Int J Clin Exp Med 2017;10(8):12807-12816
www.ijcem.com /ISSN:1940-5901/IJCEM0052274

*Original Article*

# Therapeutic and anti-metastatic effect of CIKs on cervical cancer enhanced by modified HPV16 E7-pulsed DCs with SOCS1-knockdown

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Received March 6, 2017; Accepted April 7, 2017; Epub August 15, 2017; Published August 30, 2017

**Abstract:** Cervical cancer is the second most frequent cancer and the second most common cause of cancer-related deaths among women worldwide. In this study, a modified HPV16 E7 (HPV16mE7) with reduced transformation activity and enhanced antigenicity was employed to load DCs with silenced suppressor of cytokine signaling 1 (SOCS1) gene. The prepared DCs were co-cultured with peripheral blood mononuclear cells (PBMCs)-derived CIKs. The resulted DCCIKs were then transfused into mouse models bearing tumor of HPV16 E6/E7 expressing SiHa cells for the *in vitro/in vivo* antitumor activity assay. The expression levels of IL-12, IL-6, TNF-α and IFN-γ were also evaluated. The survival days and the tumor volume from different treatment groups were measured as well as the *in vivo* heterotopic tumor metastasis rate from the liver to the lung established in the mouse models. The data demonstrated that the xenografted tumor mice treated with Ad-shSOCS1-DCCIKs exhibited better immunotherapeutic and antimetastatic effect. This method may provide a new strategy for the development of safe and effective immunotherapy for cervical cancer.

**Keywords:** Cervical cancer, HPV, DC, CIKs, SOCS1, RNAi, metastasis

## Introduction

Cervical cancer is one of the most common gynecologic malignant tumors. Molecular, clinical and epidemiological researches identify human papillomavirus (HPV) infection is responsible for nearly all cervical cancer cases [1, 2]. HPV16 and HPV18 are associated with 70% oncogenesis cause of cervical cancer cases, and therefore are considered as "high risk" (HR) viruses [3-6]. Encoded by these two viruses, the E6 and E7 oncoproteins are crucial for the viral life cycle in the transformation and maintenance of the malignant phenotype of cervical cancer [7]. Various immunotherapeutic strategies against E6 and E7 were developed, however they have largely proven ineffective in treating HPV-induced cervical neoplasia, mainly attributing to the patients' severe cellular immune function defects such as the activated immune escape and tolerance mechanisms employed by tumors and virally infected cells [8, 9].

Therefore, improving the cervical cancer patients' cellular immunity functions is essential to improve the cure rate, prolong the survival time and inhibit tumor recurrence or metastasis. In recent years, the applications of adoptive cellular immunotherapy such as CIKs, CAR T cells, DC, DCCIKs, natural killer (NK) cells, tumor-infiltrating lymphocytes (TILs), and cytotoxic T lymphocytes (CTLs) have been constantly expanding and gradually accepted [10-13].

Possessing the properties of strong antitumor activity of T lymphocytes and a non-major histocompatibility complex (MHC)-restricted tumoricidal effect, CIK cells generated from PBMCs and *ex vivo* cultured in the presence of a variety

of cytokines have become the preferred type of cells used in clinic practice [10, 12, 13]. When co-cultured with DCs which can generate antigen-specific immune responses, CIKs activated by DCs (DCCIKs) achieve the advantages of fast proliferation, high cytotoxicity, and a broad tumor-killing spectrum [11].

DCs which are the most professional antigen-presenting cells (APC) are considered at the centre of the immune system because of their ability to regulate both immune tolerance and immunity. And the generation of protective anti-tumor immunity depends on the presentation of tumor antigens by DCs [11, 14]. Activated (mature), antigen-loaded and *ex vivo* cultured DCs can induce the differentiation of antigen-specific T cells into effector T cells which can reduce the tumor mass specifically as well as control tumor relapse, in contrast to non-activated (immature) DCs which can present self-antigens to T cells, leading to immune tolerance by generating suppressor T cells or T cell deletion [14-16]. As the presentation of tumor antigens by DCs play a decisive role in generating protective anti-tumor immunity in mouse models [17, 18], therefore, the activation of the full immune stimulatory potential of DCs to achieve an effective immunity provides a promising treatment against tumor.

