogenic B16 melanoma tumors in comparison with control mice that received Stat3-<sup>+</sup> T cells (Fig. 1A). In mice receiving Stat3<sup>−/−</sup> CD8<sup>+</sup> T cells, tumor tissue was highly infiltrated by CD8<sup>+</sup> or CD8<sup>+</sup> T cells with very high efficiency. This increased (Fig. 1B, top) and Stat3<sup>−/−</sup> T-cell killing of p15E peptide-
pulsed splenocytes was significantly better (Fig. 1C). Moreover, adoptive transfer of Stat3−/− CD8+ T cells into Rag1−/− mice with established B16 melanoma tumor significantly reduced tumor growth (Fig. 1D, left). B16 tumor–bearing mice receiving Stat3−/− CD8+ T cells mounted stronger responses against the endogenous B16 tumor antigen p15E than their Stat3+/+ counterparts, as shown by IFN-γ ELISPOT (Fig. 1D, right).

We further evaluated whether genetically blocking Stat3 in antigen-specific CD8+ T cells would allow better T-cell
response in vivo. To test this, we generated ovalbumin-specific mice (CD8\textsuperscript{OVA}/TCR [OT-I]) with or without Stat3 gene ablation in T cells. B16\textsuperscript{OVA}-expressing tumor growth was significantly inhibited in Rag\textsuperscript{1}–/– mice receiving adoptive transfer of Stat3\textsuperscript{+/–} CD8\textsuperscript{+} OT-1 T cells (data not shown). B16\textsuperscript{OVA} tumor–bearing mice that received Stat3\textsuperscript{−/−}/CD8\textsuperscript{−/−} OT-1 cells could mount stronger antigen-specific T-cell responses after OVA peptide restimulation than their Stat3\textsuperscript{+/−} counterparts, as shown by IFN-γ and granzyme B ELISPOT (Fig. 1E, left and right, respectively).

**Tumor infiltration and proliferation of genetically engineered Stat3\textsuperscript{−/−} CD8\textsuperscript{+} T cells in vivo**

Although data shown in Figure 1B suggested that the lack of Stat3 in CD8\textsuperscript{+} T cells leads to efficient T-cell tumor accumulation, this could be attributed to reduced tumor growth rate. To directly assess whether inhibiting Stat3 in CD8\textsuperscript{+} T cells would increase their ability to infiltrate tumors, we adoptively transferred Stat3\textsuperscript{+/−} and Stat3\textsuperscript{−/−}/OVA-specific CD8\textsuperscript{+} T cells mixed at 1:1 ratio into the Rag\textsuperscript{1}–/– mice bearing B16\textsuperscript{OVA} tumors. To distinguish between the 2 populations in vivo, we labeled the Stat3\textsuperscript{+/−} and Stat3\textsuperscript{−/−}/OVA-specific CD8\textsuperscript{+} T cells with distinct fluorescent cell trackers before infusion into B16\textsuperscript{OVA} tumor–bearing mice. Intravital multiphoton microscopy analysis of tumors conducted at 48 and 72 hours postadoptive transfer indicated enhanced accumulation of Stat3\textsuperscript{−/−}/CD8\textsuperscript{+} OT-1 T cells in tumors (Fig. 2A). Flow cytometry to confirm the live microscopy imaging data showed significantly increased accumulation of Stat3\textsuperscript{−/−}/CD8\textsuperscript{+} T cells inside the tumors after 48 hours (Fig. 2B). This finding was further confirmed using Stat3\textsuperscript{+/−} and Stat3\textsuperscript{−/−}/OVA-specific CD8\textsuperscript{+} T cells with reverse color labeling (Fig. 1B).

Another major challenge facing adoptive T-cell therapy is the need to expand the number of T cells ex vivo. We, therefore, tested whether inhibiting Stat3 in transferred T cells would allow them to proliferate in vivo in the tumor-bearing hosts. Our in vivo experiments indicated that adoptively transferred Stat3\textsuperscript{−/−}/CD8\textsuperscript{+} OT-1 T cells proliferated better both in tumor-draining and contralateral lymph nodes (Fig. 2C). Experiments conducted in vitro confirmed higher proliferative potential of Stat3\textsuperscript{+/−}/OVA-specific CD8\textsuperscript{+} cells as well as elevated IFN-γ secretion after antigen-specific stimulation in the presence of tumor factors (Supplementary Fig. 2).

