

Regulation of Dendritic Cell Differentiation and Antitumor Immune Response in Cancer by Pharmacologic-Selective Inhibition of the Janus-Activated Kinase 2/Signal Transducers and Activators of Transcription 3 Pathway

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Abstract

Abnormal dendritic cell differentiation and accumulation of immunosuppressive myeloid cells in cancer is one of the major factors of tumor nonresponsiveness. We have previously shown that hyperactivation of the Janus-activated kinase 2/signal transducers and activators of transcription 3 (JAK2/STAT3) induced by tumor-derived factors (TDF) is responsible for abnormal dendritic cell differentiation. Here, using a novel selective inhibitor of JAK2/STAT3 JSI-124, we investigated the possibility of pharmacologic regulation of dendritic cell differentiation in cancer. Our experiments *in vitro* have shown that JSI-124 overcomes the differentiation block induced by TDF and promotes the differentiation of mature dendritic cells and macrophages. JSI-124 significantly reduced the presence of immature myeloid cells *in vivo* and promoted accumulation of mature dendritic cells. In addition to a direct antitumor effect in several animal models, JSI-124 significantly enhanced the effect of cancer immunotherapy. This indicates that pharmacologic inhibition of the JAK2/STAT3 pathway can be an important new therapeutic strategy to enhance antitumor activity of cancer immunotherapy. (Cancer Res 2005; 65(20): 9525-35)

Introduction

Dendritic cells are specialized antigen-presenting cells (APC) that recognize, acquire, process, and present antigens to naive resting T cells for the induction of an antigen-specific immune response (1–3). Dendritic cells are critically important for the induction and maintenance of antitumor immune responses both spontaneously developed and induced as a result of immunotherapy. Inadequate function of the host immune system may render all attempts to use immunotherapy ineffective. Data from different laboratories obtained during the past few years indicate that defects in the dendritic cell system is one of the main factors responsible for tumor escape. Recently accumulated evidence suggests that dendritic cell defects in cancer are systemic and are based on their abnormal differentiation. This abnormal differentiation produces at least three main results (reviewed in ref. 4): (a) decreased production of functionally competent mature dendritic cells; (b) accumulation of immature dendritic cells that have

characteristics of lineage committed dendritic cells but cannot up-regulate MHC class II and costimulatory molecules or produce appropriate cytokines (These cells are not only inefficient in T-cell stimulation but also can be involved in induction of T-cell tolerance.); (c) increased production of immature myeloid cells. Immature myeloid cells suppress antigen-specific T cells via direct cell-cell contact and contribute greatly into tumor nonresponsiveness.

It is now established that abnormal dendritic cell differentiation is mediated by soluble factors produced by tumor cells including vascular endothelial growth factor, macrophage-colony-stimulating factor (CSF), granulocyte macrophage CSF (GM-CSF), interleukin-10 (IL-10), IL-6, gangliosides, spermin, etc. (5–19). These tumor-derived factors (TDF) bind to different receptors on hematopoietic cells. This suggests that to exert similar functional effects on dendritic cell differentiation, these factors may converge at the level of signal transduction. Recent studies have identified one such pathway, Janus-activated kinase 2/signal transducers and activators of transcription 3 (JAK2/STAT3) signaling.

JAK and STAT proteins are critical components of diverse signal transduction pathways that are actively involved in cellular survival, proliferation, differentiation, and apoptosis (20). JAKs are constitutively associated with many cytokine and growth factor receptors, including those implicated in defective dendritic cell differentiation (review ref. 21). Activated JAKs eventually induce phosphorylation of STATs followed by their translocation into the nucleus, where they modulate expression of target genes. Constitutive activation of one member of the STAT family, STAT3, has been shown in many different tumors. This activation usually results in antiapoptotic effect and promotes cell proliferation (reviewed in ref. 22). Recently, we have reported that TDF-inducible activation of JAK2/STAT3 is directly involved in the abnormal dendritic cell differentiation in cancer (23, 24). Myeloid cells maintain high levels of JAK2 and STAT3 activity, which results in the accumulation of immature myeloid cells and inhibition of dendritic cell differentiation *in vitro* (24). We hypothesized that inhibition of tumor-induced JAK2/STAT3 hyperactivation in myeloid cells may improve dendritic cell differentiation and function, and ultimately, antitumor immune response.

To test this hypothesis, we used a new selective inhibitor of JAK2/STAT3 pathway, JSI-124 (cucurbitacin I). We have previously shown that JSI-124 selectively inhibited the activation of JAK2 and STAT3 but not Src, Akt, extracellular signal-regulated kinase (ERK), and *c-jun* NH₂-terminal kinase (JNK; ref. 25). JSI-124 inhibited the growth of tumors with constitutively active STAT3 but did not affect tumors without STAT3 hyperactivation (25).

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This study, for the first time, shows that inhibition of JAK2/STAT3 signaling dramatically improves differentiation of dendritic cell and eliminates immunosuppressive myeloid cells in cancer. Importantly, JSI-124 significantly enhanced the effect of cancer vaccine.

Materials and Methods

Reagents, drug, and cell culture. RPMI 1640, DMEM, fetal bovine serum (FBS), and antibiotics were obtained from Life Technologies Bethesda Research Laboratories (Grand Island, NY); recombinant murine GM-CSF and IL-4 were from RDI (Flanders, NJ); lipopolysaccharides and Concanavalin A (ConA) were from Sigma (St. Louis, MO). The following antibodies were obtained from BD PharMingen (San Diego, CA): anti-Gr-1 (anti-Ly-6G), anti-CD11b, anti-CD11c, anti-I-A^b, anti-I-A^d, anti-CD86, anti-CD40, anti-I-A/I-E, and anti-CD3, TCR V α 2. Anti-F4/80 antibody was from Serotec, Inc. (Raleigh, NC). Anti-clonotypic TCR (clone 6.5) was obtained from Caltag (Burlingame, CA); JSI-124 (cucurbitacin I) was obtained from the National Cancer Institute (Frederick, MD) and for *in vivo* experiments cucurbitacin I was obtained from Indofine Chemicals, Inc. (Hillsborough, NJ). It was dissolved in DMSO.

Murine NIH-3T3 fibroblasts and CT26 colon carcinoma cell line were obtained from American Type Culture Collection (Manassas, VA). NIH-3T3 cells stably transfected with v-Src were kindly provided by Dr. Richard Jove (H. Lee Moffitt Cancer Center, Tampa, FL). MethA (methylcholantrene induced) sarcoma cell line was obtained from Dr. Lloyd J. Old (Cancer Research Institute, New York, NY). MethA tumor was developed in BALB/c mice and passaged *in vivo* as an ascitic tumor. C3 fibrosarcoma was made by transformation of B6 mouse embryonic cells with human papillomavirus type 16 (HPV-16; ref. 26) and kindly provided by Dr. W.M. Kast (Loyola University of Chicago, Maywood, IL). To generate conditioned medium, cells were kept in medium with reduced (3%) FBS concentration. After 48 hours, supernatants were collected, filtered, and used in experiments.

All peptides were purchased from SynPep Corp. (Dublin, CA). They include H2-K^d-restricted mutant p53-derived peptide (KYICNSSCM), H2-K^d-restricted HA-derived peptide (IYSTVASSL), H-2K^b-restricted HPV-16-derived peptide (RAHYNIVTF), H-2K^b-restricted OVA-derived peptide (SIINFEKL), I-A^d-restricted hemagglutinin-derived peptide (SFERFEIFPKE). A recombinant vaccinia virus encoding hemagglutinin from the 1934 PR8 strain of influenza was a gift from F. Guarneri (John Hopkins Institute, Baltimore, MD). A recombinant adenovirus encoding full open reading frame of wild-type p53 gene was described elsewhere (27).

Generation of dendritic cells and isolation of cells. Bone marrow cells were obtained from the femurs and tibias of mice, and red cells were eliminated using ACK buffer. Cells were cultured in RPMI 1640 supplemented with 10% FBS, 20 ng/mL GM-CSF, 10 ng/mL IL-4, and 50 μ mol/L 2-mercaptoethanol alone or in the presence of control (from 3T3 fibroblasts) or tumor cell (from CT26) conditioned medium. Half of the medium was replaced every 2 days. Gr-1- or CD11c-positive cells were isolated from *in vitro* cultures or spleens of tumor-bearing or control mice using magnetic beads separation technique according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). Purity of Gr-1-positive or CD11c-positive populations was >95% as determined by flow cytometry. Infection and activation of dendritic cells as well as the description of p53-adenovirus (Ad-p53) were reported previously (27).