As an inducible negative feedback inhibitor of the JAk/STAT signal pathway [14] and negative regulator of various cytokines such as interferon (IFN)-γ, IL-2, IL-6, IL-7, IL-12, and IL-15 in DCs, suppressor of cytokine signaling 1 (SOCS1) has been implicated in exerting potent immunosuppressive effect on blocking the constitutive activation of the immune response [19]. DCs derived from $SOCS1^{-/-}$ transgenic mice were found hyper-responsive to LPS and IFN-γ, triggering an allogeneic T-cell expansion [20, 21].

In this study, HPV16mE7-loaded DCs with SOCS1-knock down were co-cultured with CIK cells prepared from human PBMCs. The generated DCCIKs were infused into mouse models bearing tumor of viral HPV16 E6E7 expressing SiHa cells. The survival days and the tumor volume from different treatment groups were investigated as well as the *in vivo* heterotopic tumor metastasis rate from liver to lung. The results demonstrated that the xenografted tumor mice treated with Ad-shSOCS1-DCCIKs exhibited better immunotherapeutic and anti-metastatic effect.

## Materials and methods

### *Cells and reagents*

SiHa and HEK293 cell lines were purchased from American Type Culture Collection (ATCC). Cells were maintained in the DMEM or RPMI-1640 culture media (Gibco, Life Technologies, US) supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, US) and 2 mM l-glutamine (Invitrogen, Carlsbad, CA) and in a humidified atmosphere containing 5% $CO_2$ at 37°C. PBMCs were generated from healthy donors whose informed consent was obtained from the participants in accordance with the study protocols approved by the Institutional Review Board of the Hospital Authority of Wuhan. rhIL-2 and rhGM-CSF were acquired from R&D Inc. (USA). rhIL-4, rhIFN-γ, and TNF-α were purchased from Peprotech Inc. Normal human AB serum were sourced from (Gibco, Life Technologies, USA). Lymphocyte separation medium Ficoll was purchased from GE Healthcare (USA). Mouse anti-human CD3 (FITC labeled) monoclonal antibody, mouse anti-human CD8 (PE labeled) monoclonal antibody, and mouse anti-human CD56 (PE labeled) monoclonal antibody were purchased from eBioscience Co, Ltd. Mouse anti-human CD80-PE monoclonal antibody, mouse anti-human CD83-PE monoclonal antibody, mouse anti-human CD86-PE monoclonal antibody, mouse antihuman CD40-FITC monoclonal antibody, and mouse antihuman CD1a-FITC monoclonal antibody were all products of Santa Cruz Co, Ltd. Female BALB/c nude mice (5-8 weeks of age, weighing 18-22 g) raised under SPF circumstance were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Science. All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

### *Recombinant adenovirus production*

Primers used for shRNA-SOCS1 are as follows: shRNA-SOCS1 (F): 5'-GATCCCTACCTGAGTTCCTTCCCCTTCAAGAGAGGGGAAGGAACTCAGGTAGTTTTTTG-3' (BamHI and EcoRI); (R): 5'-AATTCAAAAAACTACCTGAGTTCCTTCCCCTCTCTTGAAGGGGAAGGAACTCAGGTAGG3'; shRNA-Control (F): 5'-GATCCTCACAACCTCCTAGAAAGAGTA-

GATTGTACTACACAAAAGTACTATGTTTTTTG-3' (BamHI and EcoRI); (R): 5'-AATTCAAAAAACATAGTACTTTTGTGTAGTACAATCTACTCTTTCTAGGAGGTTGTGAG. The shRNA-SOCS1 and shRNA-Control were cloned into pShuttle-2 vector (BD Clontech), and then inserted into the replication-deficient pAdeno-X vector (BD Clontech). The generated recombinant adenovirus plasmids were sequenced. The recombinant adenovirus was prepared according to the manufacturer's instructions and titrated using Adeno-X Rapid Titer kits (BD Bioscience).

