



Overexpression of SOCS3 mediated by adenovirus vector in mouse and human castration-resistant prostate cancer cells increases the sensitivity to NK cells in vitro and in vivo

Yoneda, Tomomi ; Kunimura, Naoto ; Kitagawa, Koichi ; Fukui, Yuka ; Saito, Hiroki ; Narikiyo, Keita ; Ishiko, Motoki ; Otsuki, Naoki ;...

(Citation)

Cancer Gene Therapy, 26:388-399

(Issue Date)

2019-01-04

(Resource Type)

journal article

(Version)

Accepted Manuscript

(URL)

<https://hdl.handle.net/20.500.14094/90007723>



**Overexpression of SOCS3 mediated by adenovirus vector in mouse and human
castration resistant prostate cancer cells increases the sensitivity to NK cells *in vitro* and
in vivo.**

Tomomi Yoneda¹, Naoto Kunimura¹, Koichi Kitagawa¹, Yuka Fukui¹, Hiroki Saito¹, Keita
Narikiyo¹, Motoki Ishiko¹, Naoki Otsuki², Ken-ichi Nibu², Masato Fujisawa³, Satoshi
Serada⁴, Tetsuji Naka⁴, Toshiro Shirakawa^{1,3}

1. Division of Advanced Medical Science, Kobe University Graduate School of Science,
Technology and Innovation, Kobe Japan
2. Division of Otolaryngology, Kobe University Graduate School of Medicine, Kobe, Japan
3. Division of Urology, Kobe University Graduate School of Medicine, Kobe, Japan
4. Center for Intractable Immune Disease, Kochi Medical School, Kochi University, Kochi,
Japan

Corresponding author:

Toshiro Shirakawa MD, PhD

Professor

Department of Advanced Medical Science, Kobe University Graduate School of Science,
Technology and Innovation, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe, 650-0017, Japan

Tel: +81-78-382-5681

Fax: +81-78-382-5715

E-mail: toshiro@med.kobe-u.ac.jp

Running title: Ad-SOCS3 increases NK sensitivity of CRPC cells

Abstract

Prostate cancer is one of the most common cancers in men. The over-activation of IL-6/JAK/STAT3 signaling and silencing of SOCS3 are frequently observed in prostate cancer. In the present study we undertook to develop Ad-SOCS3 gene therapy for the treatment of prostate cancer and also investigated whether Ad-SOCS3 increased sensitivity to NK cells. We demonstrated that Ad-SOCS3 could significantly inhibit growth of castration-resistant prostate cancer (CRPC) cell lines expressing pSTAT3, DU-145 (at 10, 20, and 40 MOI) and TRAMP-C2 (at 40 MOI), but not the PC-3 CRPC cell line with the *STAT3* gene deleted. Ad-SOCS3 (40 MOI) could suppress IL-6 production in DU-145 cells and PD-L1 expression induced by IFN- γ in TRAMP-C2 cells, and increased the NK cell sensitivity of both TRAMP-C2 and DU-145 cells. In the DU-145 mouse xenograft tumor model, intratumoral injections (twice/week for 3 weeks) of 1×10^8 pfu of Ad-SOCS3 significantly inhibited tumor growth and combining the Ad-SOCS3 treatment with intratumoral injections (once/week for 2 weeks) of 1×10^7 human NK cells showed the highest tumor growth inhibitory effect. These results suggested that a combination of Ad-SOCS3 gene therapy and NK cell immunotherapy could be a powerful treatment option for advanced CRPC overexpressing pSTAT3.

Key word: JAK/STAT, SOCS3, Ad-SOCS3, CRPC, NK cell

INTRODUCTION

The cytokine-induced Src homology (SH2)-containing protein (CIS)/Suppressor of cytokine signaling (SOCS) family consists of 8 intracellular proteins (CIS and SOCS1-7).¹ Among CIS/SOCS family proteins, SOCS1 and SOCS3 are strictly associated with cytokine signal control including the negative regulation of Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling.² Most JAK/STAT signaling pathways are extracellularly stimulated by cytokines, including interleukin (IL)-6 and interferon (IFN)- α , β , and γ , and intracellularly mediate the cytokine signals to regulate cell proliferation, migration, differentiation, and inflammation.³ While the activation of JAK/STAT signaling is quickly suppressed by negative SOCSs feedback in normal cells,⁴ over-activation of JAK/STAT signaling and silencing of SOCSs, especially SOCS1/3, are frequently observed in variety of cancer cells.^{5,6} The anti-tumor effects of SOCS1/3 targeting the IL-6/JAK/STAT signaling have also been reported elsewhere.^{2,7,8}

Prostate cancer (PCa) is one of the most common cancers in men and the sixth leading cause of cancer-related death worldwide.⁹ Generally metastatic PCa is initially treated with androgen deprivation therapy, after which it often progresses to castration-resistant PCa (CRPC), which is resistant to androgen deprivation therapy.¹⁰ Although several chemotherapeutic agents have been developed to treat CRPC, there is still a great need for development of novel CRPC therapies to improve overall survival (OS) and quality of life (QOL) in CRPC patients. Previously, Iwahori and colleagues reported that overexpression of SOCS3 in malignant pleural mesothelioma (MPM) cells mediated by a recombinant adenovirus vector carrying human *SOCS3* gene (Ad-hSOCS3) reduced proliferation of MPM cells and induced cell apoptosis and partial G0/G1 arrest via JAK/STAT3 and another pathways.² Since the IL-6/JAK/STAT pathway is frequently over-activated in PCa cells,¹¹ we undertook to develop the Ad-SOCS3 therapy for CRPC treatment in the present study.

The biggest strategic obstacle to effective cancer gene therapy using viral vectors is how to induce systemic anti-tumor activity by local intratumoral injections of the viral vectors. Combination therapies with local injections of talimogene laherparepvec, which is a herpes simplex virus vector carrying the *granulocyte-macrophage colony-stimulating factor* (GM-CSF) gene, and systemic immunotherapies including ipilimumab and pembrolizumab are currently being developed.¹² In the phase II study of the talimogene laherparepvec and ipilimumab combination, treatment demonstrated a significantly higher objective response rate (ORR) compared to ipilimumab alone.¹³ It is crucial for the success of viral cancer therapy for local injections of viral vectors to induce systemic anti-tumor immunity.

At this moment, the roles of SOCSs in the tumor immunity are controversial. Recent studies suggest that CIS, SOCS1, and SOCS3 proteins could act as immune checkpoint molecules to regulate the activities of immune cells including T cells and macrophages.¹ In addition, the deletion of SOCS3 in T cells and macrophages reportedly induced anti-tumor effects in MC38 colon cancer and B16F10 melanoma mouse tumor models,^{1,14}. On the other hand, previous studies have shown that the activation of IL-6/JAK/STAT3 signaling suppresses the antigen presentation of dendritic cells (DCs),¹⁵ and the activation of the IFN- γ /JAK/STAT3 signaling pathway increased the expression of programmed death ligand 1 (PD-L1) in tumor cells.¹⁶ Therefore, blocking JAK/STAT3 signaling with SOCS3 might help activate antitumor immunity in the tumor microenvironment. In the present study, we evaluated the anti-tumor activity of Ad-SOCS3 in mouse and human CRPC cells, and also investigated whether the overexpression of SOCS3 in CRPC cells mediated by Ad-SOCS3 could increase sensitivity to natural killer (NK) cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell lines

Human CRPC cell lines DU-145 and PC-3 and murine CRPC cell line TRAMP-C2 were purchased from American Type Culture Collection (ATCC; Manassas, VA). DU-145, PC-3 and TRAMP-C2 cell lines were maintained in minimum essential medium (MEM; Sigma Aldrich, St. Louis, MO), Roswell Park Memorial Institute 1640 medium (RPMI-1640; Sigma Aldrich), and Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich), supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich), 100 U/mL penicillin, and 100 µg/mL streptomycin (P/S; Nacalai Tesque, Kyoto, Japan). All cell lines were grown at 37°C under a humidified atmosphere of 5% CO₂.

Adenoviral vectors

Ad-SOCS3, replication-deficient recombinant adenoviral vectors expressing human or mouse *SOCS3* gene (Ad-hSOCS3 or Ad-mSOCS3), and Ad-LacZ, a replication-deficient recombinant adenoviral vector expressing *β-galactosidase* (*LacZ*) gene, were constructed by the cosmid–adenoviral DNA terminal protein complex method.^{2,7,17} Ad-hSOCS3, Ad-mSOCS3, and Ad-LacZ were designed to express human or mouse *SOCS3* genes and *LacZ* gene, respectively, under the control of the CAG promoter (a modified chicken *β-actin* promoter with a cytomegalovirus immediate early enhancer).^{18,19} The viruses were amplified in 293 cells and purified using CsCl₂ step gradient ultracentrifugation followed by CsCl₂ linear gradient ultracentrifugation. The purified viruses were dialyzed against a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, and 10% glycerol and stored at -80°C.²⁰ Viral particle and biological titers were determined using a standard plaque-forming assay.^{21,22} This study was approved by the Committee for Safe Handling of Living Modified Organisms at Kobe University and carried out according to the committee guidelines.

Western blotting

The CRPC cells were plated in 6-well plates at a density of 5×10^5 cells/well and incubated at 37°C and 5% CO₂ for 24 hours. Then cells were infected with Ad-hSOCS3, Ad-mSOCS3 or Ad-LacZ at 40 multiplicity of infection (MOI) and incubated an additional 48 h. These cells were harvested and washed by phosphate buffered saline (PBS), then lysed in 8 M urea buffer containing 0.1% dithiothreitol, and protein concentration was determined. Equal amounts of each sample were added into sample buffer (Nacalai Tesque) and heated at 95°C for 5 minutes. The samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking with Blocking One (Nacalai Tesque) 1 hour at room temperature (RT), followed by washing, the membranes were incubated overnight at RT with anti-SOCS3 antibody (Immno-Biological Laboratories) 1:1000, anti-pSTAT3 antibody (Cell Signaling Technology, Danvers, MA) 1:1000, anti-STAT3 antibody (Santa Cruz Biotechnology, Dallas, TX) 1:1000, or anti-beta-actin antibody (Santa Cruz Biotechnology), 1:1000. pSTAT3 antibody was diluted with Can Get Signal Immunoreaction Enhancer Solution (TOYOBO, Osaka, Japan), and SOCS3, STAT3, beta-actin antibodies were diluted with PBS-Tween 20%. After another washing, membranes were incubated for 1 hour at RT with HRP conjugated goat anti-mouse IgG or anti-rabbit IgG 1:1000. Antibody binding to proteins was detected by enhanced chemiluminescence.

