Published in final edited form as:

Pharmacol Res. 2013 May ; 71: . doi:10.1016/j.phrs.2013.02.001.

Bortezomib enhances antigen-specific cytotoxic T cell responses against immune-resistant cancer cells generated by STAT3-ablated dendritic cells

Jee-Eun Kim^{a,b,c}, Dong-Hoon Jin^b, Wang Jae Lee^c, Daeyoung Hur^d, T.-C. Wu^{e,f,g,h}, and Daejin Kim^{d,*}

^aDepartment of Anatomy, Chung-Ang University, College of Medicine, Seoul, Republic of Korea

^bInstitute for Innovate Cancer Research, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

^cDepartment of Anatomy and Tumor Immunity Medical Research Center, Seoul National University, College of Medicine, Seoul, Republic of Korea

^dDepartment of Anatomy, Inje University, College of Medicine, Busan, Republic of Korea

^eDepartment of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD, USA

^fDepartment of Obstetrics and Gynecology, Johns Hopkins Medical Institutions, Baltimore, MD, USA

^gDepartment of Molecular Microbiology and Immunology, Johns Hopkins Medical Institutions, Baltimore, MD, USA

^hDepartment of Oncology, Johns Hopkins Medical Institutions, Baltimore, MD, USA

Abstract

Dendritic cell (DC)-based vaccines have received attention as a new therapeutic modality against cancer. However, increased STAT3 activity in the tumor microenvironment makes DCs tolerogenic and suppresses their antitumor activity. In this study, we explored the effects of a combination treatment consisting of a proteasome inhibitor, bortezomib, and an antigen specific STAT3-ablated (STAT3^{-/-}) DC-based vaccine on the control of TC-1(P3) tumors, a p53-degraded immune resistant cancer cells. We found that E7-antigen expressing STAT3^{-/-} DC (E7-DC-1STAT3^{-/-}) vaccination enhanced generation of E7-specific CD8⁺ T cells, but was not enough to control TC-1(P3) cancer cells. Therefore, we investigated whether bortezomib could create a synergistic effect with E7-DC-1STAT3^{-/-} vaccination. We found that apoptosis via down-regulation of STAT3 and NF- κ B and up-regulation of Fas and death receptor 5 (DR5) expression in TC-1(P3) induced by bortezomib was independent of p53 status. We also observed that TC-1(P3) cells pretreated with bortezomib had markedly enhanced anti-tumor effects on E7-specific CD8⁺ T cells through a Fas/DR5-mediated mechanism. In addition, TC-1(P3) tumor-bearing mice treated with bortezomib prior to vaccination with E7-DC-1STAT3^{-/-} demonstrated enhanced generation of E7-specific CD8⁺ T cells and prolonged survival compared to those treated with monotherapy. These results suggest that the anti-tumor effects against a p53-degraded immune resistant variant generated by antigen-expressing STAT3-ablated mature DCs may be enhanced by bortezomib via death receptor-mediated apoptosis.

© 2013 Elsevier Ltd. All rights reserved.

*Corresponding author at: Department of Anatomy, Inje University, College of Medicine, Busanjin-Gu, Busan, Republic of Korea. Tel.: +82 51 890 8675; fax: +82 51 896 6634. untung10@empal.com. .

Conflict of interest The authors declare no financial or commercial conflict of interest.

Keywords

Bortezomib; STAT3; Immune resistance; Cytotoxic T cells; Dendritic cells

1. Introduction

Dendritic cells (DCs) present antigen and prime T-cells and therefore vaccines utilizing DCs should stimulate superior protective and therapeutic immune responses in cancer patients when compared to other vaccination strategies. Furthermore, DC-based vaccines may circumvent tumor-mediated immune suppression [1]. However, it was found that constitutive STAT3 (signal transducer and activator of transcription 3) expression in tumors negatively regulates inflammation, DC maturity, and T cell immunity [2]. Moreover, STAT3 protein is over-expressed in most cancer cells. It promotes tumorigenesis by preventing apoptosis and enhancing cellular proliferation [3,4]. Therefore, blockade of constitutive STAT3 signaling results in inhibition of growth in tumor cells that activated STAT3 *in vitro* and *in vivo* [5,6]. Activated STAT3 can stimulate nuclear factor- κ B (NF- κ B), which inhibits apoptosis of cancer cells [7] and prevents p53-mediated tumor cell apoptosis by binding to the p53 promoter [8]. Nonetheless, the role of STAT3 in cell death in p53-mutated or p53-degraded cancer cells is uncertain.

Bortezomib (formerly PS-341), a proteasome inhibitor, was approved by the FDA as therapy for human multiple myeloma [9]. Proteasome inhibitors have been shown to directly suppress the growth of a variety of cancer cells and are now being investigated in combination with other chemotherapeutic agents [10,11]. Bortezomib also down-regulates STAT3 expression through the p38 MAPK or NF- κ B pathway in cancer cells [12,13]. However, proteasome inhibition has numerous effects on various cellular signaling pathways, so the precise mechanism of antitumor effects mediated by bortezomib may depend on the particular cancer cell type.

TC-1(P3) cells are a highly resistant immune escape variant generated from the TC-1/P0 cell line, which is a mouse model of human papillomavirus (HPV)-associated cervical cancer created by transducing murine lung epithelial cells with the HPV-16 E6 and E7 oncogenes [14]. HPV E6 and E7 proteins degrade p53 tumor suppressor gene and down-regulate Fas expression in TC-1(P3) cells [15]. Decreased Fas expression induces tumor immune escape and results in increased tumor resistance. Several studies show that bortezomib leads to enhancement of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL)-induced apoptosis by up-regulation of Fas and DR5 in cancer cells [16–18]. We initiated this study to determine the direct effect of bortezomib on the expression of STAT3 in TC-1(P3) cells to make them sensitive to the pro-apoptotic activities of FasL and TRAIL on cytotoxic T lymphocytes (CTLs) generated by DCs. We also investigated whether CTL-mediated cytotoxicity against TC-1(P3) cells was enhanced after treatment with bortezomib in combination with vaccination of E7-expressing DCs with down-regulated STAT3 induced by shRNA lentiviral particle instead of by bortezomib. This study suggests that STAT3 down-regulation by bortezomib, in p53-degraded immune resistant variant tumors, may induce apoptosis of cancer cells as well as enhance CTL-mediated killing generated by tumor antigen-expressing DCs with down-regulated STAT3 through Fas and DR5 expression.