Animals and tumor models. Female BALB/c and C57BL/6 mice ages 6 to 8 weeks were obtained from the National Cancer Institute. B6.SJL-PtrcaPep3b/BoyJ mice (CD45.1⁺) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and Swiss mice from Charles River Lab (Wilmington, MA). TCR-transgenic mice expressing an α/β TCR specific for MHC class II-restricted SFERFEIFPKE peptide, derived from influenza hemagglutinin, were originally obtained from Harold von Boehmer (Basel Institute for Immunology, Basel, Switzerland) and then were crossed to a BALB/c background for >10 generations. Mice were kindly provided by Dr. E. Sotomayor (H. Lee Moffitt Cancer Center, Tampa, FL). OT-1 TCR-transgenic mice [C57Bl/6-Tg(TCR α TCR β)1100mjb] were purchased from The Jackson Laboratory. All mice were kept in pathogen-free conditions and

handled in accordance with the Guidelines for Animal Experiments requirements. C3 sarcoma was established in C57BL/6 mice by s.c. inoculation of 5×10^5 tumor cells. CT26 colon carcinoma and MethA sarcoma were established in BALB/c mice by s.c. inoculation of 5×10^5 tumor cells; 3T3-v-Src tumor in Swiss mice were established by s.c. inoculation of 2.5×10^6 cells.

Adoptive transfer experiments to evaluate immature myeloid cell differentiation. C3 tumor was established in C57BL/6 (CD45.2⁺) and B6.SJL-PtrcaPep3b/BoyJ (CD45.1⁺) mice by s.c. inoculation of 5×10^5 tumor cells. After 2 weeks (tumor size, 1-1.2 cm in diameter) Gr-1-positive cells were isolated from spleens of tumor-bearing C57BL/6 mice followed by transfer of 4×10^6 of them into congenic tumor-bearing CD45.1⁺ mice. Mice were then treated i.p. with JSI-124 at dose 1 mg/kg/d or vehicle control (DMSO) for 3 days (three mice per group). On day 4, mice were sacrificed and spleens were collected. The phenotype of the cells was evaluated in the population of donor's CD45.2⁺ by flow cytometry.

Adoptive cell transfer and immunization. Purified T cells (3.5×10^6) from OT-1 or HA-TCR transgenic mice were injected i.v. into naive C57BL/6 or BALB/c mice. Two days later, these mice were immunized s.c. with 100 μ g of OVA-derived peptide SIINFEKL or with 1×10^7 plaque-forming units of recombinant vaccinia encoding hemagglutinin in 0.1 mL PBS. Ten days later, cells from lymph nodes and spleens were isolated, restimulated *in vitro* with specific or control peptide, and analyzed.

Evaluation of T-cell proliferation and cytokine production. Murine CD11c dendritic cells were used as stimulators of allogeneic T cells isolated from spleens of allogeneic mice using T-cell enrichment columns (R&D Systems, Minneapolis, MN). Cells were mixed at different ratios and incubated in triplicates in U-bottomed 96-well plates for 4 days; 1 μ Ci of [³H]-thymidine (Amersham, Arlington Heights, IL) was added per well 18 hours before cell harvest. [³H]-thymidine incorporation was measured using liquid scintillation counter. In some experiments, splenocytes were cultured for 4 days in the presence of 1 or 5 μ g/mL of ConA, or 0.5 μ g/mL anti-CD3 antibody. Antigen-specific T-cell response was evaluated using MHC class II-restricted hemagglutinin-derived peptide. Lymph node cells were cultured in the presence of 12.5 μ g/mL of control or hemagglutinin-derived peptide; supernatants were collected after 2 days of incubation; and the level of IL-2 and IFN- γ was measured using ELISA (Endogen, Woburn, MA).

ELISPOT assay. ELISPOT assay was done as described previously (28). Briefly, MultiScreen-HA plates (Millipore, Bedford, MA) were precoated with anti-mouse IFN- γ antibody (BD PharMingen) by overnight incubation at 4°C. Two hundred thousand lymph node cells were plated in quadruplicates in each well and cultured for 24 hours at 37°C in the presence of the control (RAHYNIVTF) or specific (SIINFEKL) peptides (10 μ g/mL). Cells were then washed with PBS containing 0.1% Tween, and plates were incubated overnight at 4°C with biotinylated anti-IFN- γ antibody (BD PharMingen). Results were visualized using avidin-alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Sigma-Aldrich, St. Louis, MO). The number of spots was calculated on a CTL analyzer (Cellular Technology Ltd. Analyzers, Cleveland, OH) using ImmunoSpot 2.8 version software (Cellular Technology). Results are presented as number of spots per 1×10^6 cells.

Colony formation assay. Bone marrow cells isolated from control or JSI-124 treated mice were plated in triplicates in a six-well plate at density 2×10^4 cells per well in semisolid 1% methylcellulose medium supplemented with recombinant cytokines supporting the optimal growth of myeloid and erythroid colony-forming units (MethoCult GF M3434, Stem Cell Technologies, Vancouver, Canada). The number of colonies was evaluated and counted under microscope on day 10.

Statistics. Statistical analysis was done using JMP software (SAS Institute, Cary, NC).

Results

Inhibition of Janus-activated kinase 2/signal transducers and activators of transcription 3 pathway improves differentiation of dendritic cells from immature myeloid cells. Previous studies have determined that JSI-124 inhibits STAT3 activation in

tumor cells (25). Here, we have investigated whether this compound exerts similar effects on STAT3 activation in immature myeloid cells and dendritic cells. CD11c⁺ dendritic cells were generated from bone marrow precursors in the presence of conditioned medium from CT-26 tumor cells and Gr-1⁺ immature myeloid cells were isolated from spleens of CT26 tumor-bearing mice. Cells were treated for 24 hours with different concentrations of JSI-124 in the presence of CT26 tumor cell conditioned medium and GM-CSF. JSI-124 (0.5 μmol/L) significantly reduced the level of phospho-STAT3 without affecting the level of total STAT3 in both immature myeloid cells and dendritic cells (Fig. 1A). JSI-124 did not affect the level of phospho-STAT1, phospho-STAT6, and phospho-STAT5 (Fig. 1A). Phospho-STAT6 was not detectable in immature myeloid cells. More than 85% of cells were viable at that concentration (data not shown).

Accumulation of immature myeloid cells and their inability to differentiate into mature myeloid cells is one of the hallmarks of hematologic and immunologic abnormalities in tumor-bearing mice. We asked whether the inhibition of STAT3 signaling might affect differentiation of these cells. Gr-1⁺ immature myeloid cells were isolated from spleens of CT26 tumor-bearing mice and were cultured for 7 days with GM-CSF in the presence of CT26 tumor cell conditioned medium. JSI-124 or DMSO were added on day 0 and cell phenotype was evaluated by flow cytometry. Immature

myeloid cells obtained from tumor-bearing mice retained immature phenotype (Gr-1⁺CD11b⁺) in the presence of TDF (Fig. 1B). The total number of cells collected after a 7-day treatment with JSI-124 was reduced by 50%, comparing with the number of cells treated with DMSO (data not shown). JSI-124 had a dramatic effect on the cell phenotype. The proportion of Gr-1⁺CD11b⁺ immature myeloid cells decreased almost 5-fold, whereas the proportion of dendritic cells increased >5-fold (Fig. 1B).