Cell proliferation assay

The CRPC cells were plated in 96-well plates at a density of 1×10^3 cells/well. After the tumor cells adhered, they were infected with Ad-SOCS3 at 10, 20 and 40 MOI. Six days after infection, the viable cell density was measured by Alamar Blue assay (Invitrogen, Waltham, MA). All experiments were performed in triplicate wells.

Apoptosis analysis by Annexin-V/propidium iodide assay

The CRPC cells were plated in 6-well plates at a density of 5×10^5 cells/well and incubated at 37°C and 5% CO₂ for 24 h. Then cells were infected with Ad-hSOCS3, Ad-mSOCS3 or Ad-LacZ at 20 MOI and incubated an additional 48 h. The cells with apoptosis were stained using an Annexin V-FITC Apoptosis Detection Kit (Nacalai Tesque) according to the manufacturer's instructions, and then fluorescence-activated cell sorting and analysis was performed using Explore Guava easyCyte Benchtop Flow Cytometers (Merck Millipore, Darmstadt, Germany).

Flow cytometry for PD-L1 expression

The expression of PD-L1 on the CRPC cell surface was assessed by flow cytometry analysis. The CRPC cells were plated in 6-well plates at a density of 1×10^6 cells/well and incubated at 37°C and 5% CO₂ for 24 h. Then we added IFN- γ at 0, 5, 10 or 20 ng/mL and the cells were incubated an additional 48 h. The cells were harvested and re-suspended in 80 μ L/tube of PBS (DU-145 and PC-3) or 100 μ L/tube of PBS (TRAMP-C2). Then we added 20 μ L of purified human Fc receptor binding inhibitor (eBioscience, San Diego, CA) (DU-145 and PC-3) or 1 μ L of purified anti-mouse CD16/32 (BioLegend, San Diego, CA) (TRAMP-C2). After 10 min at RT, the CRPC cells were labeled with antibody against phycoerythrin (PE)-conjugated anti-human CD274 (BioLegend) in the dark for 30 min at 4°C. After the cells were washed once with PBS, fluorescence-activated cell sorting and analysis was performed using Explore Guava easyCyte Benchtop Flow Cytometers (Merck Millipore, Darmstadt, Germany). Then the cells expressing PD-L1 were plated in 6-well plates at a density of 1×10^6 cells/well and incubated at 37°C and 5% CO₂ for 24 h. We then added IFN- γ at 10 ng/mL to cells expressing PD-L1 by IFN- γ stimulation, infected them with Ad-hSOCS3, Ad-mSOCS3

or Ad-LacZ at 40 MOI and incubated them an additional 48 h before performing flow cytometry as described above.

Enzyme-linked immunosorbent assay (ELISA)

The CRPC cells were plated in 6-well plates at a density of 5×10^5 cells/well and incubated at 37°C and 5% CO₂ for 24 h. Then cells were infected with Ad-hSOCS3, Ad-mSOCS3 or Ad-LacZ at 40 multiplicity of infection (MOI) and incubated an additional 48 h. The concentrations of IL-6 in the cell culture supernatant were measured using human IL-6 Quantikine ELISA kit for human cells and mouse IL-6 Quantikine ELISA kit for murine cells (R&D Systems, Minneapolis, MN, both) according to the manufacturer's instructions.

Cell cycle assay

The CRPC cells seeded at 1×10^6 cells in 100mm dishes were grown to confluence to attain synchronization in G1 and subcultured at a lower density (1×10^5 cells in a six-well plate) for 24 hr so that most of the cells were in the S phase. Adenoviral vectors were infected by distributing suspensions of Ad-hSOCS3 or Ad-LacZ onto cells at MOI of 40, followed by incubation at 37 °C for an additional 48 hr. Also, human STAT3 siRNA (Dharmacon Inc, Lafayette, USA) or negative siRNA (Dharmacon Inc, Lafayette, USA) was lipofected according to Lipofectamine RNAiMAX Reagent (Invitrogen, Waltham, MA). The cells were then trypsinized and collected with the supernatants, after which the cell cycle was determined by means of propidium iodide (PI) staining according to the instructions for the Guava® Cell Cycle Reagent (Merck Millipore, Darmstadt, Germany) using the Explore Guava easyCyte Benchtop Flow Cytometers (Merck Millipore, Darmstadt, Germany). This assay was performed in duplicate.

NK cells

20 mL of blood was collected from healthy adult donors. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from fresh blood by Ficoll-Paque density gradient centrifugation. The PBMCs were washed one time with PBS-FBS 4%, adjusted to a final concentration of $1-3 \times 10^6$ cells/mL with RPMI1640 with 10% FBS and incubated at 37°C and 5 % CO₂ for 2 h. Then only suspended cells were collected and cultured with KBM501 containing 1,750 JRU/mL of interleukin-2 (IL-2) (KOHJIN BIO, Tokyo, Japan) in a humidified 5% CO₂ incubator at 37°C for 2 days and KBM502 (KOHJIN BIO) in a humidified 5% CO₂ incubator at 37°C for 3 days. The ratio of NK cells (CD3⁻, CD56⁺) in the culture cells was determined by flow cytometry analysis as previously described.²¹ For mice experiment, mouse spleens were obtained from male C57BL/6 mice (CLEA Japan, Tokyo, Japan). Mice were sacrificed and aseptically isolated splenocytes were strained, hemolyzed, and suspended in RPMI-1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1000 IU/mL IL-2. After 10 days of culture, murine NK cells were generated and prepared for following assays.

Cytotoxicity assay

The cytotoxicity of NK cells against target TRAMP-C2 and DU-145 cells with or without 40 MOI of Ad-mSOCS3, Ad-hSOCS3 or Ad-LacZ infection was determined by *in vitro* cytotoxicity assay. In addition, the cytotoxicity of NK cells against DU-145 cells was also examined in the presence of 5 ng/ml of recombinant human IL-6 (Oriental Yeast Co., Ltd., Tokyo, Japan). Target cells (TRAMP-C2 and DU-145) were plated in 96-well plates at a density of 1×10^4 cells/well and incubated at 37°C and 5% CO₂ for 48 h. The supernatants were removed and human or murine NK cells were added to the plate at 1-10 times the target cell numbers respectively, and were co-cultured for 6 h in a humidified 5% CO₂ incubator at

37°C. NK cell cytotoxicity was estimated by measuring the amount of LDH from each cell type using the CytoTox 96 assay (Promega, Madison, WI). LDH measurement was performed in triplicate wells.

***In vivo* experiments**

All animal experiments were conducted according to the institutional ethical guidelines for animal experimentation of the National Institute of Biomedical Innovation (Kobe, Japan). Male Balb/c nu/nu mice, 6 to 7 weeks of age, were purchased from CLEA Japan. mice were housed for 7 days and allowed ad libitum access to food and water. For subcutaneous xenograft experiments, we injected 1×10^6 DU-145 cells in a total volume of 100 μ L of 1/1 (v/v) PBS/Matrigel (Becton Dickinson, Bedford, MA) in the right flank of the Balb/c nu/nu mice. After the tumor adhered, thirty-five mice were randomized into 5 treatment groups (n=7). 1×10^8 plaque-forming units (pfu)/100 μ L of Ad-hSOCS3 or Ad-LacZ was injected intratumorally twice/week for 3 weeks (total of 5 times) and 1×10^7 cells of human NK cells once/week for 2 weeks (total of 2 times). Tumor volumes were determined every third day in a non-blinded manner by measuring in two dimensions, length (L) and width (W), and calculating volume as $(W^2 \times L)/2$.

Immunohistochemical staining

The xenograft tumor tissues were resected from mice after 2 days of the final injection of NK cells. Paraffin embedded tumor tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed in Bond epitope retrieval buffer (Leica Microsystems, Wetzlar, Germany). Immunohistochemical staining was performed in automatic tissue processor (Leica Microsystems Bond-Max) according to manufacturer's standard protocol. Briefly, tissue sections were incubated with rabbit monoclonal anti-human CD56 antibody (Nichirei

Biosciences Inc, Tokyo, Japan) to stain NK cells. After washing, sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA. After incubation, sections were washed and incubated with R.T.U. Vectastain Elite ABC reagent (Vector Laboratories). After washing, sections were incubated with 3,3'-diaminobenzidine, and were counterstained with hematoxylin. Resulting tissue slides were observed under microscope BZ-X710.

Statistical analysis

Statistical significance was determined using a t-test for direct 2-group comparisons and analysis of variance (ANOVA) for multiple-group comparisons. All data were reported as the SD, as index of the variability of the original data points, and as the mean $\pm$ SE of 3 independent experiments if they were repeated. Statistical significance was set at $p<0.05$.

RESULTS

Ad-SOCS3 induced the over-expression of SOCS3 and suppressed the expression of pSTAT3 in TRAMP-C2 and DU-145 cells.

To determine whether overexpression of SOCS3 mediated by Ad-SOCS3 could inhibit the JAK/STAT3 signaling in the CRPC cells *in vitro*, we evaluated the expression of SOCS3, STAT3 and phosphorylated-STAT3 (pSTAT3) proteins in mouse (TRAMP-C2) and human (DU-145 and PC-3) CRPC cells with or without Ad-mSOCS3 or Ad-hSOCS3 infection by Western blotting assay. As shown in Figure 1a, Ad-mSOCS3 or Ad-hSOCS3 could induce the over-expression of SOCS3 in the all three TRAMP-C2, DU-145 and PC3, CRPC cells. The expression of STAT3 was detected in TRAMP-C2 and DU-145 cells and Ad-SOCS3 infections did not decrease the expression of STAT3 in these CRPC cells. On the other hand, the expression of pSTAT3 in TRAMP-C2 and DU-145 cells was suppressed by Ad-SOCS3 infection (Figure 1a). These results suggested that Ad-SOCS3 could regulate the over-activation of JAK/STAT3 signaling in mouse and human CRPC cells.