2. Materials and methods

2.1. Antibodies, drug, cell line and mice

The proteasome inhibitor, bortezomib, was provided by Janssen Korea. Antibodies (Abs) against CD8, IFN- γ , Fas, DR5 were purchased from BD Pharmingen. Both DR5 siRNA and Fas siRNA were purchased from Santa Cruz Biotechnology. The HPV-16 E7-expressing murine tumor model TC-1, TC-1(P3) and immortalized murine DC cell line, DC-1 have been previously described [14]. All cells were maintained in completed RPMI medium. Recombinant adenoviruses encoding wild-type p53 were purchased from Vector BioLabs (Philadelphia, PA, USA). Female C57BL/6 mice were acquired from the Chung-Ang Laboratory Animal Service (Seoul, Korea). All animal procedures were performed according to approved protocols and were in accordance with recommendations for the proper use and care of laboratory animals of our institution.

2.2. shRNA infection and siRNA transfection

2.2.1. STAT3 shRNA lentiviral particles transduction—TC-1(P3) cells or DC-1 cells were transduced with murine STAT3 (mSTAT3)-shRNA or control shRNA lentiviral particles (Santa Cruz Biotechnology Inc., CA, USA) according to the manufacturer's protocol. Target cells were incubated with a mixture of complete medium with polybrene (5 μ g/ml) and mSTAT3-shRNA or scrambled shRNA lentiviral particles. To select stable clones that express mSTAT3-shRNA, the medium was replaced with puromycin-containing medium every 3–4 days until resistant colonies could be identified.

2.2.2. siRNA transfection—Cells in the exponential phase of growth were plated in 60 mm dishes at 2×10^5 cells/well, grown for 24 h, and then transfected with 1.5 μ g of siRNA using oligofectamine and OPTI-MEMI reduced serum medium (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) following the manufacturer's protocol. The concentrations of siRNA were chosen based on dose–response studies. Transfection efficiency was examined by immunoblotting 24 h after transfection. Control cells were transfected with control siRNA with oligofectamine and serum-reduced medium (mock).

2.3. p53 adenovirus and infection conditions

Cells (2×10^5) were plated on 60 mm dishes. Twenty-four hours after plating, the cells were washed with phosphate-buffered saline (PBS) and transfected with adenovirus-expressing wild-type p53 (Ad-p53) in 2 ml medium without serum for 4 h at 37 °C in 5% CO₂/95% air, with brief agitation every 30 min. Multiplicity of infection (MOI) was based on the original cell number plated in all experiments and was established at 80 plaque-forming units/cell. After 4 h, cells were washed and fresh medium supplemented with fetal bovine serum (FBS) was added to each dish. Infected cells were cultured for 16 h before protein extraction and subsequent studies.

2.4. Recombinant FasL and TRAIL treatment

TC-1(P3) cells in 96-well plates were cultured in media in the presence of bortezomib (25 nM) for 24 h. Cells were then treated with recombinant mouse TRAIL and/or FasL (untagged form, R&D Systems, Abingdon, UK) for 20 h. Cell viability was assessed by the MTT assay.

2.5. T cell-mediated cytotoxicity assay

T cell-mediated cytotoxicity assay was performed using the PKH-26 Red Fluorescent Cell Linker Kit (Sigma, St. Louis, MO, USA) and FITC-conjugated Annexin-V (Ann-FITC) (BD Pharmingen, San Diego, CA, USA), according to the manufacturer's directions. Effector

cells, E7-specific CD8⁺ T cells, were incubated for 3 or 6 h with the PKH26-labeled target cells at effector: target ratio of 5:1. Cells were then stained with Ann-FITC. PKH26-labeled target cells were gated and further analyzed by Ann-FITC for specific lysis by CD8⁺ T-cells.

2.6. Western blot analysis

Equal amounts of total protein (30–50 µg) from each sample were separated by SDS-PAGE and then transferred onto a PolyScreen membrane (New England Nuclear, Boston, USA). Membranes were probed with one of the following primary antibodies: anti-STAT3 (sc-483), anti-NF- κ B (sc-114) and anti-I κ B (sc-847), which were purchased from Santa Cruz Biotechnology (Santa Cruz CA, USA). Anti- α -actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Cell Signaling Technology, Danvers, MA, USA) or HRP-conjugated anti-mouse IgG (Cell Signaling Technology, Danvers, MA, USA), were used as appropriate.

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Bone marrow-derived DCs isolated from C57BL/6 mice were cultured with GM-CSF (20 ng/ml) for 6 days in 24-well plates. Cells were treated with LPS (400 ng/ml) or LPS with bortezomib (25 nM) for 24 h. DCs were isolated using anti-CD11c antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). Total RNA was extracted using Trizol (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) and cDNA was synthesized using AMV RT and oligo(dT)₁₅ and amplified using the following primers: IL-12p40 (sense, 5'-CACCTGCCCAACTGCCGAGG-3'; and antisense, 5'-TAGCTCCCTGGCTCTGCGGG-3')/IL-6 (sense, 5'-ATGCTGGTGACAACCACGGCC-3'; and antisense, 5'-GGCATAACGCACTAGGTTT-GCCGA-3')/IL-10 (sense, 5'-GCAGGACTTTAAGGGTTACT-3'; and antisense, 5'-TTCATGGCCTTGTAAGACACC-3')/TNF- α (sense, 5'-AGCCCCCAGTCTGTATCCTT-3'; and antisense, 5'-CTCCCTTTGCAGAACTCAGG-3')/GAPDH (sense, 5'-GTGGAGTCTACTGGCGTCTT-3'; and antisense, 5'-GCCTGCTTCACCACCTTCTT-3'). PCR products were electrophoresed and analyzed.