To verify these findings *in vivo*, we used a previously described experimental system with adoptive transfer of Gr-1⁺ immature myeloid cells into tumor-bearing congenic hosts (29). Gr-1⁺ immature myeloid cells (4 × 10⁶ cells) isolated from spleens of C3 tumor-bearing C57BL/6 (CD45.2⁺) mice were transferred *i.v.* into congenic C3 tumor-bearing B6.SJL-PtcrcaPep3b/BoyJ (CD45.1⁺) mice. Treatment with JSI-124 (1 mg/kg/d *i.p.* for 3 days) or DMSO began 3 hours after the transfer. On day 4, mice were sacrificed and phenotype of donor's CD45.2⁺ splenocytes was evaluated by flow cytometry. In mice treated with DMSO, donor's cells (CD45.2⁺) represented 3.4 ± 0.6% of the nucleated cells in spleens, whereas in mice treated with JSI-124 donor's cells represented 1.8 ± 0.5% (*P* < 0.05). In control mice, the vast majority of donor's cells remained Gr-1⁺CD11b⁺ immature myeloid cells. However, in mice treated with JSI-124, the proportion of these cells not only decreased >3-fold, but instead, a significant increase in the proportion of

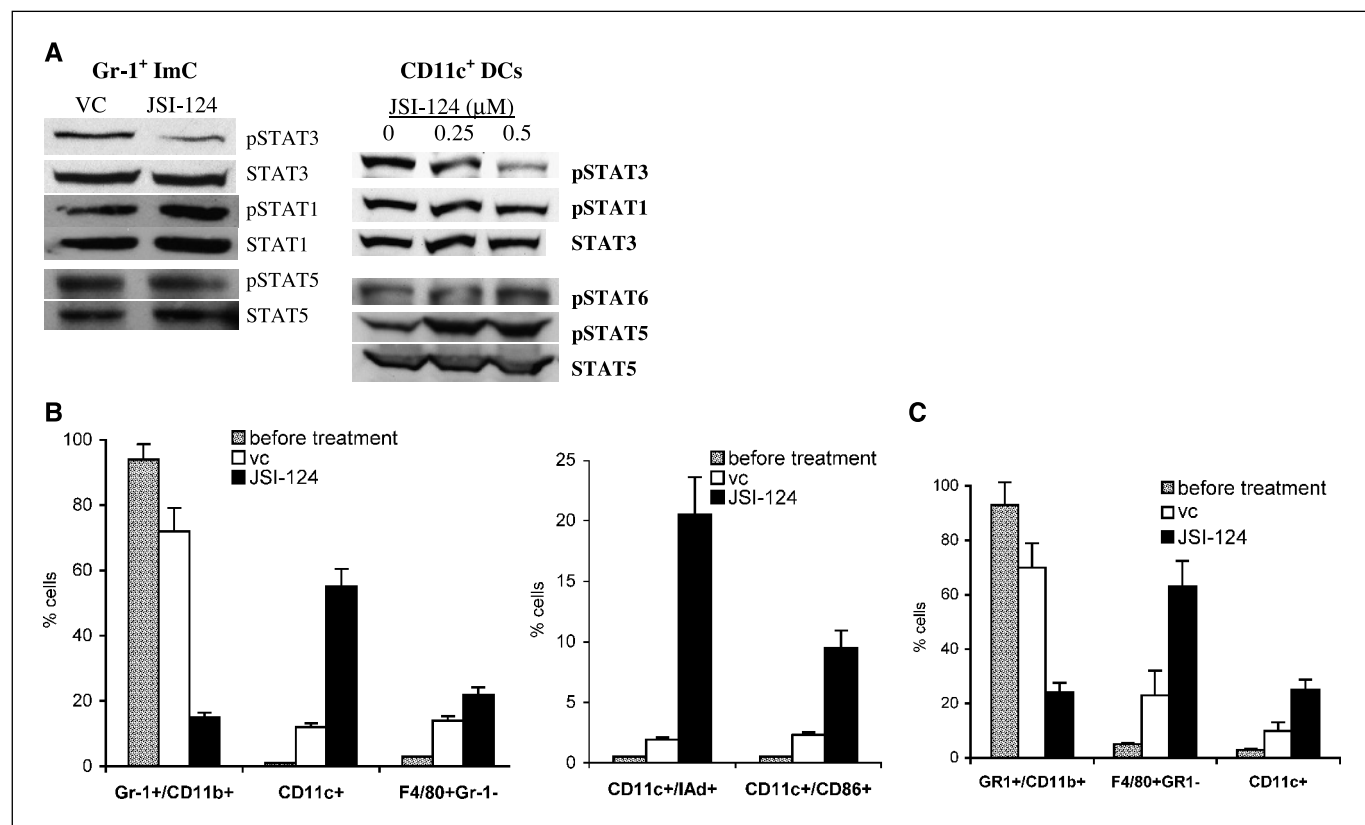


Figure 1. JSI-124 effects on immature myeloid cells (*ImC*) and dendritic cell (*DC*) differentiation *in vitro*. **A**, Gr-1⁺ immature myeloid cells were isolated from spleens of CT26 tumor-bearing mice. Dendritic cells were generated from bone marrow progenitors using GM-CSF and IL-4 in the presence of CT26 conditioned medium as described in details previously (24), and CD11c⁺ dendritic cells were isolated using magnetic beads. Dendritic cells and immature myeloid cells were treated with 0.5 μmol/L JSI-124 or vehicle control (DMSO, vc) for 24 hours in the presence of CT26 conditioned medium. Cells were then collected and Western blotting with antibodies against different members of STAT family was done. **B**, Gr-1⁺ immature myeloid cells were isolated from spleens of tumor-bearing mice, then treated with 0.5 μmol/L JSI-124 or vehicle control and cultured for 7 days in the presence of GM-CSF and CT26 conditioned medium. Cell phenotype was evaluated by flow cytometry. Cumulative results from three experiments. **C**, four million Gr-1⁺ immature myeloid cells were isolated from spleens of C3 tumor-bearing C57BL/6 (CD45.2⁺) mice and transferred into congenic C3 tumor-bearing B6.SJL-PtcrcaPep3b/BoyJ (CD45.1⁺) mice (tumor size, 1 cm in diameter). Mice were then treated with JSI-124 or DMSO for 3 days. On day 4, mice were sacrificed and phenotype of donor's CD45.2⁺ splenocytes was evaluated by flow cytometry. Each group included four mice.

F4/80⁺Gr-1⁻ macrophages and CD11c⁺ dendritic cells was observed (Fig. 1C). These data were similar to that obtained during differentiation of immature myeloid cells *in vitro* and suggested that inhibition of JAK2/STAT3 signaling allowed immature myeloid cells to differentiate towards dendritic cells.

Effect of JSI-124 on immature myeloid cell and dendritic cell differentiation *in vivo*. We investigated the effect of JAK2/STAT3 inhibitor *in vivo* in tumor-free naive mice. JSI-124 was delivered via daily i.p. injections at dose 1 mg/kg/d. This dose was selected after preliminary experiments and was similar to the dose used in our previous study of antitumor activity of this compound (25). Mice were sacrificed 15 to 16 days after start of the treatment; spleens and lymph nodes were collected and the presence of different cell populations was evaluated using multicolor flow cytometry. Effect of JSI-124 on splenocytes of naive tumor-free mice was rather modest. Only a slight increase in the number of CD11c⁺ and CD11c⁺IA^{d+} dendritic cells was observed. No statistically significant differences were seen in any tested populations of dendritic cells, macrophages (F4/80⁺ and CD11c⁻CD11b⁺ cells), immature myeloid cells (Gr-1⁺CD11b⁺), or granulocytes (Gr-1⁺CD11b⁻; Fig. 2A; data not shown). In contrast, a significant increase in the number of dendritic cells and macrophages was observed in lymph nodes of these mice (Fig. 2B).

Next, we studied the effect of JSI-124 in tumor-bearing mice. Several tumor models were selected. 3T3 fibroblasts transformed with v-src have high level of STAT3 activity; CT-26 colon adenocarcinoma has moderate level of STAT3 activity; C3 and MethA sarcomas do not have hyperactivated STAT3 (23, 30). Tumors were established s.c. and treatment with JSI-124 (1 mg/kg/d i.p.) was started when tumor size reached 30 to 100 mm². JSI-124 had different effect in four tested models. Tumor growth in 3T3-v-src tumor-bearing mice was completely blocked and by day 11, tumor size in JSI-124-treated mice was >4-fold lower than in DMSO-treated mice (Fig. 2C). Effect of JSI-124 on CT-26 tumor-bearing mice was less pronounced. However, tumor growth was also significantly inhibited. No effect of the compound was evident in C3 and MethA sarcoma-bearing mice (Fig. 2C). These results are consistent with our previous data (25) and the mechanism of JSI-124 action on tumor cells. If tumor cells have hyperactivated STAT3 and their growth is dependent on STAT3 activity (3T3-v-src or CT-26), then JAK2/STAT3 inhibition blocks or slows tumor progression. If tumor cells do not have STAT3 hyperactivation, then JSI-124 did not affect tumor growth.