Ad-SOCS3 inhibited the cell proliferation of TRAMP-C2 and DU-145 cells *in vitro*

We evaluated the *in vitro* inhibitory effects of Ad-mSOCS3 or Ad-hSOCS3 on cell proliferation of TRAMP-C2, DU-145 and PC-3 cells using Alamar blue assay. As shown in Figure1b, Ad-mSOCS3 significantly inhibited TRAMP-C2 cell proliferation at 40 MOI on day 6. Also, Ad-hSOCS3 significantly inhibited DU-145 cell proliferation at 10, 20 and 40 MOIs, but did not show any inhibitory effects on the proliferation of PC-3 cells. These results suggested that Ad-SOCS3 could inhibit cell proliferation of JAK/STAT3 over-activating CRPC cells.

Ad-SOCS3 induced cell apoptosis in TRAMP-C2 and DU-145 cells *in vitro*

To determine whether Ad-SOCS3 could induce cell apoptosis in CRPC cells *in vitro*, we performed an Annexin-V/propidium iodide (PI) assay using flow cytometry. Annexin-V preferentially binds to phosphatidylserine (PS) expressed on the cell surface of apoptotic cells; PI is not taken up by live or early apoptotic cells due to their intact plasma membranes.²³ Therefore, the early apoptotic cells were stained with annexin-V but not with PI (upper left, Figure 2a), and the late apoptotic cells were stained with both annexin-V and PI (upper right, Figure 2a). As shown in Figure 2a and b, Ad-mSOCS3 increased the ratio of late apoptotic TRAMP-C2 cells from 12.1% to 29.2%, and the total apoptotic cells (early and late apoptotic cells) from 33.1% to 63.1%. Also, Ad-hSOCS3 increased the ratio of late apoptotic DU-145 cells from 16.4% to 56.5%, and the total apoptotic cells from 51.2% to 75.4%. Ad-hSOCS3 did not show any substantial change in the numbers of apoptotic PC-3 cells. These results suggested that Ad-SOCS3 could induce cell apoptosis in the JAK/STAT3 over-activating CRPC cells.

Ad-SOCS3 suppressed IL-6 production in DU-145 cells

We performed ELISA assays to determine whether Ad-SOCS3 could suppress IL-6 production in CRPC cells *in vitro*. While TRAMP-C2 did not produce a substantial level of IL-6 protein, DU-145 and PC-3 cells produced a high level of IL-6 protein (Figure 3a). As shown in Figure 3b, Ad-hSOCS3 significantly suppressed IL-6 protein production in DU-145 cells compared with the no-infection or Ad-LacZ infection groups, but not in PC-3 cells. These results suggested that Ad-hSOCS3 could downregulate JAK/STAT3 signaling and also IL-6 production in DU-145 cells overexpressing pSTAT3, but Ad-hSOCS3 could not downregulate IL-6 protein production in PC-3 cells, which did not express pSTAT3.

Ad-SOCS3 induced G0/G1 cell cycle arrest in DU-145 cells

The cell cycle regulation is one of the important mechanisms for inhibition of cell growth. To analyze the effect of Ad-SOCS3 on cell cycle regulation, we infected DU-145 and PC-3 cells with Ad-SOCS3 and then analyzed cell cycle distribution by flow cytometry. In addition, siRNA was introduced to suppress STAT3 expression and cell cycle distribution was determined. Ad-SOCS3 significantly increased the G0/G1 cell population compared with those treated with Ad-LacZ or no virus in DU-145 cells but not in PC-3 cells. Similarly, the human STAT3 siRNA significantly increased the G0/G1 cell population in DU-145 but not in PC-3 cells (Figure 3 c and d).

Ad-SOCS3 suppressed the PD-L1 expression induced by IFN- γ in TRAMP-C2 cells but could not suppress PD-L1 expression in DU-145 cells *in vitro*

To investigate the effect of Ad-SOCS3 infection on the expression of PD-L1 in the CRPC cells overexpressing pSTAT3, we evaluated the expression of PD-L1 with or without IFN- γ stimulation in the Ad-SOCS3 infected or non-infected TRAMP-C2 and DU-145 cells by flow cytometry. As shown in Figure 4a, without IFN- γ , DU-145 cells expressed PD-L1 (77.20%), but TRAMP-C2 cells did not express PD-L1 (only 3.14%). Stimulation with IFN- γ increased the expression of PD-L1 in TRAMP-C2 cells (90.86%) but not in DU-145 cells (77.89%). Ad-mSOCS3 decreased the expression of PD-L1 induced by IFN- γ in TRAMP-C2 cells from 90.86% to 42.26%, but Ad-hSOCS3 could not decrease the expression of PD-L1 in DU-145 cells (Figure 4b). These results indicated that Ad-SOCS3 could suppress PD-L1 expression via the IFN- γ /JAK/STAT pathway, while Ad-SOCS3 could not suppress IFN- γ independent PD-L1 overexpression.

Ad-SOCS3 increased the sensitivity of TRAMP-C2 and DU-145 cells to NK cells *in vitro*

To investigate the effect of Ad-SOCS3 infection on the NK cell sensitivity of CRPC cells *in vitro*, we performed an *in vitro* cytotoxicity assay. Prior to the *in vitro* cytotoxicity assay, we confirmed that 24.7% of the cultured PBMCs with IL-2 had the NK cell phenotype (CD3⁻, CD56⁺). The *in vitro* cytotoxicity assay showed that Ad-mSOCS3 significantly increased the cytotoxicity of murine NK cells (effector cells) against TRAMP-C2 cells (target cells) at effector:target cell ratio 10:1 compared with no-infection group (Figure 5). Also, Ad-hSOCS3 significantly increased the *in vitro* cytotoxicity of human NK cells against DU-145 cells at cell ratios of 5:1 and 10:1 compared with no-infection or Ad-LacZ infection groups (Figure 5). However, in the presence of recombinant IL-6, Ad-hSOCS3 could not increase the cytotoxicity of NK cells against DU-145 cells (Figure 5). These results indicated that Ad-SOCS3 infection could increase sensitivity to NK cells in JAK/STAT3 over-activated CRPC cells by suppressing the PD-L1 expression or IL-6 production.

Combining Ad-SOCS3 and NK cells had synergistic anti-tumor effects in an *in vivo* DU-145 xenograft tumor model

To investigate the synergistic anti-tumor activity of combining Ad-SOCS3 and NK cells *in vivo*, we treated mice xenografted with DU-145 subcutaneous tumors with PBS, Ad-LacZ, NK cells, Ad-hSOCS3, and combination of Ad-hSOCS3 and NK cells. As shown in Figure 6, the combination of Ad-hSOCS3 and NK cells significantly inhibited the growth of DU-145 subcutaneous tumors compared with the PBS, Ad-LacZ, and NK cell treatment groups at day 42. These results indicated that a combination of Ad-SOCS3 and NK cells treatment could markedly inhibit CRPC tumor growth in a mouse xenograft model.

Ad-SOCS3 could prolong the survival of tumor-infiltrating NK cells *in Vivo*

As the result of immunohistochemical study, CD56-positive lymphocytes were remarkably detected in tumor tissues of mice that were treated with Ad-SOCS3 and NK cells after 2 days of the final NK injection but not in tumor tissues of mice treated with NL cells alone (Fig. 7). These results indicated that Ad-SOCS3 treatment could prolong the survival of tumor-infiltrating NK cells in CRPC tumors and enhance the antitumor activity of NK cells.

DISCUSSION

IL-6 is a multifunctional inflammatory cytokine that is frequently overexpressed in several kinds of cancer including prostate cancer.²⁴ The association between IL-6 and prostate cancer was originally discovered by elevated IL-6 serum levels in patients with metastatic CRPC compared with healthy or localized prostate cancer patients.²⁵ Binding of IL-6 to the IL-6 receptor (IL6R) activates JAK/STAT signaling and STAT3 is most commonly phosphorylated and activated in IL-6/JAK/STAT signaling.²⁶ Reportedly, pSTAT3 and IL6R expression was detected in 95% of metastatic CRPC, and the bone metastases showed significantly higher expressions of pSTAT3 and IL6R compared to lymph node and visceral metastases.²⁷ In addition, the most undifferentiated stem-like PC cells expressed the highest levels of IL-6 and IL6R, and blockade of JAK/STAT3 signaling could inhibit colony-forming and tumor initiation by prostate cancer stem-like cells.²⁸ Moreover, IL-6/JAK/STAT3 signaling could induce the overexpression of pituitary tumor transforming gene 1 (PTTG1) and subsequently induce epithelial-mesenchymal transition (EMT) to increase the cancer stem cell population in the LNCaP androgen-dependent prostate cancer cell line, and STAT3 inhibition significantly increased the sensitivity of the DU-145 androgen-independent prostate cancer (CRPC) cell line to the antiandrogen agent bicaltamide.²⁹

In the present study, we employed one mouse CRPC cell line, TRAMP-C2, and two human CRPC cell lines, DU-145 and PC-3, to investigate whether the blockade of IL-

6/JAK/STAT3 signaling by the overexpression of SOCS3 mediated via Ad-SOCS3 could inhibit CRPC cell proliferation and increase the sensitivity of CRPC cells to NK cells. Previously, it was reported that the DU-145 and PC-3 human CRPC androgen-receptor negative cell lines expressed high levels of IL-6,³⁰ while the *STAT3* gene was completely deleted in the PC-3 cell line.³¹ In our results, Ad-SOCS3 successfully induced the overexpression of SOCS3 protein in all three CRPC cells, and suppressed the expression of pSTAT3 protein in TRAMP-C2 and DU-145 cells (Figure 1a). In PC-3 cells, we did not detect pSTAT3 protein expression even without Ad-hSOCS3 infection (Figure 1a). In cell proliferation assays, Ad-SOCS3 significantly inhibited the cell proliferation of TRAMP-C2 and DU-145 cells compared to Ad-LacZ, but not that of the PC-3 cell line (Figure 1b). Also, in a cell apoptosis analysis using annexin-V and propidium iodide, Ad-SOCS3 increased both early and late apoptosis cells in TRAMP-C2 cells and increased late apoptosis and total apoptosis in DU-145 cells, but Ad-SOCS3 could not increase apoptosis in PC-3 cells (Figures 2a and b). Since the *STAT3* gene is deleted in PC-3 cells, Ad-SOCS3 could not inhibit cell growth or induce apoptosis in PC-3 cells. However, PC-3 cells produced the high level of IL-6 in our ELISA assay (Figure 3a) and in other previous reports.^{32,33} IL-6 may stimulate PC-3 cell growth through other signaling pathways such as the extracellular signal-regulated kinase 1 and 2 (ERK1/2)-mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide 3-kinase (PI3-K) pathway.²⁶ In the ELISA assay, both human CRPC cell lines, DU-145 and PC-3, produced a high level of IL-6 protein, but the TRAMP-C2 mouse CRPC cell line did not produce a substantial level of IL-6 protein (Figure 3a). Interestingly, Ad-SOCS3 could suppress IL-6 production in DU-145 cells but not in PC-3 cells (Figure 3b). It seems likely that overexpression of SOCS3 via Ad-SOCS3 infection can interrupt the autocrine loop of IL-6/JAK/STAT3 signaling in DU-145 cells. In addition, we confirmed that both of Ad-SOCS3 and STAT3 siRNA could induce G0/G1 arrest in DU-145 cells but not in

PC-3 cells (Figure 3 c and d). These results suggested that Ad-SOCS3 could induce the G0/G1 cell cycle arrest by the suppression of STAT3 expression.