2.8. Characterization of immune responses with intracellular cytokine staining and flow cytometry analysis

E7 peptide (aa49–57, RAHYNIVTF), pulsed STAT3^{+/+} (wild type) or STAT3^{-/-} DC-1 cells (1.0×10^6 cells/mouse) were injected into the footpads of each mouse. One week later, the mice were boosted with the same dose and immunization regimens. Pooled splenocytes (7.0×10^6 /mouse) harvested from mice (three per group) were incubated with E7 peptide (1 µg/ml) for the detection of E7-specific CD8⁺ T-cell precursors in the presence of GolgiPlug (BD Pharmingen, San Diego, CA, USA). Stimulated splenocytes were stained using anti-CD8 and anti-IFN- γ antibodies. The numbers of IFN- γ secreting CD8⁺ T-cells were quantified using flow cytometry.

2.9. In vivo tumor treatment and the antibody depletion experiment using TC-1(P3) tumor cells

C57BL/6 mice (five per group) were challenged with subcutaneous injection of TC-1(P3) tumor cells (1×10^5 /mouse) and treated with bortezomib (0.5 mg/kg) twice prior to co-treatment with E7 peptide-pulsed DC-1 cells twice (day 3 and day 10) after tumor challenge. Mice were monitored for evidence of tumor growth by inspection and palpation twice per week. Tumor volumes were measured on day 7 after tumor challenge. To determine the subset of lymphocytes that are important for the antitumor effects, in vivo antibody depletions were started immediately after the last vaccination. Monoclonal Ab (MAb)

GK1.5 was used for CD4 depletion, MAb 53-6.7 was used for CD8 depletion and MAb PK136 was used for NK1.1 depletion. All the antibodies were purchased from eBioscience (San Diego, CA, USA). Flow cytometry analysis revealed that the >95% of the appropriate lymphocyte subsets were depleted while a normal level of the other subsets was maintained. Antibodies (100 µg/mouse) were injected three times every other day after prime and boost vaccination.

2.10. Tumor measurement and conditional survival

Tumors were measured every other day at day 7 post-challenge, and mice with tumor sizes >18 mm in diameter, projected tumor volumes >10% body weight, or >2500 mm³ were euthanized. Tumor volumes were calculated with the following formula: $V = (L \times W \times D)$; V = tumor volume, L = length, W = width, and D = depth.

2.11. Statistical analysis

All data were expressed as mean ± standard deviation (SD) values and were representative of at least two different experiments. Comparisons between individual data points were made using Student's t-test. Null hypotheses of no difference were rejected if p -values were less than .05.

3. Results

3.1. STAT3 down-regulation in DC-1 cells generates more E7-specific CD8⁺ T cells, but does not prolong survival in TC-1(P3) tumor-bearing mice

DCs are antigen-presenting cells (APCs) closely involved in the generation of CTLs and thereby contribute to the production of immune responses against tumors. However, increased STAT3 activity in DCs induced by tumor-derived factors was found to make DCs tolerogenic and suppress their antitumor activity by inhibiting DC maturation [19]. From this result, we first generated stable STAT3-knockdown DC-1 cells, using lentiviral particles containing STAT3-shRNA to clarify the role of STAT3 in DCs generating anti-tumor immune responses. The growth rate after the infection and selection processes did not change significantly compared to that of original DC-1 (data not shown) and the expression of cell surface markers, including co-stimulatory molecules did not change (Fig. 1A and B). Next, we determined the effect of E7-antigen expressing STAT3-ablated DC-1 cells (E7-DC-1STAT3^{-/-}) on the generation of E7-specific CD8⁺ T-cells. Mice vaccinated subcutaneously twice at 1-week intervals with E7-DC-1STAT3^{-/-} generated twice the number of E7-specific CD8⁺ T-cells compared to the mice vaccinated with antigen-expressing wild type DC-1 or DC-1 transfected with control shRNA (Fig. 1C and D). We also performed *in vivo* tumor treatment experiments using TC-1(P3) cells to determine whether the increased numbers of E7-specific CD8⁺ T-cells generated by E7-DC-1STAT3^{-/-} could translate into therapeutic anti-tumor effects. Unfortunately, the mice primed and boosted with E7-DC-1STAT3^{-/-} did not survive longer than mice vaccinated with E7-expressing wild type DC-1 or DC-1 transfected with control shRNA (Fig. 1E and F). These results suggest that antigen-expressing STAT3-inhibited mature DCs should be combined with other treatment measures to eradicate immune resistant TC-1(P3) tumors.

3.2. p53 status affects bortezomib-induced apoptosis in TC-1(P3) cells but is independent of STAT3 expression

We next determined whether the effect of bortezomib on cell viability and STAT3 expression is dependent on p53 status in immune resistant cancer cells. TC-1(P3) cells infected with or without Ad-p53 were incubated with various doses of bortezomib for 24 h, stained with Annexin V-FITC and followed by MTT assay and flow cytometric analysis to

detect cell viability. We observed that p53 status had no effect on STAT3 expression in TC-1(P3) cells after transduction (Fig. 2A). The viability of p53-degraded TC-1(P3) cells was reduced after treatment with more than 25 nM of bortezomib, while the MTT assay showed that the viability of Adp53 infected TC-1(P3) cells significantly decreased after treatment with more than 10 nM of bortezomib. Similarly, the apoptosis of Ad-p53 infected TC-1(P3) cells was more significantly increased compared to that of p53-degraded TC-1(P3) in a dose-dependent manner (Fig. 2B and C). These results suggest that the p53 status of cancer cells might alter the effects of bortezomib on cell viability and apoptosis, but that it does not modify the expression of STAT3.