*Preparation of DCs and CIKs*

Using Ficoll-Paque, PBMCs were separated from 70 mL peripheral blood and cultured in RPMI-1640 supplemented with 10% (V/V) autologous serum at $1\times10^6$/mL. Informed consent was obtained from the participants in accordance with the study protocols approved by the Institutional Review Board of Hubei University of Arts and Science. Subsequently, the obtained cells were transferred into culture flasks and cultured for 2 hrs. Nonadherent cells collected as the progenitor of CIKs were resuspended at $1\times10^6$ cells/mL in RPMI-1640 medium supplemented with 10% human AB serum and cultured with immobilized anti-CD3 antibody (10 μg/mL, BD Pharmingen, NJ, USA) and rhIL-2 (500 U/mL) as well as rhIFN-γ (1000 U/mL) in culture flask. Half the amount of medium was exchanged every three days and supplemented with rhIL-2 and rhIFN-γ. The remaining adherent cells were cultured in RPMI-1640 medium (containing 40 ng/mL rhGM-CSF and 40 ng/mL rhIL-4). The supernatant was replaced with fresh medium containing rhGM-CSF and rhIL-4 every 3 days. All cultures were incubated at 37°C in 5% humidified $CO_2$. After 7 days of culture, flow cytometric analysis demonstrated that >80% of the cells expressed characteristic DC-specific markers.

*Analysis of SOCS1 silencing by western blot*

After 7 days of culture, the *ex vivo* cultured DCs were washed and placed in 6-well plates at $2.5\times10^5$ cells/well with 600 μL RPMI-1640. The cells were exposed to Ad-shSOCS1 or Ad-shControl at 100 MOI. After 8 h transduction, the cells were washed with PBS and further incubated in fresh medium for 48 h, and then collected and lysed with cell lysis buffer. The protein samples separated via SDS-PAGE were transferred onto immobilon membranes (Millipore, MA, USA). SOCS1 and β-actin were identified by anti-SOCS1 polyclonal and anti-β-actin monoclonal antibodies (Santa Cruz Biotechnology, USA), respectively.

*Maturation and cytokine analysis of DCs via ELISA*

After 7 days of culture as described in "*Preparation of DCs and CIKs*". The DCs were washed and placed in 12-well plates at a concentration of $1\times10^5$ cells/well in 400 μL RPMI-1640. The DCs were exposed to Ad-shRNA-SOCS1 or ad-shRNA-Control at 100 MOI. After 8 h transduction, the cells were washed with PBS and further incubated in fresh culture medium. The DCs were then pulsed with the HPVm16E7 protein at 25 μg/mL (HPVm16E7 was prepared as we previously reported in [22]) for 6 h, followed by stimulation with TNF-α (10 ng/mL) for 24 h to develop mature DCs. IL-12p70 and IL-6 contents (absorbance) in the supernatants were quantitated separately by ELISA (Dakewe Biotech Co. Ltd., China) according to the manufacturer's instructions. Using FACS, the phenotype molecules of HLA-DR/CD1a/CD80/CD83/CD86-flurescein isothiocyanate (FITC) were measured to investigate DC maturation (Santa Cruz Co, Ltd.), three repeats for each group.