Programmed death 1 (PD-1) is a member of the B7-CD28 family and an immune inhibitory receptor expressed on many types of immune cells including CD8⁺ and CD4⁺ T cells and NK cells.³⁴ PD-1 signaling directly suppresses the activation of T cells and NK cells, and PD-1 receptor has two corresponding ligands, PD-1 ligand 1 (PD-L1) and PD-1 ligand 2 (PD-L2).³⁵ While PD-L1 is expressed on a variety of cell types including hematopoietic cells, parenchymal cells and tumor cells, PD-L2 is expressed only on professional antigen-presenting cells (APCs), which are dendritic cells and macrophages.³⁶ Recent evidences strongly suggested that the expression of PD-L1 by tumor cells could induce immune escape through PD-1/PD-L1 signaling³⁴. Previously it was reported that IFN- γ , which is an inflammatory cytokine expressed by CD8⁺ T cells, NK cells and macrophages, induces PD-L1 expression and the consequent immune escape of tumor cells.³⁷ In our results, PD-L1 was highly expressed in DU-145 cells but not in TRAMP-C2 cells without IFN- γ stimulation, and IFN- γ could induce PD-L1 expression in TRAMP-C2 cells but not increase PD-L1 expression in DU-145 cells (Figure 4a). Previously Zhang et al reported that blockade of JAK/STAT3 signaling could suppress PD-L1 expression by IFN- γ in A549 human lung cancer cells.³⁸ Similarly, Ad-SOCS3 could suppress only the PD-L1 induced by IFN- γ in TRAMP-C2 cells but not suppress the PD-L1 naturally overexpressed in DU-145 cells (Figure 4b).

In the present study, we investigated whether Ad-SOCS3 could increase the sensitivity of CRPC cells to NK cells. In our *in vitro* cytotoxicity assay, Ad-SOCS3 significantly increased the sensitivity of both TRAMP-C2 and DU-145 cells to NK cells (Figure 5). Especially, Ad-hSOCS3 greatly increased the sensitivity of DU-145 cells to NK cells (Figure 5). Kang et al. demonstrated that IL-6 inhibited the functional activity of NK cells

concomitantly with the downregulation of granzyme B and perforin.³⁹ This finding may support our results that Ad-SOCS3 decreased IL-6 production in DU-145 and increased NK activity against DU-145 cells, but Ad-SOCS3 could not increase NK activity against DU-145 cells in the presence of recombinant IL-6. In addition, Ad-SOCS3 suppressed the PD-L1 expression induced by IFN- γ in TRAMP-C2 cells, and increased NK activity against TRAMP-C2 cells. We also investigated the *in vivo* antitumor activity of Ad-SOCS3 and synergistic effect of Ad-SOCS3 and NK cells in a DU-145 mouse xenograft model. In our model Ad-hSOCS3 significantly inhibited DU-145 tumor growth and the combination of Ad-SOCS3 and NK cells showed the highest antitumor activity (Figure 6). Furthermore, the immunohistochemical study revealed that the combination of NK cell injection and Ad-SOCS3 could prolong the survival of tumor-infiltrating NK cells in tumors infected with Ad-SOCS3, while NK cells in tumors without Ad-SOCS3 infection disappeared at two days after the NK cells injection (Figure 7). Recently, Nakagawa et al. showed adenovirus-mediated SOCS-1 gene delivery (AdSOCS-1) in several human cancer cell lines *in vitro* potently inhibited IFN- γ -induced STAT1 phosphorylation and PD-L1 upregulation. In addition, intratumoral injection of AdSOCS-1 significantly inhibited the tumor growth of those cancer cells in mice, reduced PD-L1 expression on tumors and restored the activation of tumor-infiltrating CD8 T cells, supporting that the inhibition of JAK-STAT signaling pathway attributable to enhancement of tumor immunity involving T cells and NK cells.⁴⁰

In conclusion, we demonstrated that Ad-SOCS3 can inhibit cell growth in CRPC cell lines expressing pSTAT3, DU-145 and TRAMP-C2, but not the PC-3 CRPC cell line with *STAT3* gene deletion. Ad-SOCS3 could suppress IL-6 production in DU-145 cells and PD-L1 expression induced by IFN- γ in TRAMP-C2 cells, and increased the NK cell sensitivity of both DU-145 and TRAMP-C2 cells. Ad-SOCS3 showed *in vivo* antitumor activity and synergistic effects when combined with NK cells in a DU-145 xenograft tumor model. These

results suggested that the combination of Ad-SOCS3 gene therapy and NK cell immunotherapy could be a powerful treatment option for advanced CRPC overexpressing pSTAT3.

ACKNOWLEDGEMENTS

This research was supported by Japan Agency for Medical Research and Development (AMED) under Grant Number JP16ck0106106. The authors wish to acknowledge Mr. Gary Mawyer for his great support for proofreading.

CONFLICT OF INTEREST

All authors have no conflict of interest.

REFERENCES

1. Chikuma S, Kanamori M, Mise-Omata S, Yoshimura A. Suppressors of cytokine signaling: Potential immune checkpoint molecules for cancer immunotherapy. *Cancer Sci.* 2017; 108: 574-580.
2. Iwahori K, Serada S, Fujimoto M, Nomura S, Osaki T, Lee CM, *et al.* Overexpression of SOCS3 exhibits preclinical antitumor activity against malignant pleural mesothelioma. *Int J Cancer.* 2011; 129: 1005-1017.
3. Yu H, Jove R. The STATs of cancer--new molecular targets come of age. *Nat Rev Cancer.* 2004; 4: 97-105.
4. Zhang J, Li H, Yu JP, Wang SE, Ren XB. Role of SOCS1 in tumor progression and therapeutic application. *Int J Cancer.* 2012; 130: 1971-1980.
5. Kim MH, Kim MS, Kim W, Kang MA, Cacalano NA, Kang SB, *et al.* Suppressor of cytokine signaling (SOCS) genes are silenced by DNA hypermethylation and histone deacetylation and regulate response to radiotherapy in cervical cancer cells. *PLoS One.* 2015; 10:e0123133.
6. He B, You L, Xu Z, Mazieres J, Lee AY, Jablons DM. Activity of the suppressor of cytokine signaling-3 promoter in human non-small-cell lung cancer. *Clin Lung Cancer.* 2004; 5: 366-370.
7. Sugase T, Takahashi T, Serada S, Nakatsuka R, Fujimoto M, Ohkawara T, *et al.* Suppressor of cytokine signaling-1 gene therapy induces potent antitumor effect in patient-derived esophageal squamous cell carcinoma xenograft mice. *Int J Cancer.* 2017; 140: 2608-2621.
8. Wen W, Wu J, Liu L, Tian Y, Buettner R, Hsieh MY, *et al.* Synergistic anti-tumor effect of combined inhibition of EGFR and JAK/STAT3 pathways in human ovarian cancer. *Mol Cancer.* 2015; 14: 100.

9. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, *et al.* Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*. 2015; 136: E359-386.
10. Karantanos T, Corn PG, Thompson TC. Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches. *Oncogene*. 2013; 32: 5501-5511.
11. Lou W, Ni Z, Dyer K, Tweardy DJ, Gao AC. Interleukin-6 induces prostate cancer cell growth accompanied by activation of stat3 signaling pathway. *Prostate*. 2000; 42: 239-242.
12. Ribas A, Dummer R, Puzanov I, VanderWalde A, Andtbacka RHI, Michielin O, *et al.* Oncolytic Virotherapy Promotes Intratumoral T Cell Infiltration and Improves Anti-PD-1 Immunotherapy. *Cell*. 2017; 170: 1109-1119.
13. Chesney J, Puzanov I, Collichio F, Singh P, Milhem MM, Glaspy J, *et al.* Randomized, Open-Label Phase II Study Evaluating the Efficacy and Safety of Talimogene Laherparepvec in Combination With Ipilimumab Versus Ipilimumab Alone in Patients With Advanced, Unresectable Melanoma. *J Clin Oncol*. 2018; 36: 1658-1667.
14. Hiwatashi K, Tamiya T, Hasegawa E, Fukaya T, Hashimoto M, Kakoi K, *et al.* Suppression of SOCS3 in macrophages prevents cancer metastasis by modifying macrophage phase and MCP2/CCL8 induction. *Cancer Lett*. 2011; 308: 172-180.
15. Kitamura H, Ohno Y, Toyoshima Y, Ohtake J, Homma S, Kawamura H, *et al.* Interleukin-6/STAT3 signaling as a promising target to improve the efficacy of cancer immunotherapy. *Cancer Sci*. 2017; 108: 1947-1952.
16. Zhang X, Zeng Y, Qu Q, Zhu J, Liu Z, Ning W, *et al.* PD-L1 induced by IFN- γ from tumor-associated macrophages via the JAK/STAT3 and PI3K/AKT signaling pathways promoted progression of lung cancer. *Int J Clin Oncol*. 2017; 22: 1026-1033.