3.3. Bortezomib increases Fas and DR5 expression and decreases STAT3 and NF- κ B in TC-1(P3) cells

Several previous studies reported that bortezomib induces DR5- and TRAIL-mediated apoptosis in several types of cancer cells [16–18]. Thus, we hypothesized that treatment with a combination of bortezomib and E7-DC-1STAT3^{-/-} vaccination could generate a better therapeutic effect against immune resistant TC-1(P3) cells compared to monotherapy. We first investigated whether the p53 expression might modify the effect of bortezomib on death receptor expression on cancer cells. Bortezomib increased the expression of Fas and DR5 in a dose-dependent manner in both p53-degraded and Ad-p53 infected TC-1(P3) cells (Fig. 3A and Supplemental Fig. 1). We also observed that STAT3 and p-STAT3 were decreased by bortezomib treatment in both p53-degraded and Adp53 infected TC-1(P3) cells. Moreover, expression of NF- κ B, a target of STAT3, decreased and expression of I κ B, an endogenous inhibitor of NF- κ B, increased with bortezomib treatment regardless of p53 status (Fig. 3B). These results showed that bortezomib acted independently of p53 in immune resistant cancer cells. From these results, we chose to use p53-degraded immune resistant variant TC-1(P3) cells, which are less sensitive to bortezomib than Ad-p53 infected TC-1(P3) cells, to examine the effects of combination treatment with bortezomib and E7-DC-1STAT3^{-/-} vaccination. Next, we examined the relationship between the level of STAT3 and the expression of Fas and DR5. We established STAT3-knockdown p53-degraded TC-1(P3) cells using lentiviral particles containing STAT3-shRNA (Fig. 4A). Fas and DR5 expression levels increased significantly in stable STAT3-knockdown TC-1(P3) cells compared to those transfected with control shRNA particles (Fig. 4B). Based on the decrease of STAT3 induced by bortezomib treatment in TC-1(P3) cells, we expected that DCs treated with low doses of bortezomib (25 nM or less) could generate more tumor-specific CD8⁺ T cells through the down-regulation of STAT3. Unfortunately, only high doses (100 nM) of bortezomib decreased the STAT3 expression in DC-1 cells, a model for mature DCs, and also reduced cell viability (Supplemental Fig. 2A and B). RT-PCR was also performed with separated bone marrow-derived dendritic cells (BMDCs) to check whether bortezomib affected the secretion of immune-reactive cytokines. IL-6, TNF- α , IL-10 and IL-12 p40 mRNA levels were found to be slightly down-regulated even after co-treatment with LPS and bortezomib (Supplemental Fig. 2C). Collectively, these results suggest that bortezomib could increase surface expression of both Fas and DR5 by inhibiting NF- κ B and STAT3 expression, not in DCs but in p53-degraded immune resistant cancer cells.

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phrs.2013.02.001>.

3.4. Bortezomib pretreatment of TC-1(P3) cells results in apoptosis through Fas-FasL DR5–TRAIL interactions

Because bortezomib and STAT3 knockdown increase the expression of Fas and DR5, we also examined whether bortezomib enhances FasL and TRAIL-induced apoptosis via

interaction with Fas and DR5, respectively. TC-1(P3) cells were pre-exposed to bortezomib for 24 h to enhance the surface expression of Fas and DR5, and then co-cultured with E7-specific CD8⁺ T cells, which transiently expressed FasL and TRAIL during active conditions, in order to examine apoptosis. We observed that the combination of pre-exposure to bortezomib and E7-specific CD8⁺ T cells (at 5:1 E:T ratio) enhanced the apoptosis rate compared to the death rate of TC-1(P3) cells treated with bortezomib alone or E7-specific CD8⁺ T cells alone after 6 h (Fig. 5A). We observed that bortezomib pretreatment for 24 h prior to the addition of recombinant FasL and TRAIL had a superior ability to induce apoptosis in TC-1(P3) cells compared to single treatment with FasL or TRAIL (Fig. 5B). We confirmed that the increased apoptosis induced by treatment with recombinant FasL, TRAIL and bortezomib was abolished by blocking the expression of Fas and DR5 with siRNA (Supplemental Fig. 3A and B). These results suggest that bortezomib had the potential to generate more effective therapeutic effects against TC-1(P3) cells when combined with immune therapy by enhancing of antigen-specific CD8⁺ T cells via induction of Fas/DR5 expression.

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phrs.2013.02.001>.

3.5. The combination of bortezomib with STAT3 down-regulated DC-1 cells contributes to prolonged survival of TC-1(P3) tumor-bearing mice

The previous results indicate that bortezomib induced apoptosis in immune resistant TC-1(P3) cells through a Fas/DR5-mediated mechanism by inhibiting the expression of STAT3 and that antigen-expressing STAT3-ablated mature DCs enhanced the generation of antigen-specific CD8⁺ T cells. Thus, we further determined whether bortezomib-induced Fas/DR5-dependent apoptosis could be enhanced by E7-DC-1STAT3^{-/-} vaccination against TC-1(P3) tumors. The TC-1(P3) tumor-bearing mice were administered with bortezomib (0.5 mg/kg) intraperitoneally two times every day prior to vaccination with E7-DC-1STAT3^{-/-}. Moreover, to address whether the combinatory effects of bortezomib and E7-DC-1STAT3^{-/-} vaccination were effective in other cell lines, we performed the same experiments using the parental TC-1 cell line. The mice pre-treated with bortezomib followed by vaccination with E7-DC-1STAT3^{-/-} demonstrated slightly enhanced antigen-specific CD8⁺ T-cell immune responses compared to mice vaccinated with E7-DC-1STAT3^{-/-} only in both TC-1 and TC-1(P3)-tumor bearing mice. (Fig. 6A and B, Supplemental Fig. 4A and B). Interestingly, the mice treated with bortezomib prior to vaccination with E7-DC-1STAT3^{-/-} generated the best therapeutic anti-tumor effects and demonstrated prolonged survival compared to mice vaccinated with E7-DC-1STAT3^{-/-} only in *in vivo* tumor treatment experiments (Fig. 6C and D). The selective depletion of CD8⁺ T cells, but not of NK cells or CD4⁺ T cells, by *in vivo* injection of specific mAbs abolished the anti-tumor immune responses generated by vaccination with E7-DC-1STAT3^{-/-} (Fig. 6E). Therefore, our data suggest that pre-exposure to bortezomib could significantly improve the tumor specific immune responses induced by vaccination with E7-DC-1STAT3^{-/-} in tumor-bearing mice.

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phrs.2013.02.001>.