*Phenotype detection of DCCIKs and secretion levels of TNF-α and IFN-γ*

CIKs cultured without rhIFN-γ were mixed with HPVm16E7-pulsed DCs which had been transducted with Ad-shRNA-SOCS1 or Ad-shRNA-Control at 100 MOI, stimulated by TNF-α, and cultured for 8 days at a ratio of 10:1. The cell mixtures were then cultured in CIKs medium for 3 days. CIKs, Ad-shSOCS1-DCCIKs, and Ad-shControl-DCCIKs were collected for the detection of the CIKs surface markers. CD3, $CD3^+CD56^+$, and $CD3^+CD8^+$ were examined by FACS analysis. The effector cells cultivated without rhIFN-γ was also cocultured with target SiHa cells constitutively expressing HPV16 E6 and E7 at effector/target (E/T) ratios of 10:1, 30:1, and 90:1 for 24 h. Levels of TNF-α and IFN-γ in the supernatant of CIKs, Ad-shSOCS1-DCCIKs, and Ad-shControl-DCCIKs at 10:1, 30:1 and 90:1 E/T ratios were tested by ELISA (R&D Systems, Minneapolis, MN). The

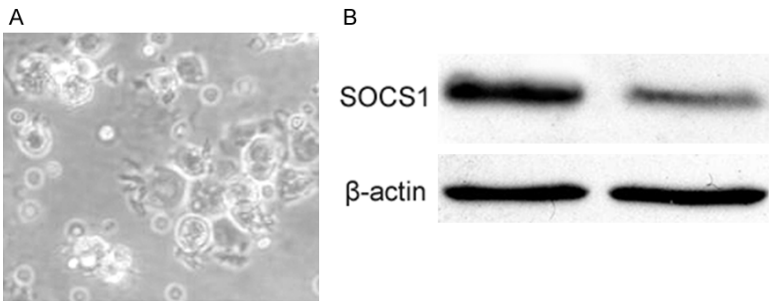


**Figure 1.** A. After pulsed with HPVm16E7 and stimulated with TNF-α, DCs in irregular shape presented mature characteristics of larger soma with plenty of dendritic long and samll bulges on their surfaces (400× magnification). B. The inhibition of SOCS1 expression in DCs. Lane 1, Ad-shControl (MOI 100); lane 2, Ad-shSOCS1 (MOI 100).

TNF-α and IFN-γ ELISA assays were performed following the instruction manual.

*In vitro antitumor activity analysis*

The *in vitro* cytotoxicity of the CIKs, Ad-shSOCS1-DCCIKs, and Ad-shControl-DCCIKs against SiHa cells was determined using a CCK8 kit, (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The CIKs, Ad-shSOCS1-DCCIKs and Ad-shControl-DCCIKs harvested 11 days after induction were used as effector cells. The SiHa cells were used as target cells. The effector and target cells were added to 96-well plates at a ratio of 10:1, 30:1 and 90:1. The cells were incubated for 24 h. The groups comprising a mixture of cell types were the experimental groups, while the control groups contained only one cell type of the SiHa, CIKs, Ad-shSOCS1-DCCIKs, Ad-shControl-DCCIKs or 1640-RPMI cultivating solution. The CCK8 assay was performed in triplicate to evaluate cell viability, and optical density (OD) was read at 570 nm.

*In vivo antitumor assay*

Xenografted tumor mouse models were established by abdominal subcutaneous injection of $1\times10^6$ SiHa cells into 6-week-old female BALB/c nude mice. On day 9, all mice had palpable tumors were assigned to 4 groups: PBS (served as control), CIKs, Ad-shSOCS1-DCCIKs, and Ad-shControl-DCCIKs (10 mice in each group). Therapeutic treatments were initiated with a total of $1.0\times10^7$ CIKs, Ad-shSOCS1-DCCIKs, Ad-shControl-DCCIKs or PBS, respectively. $2.5\times10^6$ cells (per dose) on the day of 9, 13, 17 and 21 were administered intravenously into the mice tail for a total of 4 injections. The tumor volume was calculated by the following formula: (major axis × minor axis$^2$) ×0.52. When the tumor volume reached up to 2,500 mm$^3$, the tumor-bearing mice were euthanized to avoid unnecessary pain.