17. Miyake S, Makimura M, Kanegae Y, Harada S, Sato Y, Takamori K, *et al.* Efficient generation of recombinant adenoviruses using adenovirus DNA terminal protein complex and a cosmid bearing the full-length virus genome. *Proc Natl Acad Sci USA*. 1996; 93: 1320-1324.
18. Goto H, Osaki T, Kijima T, Nishino K, Kumagai T, Funakoshi T, *et al.* Gene therapy utilizing the Cre/loxP system selectively suppresses tumor growth of disseminated carcinoembryonic antigen-producing cancer cells. *Int J Cancer*. 2001; 94: 414-419.
19. Nishino K, Osaki T, Kumagai T, Kijima T, Tachibana I, Goto H, *et al.* Adenovirus-mediated gene therapy specific for small cell lung cancer cells using a Myc-Max binding motif. *Int J Cancer*. 2001; 91: 851-856.
20. Kijima T, Osaki T, Nishino K, Kumagai T, Funakoshi T, Goto H, *et al.* Application of the Cre recombinase/loxP system further enhances antitumor effects in cell type-specific gene therapy against carcinoembryonic antigen-producing cancer. *Cancer Res*. 1999; 59: 4906-4911.
21. Saito H, Kitagawa K, Yoneda T, Fukui Y, Fujisawa M, Bautista D, *et al.* Combination of p53-DC vaccine and rAd-p53 gene therapy induced CTLs cytotoxic against p53-deleted human prostate cancer cells in vitro. *Cancer Gene Ther*. 2017; 24: 289-296.
22. Kanegae Y, Makimura M, Saito I. A simple and efficient method for purification of infectious recombinant adenovirus. *Jpn J Med Sci Biol*. 1994; 47: 157-166.
23. Riccardi C, Nicoletti I. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat Protoc*. 2006; 1: 1458-1461.
24. Sansone P, Bromberg J. Targeting the interleukin-6/Jak/stat pathway in human malignancies. *J Clin Oncol*. 2012; 30: 1005-1014.

25. Adler HL, McCurdy MA, Kattan MW, Timme TL, Scardino PT, Thompson TC.
Elevated levels of circulating interleukin-6 and transforming growth factor-beta1 in
patients with metastatic prostatic carcinoma. *J Urol.* 1999; 161: 182-187.
26. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Müller-Newen G, Schaper F.
Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J.*
2003; 374: 1-20.
27. Don-Doncow N, Marginean F, Coleman I, Nelson PS, Ehrnström R, Krzyzanowska A, *et al.*
Expression of STAT3 in Prostate Cancer Metastases. *Eur Urol.* 2017; 71: 313-316.
28. Kroon P, Berry PA, Stower MJ, Rodrigues G, Mann VM, Simms M, Bhasin D, Chettiar
S, Li C, Li PK, Maitland NJ, Collins AT. JAK-STAT blockade inhibits tumor initiation
and clonogenic recovery of prostate cancer stem-like cells. *Cancer Res.* 2013; 73: 5288-
5298.
29. Huang S, Liu Q, Liao Q, Wu Q, Sun B, Yang Z, *et al.* Interleukin-6/signal transducer and
activator of transcription 3 promotes prostate cancer resistance to androgen deprivation
therapy via regulating pituitary tumor transforming gene 1 expression. *Cancer Sci.* 2018;
109: 678-687.
30. Chung TD, Yu JJ, Spiotto MT, Bartkowski M, Simons JW. Characterization of the role
of IL-6 in the progression of prostate cancer. *Prostate.* 1999; 38: 199-207.
31. Clark J, Edwards S, Feber A, Flohr P, John M, Giddings I, *et al.* Genome-wide screening
for complete genetic loss in prostate cancer by comparative hybridization onto cDNA
microarrays. *Oncogene.* 2003; 22: 1247-1252.
32. Keller ET, Chang C, Ershler WB. Inhibition of NFkappaB activity through maintenance
of IkappaBalpha levels contributes to dihydrotestosterone-mediated repression of the
interleukin-6 promoter. *J Biol Chem.* 1996; 271: 26267-26275.

33. Asbagh LA, Uzunoglu S, Cal C. Zoledronic acid effects interleukin-6 expression in hormone-independent prostate cancer cell lines. *Int Braz J Urol.* 2008; 34: 355-363.
34. Zitvogel L, Kroemer G. Targeting PD-1/PD-L1 interactions for cancer immunotherapy. *Oncoimmunology.* 2012; 1: 1223-1225.
35. Beldi-Ferchiou A, Caillat-Zucman S. Control of NK Cell Activation by Immune Checkpoint Molecules. *Int J Mol Sci.* 2017; 18. pii: E2129.
36. Rozali EN, Hato SV, Robinson BW, Lake RA, Lesterhuis WJ. Programmed death ligand 2 in cancer-induced immune suppression. *Clin Dev Immunol.* 2012; 2012: 656340.
37. Mandai M, Hamanishi J, Abiko K, Matsumura N, Baba T, Konishi I. Dual Faces of IFN γ in Cancer Progression: A Role of PD-L1 Induction in the Determination of Pro- and Antitumor Immunity. *Clin Cancer Res.* 2016; 22: 2329-2334.
38. Zhang X, Zeng Y, Qu Q, Zhu J, Liu Z, Ning W, *et al.* PD-L1 induced by IFN- γ from tumor-associated macrophages via the JAK/STAT3 and PI3K/AKT signaling pathways promoted progression of lung cancer. *Int J Clin Oncol.* 2017; 22: 1026-1033.
39. Kang YJ, Jeung IC, Park A, Park YJ, Jung H, Kim TD, *et al.* An increased level of IL-6 suppresses NK cell activity in peritoneal fluid of patients with endometriosis via regulation of SHP-2 expression. *Hum Reprod.* 2014; 29: 2176-2189.
40. Nakagawa S, Serada S, Kakubari R, Hiramatsu K, Sugase T, Matsuzaki S, *et al.* Intratumoral delivery of an adenoviral vector carrying the SOCS-1 gene enhances T cell-mediated anti-tumor immunity by suppressing PD-L1. *Mol Cancer Ther.* 2018. pii: molcanther.0822.2017.

FIGURE LEGENDS

Figure 1. Expression of JAK/STAT-pathway related proteins in androgen-independent PCa cell lines and determining cell proliferation after SOCS3 gene transduction with Ad-SOCS3.

(a) After transduction of SOCS3 gene into androgen-independent PCa cell lines with Ad-hSOCS3, Ad-mSOCS3 or Ad-LacZ, the expression of pSTAT3, STAT3 and SOCS3 in cells were determined by western blotting. The expression of phosphorylated STAT3 protein was suppressed by over expression of SOCS gene in TRAMP-C2 and DU-145, which were expressing STAT3. **(b)** TRAMP-C2, DU-145 and PC-3 cells were infected with any of Ad-hSOCS3, Ad-mSOCS3 and Ad-LacZ as control at MOI of 10-40. After a 6-day culture, Alamar blue assay was performed to determine cell proliferation. Transduction of SOCS3 gene significantly inhibited the growth in TRAMP-C2 and PC-3 cells which were expressing pSTAT3 protein compared with LacZ. Figures show the average (points) of 4 wells $\pm$ SE (bars); (* p <0.05, ** p <0.01).

Figure 2. (a). CRPC cell lines (TRAMP-C2, DU-145 and PC-3) were stained with annexin V-FITC and PI, following infection with 40 MOI of Ad-hSOCS3, Ad-mSOCS3 or Ad-LacZ, and analyzed by flow cytometry. In each panel, lower left quadrant shows cells which are negative for both annexin V-FITC and PI, lower right shows PI positive cells, upper left shows annexin-V positive cells which are in the early stage of apoptosis, and upper right shows both annexin V and PI positive, which are in the late stage of apoptosis. **(b).** Bar diagram showing the distribution of early and late apoptotic cell populations. Infection of Ad-SOCS3 increased the ratios of late and total (late and early) apoptotic cells in TRAMP-C2 and DU-145 cells, which were expressing pSTAT3 protein, compared with non-infection or Ad-LacZ infection groups.

Figure 3. IL-6 production and cell cycle distribution in CRPC cell lines

(a) IL-6 production from TRAMP-C2, DU-145 and PC-3 cells was determined by ELISA.

(b) After transduction of SOCS3 gene with Ad-SOCS3 into DU-145 and PC-3, production of IL-6 was determined. Over expression of SOCS3 gene significantly suppressed the production of IL-6 from DU-145 which were expressing pSTAT3 (** $p < 0.01$).

(c) and (d) DU-145 cells (c) or PC-3 cells (d) were infected with either Ad-hSOCS3 or Ad-LacZ as control at MOI of 40. Also, these cells were lipofected with either human STAT3 siRNA or negative siRNA as control at 10 μ M. The cell cycle was determined by means of propidium iodide (PI) staining using flow cytometry. Figures show the average (columns) of duplicate wells + SD (bars). (* $p < 0.05$, ** $p < 0.01$)

Figure 4. PD-L1 expression on CRPC cell lines

The expression of PD-L1 with or without IFN- γ stimulation in the Ad-SOCS3 infected or non-infected TRAMP-C2 and DU-145 cells by flow cytometry. **(a)** without IFN- γ , DU-145 cells expressed PD-L1, but TRAMP-C2 cells did not express PD-L1. Stimulation with IFN- γ increased the expression of PD-L1 in TRAMP-C2 cells but not in DU-145 cells. **(b)** Ad-mSOCS3 decreased the expression of PD-L1 induced by IFN- γ in TRAMP-C2 cells, but Ad-hSOCS3 could not decrease the expression of PD-L1 in DU-145 cells.

Figure 5. Cytotoxic effect of NK cells against TRAMP-C2 and DU-145 which were transduced SOCS3 gene.