4. Discussion

STAT3 plays an important role in the regulation of cell proliferation, survival and apoptosis. Whereas STAT3 activation in normal cells is transient and tightly controlled, STAT3 is persistently activated in many cancer cell lines and tumors [20]. In particular, activated STAT3 has been shown to protect tumor cells from apoptosis and promote cell proliferation

[3,5,21]. STAT3 is constitutively activated in both tumor cells and tumor-associated immune cells [22,23], whose products, including VEGF and IL-10, inhibit the functional maturation of DCs and other tumor-infiltrating hematopoietic immune cells. Ablation of STAT3 in immune cells elicits T cell- and natural killer cell-dependent anti-tumor immune responses [23]. Treatment of myeloid DCs with tumor-conditioned medium activates STAT3 in DCs and maintains their immature status with low class II MHC, CD40, CD80, CD86 expression [24]. STAT3 down-regulated DCs have no change in proliferation, survival, co-stimulatory molecule production or other phenotypes compared to mature DCs after ablating STAT3 selectively in order to enhance immune responses. However, the enhanced E7-specific CD8⁺ T cells could not be translated into therapeutic effects against immune-resistant variant TC-1(P3) tumor cells derived from TC-1 *in vivo*. Chemo-immunotherapy with bortezomib and CRT/E7 (detox) DNA also generates more potent E7-specific CD8⁺ T cell immune responses and better therapeutic effects against TC-1 tumors in mice compared to monotherapy [25]. However, the possible mechanism of apoptotic death in cancer cells after combinational treatment is not understood.

Inactivation of apoptotic processes and sustained expression of oncogenes has been linked to cancer pathogenesis and resistance to cytotoxic therapies. Activation of pro-survival signal transcription factor STAT3 and alterations of the most common tumor suppressor, p53, have been identified in many cancer cases. Several studies have reported the potential interactions between the dysfunction of p53 and either the expression of STAT3 or activation of NF- κ B [26,27]. However, the effects of mutated or degraded p53 are conditional upon STAT3 level and immunotherapies against cancer cells have not been fully understood. TC-1(P3) cells infected with Ad-p53 became more sensitive to bortezomib and increased their expression of p53 after treatment without modification of STAT3 expression. Previous studies also reported that bortezomib increased p53 expression in cancer [28,29] and cancer cell susceptibility to bortezomib is associated with survivin expression and p53 status [30]. However, there are studies indicating that bortezomib induces apoptosis of tumor cells independent of p53 status [31,32]. Additionally, there is a report that bortezomib inhibits cell survival by decreasing phosphorylated STAT3 in Jeko-1 mantle cell lymphoma cell line [33], but other research has shown that STAT3 is up-regulated to promote cell death in head and neck squamous cell carcinomas after co-treatment with bortezomib and guggulsterone, a naturally occurring compound known to inhibit STAT3 activation [34]. We also determined whether STAT3 expression modulated by bortezomib is dependent on cell lines. We observed that STAT3 and p53 were increased by bortezomib treatment in B16F1 mouse melanoma cells, contrary to our results in TC-1(P3) cells. Bortezomib decreased the expression of p53 and STAT3 in MOSEC (mouse ovarian surface epithelial cancer) cells. The viability of both cancer cells was reduced, while the effect of bortezomib on p53 and STAT3 expression was inconsistent (Supplemental Fig. 5). Based on this study and previous results, it will be important to further investigate the causes of resistance or susceptibility to bortezomib related to p53 status in cancer cells.

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phrs.2013.02.001>.

The DR5/TRAIL apoptotic pathway potently induces rapid apoptosis in tumor cell lines, although it lacks cytotoxicity against normal cells. Furthermore, death receptors can elicit apoptosis in cancer cells independent of their p53 level [35,36]. DR5/TRAIL-mediated apoptosis by bortezomib has been shown in several types of cancer cells [16–18]. Bortezomib combined with TRAIL further enhances apoptosis of human multiple myeloma cells *in vitro* [18]. DR5 expression is also regulated through p53-dependent and p53-independent mechanisms [37,38]. From these reports, we suspect that TRAIL may induce apoptosis in p53 down-regulated tumor cells. However, the mechanism by which

bortezomib induces up-regulation of DR5 in p53-degraded immune resistant TC-1(P3) cells still remains unclear. Our results showed that bortezomib up-regulated expression of Fas and DR5 irrespective of p53 expression through the down-regulation of STAT3 and NF- κ B in immune resistant cancer cells TC-1(P3) tumor cells, although many studies have reported that p53 suppressed STAT3 and NF- κ B after bortezomib treatment [26,27]. We also confirmed that knockdown of STAT3 expression by shRNA enhanced the expression of Fas and DR5 in TC-1(P3) cells. These findings are consistent with those of previous studies reporting that decreased STAT3 induces expression of Fas [39], DR4 and DR5 [40,41]. But to our knowledge, there is no report that bortezomib induces the increase of both Fas and DR5 expression through inhibition of STAT3. These data suggest that STAT3 regulation by bortezomib controls Fas and DR5 expression through a p53-independent mechanism in TC-1(P3) cells. We also observed that TC-1(P3) cells pre-exposed to bortezomib for 24 h before treatment with recombinant FasL or TRAIL were triggered into apoptosis and had reduced viability compared to TC-1(P3) cells not treated with bortezomib or treated with a single recombinant protein. Unfortunately, we could not detect an additive effect of co-treatment with recombinant FasL and TRAIL on apoptosis in bortezomib treated TC-1(P3) cells. It was thought that both Fas and DR5 recruit the adaptor protein FADD, which then binds caspase-8 via a homotypic interaction involving “death effector domains” [42]. Moreover, each Fas-siRNA and DR5-siRNA may affect the other’s expression.