*In vivo metastasis assay by heterotopic liver implantation*

To observe the reversed metastatic potential by HPVm16-E7-pulsed Ad-shSOCS1-DCCIKs on SiHa cells, $3\times10^6$ SiHa cells were injected subcutaneously into nude mice. As the tumor cube implantation in the liver has advantages over injecting cells to ensure tumor formation in liver in order to observe heterotopic tumor metastasis from the liver to the lung, the subcutaneous formed tumors were then implanted as 1-2 mm$^3$ cubes into the left liver lobes of nude mice. Post 9 days of implantation, Therapeutic treatments of PBS, CIKs, Ad-shSOCS1-DCCIKs, and Ad-shControl-DCCIKs with a total of $1.0\times10^7$ cells were administered (10 mice in each group). A total of 4 consecutive injections every 5 days were administered with $2.5\times10^6$ cells (per dose). After 6 weeks, the mice were sacrificed for lung metastasis assay examined by sectioning and microscopy. All groups were euthanized simultaneously at 6 weeks after tumor cube implantation.

## Results

*Morphological observation of DCs*

After pulsed with HPVm16E7 and stimulated with TNF-α, DCs in irregular shape presented mature characteristics of larger soma with plenty of dendritic long and small bulges on their surfaces under scanning microscopy (**Figure 1**).

*Inhibition of SOCS1 expression*

Constructs of Ad-shSOCS1 and Ad-shRNA-Control were verified in DCs (**Figure 1**). Western blot analysis demonstrated that Ad-shControl infection at MOI 100 greatly reduced SOCS1 expression compared with Ad-shControl.

**Table 1.** Percentage of DCs expressing CD1a, CD80, CD83, CD86, HLA-DR

| | CD1a | CD80 | CD83 | CD86 | HLA-DR |
|---|---|---|---|---|---|
| Control | 27.2±3.3 | 31.2±6.5 | 38.9±6.3 | 42.5±5.4 | 57.6±5.8 |
| TNF-α | 82.4±5.6[a] | 72.4±8.6[a] | 79.2±8.2[a] | 84.3±6.2[a] | 90.3±5.2[a] |
| Ad-shControl-DCs | 83.1±7.4[a,b] | 76.1±7.5[a,b] | 81.2±5.2[a,b] | 87.3±4.3[a,b] | 92.3±4.3[a,b] |
| Ad-shSOCS1-DCs | 93.6±4.1[a,c] | 89.6±6.1[a,c] | 92.3±3.7[a,c] | 92.6±2.7[a,d] | 94.6±2.5[a,d] |

Immature DCs generated from PBMCs were stimulated by GM-CSF and IL-4 for 7 days and further stimulated by Ad-shSOCS1 or Ad-shControl. Percent of DCs expressing CD1a, CD80, CD83, CD86 and HLA-DR were detected using flow cytometry before and after 24 h TNF-α stimulation and analyzed by SPSS one-way ANOVA (Student's t test) analysis. [a]Significant differences compared with that of the DCs control, P<0.001; [b]No significant differences compared with that of the TNF-α control, P>0.05; [c]Significant differences compared with that of the TNF-α control or Ad-shControl group, P<0.05; [d]Significant differences compared with that of the TNF-α control, P<0.05, but no significant differences compared with that of the Ad-shControl group, P>0.05.