After transduction of SOCS3 gene with Ad-SOCS3 into TRAMP-C2 and DU-145, these cells were co-cultured with NK cells *in vitro* and cytotoxicity was determined. Ad-mSOCS3 significantly increased the cytotoxicity of murine NK cells against TRAMP-C2 cells at effector:target cell ratio of 10:1 (* $p < 0.05$). Ad-hSOCS3 significantly increased the *in vitro*

cytotoxicity of human NK cells against DU-145 cells at cell ratios of 5:1, and 10:1 ($**p<0.01$, $\ddagger p<0.005$). In the presence of recombinant IL-6, Ad-hSOCS3 could not increase the *in vitro* cytotoxicity of NK cells against DU-145 cells at cell ratio of 10:1. Figures show the average (columns) of triplicate wells + SD (bars) ($**p<0.01$, $\ddagger p<0.005$).

Figure 6. Tumor volume curve in DU-145 xenograft model and the synergistic anti-tumor activity of combining Ad-SOCS3 and NK cells *in vivo*.

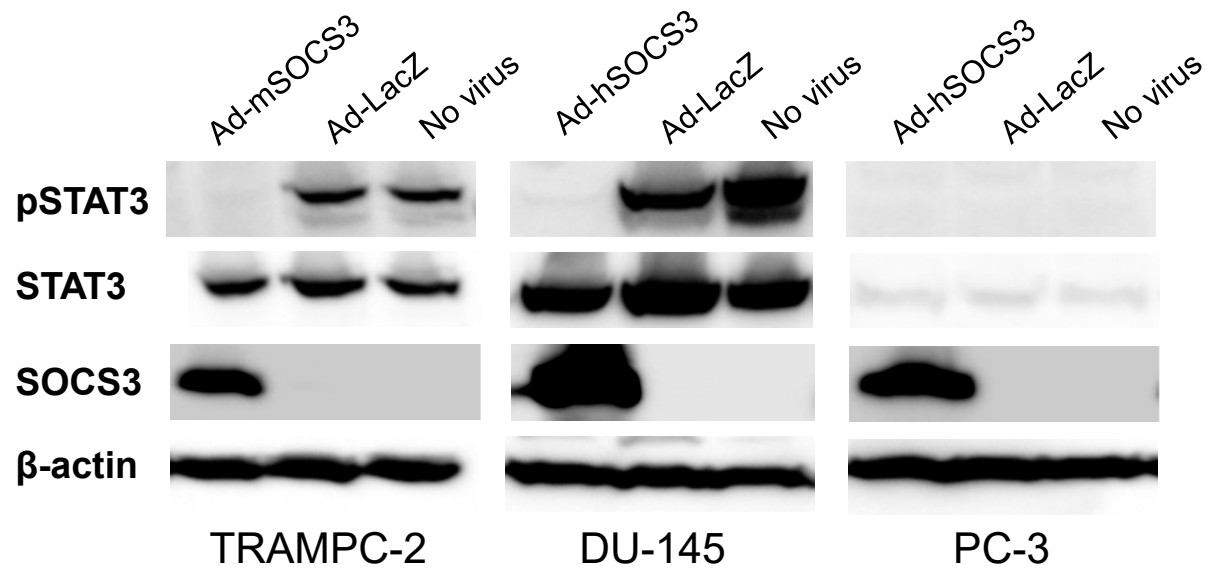
After subcutaneous transplantation of DU-145, PBS, Ad-LacZ, NK cells, Ad-hSOCS3, and combination of Ad-hSOCS3 and NK cells were intratumorally administered for five times (days 0, 3, 7, 10 and 14). The combination of Ad-hSOCS3 and NK cells significantly inhibited the growth of DU-145 subcutaneous tumors compared with the PBS, Ad-LacZ, and NK cell treatment groups at day 42 ($**p<0.01$).

Figure 7. Immunohistochemical staining with anti-CD56 antibody in DU-145 xenograft tumor tissues.

Two days after the final injection of NK cells, tumor tissues were resected and stained with anti-CD56 antibody. The combination of Ad-SOCS3 and NK cells treatment remarkably sustained CD56-positive NK cells in tumor tissues (e), whereas NK cells were not detected in other treatment groups; (a) PBS, (b) Ad-LacZ, (c) Ad-SOCS3 alone, and (d) NK cells alone.

Fig 1

(a)



(b)

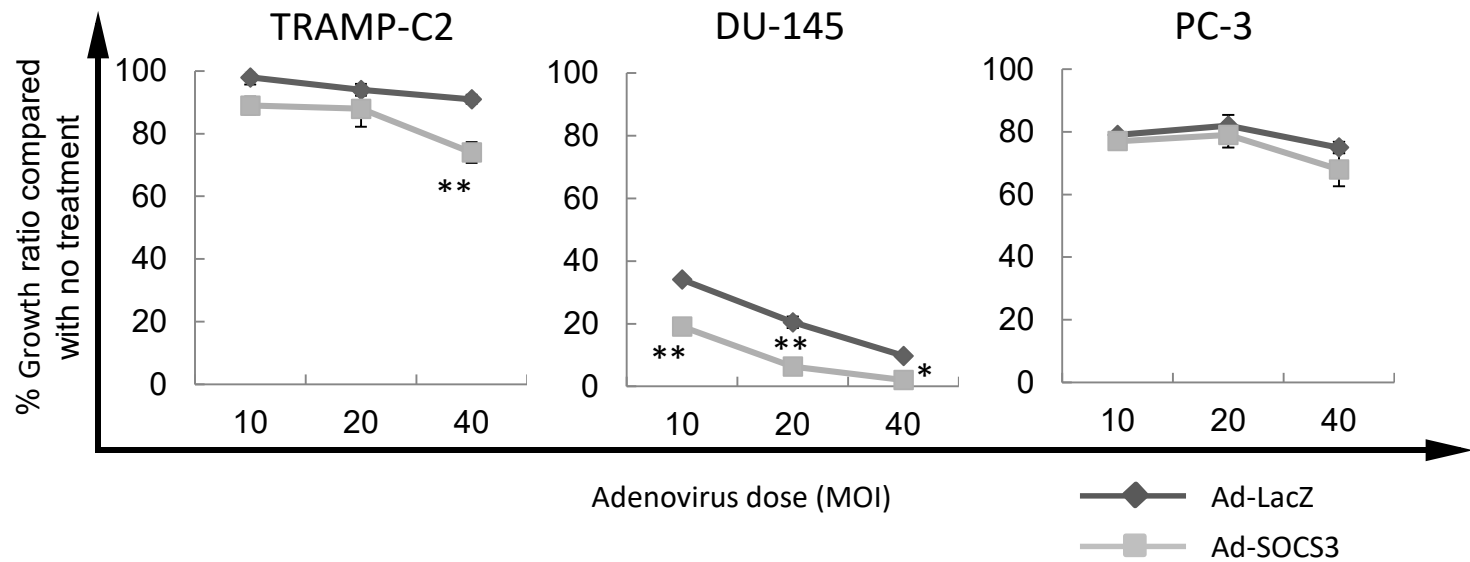
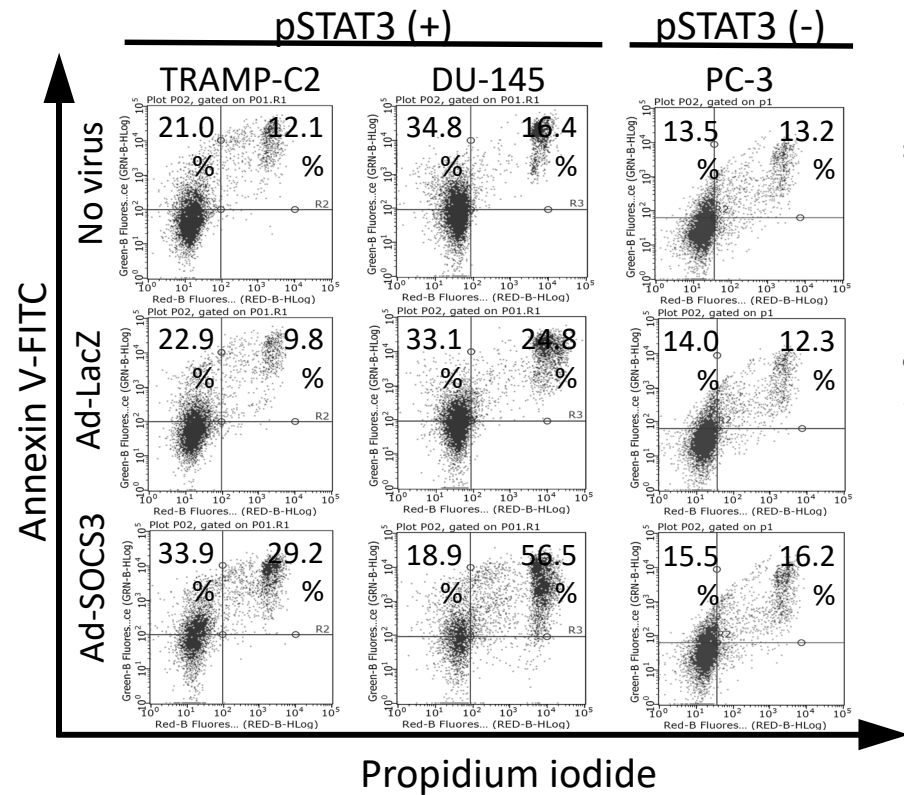


Fig 2

(a)



(b)