The question remains how JAK2/STAT3 inhibition may affect antitumor immune response. It is well known that tumor burden has direct effect on immune system (31, 32). Therefore, interpretation of the effect of JSI-124 on the function of dendritic cell and T cells will be very difficult in the models where this compound significantly reduced tumor burden. We focused on MethA sarcoma model where JSI-124 did not affect tumor growth. Mice were sacrificed 1 to 2 days after finish of the treatment; spleens and lymph nodes were collected; and the presence of different cell populations was evaluated using multicolor flow cytometry. Spleens of tumor-bearing mice had a large number of Gr-1⁺CD11b⁺ immature myeloid cells (7×10^7 compared with 5×10^6 in tumor-free mice). JSI-124 dramatically reduced the absolute number of these cells in spleens (Fig. 2D). In lymph nodes, the presence of immature myeloid cells was only moderately increased and JSI-124 did not affect it. However, the number of CD11c⁺IA^{d+} dendritic cells was significantly increased in JSI-treated mice similar to the results in tumor-free mice (Fig. 2E).

Effect of JSI-124 on the function of dendritic cells and T cells in tumor-bearing mice. Next, we asked whether JSI-124 affected the function of dendritic cells in tumor-bearing mice. CD11c⁺ were isolated from lymph nodes of MethA sarcoma-bearing mice treated with either DMSO or JSI-124 and used as stimulators of allogeneic T cells. Allostimulatory activity of dendritic cells isolated from JSI-124 treated mice was significantly higher than that of dendritic cells isolated from DMSO-treated mice (Fig. 3A). CD11c⁺ dendritic cells isolated from JSI-124-treated mice induced dramatic increase in the proportion of allogeneic CD4⁺ T cells producing IL-2, IFN- γ , and less prominent but nevertheless significant increase in the production of IL-10 (Fig. 3B). This pattern of cytokine production is typical for activated T cells without preferential shift towards Th1 or Th2 cells.

We studied the possibility that inhibition of JAK2/STAT3 pathway may induce apoptosis of T cells. Mice were injected with DMSO or JSI-124; splenocytes were collected 6 hours later and analyzed using Annexin V/7-AAD staining and multicolor flow cytometry. JSI-124 induced significant increase in the level of early apoptosis (Annexin-V⁺/7-AAD⁻ cells) in the population of Gr-1⁺CD11b⁺ immature myeloid cells. The effect of the compound on the populations of CD11c⁺ dendritic cells and CD4⁺ and CD8⁺ T cells was not significant (Fig. 3C). In the next set of experiments, apoptosis was measured 24 hours after the injection. No increase of apoptosis was observed in any of the tested population (data not shown). We also investigated the effect of JSI-124 on T-cell activity in tumor-bearing mice. Splenocytes were isolated from naive tumor-free mice or tumor-bearing mice treated with DMSO or JSI-124. As shown in Fig. 3D, tumor-bearing mice had decreased T-cell response to anti-CD3 stimulation. Treatment of the mice with JSI-124 significantly up-regulated this response. Similar results were obtained after stimulation with ConA (Fig. 3E). Taken together, this data indicate that JSI-124 improves dendritic cell function and overall T-cell response in tumor-bearing mice.

A 2-week treatment with JSI-124 was well tolerated. A complete necropsy was done in mice with careful gross examination of organs and cavities. No significant alterations were found. Microscopic examination failed to reveal abnormalities in any of the animals in the following organs: brain, heart, lung, kidney, adrenal gland, gastrointestinal tract, lymph nodes, thymus, skin, and adipose tissue (data not shown).

JAK2/STAT3 signaling is very important for hematopoiesis. To investigate possible hematologic toxicity of JSI-124, bone marrow cells isolated from control or treated mice were placed in semisolid methylcellulose medium supplemented with cytokines supporting the growth of myeloid and erythroid colonies (MethoCult GF M3434, Stem Cell Technologies). No differences in the number of colonies have been found (Fig. 3F-G).

Effect of Janus-activated kinase 2/signal transducers and activators of transcription 3 inhibition on the development of antigen-specific CD4⁺ and CD8⁺ T-cell responses. Improvement of dendritic cell function by itself was not sufficient to affect tumor growth of MethA sarcoma-bearing mice. It is known that to control tumor growth, the immune system should be stimulated with tumor-specific antigen. MethA sarcoma contains two point mutations in the p53 gene and responds to immunization with wild-type p53 (27, 33). However, one of the main limitations of cancer immunotherapy in a tumor-bearing host is that antitumor effect is short-lived and tumor growth resumed in about a week after termination of the treatment. We hypothesized that a positive effect of JSI-124 could be translated into antitumor activity in combination with cancer vaccine.