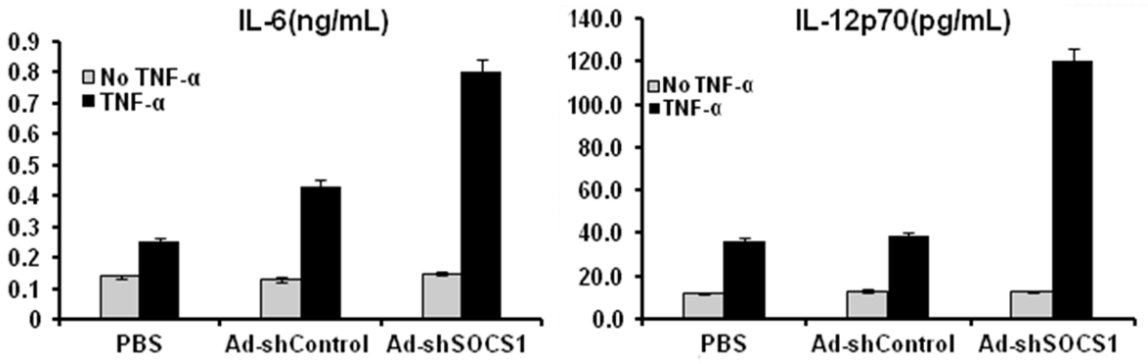


**Figure 2.** Cytokines secreted by transfected DCs after TNF-α (10 ng/mL) stimulation for 24 hrs were measured via ELISA assays. Data were representative of three independent assays and analyzed using Student's t-test. *P<0.01 versus Ad-shSOCS1-DCs.

**Table 2.** Phenotype detection of CIKs and DCCIKs

| | CD3 | $CD3^+CD8^+$ | $CD3^+CD56^+$ |
|---|---|---|---|
| CIKs | 98.7±0.04 | 76.7±2.3 | 16.9±1.3 |
| Ad-shControl DCCIKs | 99.3±0.03[b] | 85.6±2.3[a] | 20.7±2.5[a] |
| Ad-shSOCS1 DCCIKs | 99.40±0.01[b] | 89.5±1.5[a] | 29.5±3.6[a,c] |

After cocultivation with Ad-shControl-DCs or Ad-shSOCS1-DCs in CIKs culture medium containing INF-γ, both $CD3^+CD8^+$ and $CD3^+CD56^+$ double positive cells were significantly improved in respect to CIKs. Further, the $CD3^+CD56^+$ population in the Ad-shSOCS1-DCCIKs were improved about 42.5% compared to that of Ad-shControl-DCCIKs. Datas were analyzed by SPSS one-way ANOVA (Student's t test) analysis. [a]Significant differences compared with that of the CIKs control, P<0.05; [b]No significant differences compared with that of the CIKs control, P>0.05; [c]Significant differences compared with that of the CIKs control or Ad-shControl-DCCIKs group, P<0.05.

*Evaluation of DCs maturation*

Immature DCs (iDCs) produced from PBMCs were stimulated with GM-CSF and IL-4 for 7 days, and further stimulated with Ad-shSOCS1 and HPVm16E7. Before and after 24 h of TNF-α stimulation, the expression levels of the surface markers CD1a, CD80, CD83, CD86 and HLA-DR were analyzed by flow cytometry and SPSS one-way ANOVA analysis. The results in **Table 1** demonstrated that the expression levels of all antigens significantly increased by 2-4 folds during maturation in both Ad-shSOCS1 and Ad-shControl infected DCs with respect to control. The expression levels of CD1a, CD80 and CD83 increased about 10% in the former more than the latter. In addition, although TNF-α greatly induced the expression of these CD markers, it was lower than that of the Ad-shSOCS1 group in CD1a, CD80 and CD83 expression.

*IL-12 and IL-6 analysis in DCs*

Such proinflammatory cytokines as IL-12 (IL-12p70) and IL-6 induced by HPV16mE7-pulsed DCs with or without TNF-α stimulation were quantitated by ELISA analysis kits (**Figure 2**). Stimulated by TNF-α, the expression of IL-12p70 and IL-6 was considerably enhanced in all groups, while silencing SOCS1 in HPV16-