**Targeting Stat3 systemically by sunitinib improves T-cell therapy**

Although genetic engineering of T cells prior to adoptive transfer has been an important approach for T-cell therapy (28), the availability of some Food and Drug Administration–approved, small-molecule drugs with capacity to inhibit angiogenesis, kill tumor cells, and modulate the tumor immunologic microenvironment, in part through Stat3 inhibition, may lead to new paradigm for T-cell therapy. We therefore tested in vitro whether sunitinib, which inhibits Stat3 (26), could effectively inhibit the activation of Stat3 by tumor-derived factors. Data shown in Figure 3A indicated that tumor factor–induced Stat3 activation in CD8\textsuperscript{+} T cells was inhibited by sunitinib. To assess whether sunitinib might improve adoptively transferred CD8\textsuperscript{+} T-cell antitumor efficacy in vivo, Rag2\textsuperscript{−/−} mice (Balb/c) with established syngeneic Renca mouse RCC were given vehicle control or sunitinib 2 days prior to T-cell transfer. Results from these experiments indicated that sunitinib treatment combined with CD8\textsuperscript{+} T-cell adoptive transfer inhibited growth of Renca tumor significantly better than either treatment alone (Fig. 3B, left). Moreover, systemic treatment with sunitinib inhibited Stat3 activity in tumor-infiltrating dendritic cells (DC), as shown by intracellular flow cytometry (Fig. 3B, right).

Inhibiting Stat3 activity by sunitinib led to increased tumor infiltration and activation of the transferred CD8\textsuperscript{+} T cells in tumor-draining lymph nodes (Fig. 3C, left and right, respectively). To assess whether the positive effects of sunitinib on CD8\textsuperscript{+} T cells were at least partially through modulating Stat3 within CD8\textsuperscript{+} T cells, we combined sunitinib treatment with adoptively transferred Stat3\textsuperscript{+/−} and Stat3\textsuperscript{−/−}/CD8\textsuperscript{+} T cells. Subsequent analysis of CD8\textsuperscript{+} T-cell tumor infiltration and activation suggested that systemic sunitinib treatment did affect CD8\textsuperscript{+} T cells because treating mice receiving Stat3\textsuperscript{−/−}/CD8\textsuperscript{+} T cells with sunitinib did not further significantly enhance the T-cell functions (Supplementary Fig. 3).

We also combined sunitinib treatment with adoptively transferring antigen-specific CD8\textsuperscript{+} T cells. C57BL6 mice with progressively growing B16\textsuperscript{OVA} tumor (4–6 mm in diameter) were treated with either vehicle or sunitinib, followed by adoptive transfer of naïve OVA-specific CD8\textsuperscript{+} T cells. In mice that received the combination of both antigen-specific T cells and sunitinib, tumor growth was significantly reduced (Fig. 4A). Moreover, sunitinib-treated mice showed an
increased number of activated total CD8\(^+\) T cells in tumors (Fig. 4B). To distinguish the effect of sunitinib treatment on the activation of host versus transferred antigen-specific T cells, we conducted experiments similar to those shown in Figure 4B but in GFP\(^+\) transgenic mice. Again, sunitinib treatment caused increased infiltration of CD8\(^+\) T cells into the tumor tissue and induced activation of both host and transferred T cells (Fig. 4C).

\textit{In vitro} analysis indicated that sunitinib can inhibit tumor factor–induced Stat3 activity in CD8\(^+\) T cells (Fig. 3A). We next evaluated whether sunitinib could also reduce activated Stat3 in CD8\(^+\) T cells in tumors \textit{in vivo}. Tumor tissue sections from mice receiving transfer of naive OVA-specific CD8\(^+\) T cells and treatment with either vehicle or sunitinib were subject to immunofluorescent staining, followed by confocal microscopic analysis. Representative photographs of the tumor tissue areas with comparable numbers of CD8\(^+\) T cells indicated higher nuclear phosphorylated Stat3 (p-Stat3) in CD8\(^+\) T cells in mice receiving vehicle than their sunitinib-treated counterparts (Fig. 4D). Mean fluorescence intensity of the tumor CD8\(^+\) T cells p-Stat3 is also shown (Fig. 4D, right).

Sunitinib abrogates tumor-induced Treg cell conversion from CD4\(^+\) FoxP3\(^+\) T cells

It has been shown that depletion of endogenous Tregs prior to T-cell transfer can improve the outcome of T-cell therapy (31). However, the tumor microenvironment can continually convert non-Treg (CD4\(^+\) FoxP3\(^-\)) T cells into Treg (CD4\(^+\) FoxP3\(^+\)) T cells even after initial depletion.
Although recent studies indicated that sunitinib treatment diminished Treg cells in mouse tumor models and in RCC patients (23, 25), whether sunitinib effects on Tregs were indirect or through inhibiting Treg conversion remains unknown. We have previously shown that sunitinib treatment was associated with a reduction in tumor Stat3 activity and the number of Tregs in Renca murine tumor model (26).