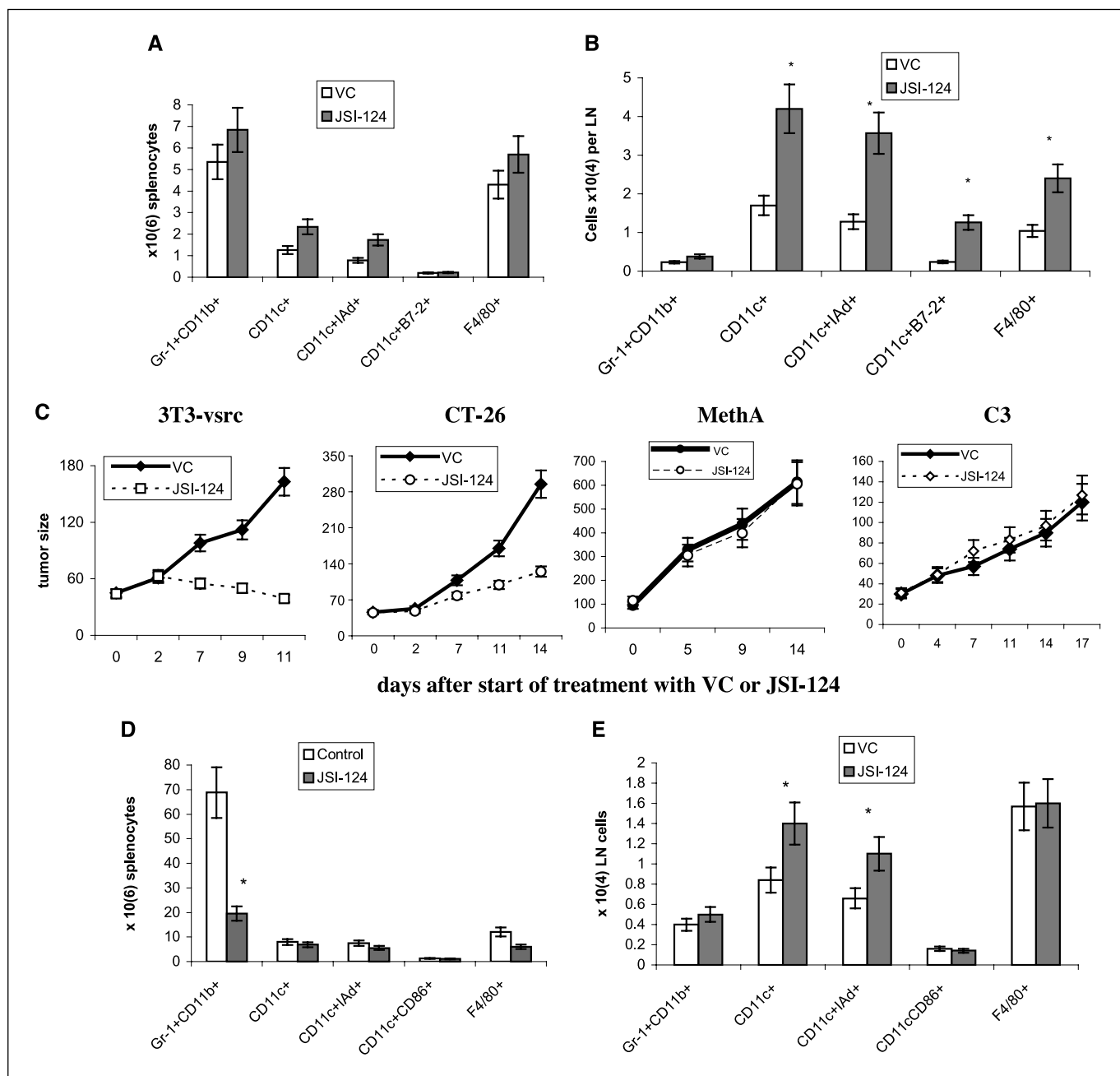


Figure 2. *In vivo* effect of JSI-124 on dendritic cell differentiation. Tumor-free BALB/c mice were treated with JSI-124 (1 mg/kg/d i.p.) for 14 days. Mice were sacrificed on day 15 to 16, and phenotype of cells in spleens (A) and lymph nodes (B) was evaluated using flow cytometry. Absolute number of cells was calculated. Cumulative results of two experiments (three mice per group in each experiment). *, $P < 0.05$, statistically significant differences between control (VC) and treated groups. C, 3T3-v-*Src* cells (2.5×10^5) were implanted s.c. into Swiss mice CT-26 carcinoma was established in BALB/c mice by s.c. inoculation of 5×10^5 cells. MethA sarcoma was established in BALB/c mice by s.c. administration of 5×10^5 tumor cells. C3 tumor was established in C57BL/6 mice by s.c. inoculation of 5×10^5 cells. Injections of JSI-124 (1 mg/kg/d, once a day, i.p.) or VC (DMSO) were started in 7 to 10 days when tumor reached ~ 30 to 100 mm^2 . Tumor size in mice was continuously monitored during treatment. D-E, MethA sarcoma mice (six mice per group) were sacrificed on days 15 to 16, and phenotype of cells in spleens (D) and lymph nodes (E) was evaluated using flow cytometry. Absolute number of cells was calculated. Cumulative results of two experiments (three mice per group in each experiment). *, $P < 0.05$, statistically significant differences between control (VC) and treated groups.

The development of an antitumor immune response requires adequate function of T cells. We asked what effect could the inhibition of JAK2/STAT3 signaling have on T cells during induction of antigen-specific immune response and initial expansion of antigen-specific T cells. It is especially important for combination of this treatment with immunotherapy. To address this question, we used two different experimental systems. Effect of JSI-124 on

induction of antigen-specific CD4⁺ T cells was evaluated using adoptive transfer of transgenic T cells specific of I-A^d-matched hemagglutinin-derived peptide to syngeneic control recipient. Two days after, the transfer mice were immunized with vaccinia-HA. Our previous studies showed that this protocol results in significant accumulation of antigen-specific CD4⁺ T cells and increased production of IL-2 and IFN- γ (28, 34). JSI-124 was

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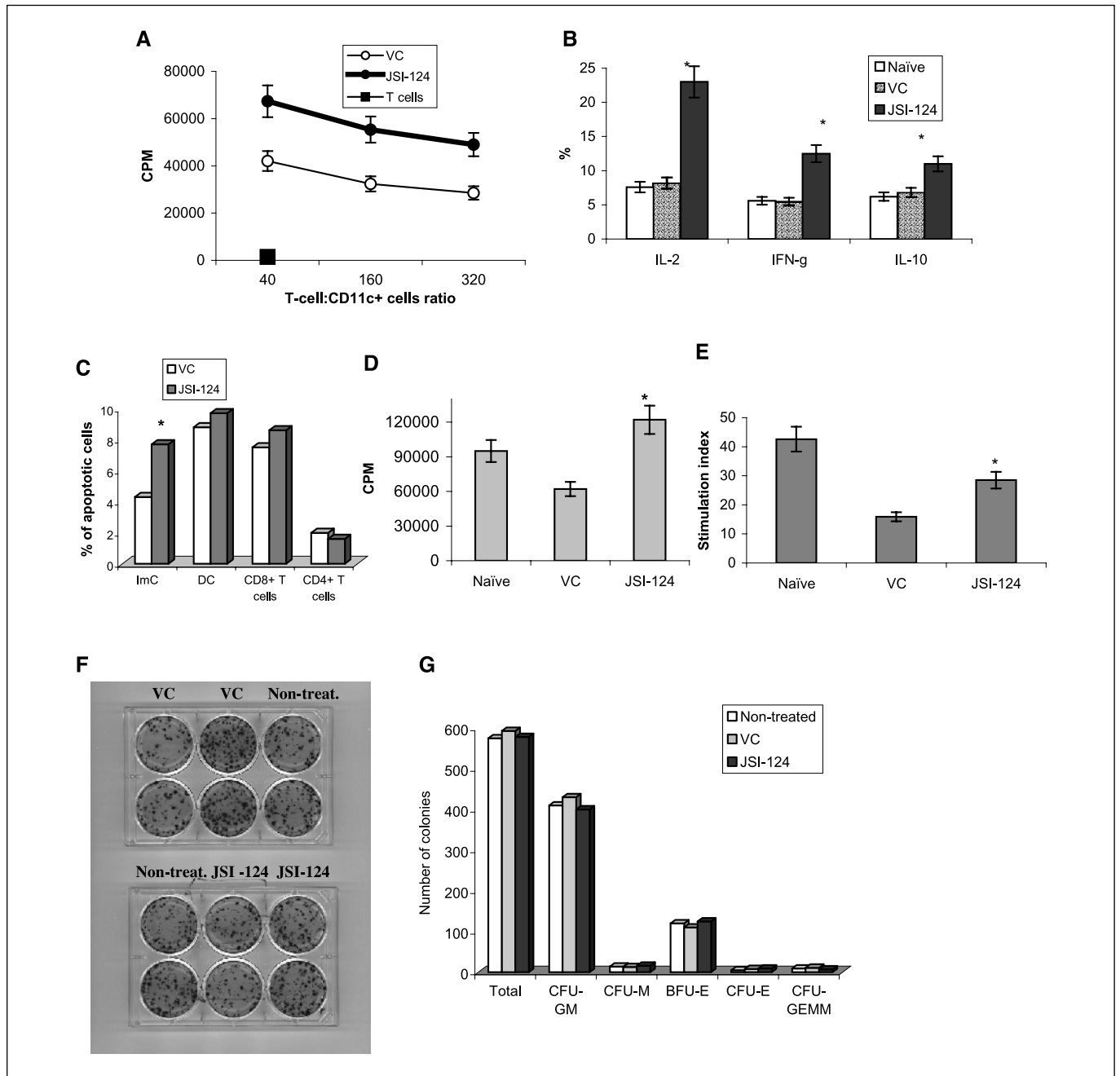


Figure 3. *In vivo* effect of JSI-124 on dendritic cell (DC) function. CD11c⁺ dendritic cells were isolated from lymph nodes (A) or spleens (B) of MethA sarcoma-bearing mice treated with either vehicle control (VC) or JSI-124, irradiated and mixed together with T cells isolated from allogeneic C57BL/6 mice using T-cell enrichment columns (R&D Systems). A, cell proliferation was measured in triplicates after a 4-day incubation using uptake of ³H-thymidine as described in Materials and Methods. Two experiments with similar results were done. B, after a 48-hour incubation of T cells with dendritic cells at 50:1 ratio, cells were fixed, permeabilized, and stained with anti-IL-2, IFN- γ , and IL-10 antibody in combination with anti-CD4 antibody. The proportion of cytokine-producing cells was evaluated within the population of CD4⁺ cells by flow cytometry. Each group included three mice. *, $P < 0.05$, statistically significant differences between control (vehicle control) and treated groups. C, MethA sarcoma-bearing mice were injected with DMSO (vehicle control) or JSI-124, and 6 hours later, splenocytes were collected and stained with Annexin V-PE/7-AAD and antibodies against different cell populations: CD11c-APC and IA^d-FITC for dendritic cells, Gr-1-APC and CD11b-FITC for immature myeloid cells, and CD4-FITC and CD8-APC for T cells. Apoptosis was measured within separate cell populations. Three mice formed each treated group. *, $P < 0.05$, statistically significant differences between control (vehicle control) and treated groups. D-E, splenocytes were isolated from MethA sarcoma-bearing mice treated with DMSO or JSI-124. As a control, splenocytes from naive, tumor-free mice were also used in these experiments. Splenocytes (2×10^5) were stimulated for 4 days with either (D) 0.5 μ g/mL anti-CD3 antibody or (E) 5 μ g/mL ConA, and proliferation was measured in triplicates using uptake of ³H-thymidine. Cumulative results of two experiments. *, $P < 0.05$, statistically significant differences between control (vehicle control) and treated groups. E, stimulation index = cell proliferation in response to ConA / cell proliferation in nonstimulated cells. F-G, bone marrow cells were collected from MethA sarcoma-bearing mice after 14 days of treatment with JSI-124 or DMSO (vehicle control). In parallel, bone marrow cells were collected from nontreated MethA sarcoma-bearing mice. Colony formation assay was done in semisolid medium as described in Materials and Methods. Bone marrow cells (2×10^4) were plated per well. F, for the demonstration of the overall number of colonies, cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye. Please note that only large colonies could be visible. G, unstained colonies were scored using inverted microscope. The numbers of colonies per 2×10^4 bone marrow cells are shown. Experiment was done in duplicates and included two mice per group. Abbreviations: CFU, colony-forming units; M, macrophage; E, erythrocyte; GEMM, mixed granulocyte, erythrocyte, and macrophage; BFU-E, burst-forming units-erythroid.