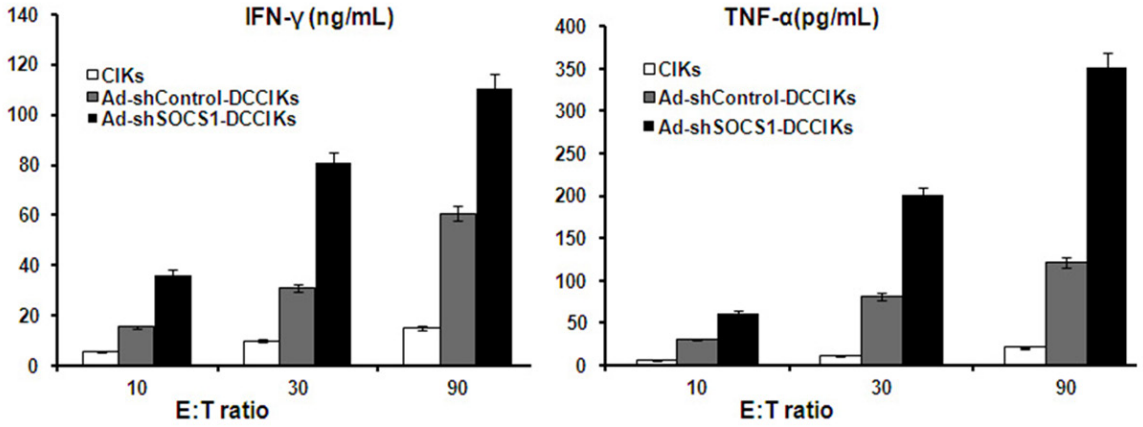


**Figure 3.** Co-cultured with target SiHa cells constitutively expressing HPV16 E6 and E7 at E/T ratios of 10:1, 30:1, and 90:1 for 24 hrs, levels of TNF-α and IFN-γ in the supernatant of CIKs, Ad-shSOCS1-DCCIKs, and Ad-shControl-DCCIKs were tested by ELISA. The production levels of TNF-α and IFN-γ were higher in group Ad-shSOCS1-DCs. *P<0.01 versus Ad-shSOCS1-DCs, analyzed by Student's t-test.

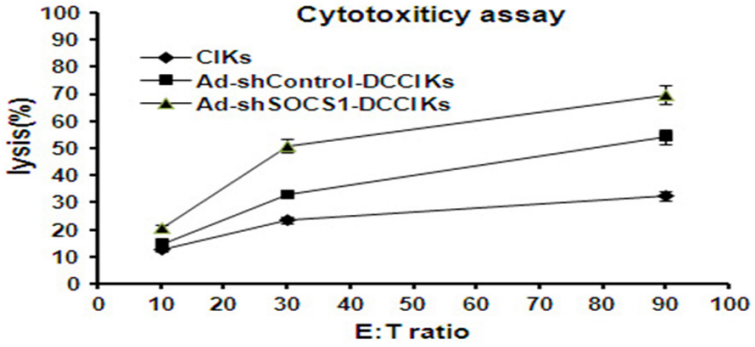


**Figure 4.** Using CCK-8 assay, the cytotoxicity of effector cells at 10:1, 30:1 and 90:1 E/T ratios against SiHa cells was analyzed. It revealed that Ad-shSOCS1-DCCIKs lymphocytes exhibited the highest cytotoxic activity against SiHa cells compared with Ad-shControl-DCCIKs or CIKs. *P<0.01, analyzed by Student's t-test.

mE7-pulsed DCs induced far more cytokines than that of other groups.

*Cellular phenotype detection of DCCIKs and secretion levels of TNF-α and IFN-γ*

Both $CD3^+CD8^+$ and $CD3^+CD56^+$ double positive cells were significantly improved after co-cultivation with Ad-shControl-DCs orAd-shSOCS1-DCs in CIKs culture medium. Further, the $CD3^+CD56^+$ population in the Ad-shSOCS1-DCCIKs were improved about 42.5% compared to that of Ad-shControl-DCCIKs (**Table 2**). The TNF-α and IFN-γ levels in the supernatant of group Ad-shSOCS1-DCCIKs were highest, versus Ad-shControl-DCCIKs and CIKs in CIKs culture medium without IFN-γ. And levels in group Ad-shControl-DCCIKs were also higher than those of CIKs. It was demonstrated that after co-culture of CIKs and Ad-shSOCS1-DCs or Ad-shControl-DCs, IFN-γ and TNF-α levels in the supernatants were apparently increased (**Figure 3**).