In this study, we tested whether sunitinib, through inhibition of Stat3 phosphorylation, hindered tumor-induced conversion of CD4+FoxP3+/GFP cells into CD4+FoxP3+ Treg cells in vitro and in vivo. Isolated CD4+FoxP3+/GFP cells from FoxP3-GFP knock-in C57BL/6 mice that express GFP under the control of the promoter of FoxP3 were cocultured with DCs and Renca tumor cells in the presence of sunitinib or vehicle (DMSO). After 3 days in culture, FoxP3 expression in CD4+ T cells was examined by flow cytometry. In comparison with the control treatment, sunitinib inhibited Treg conversion in a dose-dependent manner (Fig. 5A). To evaluate the effects of sunitinib on Stat3 signaling, we determined levels of Stat3 phosphorylation in CD4+ T cells by Western blot and flow cytometry. A significant and dose-dependent inhibition of Stat3 activity in CD4+ T cells treated with sunitinib was detected by both methods (Fig. 5B, right and left, respectively).

To investigate the effect of sunitinib on Treg conversion in vivo, sorted CD4+FoxP3+/GFP cells from FoxP3-GFP knock-in C57BL/6 mice were adoptively transferred into Rag1−/− mice. Mice were then challenged with B16 tumor cells. After tumors grew 5 to 7 mm in diameter, sunitinib or control vehicle was administered orally. To evaluate Treg conversion in tumors, intravital multiphoton microscopy of tumors was used to detect CD4+FoxP3+ T cells. FoxP3+ Treg cells were observed in control group but absent in the sunitinib treatment group at various time points (Fig. 5C). Because IVMPM evaluates only the surface layers of the tumor tissue, we carried out immunofluorescence staining of tumor tissues, which confirmed a reduction in CD4+FoxP3+ cells infiltrating tumors in the sunitinib-treated group (Fig. 5D and Supplementary Fig. 3).
Discussion

The tumor microenvironment severely limits the efficacy of various immunotherapies. For adoptive T-cell therapy, the need to select/engineer and expand T cells with targeted antigen specificity while still preserving their effector function and homing capacities poses additional challenges. The current study has identified strategies to overcome some of
these obstacles facing adoptive T-cell therapy. We show that genetically engineered Stat3−/− CD8+ T cells can, without any ex vivo expansion, efficiently proliferate, infiltrate, and inhibit tumor growth. Furthermore, systemic treatment with sunitinib, a tyrosine kinase inhibitor that affects Stat3 signaling pathway, can enhance adoptive T-cell therapy and effectively inhibit tumor Treg conversion. These studies provide direct evidence that blocking Stat3 in either T cells prior to in vivo infusion or systemically in the tumor microenvironment before and/or during T-cell therapy allows in vivo expansion and improved antitumor functions of the transferred T cells in lymphopenic mice.

We and others have shown that Stat3 is a key oncogenic transcription factor persistently activated in diverse cancer cells and important for tumor cell survival. Stat3 is also activated in tumor stromal immune cells and potently immunosuppressive (16, 27). Multiple studies have shown that Stat3 activity in both tumor cells and tumor-associated myeloid cells plays a critical role in inducing immunosuppression and that targeted Stat3 gene ablation in myeloid cells results in tumor DC activation, tumor Treg cell reduction, and heavy infiltration of CD8+ T cells in tumors, leading to effective antitumor immune responses (4, 11). Several recent studies further showed the feasibility of targeting Stat3 in the tumor microenvironment to enhance various immunotherapeutic methods (17, 32, 33). Related to current study, it has been shown that targeting Stat3 systemically by an experimental Jak inhibitor altered the tumor immunologic environment, leading to improved antitumor effects of transferred CTLs (34). However, the potential direct effects on T cells and Tregs by the Jak inhibitor were not assessed in the study.

Our results show that ablating Stat3 in CD8+ T cells prior to transfer allows more efficient T-cell tumor infiltration, proliferation, and antitumor effector functions. Genetically modifying selected T cells is a key part of current adoptive T-cell therapy, and several approaches to engineer T cells have led to important advancement for the treatment modality (30, 35). On the basis of our results, it may be highly desirable to modify T cells to render the selected T cells Stat3 null before infusion into patients. This can be achieved by transducing T cells with retroviral and possibly other vectors encoding either Stat3 siRNA or dominant-negative Stat3 mutant. Furthermore, it is likely that additionally silencing/inhibiting Stat3 in the genetically engineered T cells with the capacity to home into specific tumor sites and/or antitumor properties will improve current T-cell therapeutic approaches. The improvements can possibly include shortened time for ex vivo expansion, resistance to the tumor immunosuppressive effects, and better antitumor effector functions.