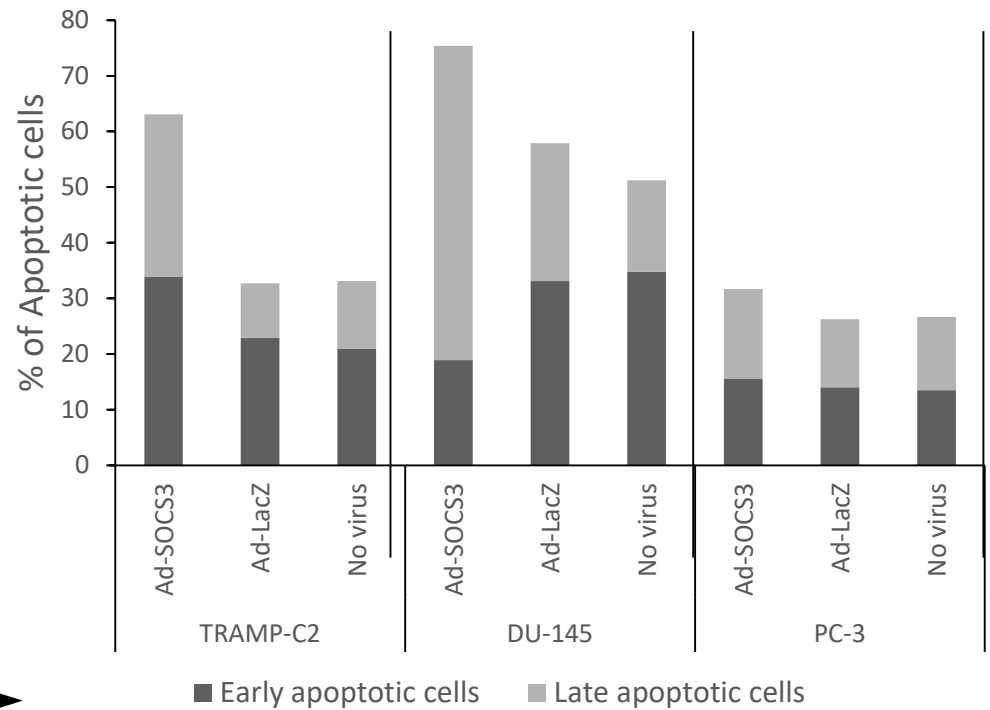


Fig 3

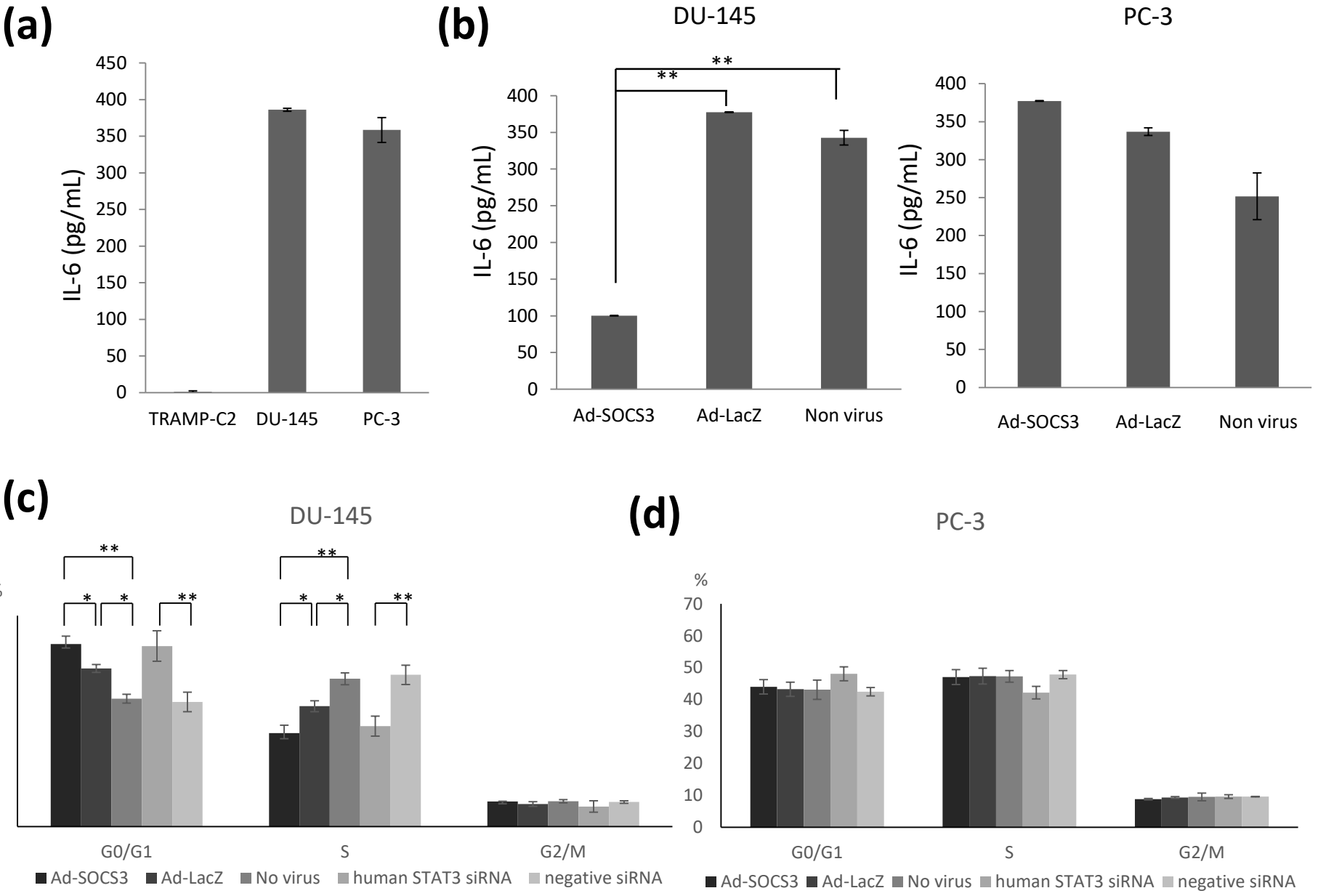
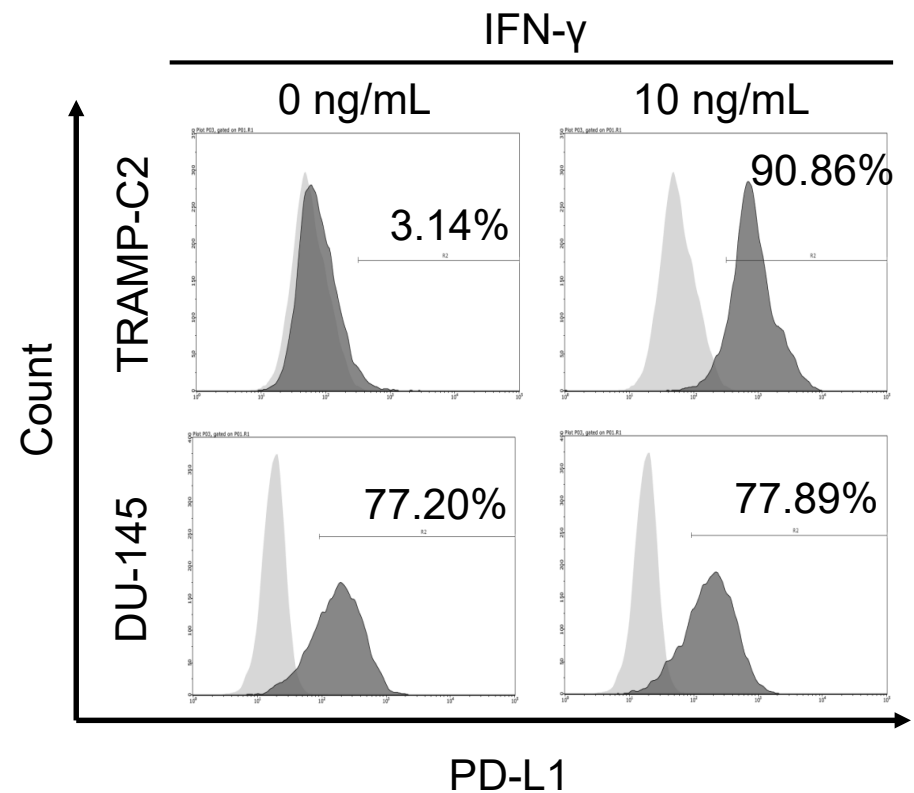


Fig 4

(a)



(b)