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injected (1 mg/kg/d i.p.) for four consecutive days. One group of mice was treated on days 2 to 5 after immunization; the second group on days 4-7; and the third group on days 6 to 9 (Fig. 4A). Untreated mice were used as control. All mice were sacrificed on day 10 after immunization; lymph node cells were isolated and restimulated with either control or hemagglutinin-specific MHC

class II-restricted peptide. JSI-124 treatment during days 2 to 5 and 4 to 7 after immunization resulted in a significant decrease in the presence of antigen-specific CD4⁺ T cells and specific antigen-inducible production of IL-2 (Fig. 4B-C). No effect was observed when JSI-124 was injected on days 6 to 9. In contrast, no decrease in IFN- γ production was seen at any time point. Moreover, JSI-124

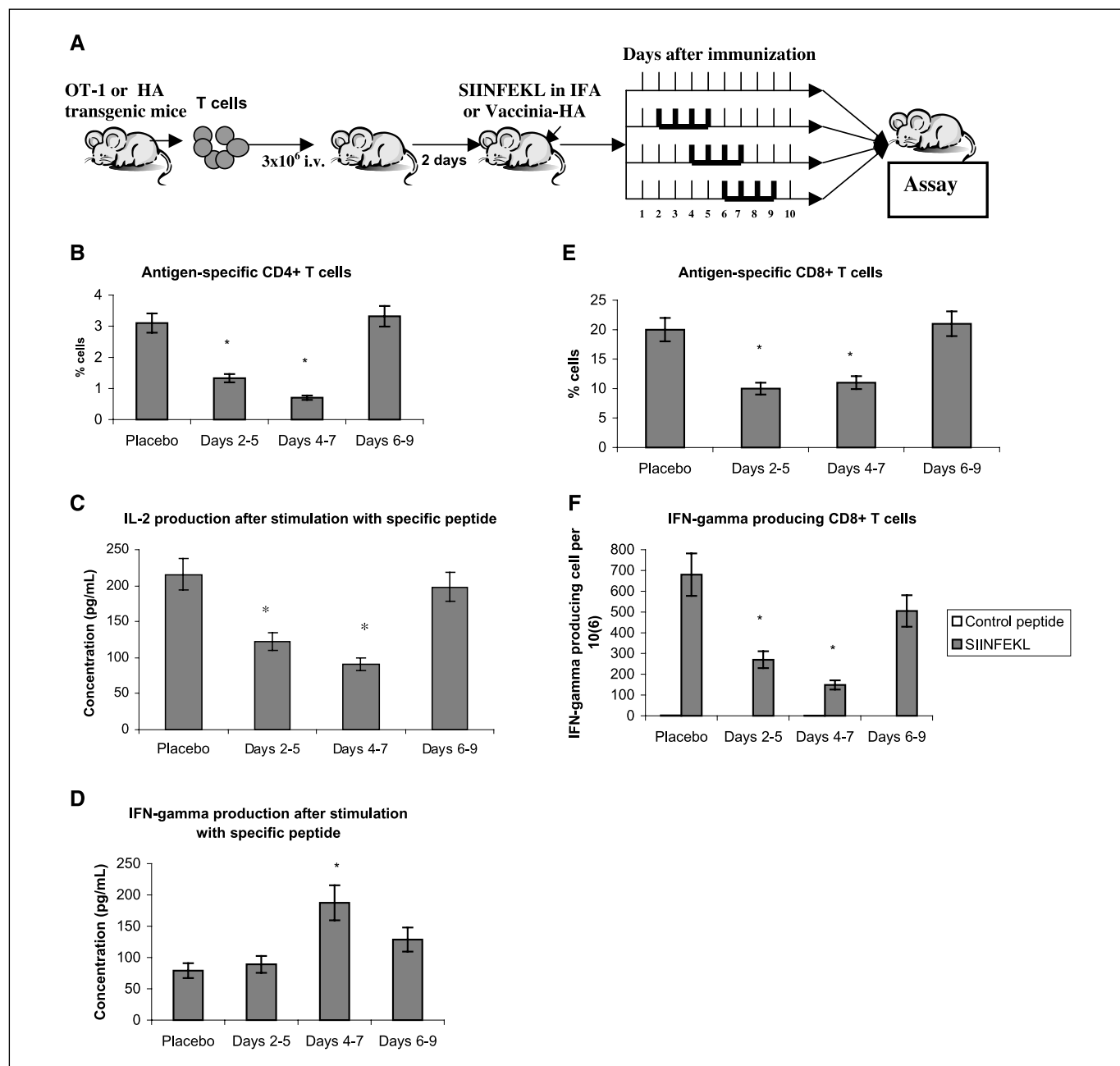
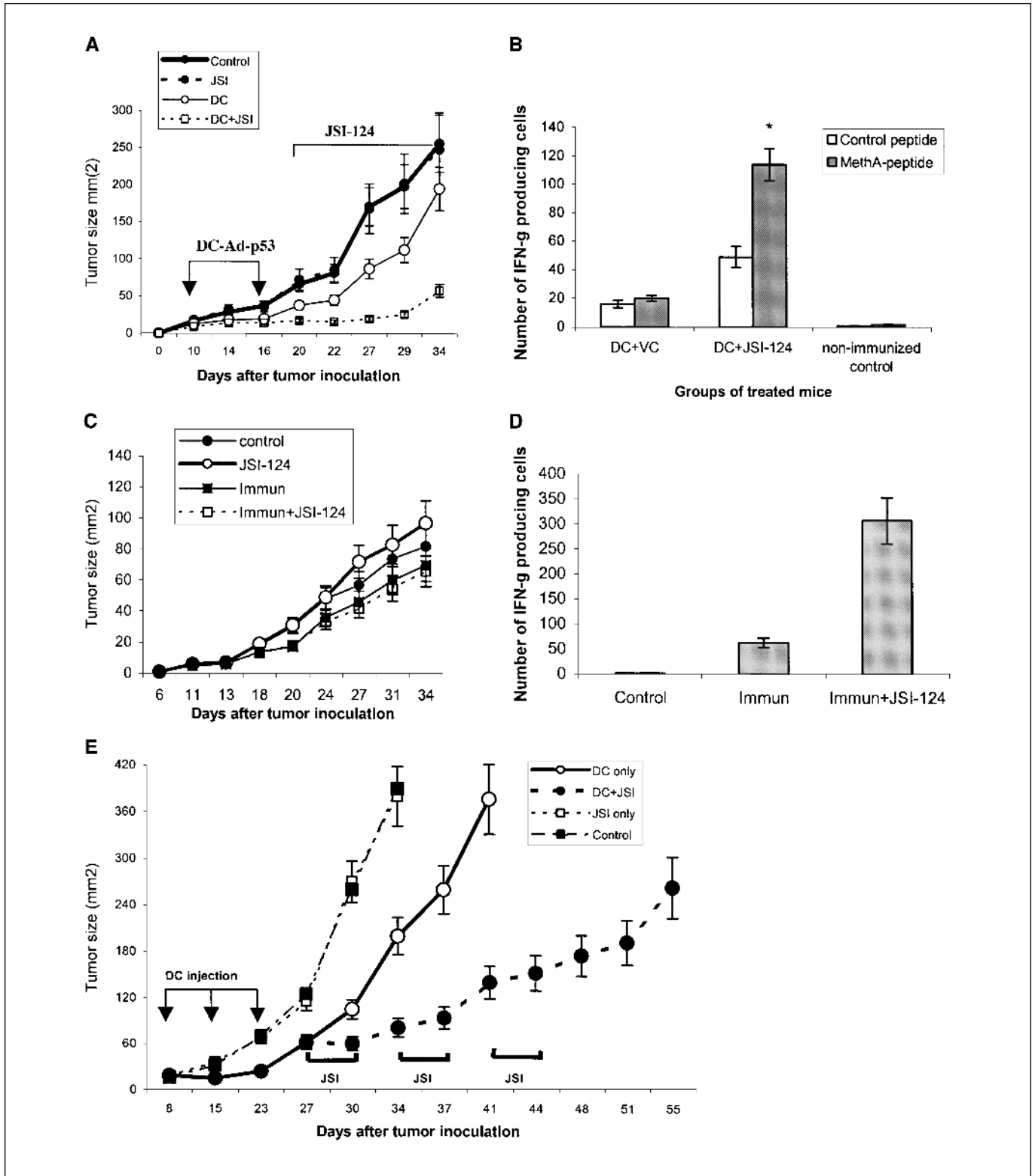