Combining immunotherapy with other anticancer therapeutical modalities can potentiate its antitumor activity by multiple mechanisms, including induced lymphodepletion, activation of antigen-presenting cells, or elimination of immunosuppressive populations of cells infiltrating the tumor microenvironment (29, 36, 37). Recent data have shown that sunitinib can modulate immunosuppression within the tumor microenvironment by decreasing MDSCs and Tregs (22, 23, 25, 26). Furthermore, we have shown that inhibition of Stat3 signaling by sunitinib is a mechanism for this activity that leads to a reduction in angiogenesis, increased apoptosis, and decreased tumor growth (26). Our results suggest that Stat3 activity not only is important for direct RCC response to sunitinib through apoptosis but is also involved in the regulation of DC activation, MDSC infiltration, and Treg accumulation in the tumor tissue. Systemic treatment of RCC and melanoma in immunodeficient mice combined with adoptive transfer of naïve CD8+ T cells significantly inhibited their growth. Furthermore, adoptive transfer of Stat3-negative CD8+ T cells enhanced the antitumor responses, indicating the importance of inhibition of Stat3 signaling in all compartments of immune system.

Sunitinib treatment significantly improved antitumor responses with adoptive transfer of antigen-specific CD8+ T cells. This observation is in agreement with studies by Ozao-Choy et al., who showed that sunitinib improved antitumor responses when using tumor-specific CD4+ T cells (25). Released factors such as TGF-β can convert Th1 CD4+ T cells into Tregs, which actively suppress anti-tumor immune response (38). In this report, we show that sunitinib treatment inhibits Stat3 phosphorylation and abrogates tumor microenvironment-induced conversion of CD4+ cells into Tregs both in vitro and in vivo. These observations concur with recent studies that implicate sunitinib Stat3 inhibition in the reduction of Tregs at the tumor site (25, 39) and provide a potential mechanism whereby Tregs not only are depleted by Stat3 inhibition but their conversion is also blocked.

Enthusiasm for adoptive T-cell therapy remains high, and as we better understand how to generate T cells with improved specificity, durability, and cytotoxicity, we must also determine appropriate and clinically feasible methods to address immunosuppression within the tumor microenvironment.

Figure 4. Sunitinib enhances antigen-specific tumor response of adoptively transferred CD8+ T cells. A, sunitinib treatment combined with adoptive T-cell therapy significantly reduced growth of B16OVA tumor. C57BL6 mice were treated with sunitinib administrated orally at a dose of 40 mg/kg of body weight once a day. Two days later, some of the mice received adoptive transfer of 1×10⁷ OVA-specific CD8+ T cells. Shown representative tumor growth curve of 3 experiments, n = 5–6 animals per group. ***, P < 0.001. B, flow cytometric analysis of tumor tissues indicated increased expansion, efficiently proliferate, infiltrate, and inhibit tumor growth. Furthermore, systemic treatment with sunitinib, a tyrosine kinase inhibitor that affects Stat3 signaling pathway, can enhance adoptive T-cell therapy and effectively inhibit tumor Treg conversion. These studies provide direct evidence that blocking Stat3 in either T cells prior to in vivo infusion or systemically in the tumor microenvironment before and/or during T-cell therapy allows in vivo expansion and improved antitumor functions of the transferred T cells in lymphopenic mice.

We and others have shown that Stat3 is a key oncogenic transcription factor persistently activated in diverse cancer cells and important for tumor cell survival. Stat3 is also activated in tumor stromal immune cells and potently immunosuppressive (16, 27). Multiple studies have shown that Stat3 activity in both tumor cells and tumor-associated myeloid cells plays a critical role in inducing immunosuppression and that targeted Stat3 gene ablation in myeloid cells results in tumor DC activation, tumor Treg cell reduction, and heavy infiltration of CD8+ T cells in tumors, leading to effective antitumor immune responses (4, 11). Several recent studies further showed the feasibility of targeting Stat3 in the tumor microenvironment to enhance various immunotherapeutic methods (17, 32, 33). Related to current study, it has been shown that targeting Stat3 systemically by an experimental Jak inhibitor altered the tumor immunologic environment, leading to improved antitumor effects of transferred CTLs (34). However, the potential direct effects on T cells and Tregs by the Jak inhibitor were not assessed in the study.