One of the major effects of proteasome inhibition in several cancer cells is the down-regulation of NF- κ B activation, which can induce anti-apoptotic genes. Blocking NF- κ B using a resistant I κ B construct has been shown to enhance the sensitivity of various cancer cells to TRAIL-mediated apoptosis [43,44]. However, NF- κ B activation does not protect all cells from apoptosis [45,46]. Previous reports showed that bortezomib could sensitize a mouse myeloid leukemia to TRAIL-mediated apoptosis independent of NF- κ B [47]. Furthermore, we treated DC-1 cells with bortezomib to reduce the level of STAT3 and increase immune responses in TC-1(P3) cells. Unfortunately, blockade of STAT3 signaling in DCs treated with high doses of bortezomib resulted in dramatically higher induction of DC-1, a model for mature DCs, apoptosis. We also observed that mRNA levels of immune-regulatory cytokines were slightly down-regulated in BMDCs after co-treatment with LPS and bortezomib. STAT3-deficient T cells display a severely impaired proliferative response to IL-6 due to a defect in IL-6-mediated anti-apoptosis [35]. Interestingly, the mice pre-exposed to bortezomib before vaccination with E7-DC-1STAT3^{-/-} showed prolonged survival compared to mice vaccinated with E7-DC-1STAT3^{-/-} only or bortezomib with E7-pulsed wild type DC-1. Therefore, targeted down-regulation or re-balancing of STAT3 in cancer cells and antigen-expressing mature DCs should be explored for induction of anti-tumor immune responses in tumor-bearing hosts.

The results of this study demonstrate that T cell-mediated anti-tumor effects against p53-degraded tumor cells can be augmented by pre-exposure to bortezomib followed by treatment with antigen-expressing STAT3-selectively ablated mature DCs. Using the STAT3-ablated DC vaccination with bortezomib in cancer cells may represent another strategy to achieve synergistic anti-tumor immune responses because DCs are the most potent antigen-presenting cells and mediate effective in vivo and in vitro immune responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Jayne Knoff for her help with the preparation of this manuscript. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (MEST-2010-0022674) and the National Institutes of Health National Cancer Institute through the Cervical Cancer SPORE (P50-CA098252) and RO1 grant (CA114425-06).

References

- [1]. Nefedova Y, Cheng P, Gilkes D, Blaskovich M, Beg AA, Sefti SM, et al. Activation of dendritic cells via inhibition of Jak2/STAT3 signaling. *Journal of Immunology*. 2005; 175:4338–46.
- [2]. Cheng F, Wang HW, Cuenca A, Huang M, Ghansah T, Brayer J, et al. A critical role for STAT3 signaling in immune tolerance. *Immunity*. 2003; 19:425–36. [PubMed: 14499117]
- [3]. Yu H, Jove R. The STATs of cancer-new molecular targets come of age. *Nature Reviews Cancer*. 2004; 4:97–105.
- [4]. Huang S. Regulation of metastases by signal transducer and activator of transcription 3 signaling pathway: clinical implications. *Clinical Cancer Research*. 2007; 13:1362–6. [PubMed: 17332277]
- [5]. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, et al. STAT3 as an oncogene. *Cell*. 1999; 98:295–303. [PubMed: 10458605]
- [6]. Dechow TN, Pedranzini L, Leitch A, Leslie K, Gerald WL, Linkov I, et al. Requirement of matrix metalloproteinase-9 for the transformation of human mammary epithelial cells by STAT3-C. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:10602–7. [PubMed: 15249664]
- [7]. Nadiminty N, Lou W, Lee SO, Lin X, Trump DL, Gao AC. STAT3 activation of NF- κ B p100 processing involves CBP/p300-mediated acetylation. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103:7264–9. [PubMed: 16651533]
- [8]. Niu G, Wright KL, Ma Y, Wright GM, Huang M, Irby R, et al. Role of STAT3 in regulating p53 expression and function. *Molecular and Cellular Biology*. 2005; 25:7432–40. [PubMed: 16107692]
- [9]. Ma MH, Yang HH, Parker K, Manyak S, Friedman JM, Altamirano C, et al. The proteasome inhibitor PS-341 markedly enhances sensitivity of multiple myeloma tumor cells to chemotherapeutic agents. *Clinical Cancer Research*. 2003; 9:1136–44. [PubMed: 12631619]
- [10]. Voorhees PM, Orlowski RZ. The proteasome and proteasome inhibitors in cancer therapy. *Annual Review of Pharmacology and Toxicology*. 2006; 46:189–213.
- [11]. Mateos MV, Hernández JM, Hernández MT, Gutiérrez NC, Palomera L, Fuertes M, et al. Bortezomib plus melphalan and prednisone in elderly untreated patients with multiple myeloma: results of a multicenter phase 1/2 study. *Blood*. 2006; 108:2165–72. [PubMed: 16772605]
- [12]. Wen J, Feng Y, Huang W, Chen H, Liao B, Rice L, et al. Enhanced antimyeloma cytotoxicity by the combination of arsenic trioxide and bortezomib is further potentiated by p38 MAPK inhibition. *Leukemia Research*. 2010; 34(1):85–92. [PubMed: 19608275]
- [13]. Malara N, Focà D, Casadonte F, Sesto MF, Macrina L, Santoro L, et al. Simultaneous inhibition of the constitutively activated nuclear factor κ B and of the interleukin-6 pathways is necessary and sufficient to completely overcome apoptosis resistance of human U266 myeloma cells. *Cell Cycle*. 2008; 7(20):3235–45. [PubMed: 18931595]
- [14]. Lin KY, Lu D, Hung CF, Peng S, Huang L, Jie C, et al. Ectopic expression of vascular cell adhesion molecule-1 as a new mechanism for tumor immune evasion. *Cancer Research*. 2007; 67(4):1832–41. [PubMed: 17308126]
- [15]. Cheng WF, Lee CN, Chang MC, Su YN, Chen CA, Hsieh CY. Antigen-specific CD8⁺ T lymphocytes generated from a DNA vaccine control tumors through the Fas-FasL pathway. *Molecular Therapy*. 2005; 12:960–8. [PubMed: 15979942]
- [16]. Kabore AF, Sun J, Hu X, McCrea K, Johnston JB, Gibson SB. The TRAIL apoptotic pathway mediates proteasome inhibitor induced apoptosis in primary chronic lymphocytic leukemia cells. *Apoptosis*. 2006; 11:1175–93. [PubMed: 16699949]