Figure 4. Effect of JSI-124 on induction of antigen-specific T-cell response. *A*, description of experimental model. *B-D*, experimental model with adoptive transfer of hemagglutinin TCR-transgenic T cells specific for MHC class II-restricted hemagglutinin-derived peptide (SFERFEIPKE). *B*, lymph node cells were labeled with APC-conjugated anti-CD4 antibody and FITC-conjugated anti-clonotypic (clone 6.5) antibody. The proportion of clonotypic positive cells within the population of CD4⁺ T cells was calculated. Each group included three mice. *, $P < 0.05$, statistically significant difference from control mice (placebo group). *C-D*, lymph node cells (10⁶/mL) were stimulated with 12.5 μ g/mL of control or specific peptide for 48 hours; supernatants were collected and the levels of IL-2 (*C*) or IFN- γ (*D*) were measured by ELISA. *, $P < 0.05$, statistically significant difference from control mice (placebo group). Only the results of stimulation with the specific peptide are shown. After the stimulation with control peptide, the levels of IL-2 production was <50 pg/mL and levels of IFN- γ was <15 pg/mL. *E-F*, experimental model with adoptive transfer of OT-1 transgenic T cells specific for MHC class I-restricted OVA-derived peptide (SIINFEKL). *E*, splenocytes were labeled with APC-conjugated anti-CD8 antibody and PE-conjugated anti-TCRV α 2 antibody. The proportion of V α 2-positive cells within the population of CD8⁺ T cells was evaluated. Each group included three mice. *, $P < 0.05$, statistically significant difference from control mice (placebo group). *F*, splenocytes were stimulated for 24 hours with specific (SIINFEKL) or control (RAHYNIVTF) peptides, and the number of IFN- γ -producing cells was evaluated in ELISPOT assay as described in Materials and Methods. Each group included three mice. *, $P < 0.05$, statistically significant difference from control mice (placebo group).

increased it when delivered on days 4 to 7 after immunization (Fig. 4D).

In a different model, we tested the effect of JAK2/STAT3 inhibition on CD8⁺ T cells. OT-1 TCR-transgenic T cells specific to H2K^b-matched OVA-derived peptide were adoptively transferred into naive C57BL/6 mice. After 2 days, these mice were immunized

s.c. with this peptide in incomplete Freund's adjuvant. JSI-124 was injected exactly as described above (Fig. 4A). Cells from spleens were collected and restimulated *in vitro* with cognate or irrelevant control peptides. As in the case of CD4⁺ T cells, JSI-124 significantly inhibited presence of antigen-specific CD8⁺ T cells when administered at early time points after immunization. No inhibition was



seen when the compound was administered on days 6 to 9 (Fig. 4E). IFN- γ production by CD8⁺ T cells in response to the specific peptide showed the same pattern (Fig. 4F). Thus, inhibition of JAK2/STAT3 signaling significantly reduced the level of antigen-specific CD4⁺ or CD8⁺ T cells. However, that inhibition occurred only during early phases of T-cell stimulation. Six days after immunization, the negative effect of JSI-124 on T-cell response was not detected, suggesting that this time frame may be used in cancer immunotherapy.

Effect of JSI-124 on the effect of immunotherapy in tumor-bearing mice. To test the hypothesis that JSI-124 can be useful in cancer immunotherapy, we used a MethA sarcoma tumor model. MethA sarcoma was established in BALB/c mice. When tumor became palpable, mice were immunized with dendritic cells transduced with wild-type *p53* gene using the adenoviral construct described earlier (Ad-p53; ref. 27). Immunization was repeated 6 days later. Treatment with JSI-124 (1 mg/kg/d i.p.) or DMSO was started 4 days after the second immunization and continued for 14 days. Immunization with Ad-p53 dendritic cells substantially decreased tumor growth. However, as expected, it resumed soon after second immunization (Fig. 5A). Treatment of immunized mice with JSI-124 dramatically reduced tumor growth. By the end of the fifth week after tumor inoculation, tumor size in mice treated with combination of JSI-124 and DC-Ad-p53 vaccine was >4-fold smaller than in mice treated with the vaccine alone (Fig. 5A). To evaluate tumor-specific immune response in treated mice, lymph nodes were collected at the end of the study, and response of CD8⁺ T cells to MHC class I-bound MethA sarcoma-specific peptide was measured in ELISPOT assay. Only mice treated with the vaccine and JSI-124 showed significant IFN- γ production by CD8⁺ T cells in response to the specific peptide (Fig. 5B). This data indicate that JAK2/STAT3 inhibitor enhances the immunologic and antitumor effects of cancer immunotherapy. Combined treatment resulted in a significant delay in tumor growth. It is still possible that the immune response was observed not because of a direct effect of JSI-124 on immune system but because of lower tumor burden. We addressed this question using different tumor model. During previous studies, we have developed a subline of C3 cells (C3R), which in contrast to original C3 tumor cells, were not recognized by CTLs specific for the H2K^b-restricted peptide RAHYNIVTF. Immunization of C3R tumor-bearing mice with this peptide did not affect tumor growth. This provides us with an opportunity to evaluate the direct effect of JSI-124 on the development and maintenance of immune response. C3R tumor-bearing mice were either immunized thrice with s.c. injection of RAHYNIVTF in incomplete Freund's adjuvant, treated with JSI-124 alone, or with combination of these regimens. As expected, tumor

growth was not affected by any of these treatments (Fig. 5C). Mice were sacrificed 5 weeks after tumor inoculation, and lymph node cells were restimulated with specific or control peptides. Mice treated with combination of JSI-124 and immunization had substantially higher level of CD8⁺T-cell response in IFN- γ ELISPOT assay to the specific peptide than mice treated with immunization alone (Fig. 5D).

To investigate the possibility of prolonged treatment of tumor-bearing mice with JSI-124, we used intermittent schedule of administration. Mice were treated 4 days in the row with 3-day interval. This cycle was repeated twice (total of three cycles). The goal was to decrease potential toxicity, because prolonged (>3 weeks) continuous administration of JSI-124 resulted in the development of ascites. Gross and microscopic examination of the different tissues from these mice showed lack of any abnormalities (data not shown). Mice were immunized with DC-Ad-p53 vaccine thrice followed by administration of JSI-124. JSI-124 significantly improved the effect of immunotherapy (Fig. 5E). By day 34, all mice from "control" and "JSI-124 alone" groups had to be euthanized because of a bulky tumor and mice from immunized group were euthanized 10 days later. However, mice in the group with combined treatment retained relatively small tumor size by day 51 (Fig. 5E). Tumor growth resumed 7 days after the last administration of JSI-124, which probably reflect continues accumulation of immature myeloid cells. Thus, these data indicate that at least in the MethA sarcoma model, the treatment with JSI-124 can markedly augment immunotherapy during relatively long period of time (8 weeks after tumor inoculation).