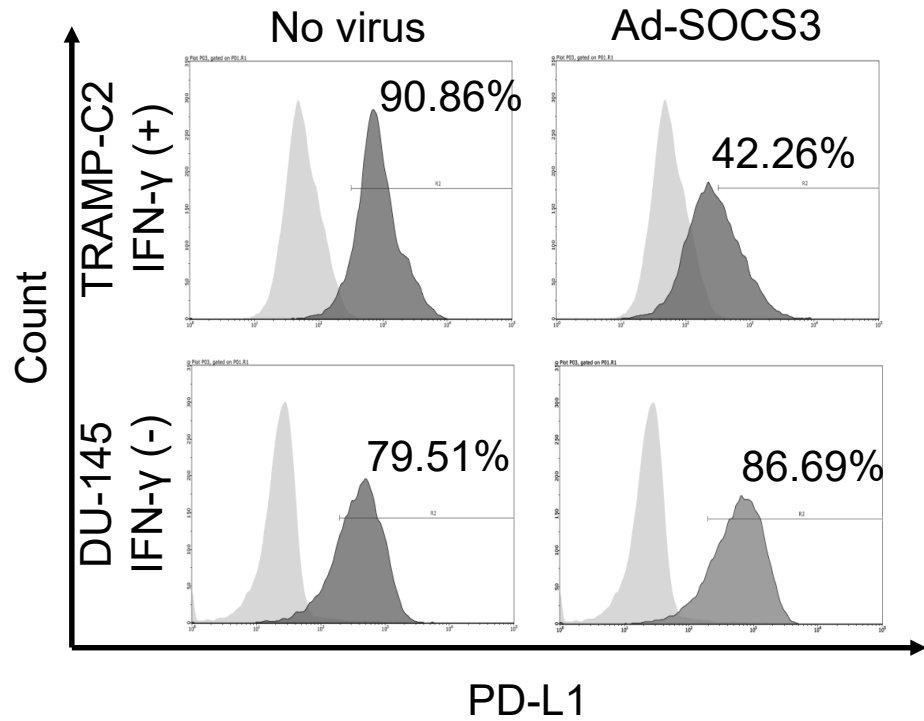


Fig 5

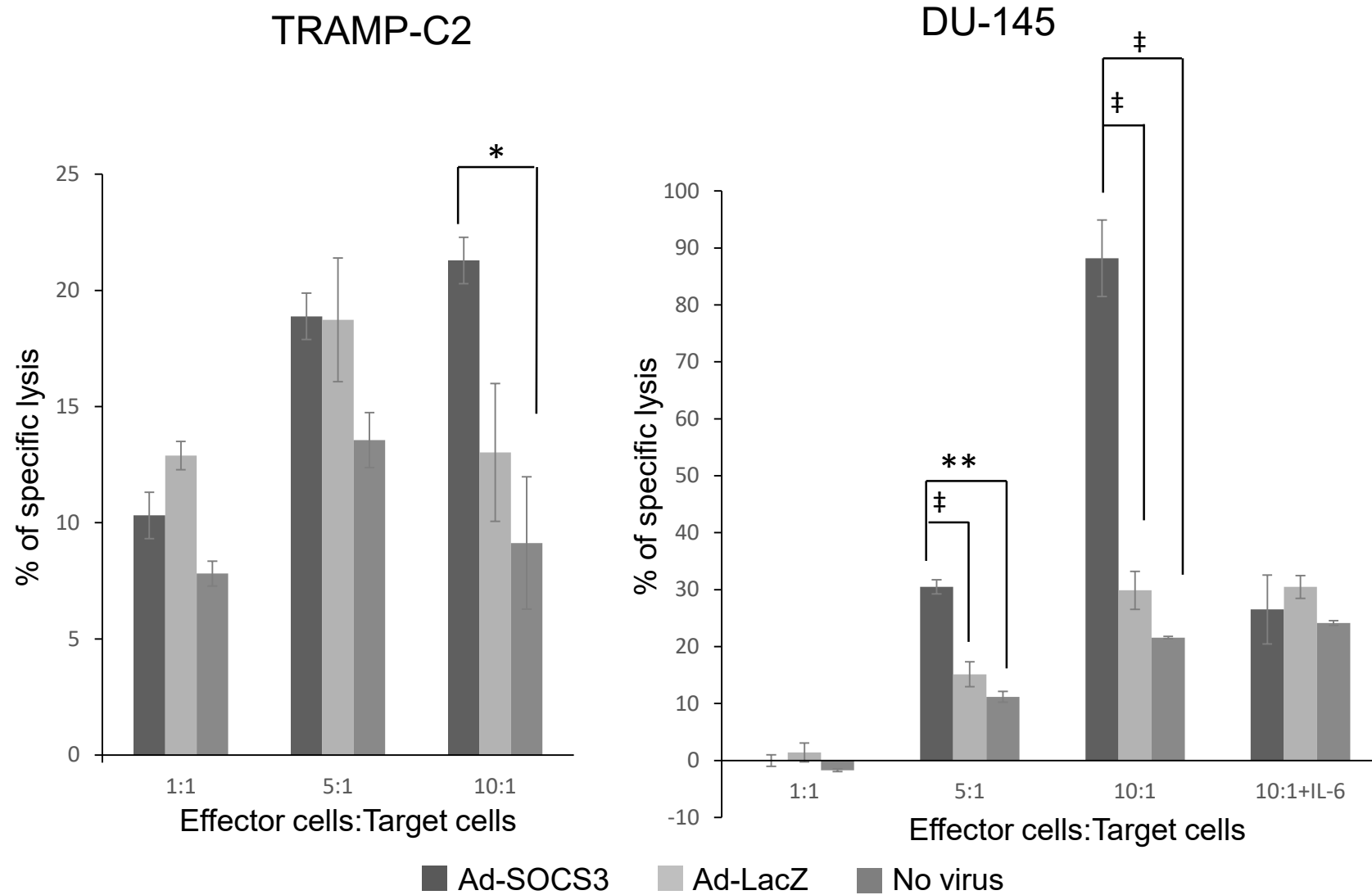


Fig 6

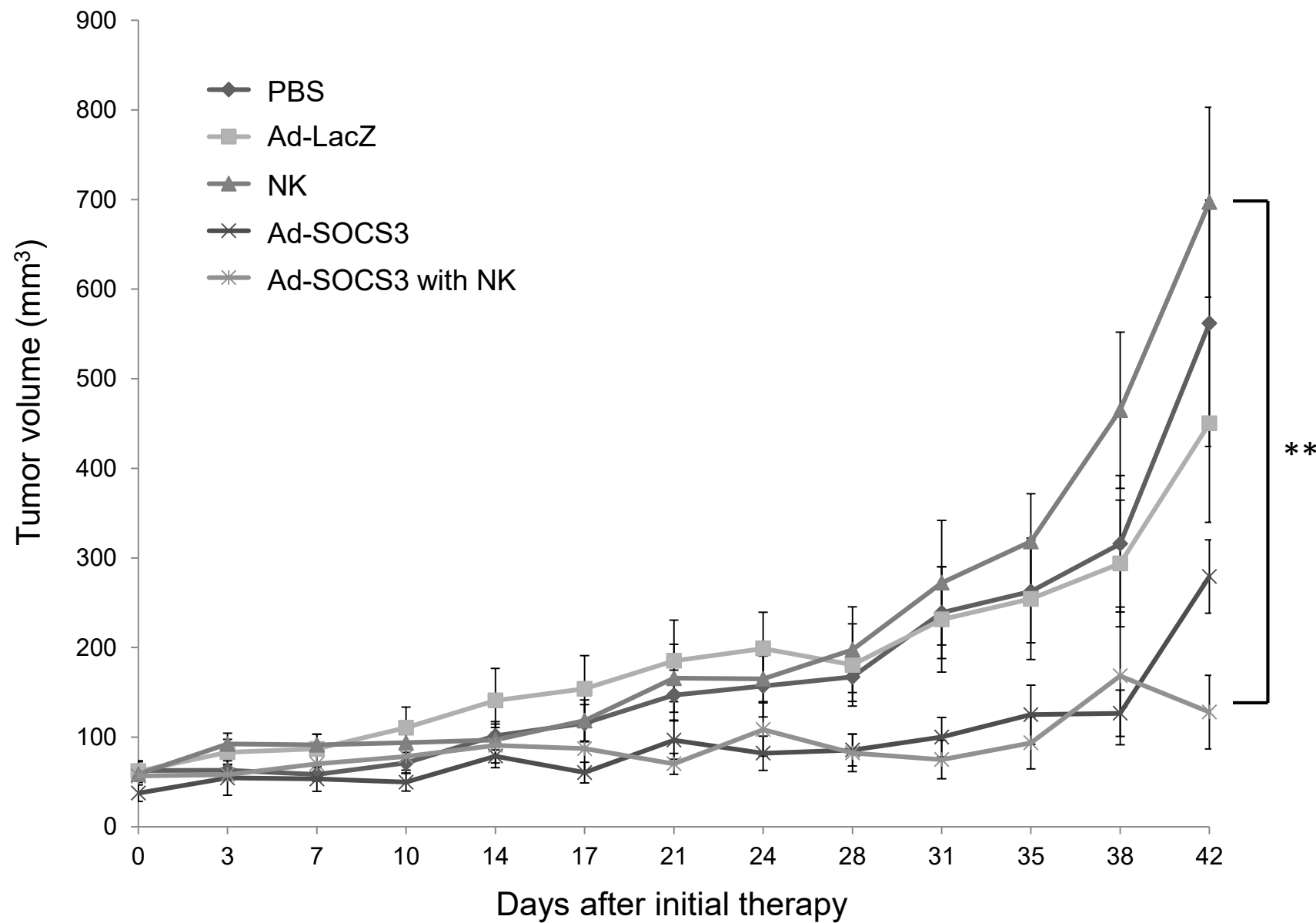
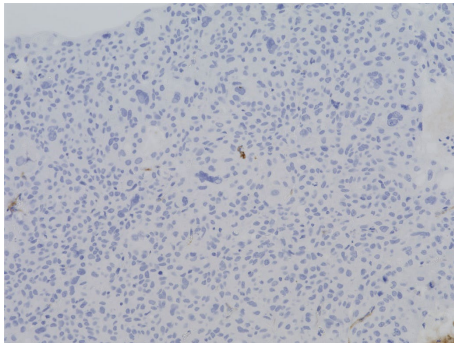
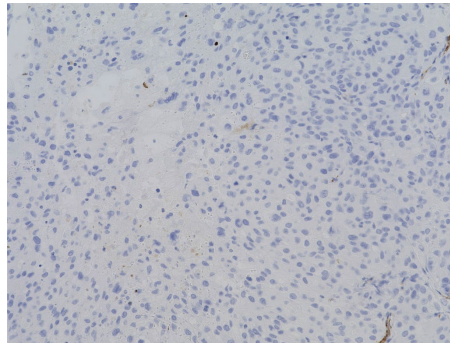


Fig 7

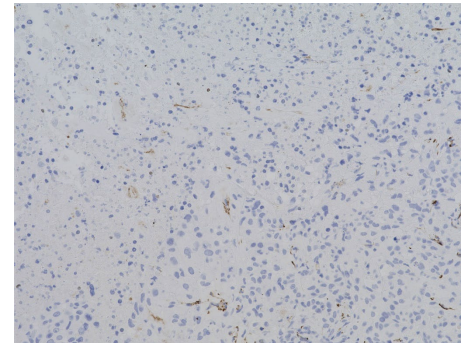
(a) PBS



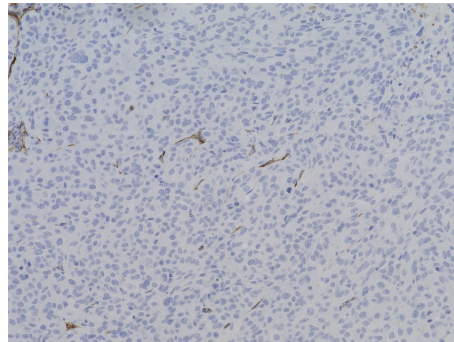
(b) Ad-LacZ



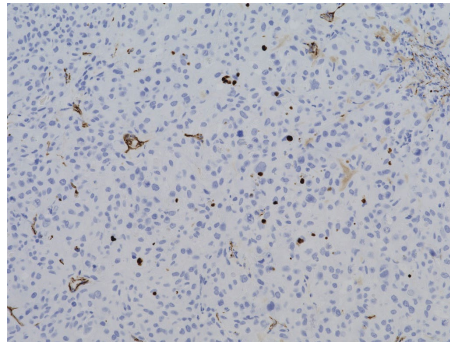
(c) Ad-SOCS3 alone



(d) NK alone



(e) Ad-SOCS3 with NK



(200x)