- [17]. Johnson TR, Stone K, Nikrad M, Yeh T, Zong WX, Thompson CB, et al. The proteasome inhibitor PS-341 overcomes TRAIL resistance in Bax and caspase 9-negative or Bcl-xL overexpressing cells. *Oncogene*. 2003; 22:4953–63. [PubMed: 12902978]
- [18]. Naumann I, Kappler R, von Schweinitz D, Debatin KM, Fulda S. Bortezomib primes neuroblastoma cells for TRAIL-induced apoptosis by linking the death receptor to the mitochondrial pathway. *Clinical Cancer Research*. 2011; 17:3204–18. [PubMed: 21459798]
- [19]. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nature Reviews Immunology*. 2007; 7(1):41–51. (Review).
- [20]. Levy DE, Lee CK. What does STAT3 do? *Journal of Clinical Investigation*. 2002; 109:1143–8. [PubMed: 11994402]
- [21]. Bromberg J. Stat proteins and oncogenesis. *Journal of Clinical Investigation*. 2002; 109:1139–42. [PubMed: 11994401]
- [22]. Wang T, Niu G, Kortylewski M, Burdelya L, Shain K, Zhang S, et al. Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nature Medicine*. 2004; 10:48–54.
- [23]. Kortylewski M, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, et al. Inhibiting STAT3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nature Medicine*. 2005; 11:1314–21.
- [24]. Cheng P, Corzo CA, Luetke N, Yu B, Nagaraj S, Bui MM, et al. Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. *Journal of Experimental Medicine*. 2008; 205(10):2235–49. [PubMed: 18809714]
- [25]. Tseng CW, Monie A, Wu CY, Huang B, Wang MC, Hung CF, et al. Treatment with proteasome inhibitor bortezomib enhances antigen-specific CD8⁺ T-cell-mediated antitumor immunity induced by DNA vaccination. *Journal of Molecular Medicine*. 2008; 86:899–908. [PubMed: 18542898]
- [26]. Orlowski RZ, Baldwin AS Jr. NF-kappaB as a therapeutic target in cancer. *Trends in Molecular Medicine*. 2002; 8:385–9. [PubMed: 12127724]
- [27]. Ryan KM, Ernst MK, Rice NR, Vousden KH. Role of NF-kappaB in p53-mediated programmed cell death. *Nature*. 2000; 404:892–7. [PubMed: 10786798]
- [28]. Yang Y, Ikezoe T, Saito T, Kobayashi M, Koeffler HP, Taguchi H. Proteasome inhibitor PS-341 induces growth arrest and apoptosis of non small cell lung cancer cells via the JNK/c-Jun/AP-1 signaling. *Cancer Science*. 2004; 95:176–80. [PubMed: 14965369]
- [29]. Ling YH, Liebes L, Jiang JD, Holland JF, Elliott PJ, Adams J, et al. Mechanisms of proteasome inhibitor PS-341-induced G(2)-M phase arrest and apoptosis in human non-small cell lung cancer cell lines. *Clinical Cancer Research*. 2003; 9:1145–54. [PubMed: 12631620]
- [30]. Ling X, Calinski D, Chanan-Khan AA, Zhou M, Li F. Cancer cell sensitivity to bortezomib is associated with survivin expression and p53 status but not cancer cell types. *Journal of Experimental & Clinical Cancer*. 2010; 29:8.
- [31]. Pérez-Galán P, Roué G, Villamor N, Montserrat E, Campo E, Colomer D. The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status. *Blood*. 2006; 107:257–64. [PubMed: 16166592]
- [32]. Strauss SJ, Higginbottom K, Jülicher S, Maharaj L, Allen P, Schenkein D, et al. The proteasome inhibitor bortezomib acts independently of p53 and induces cell death via apoptosis and mitotic catastrophe in B-cell lymphoma cell lines. *Cancer Research*. 2007; 67:2783–90. [PubMed: 17363600]
- [33]. Baran-Marszak F, Boukhari M, Harel S, Laguillier C, Roger C, Gressin R, et al. Constitutive and B-cell receptor-induced activation of STAT3 are important signaling pathways targeted by bortezomib in leukemic mantle cell lymphoma. *Haematologica*. Nov; 2010 95(11):1865–72. [PubMed: 20663948]
- [34]. Li C, Zang Y, Sen M, Leeman-Neill RJ, Man DS, Grandis JR, et al. Bortezomib up-regulates activated signal transducer and activator of transcription-3 and synergizes with inhibitors of signal transducer and activator of transcription-3 to promote head and neck squamous cell carcinoma cell death. *Molecular Cancer Therapeutics*. 2009; 8:2211–20. [PubMed: 19638453]

- [35]. Almasan A, Ashkenazi A. Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. *Cytokine & Growth Factor Reviews*. 2003; 14:337–48. [PubMed: 12787570]
- [36]. Yagita H, Takeda K, Hayakawa Y, Smyth MJ, Okumura K. TRAIL and its receptors as targets for cancer therapy. *Cancer Science*. 2004; 95:777–83. [PubMed: 15504243]
- [37]. Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene*. 2003; 22:8628–33. [PubMed: 14634624]
- [38]. Sheikh MS, Fornace AJ Jr. Death and decoy receptors and p53-mediated apoptosis. *Leukemia*. 2000; 14:1509–13. [PubMed: 10942251]
- [39]. Kunigal S, Lakka SS, Sodadasu PK, Estes N, Rao JS. Stat3-siRNA induces Fas-mediated apoptosis in vitro and in vivo in breast cancer. *International Journal of Oncology*. 2009; 34(5): 1209–20. [PubMed: 19360334]
- [40]. Tanaka F, Kawakami A, Tamai M, Nakamura H, Iwanaga N, Izumi Y, et al. IFN-gamma/JAK/STAT pathway-induced inhibition of DR4 and DR5 expression on endothelial cells is cancelled by cycloheximide-sensitive mechanism: novel finding of cycloheximide-regulating death receptor expression. *International Journal of Molecular Medicine*. 2005; 15(5):833–9. [PubMed: 15806306]
- [41]. Chen KF, Chen HL, Liu CY, Tai WT, Ichikawa K, Chen PJ, et al. Dovitinib sensitizes hepatocellular carcinoma cells to TRAIL and tigatuzumab, a novel anti-DR5 antibody, through SHP-1-dependent inhibition of STAT3. *Biochemical Pharmacology*. 2012; 83(6):769–77. [PubMed: 22230479]
- [42]. Thorburn A. Death receptor-induced cell killing. *Cellular Signalling*. 2004; 162:139–44. [PubMed: 14636884]
- [43]. Keane MM, Rubinstein Y, Cuello M, Ettenberg SA, Banerjee P, Nau MM, et al. Inhibition of NF-kappaB activity enhances TRAIL mediated apoptosis in breast cancer cell lines. *Breast Cancer Research and Treatment*. 2000; 64:211–9. [PubMed: 11194457]
- [44]. Jeremias I, Kupatt C, Baumann B, Herr I, Wirth T, Debatin KM. Inhibition of nuclear factor kappaB activation attenuates apoptosis resistance in lymphoid cells. *Blood*. 1998; 91:4624–31. [PubMed: 9616159]
- [45]. Pawlowski JE, Nesterov A, Scheinman RI, Johnson TR, Kraft AS. NF-kappa B does not modulate sensitivity of renal carcinoma cells to TNF alpha-related apoptosis-inducing ligand (TRAIL). *Anticancer Research*. 2000; 20:4243–55. [PubMed: 11205254]
- [46]. Hu WH, Johnson H, Shu HB. Tumor necrosis factor related apoptosis-inducing ligand receptors signal NF-kappaB and JNK activation and apoptosis through distinct pathways. *Journal of Biological Chemistry*. 1999; 274:30603–10. [PubMed: 10521444]
- [47]. Sayers TJ, Brooks AD, Koh CY, Ma W, Seki N, Raziuddin A, et al. The proteasome inhibitor PS-341 sensitizes neoplastic cells to TRAIL-mediated apoptosis by reducing levels of c-FLIP. *Blood*. 2003; 102(1):303–10. [PubMed: 12637321]