Discussion

Previous studies have shown a critical role of JAK2/STAT3 hyperactivation in abnormalities observed in dendritic cell differentiation in cancer (23, 24). Signaling from different TDF may converge on STAT3, and hyperactivation of STAT3 results in accumulation of immature myeloid cells and decreased production of mature dendritic cells, which contribute greatly into tumor nonresponsiveness (4). Therefore, inhibition of JAK/STAT3 pathway may be an attractive therapeutic approach to improve the differentiation and function of dendritic cells in cancer. To test this hypothesis, we used JSI-124 a recently discovered selective JAK/STAT3 signaling pathway inhibitor with potent antitumor activity against human tumors in immune deficient as well as immune-competent mouse models (25). JSI-124 (cucurbitacin I) is a member of the cucurbitacin family of compounds that are isolated from various plant families such as the Cucurbitaceae and

Figure 5. Combination of JSI-124 treatment and immunotherapy. *A*, MethA sarcoma was established in BALB/c mice by s.c. inoculation of 5×10^5 cells. When tumors became palpable, mice were split into four groups (five mice per group) with equal size of the tumors. Immunization with DC-Adp-53 and treatment with JSI-124 or DMSO (vehicle control, VC) was done as described in Materials and Methods. Tumor size was monitored constantly during mice treatment. *B*, mice were sacrificed 5 weeks after tumor inoculation. Lymph node cells were isolated from DC-Ad-p53-immunized mice treated with JSI-124 or vehicle control, or from tumor-bearing nonimmunized mice and used in ELISPOT assay. Cells were stimulated with MHC class I-bound control or MethA-specific peptides. Number of spots was measured using CTL reader. *Columns*, average number of INF- γ -producing cells per 1×10^6 cells per group. *, $P < 0.05$, statistically significant difference between cells stimulated with control and MethA-specific peptides. *C*, C3R tumor was established by s.c. inoculation of 5×10^5 cells. Mice were immunized in opposite flank with 100 μ g of RAHYNIVTF peptide in incomplete Freund's adjuvant (*Immun*). In control, mice were injected with incomplete Freund's adjuvant alone. Immunizations were done on days 6, 11, and 14. JSI-124 (Immun + JSI-124, JSI-124) or DMSO (Control, Immun) were administered i.p. from day 18 until day 30. Each group included five mice. *D*, mice described above were sacrificed 34 days after tumor inoculation; lymph node cells were stimulated in quadruplicates with specific (RAHYNIVTF) or irrelevant control peptide (SIINFELK). Both peptides bind H2K^b. The number of IFN- γ -producing cells was evaluated in ELISPOT assay. The number of spots in response to control peptide was subtracted from the number of spots in response to the specific peptide and calculated per 10^6 cells. Each group included three mice. *Columns*, means; *bars*, \pm SE. Abbreviations: Immun., mice immunized with RAHYNIVTF; Immun. + JSI-124, mice immunized with the peptide and treated with JSI-124. *E*, treatment of MethA sarcoma-bearing mice was done essentially as described in (*A*). Mice were treated with DC-control or with DC-Ad-p53 vaccine as indicated. JSI-124 was administered in three cycles, 4 days each with 3-day break. Tumor size was monitored. Each group included six mice. *Points*, mean; *bars*, \pm SE.

Cruciferae. Previous study has shown that JSI-124 inhibits the cellular levels of phosphotyrosine-STAT3 and phospho-JAK2 but not phospho-ERK1/2, phospho-JNK, and phospho-Akt (25). Importantly, although JSI-124 is very effective at suppressing the levels of tyrosine-phosphorylated STAT3 and JAK2, it is unable to directly inhibit Src or JAK2 kinase activities *in vitro*, whereas AG-490 (known inhibitor of JAKs) inhibited kinase activity of both JAK1 and JAK2 (25). JSI-124 could down-regulate phosphotyrosine-STAT3 levels by promoting the protein phosphatase activities of SHP-1 and SHP-2 (35, 36). Alternatively, JSI-124 could also activate physiologic inhibitors that are known to directly or indirectly down-regulate STAT3 activation (37). Current efforts are geared at identifying the actual biochemical target of JSI-124. However, regardless of the precise molecular mechanism of action, it is clear that JSI-124 provides a significant selective inhibition of JAK2/STAT3 pathway in tumor cells.

JSI-124 dramatically reduced the presence of Gr-1⁺CD11b⁺ immature myeloid cells *in vitro* and after adoptive transfer *in vivo*. It seems that inhibition of JAK2/STAT3 pathway has two major effects on immature myeloid cells. The total number of cells was reduced almost by half suggesting that substantial proportion of these cells were killed by this drug. This was directly confirmed by the increased level of apoptosis in immature myeloid cells 2 to 3 days after exposure to JSI-124 (data not shown) and 6 hours after JSI-124 administration *in vivo*. These findings were not surprising, because antiapoptotic effects of STAT3 are well established (37). Furthermore, STAT3 inhibition also promoted differentiation of immature myeloid cells. In our *in vitro* experiments before start of the treatment, the population of immature myeloid cells contained <1% of CD11c⁺ dendritic cells and <2% Gr-1⁻F4/80⁺ macrophages. After the treatment with JSI-124, dendritic cells represented >50% of cells and macrophages represented almost 20% of cells. The absolute number of dendritic cells in culture had increased >30-fold and macrophages increased >10-fold. These dramatic changes could not be explained by simple redistribution of the cells populations caused by loss of cells. These results suggest that inhibition of JAK2/STAT3 signaling in immature myeloid cells promotes differentiation of these cells towards mature myeloid cells.

A recent study from Laouar et al. (38) has shown using conditional knockout mice that STAT3 is necessary for normal dendritic cell differentiation. In other studies, this group reported accumulation of myeloid cells in STAT3-deficient mice (39). We believe that there is no contradiction between our results. In conditional knockout mice, STAT3 was targeted on early stages of myeloid cell differentiation. STAT3 activity in early progenitors is critically important for the development of dendritic cells. In our experiments, STAT3 inhibitor predominantly targeted population of immature myeloid cells, which is represented by a mixed group of myeloid cells primarily in the late stages of myeloid cell differentiation. It is likely that the effect of STAT3 on myeloid cells depends on the stage of cell development. At present, the molecular mechanisms of the effect of STAT3 inhibition on myeloid cell differentiation are under investigation.

One of the main advantages of JSI-124 is that this compound can be potentially used in clinical trials. We tested its activity *in vivo* on several tumor models. As expected, JSI-124 inhibited growth of tumors with hyperactivated STAT3 (v-src-transformed fibrosarcoma, CT26 colon adenocarcinoma) and did not affect the tumor without STAT3 hyperactivation (MethA sarcoma, C3R tumor). It is

known that elimination of tumor (surgical resection) improves dendritic cell differentiation (4), which will obscure (or bring about a very difficult interpretation of) the direct effect of JSI-124 on dendritic cells *in vivo*. Therefore, we only focused on tumor models where JSI-124 did not directly affect tumor growth. JSI-124 induced a dramatic decrease in the presence of immunosuppressive immature myeloid cells in MethA-bearing mice. It was associated with a substantial increase in the presence and functional activity of dendritic cells in lymph nodes. However, simple improvement of dendritic cell function in cancer is not sufficient to reject established tumors. It is evident that inhibition of the JAK2/STAT3 pathway needs to be combined with adequate immunization strategy. JAK2/STAT3 inhibition may cause significant decrease in T-cell proliferation, which may blunt any potential benefit of improvement of dendritic cell function. Our experiments showed that it happens only during early expansion of antigen-specific T cells. It suggested that JSI-124 could be combined with immunotherapy. To address this question, we used our previously developed approach, which uses immunization of MethA sarcoma-bearing mice with dendritic cells transduced with wild-type p53. MethA sarcoma responds to such therapy (27, 40). Importantly, as it is the case with many other tumors, the vaccination results in only temporary decrease in tumor growth. Combination of JSI-124 with immunotherapy dramatically reduced tumor growth. The effect was observed for >4 weeks after last immunization and was associated with tumor antigen-specific CD8⁺ T-cell response. It seems that the level of JAK2/STAT3 inhibition *in vivo* provided by JSI-124 was not sufficient to cause any detectable toxicity in mice, which suggest that it can be potentially used in clinical settings. Our experiments showed that intermittent administration of the compound provides the same if not a better effect than continuous treatment, which suggests another approach for reducing potential toxicity.

In recent years, several other strategies were developed to block IL-10-induced STAT3 signaling, which may also have potential therapeutic implications. Tellurium compound AS101 was shown to inhibit IL-10 in several tumor cell lines, which resulted in dephosphorylation of STAT3. AS101 sensitized tumor cells to chemotherapeutic drugs, resulting in their increased apoptosis (41). Immunosuppressant rapamycin was shown to inhibit of IL-10 secretion by B-cell lymphomas. The reduced IL-10 production was accompanied by corresponding decreases in the constitutive activation of STAT1 and STAT3 (42).

Thus, this has shown that selective inhibition of JAK2/STAT3 pathway with a novel pharmacologic agent, JSI-124, significantly improved dendritic cell differentiation and decreased presence of immunosuppressive immature myeloid cells in tumor-bearing hosts and suggested that pharmacologic inhibition of the JAK2/STAT3 pathway may be potentially useful in cancer immunotherapy.

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