Our results show that ablating Stat3 in CD8+ T cells prior to transfer allows more efficient T-cell tumor infiltration, proliferation, and antitumor effector functions. Genetically modifying selected T cells is a key part of current adoptive T-cell therapy, and several approaches to engineer T cells have led to important advancement for the treatment modality (30, 35). On the basis of our results, it may be highly desirable to modify T cells to render the selected T cells Stat3 null before infusion into patients. This can be achieved by transducing T cells with retroviral and possibly other vectors encoding either Stat3 siRNA or dominant-negative Stat3 mutant. Furthermore, it is likely that additionally silencing/inhibiting Stat3 in the genetically engineered T cells with the capacity to home into specific tumor sites and/or antitumor properties will improve current T-cell therapeutic approaches. The improvements can possibly include shortened time for ex vivo expansion, resistance to the tumor immunosuppressive effects, and better antitumor effector functions.

Combining immunotherapy with other anticancer therapeutical modalities can potentiate its antitumor activity by multiple mechanisms, including induced lymphodepletion, activation of antigen-presenting cells, or elimination of immunosuppressive populations of cells infiltrating the tumor microenvironment (29, 36, 37). Recent data have shown that sunitinib can modulate immunosuppression within the tumor microenvironment by decreasing MDSCs and Tregs (22, 23, 25, 26). Furthermore, we have shown that inhibition of Stat3 signaling by sunitinib is a mechanism for this activity that leads to a reduction in angiogenesis, increased apoptosis, and decreased tumor growth (26). Our results suggest that Stat3 activity not only is important for direct RCC response to sunitinib through apoptosis but is also involved in the regulation of DC activation, MDSC infiltration, and Treg accumulation in the tumor tissue. Systemic treatment of RCC and melanoma in immunodeficient mice combined with adoptive transfer of naïve CD8+ T cells significantly inhibited their growth. Furthermore, adoptive transfer of Stat3-negative CD8+ T cells enhanced the antitumor responses, indicating the importance of inhibition of Stat3 signaling in all compartments of immune system.

Sunitinib treatment significantly improved antitumor responses with adoptive transfer of antigen-specific CD8+ T cells. This observation is in agreement with studies by Ozao-Choy et al., who showed that sunitinib improved antitumor responses when using tumor-specific CD4+ T cells (25). Released factors such as TGF-β can convert Th1 CD4+ T cells into Tregs, which actively suppress anti-tumor immune response (38). In this report, we show that sunitinib treatment inhibits Stat3 phosphorylation and abrogates tumor microenvironment-induced conversion of CD4+ cells into Tregs both in vitro and in vivo. These observations concur with recent studies that implicate sunitinib Stat3 inhibition in the reduction of Tregs at the tumor site (25, 39) and provide a potential mechanism whereby Tregs not only are depleted by Stat3 inhibition but their conversion is also blocked.

Enthusiasm for adoptive T-cell therapy remains high, and as we better understand how to generate T cells with improved specificity, durability, and cytotoxicity, we must also determine appropriate and clinically feasible methods to address immunosuppression within the tumor microenvironment.
Figure 5. Sunitinib abrogates tumor-induced conversion of CD4⁺FoxP3⁻ T cells into CD4⁺FoxP3⁺ Treg cells in vitro and in vivo. A, sunitinib abrogates tumor-induced Tregs conversion in vitro. CD4⁺FoxP3⁻ T cells from FoxP3-GFP knock-in mice were sorted and cocultured with Renca tumor cells and DCs with or without sunitinib at indicated concentrations. The percentage of CD4⁺ FoxP3⁺ Tregs was analyzed by FACS. Shown are representative flow cytometric results, with calculated mean (SEM) values from 3 experiments (right and left, respectively). B, sunitinib treatment causes dose-dependent inhibition of Stat3 activity in CD4⁺ T cells. Representative Western blotting and flow analysis of CD4⁺ T cells are shown (left and right, respectively). C, IVMPM analysis to detect the conversion of CD4⁺ FoxP3⁻ to CD4⁺ FoxP3⁺ Tregs (green) in B16 tumors at 7 and 10 days since initial sunitinib treatment. Vasculature is visualized by intravenous injection of dextran-rhodamine (red color) 15 minutes before imaging. D, immunofluorescence staining of B16 tumors to confirm reduction of tumor-associated Treg conversion in mice treated with sunitinib; FoxP3 (green) and CD4 (red).
environment. Our data show that sunitinib therapy can enhance adoptive T-cell therapy, at least partially, through the inhibition of Stat3 signaling, with subsequent reversal of immunosuppression at the tumor site. These results present a paradigm that may be used in the clinic: blocking Stat3 in T cells prior to transfer and/or using Stat3 inhibitory tyrosine kinase inhibitors to improve the efficacy of T-cell therapy.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interests were disclosed.

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