Fig. 1.

Characterization of E7-specific CD8⁺ T cell response after vaccination with STAT3 down-regulated DC-1 cells. STAT3 down-regulated DC-1 cells were generated by transduction with lentiviral particles containing STAT3-shRNA. STAT3 expression was detected by Western blot (A) and the surface markers of STAT3^{+/+} or STAT3^{-/-} DC-1 cells were analyzed by FACS analysis (B). E7-specific CD8⁺ T-cells were analyzed with intracellular IFN- γ staining and flow cytometry (C). Bar graphs depicting the numbers of E7-specific IFN- γ -secreting CD8⁺ T-cells per 3×10^5 splenocytes (mean $\pm$ SD) (D). TC-1(P3) (1×10^5 /mouse) challenged mice were monitored for tumor growth by inspection and palpation after vaccination with E7-DC-1STAT3^{-/-}. Line graphs depict tumor volumes in different treatment categories (E). Kaplan-Meier survival analysis in tumor treatment categories (F). Data are from one of two representative experiments.

Fig. 2.

The effects of p53 status on cell viability in both TC-1(P3) cells and Ad-p53 infected TC-1(P3) cells. The level of p53 protein was analyzed by Western blot in TC-1(P3) cells after transduction with Ad-p53. (A). MTT assay (B) and Annexin V staining (C) were performed to detect cell viability and rate of apoptosis after bortezomib treatment for 24 h in TC-1(P3) and Ad-p53 infected TC-1(P3) cells.

Fig. 3.

The effects of bortezomib on Fas, TRAIL and STAT3-related protein expression in TC-1(P3) cells. Expressional change of Fas and DR5 (A) on TC-1(P3) cells after treatment with each dose of bortezomib. Mean fluorescence intensity (MFI) is indicated below each histogram. STAT3-related proteins in TC-1(P3) and Ad-p53 infected TC-1(P3) cells after treatment with each dose of bortezomib were analyzed by immunoblot (C). Data are from one of two representative experiments.

Fig. 4. The effect of silencing STAT3 on the expression of Fas and DR5 in TC-1(P3) cells. (A) TC-1(P3) cells were transfected with lentiviral particles containing STAT3-shRNA or a control vector containing scrambled-shRNA and the expression of STAT3 was analyzed by Western blot. (B) The expression of the Fas and DR5 in TC-1(P3) cells after transfection with lentiviral particles containing STAT3-shRNA or a control vector were analyzed by flow cytometric analysis. Data are from one of two representative experiments.

Fig. 5.

The effect of bortezomib on viability of STAT3^{-/-} TC-1(P3) cells after treatment with FasL and TRAIL. (A) Bortezomib (25 nM) pre-treated PKH positive TC-1(P3) cells were co-cultured with E7-specific CD8⁺ T cells for 6 h and labeled with FITC-Annexin V followed by flow cytometric analysis to detect cell death. Mean fluorescence intensity (MFI) is indicated below histogram. (B) The viability of bortezomib (25 nM) pre-treated TC-1(P3) cells after post-treatment with each dose of recombinant Fas L and TRAIL was assessed by MTT assay. Data are from one of two representative experiments.

Fig. 6.

The effect of combination treatment with bortezomib and STAT3-ablated DC-1 vaccination against TC-1(P3) tumors. C57BL/6 mice ($n = 5$ per group) were challenged with TC-1(P3) tumor cells (1×10^5 /mouse) by a subcutaneous injection. Mice were treated with bortezomib (0.5 mg/kg) intraperitoneally prior to co-treatment of bortezomib (0.5 mg/kg) and E7-pulsed STAT3^{+/+} or STAT3^{-/-} DC-1 cells. Mice were injected with the same combination regimen 1 week later. (A) E7-specific CD8 + T-cells were characterized by intracellular IFN- staining and flow cytometry at 1 week after boost vaccination. (B) Bar graphs depicting the numbers of E7-specific IFN- -secreting CD8⁺ T-cells per 3×10^5 splenocytes. Mice were monitored for tumor growth by inspection and palpation. (C) Line graphs depict tumor volumes in different treatment categories. Arrowhead indicates time schedule of vaccination with anti-expressing STAT3 knocked down-DC1. (D) Kaplan–Meier survival analysis in tumor treatment categories. (E) Kaplan–Meier survival analysis in in vivo antibody depletion experiments categories. Antibodies (100 μ g/mouse) for CD4, CD8 or NK depletion were injected three times every other day after prime and boost vaccinations. Data are from one of two representative experiments.