*In vitro antitumor activity analysis*

Using CCK-8 assay, the cytotoxicity of effector cells at 10:1, 30:1 and 90:1 E/T ratios against SiHa cells was analyzed. It revealed that Ad-shSOCS1-DCCIKs lymphocytes exhibited the highest cytotoxic activity against SiHa cells. The cytotoxic activity against SiHa cells induced by Ad-shSOCS1-DCCIKs and Ad-shControl-DCCIKs was not linear, but it increased with E:T ratio compared to the linear increase of the control group, indicating a dosage-dependent manner (**Figure 4**).

*In vivo antitumor activity analysis*

The therapeutic effect of the Ad-shSOCS1-DCCIKs lymphocytes against mice bearing tumors was investigated in BALB/C nude mice received $1\times10^6$ SiHa cells. When all mice had palpable tumors on day 9, the therapeutic treatments were initiated with different T cells

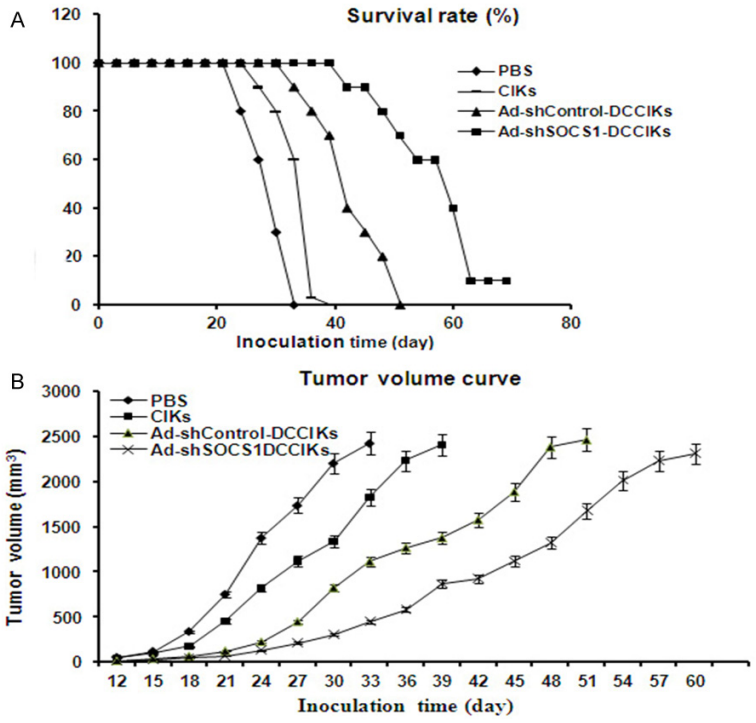


**Figure 5.** In vivo antitumor activity analysis. A. The therapeutic effect of the Ad-shSOCS1-DCCIKs lymphocytes against mice bearing tumors was investigated in the groups of 10 BALB/C nude mice received $1\times10^6$ SiHa cells. The therapy was initiated with PBS, CIKs, Ad-shControl-DCCIKs or Ad-shSOCS1-DCCIKs on day 9. Survival rates were analyzed using the Kaplan-Meier method (log-rank test). *P<0.01 (Ad-shSOCS1-DCCIKs vs PBS or CIKs or Ad-shRNAControl-DCCIKs), P<0.05 (Ad-shControl-DCCIKs vs PBS or CIKs). B. Tumor volume and time experiment demonstrated that Ad-shSOCS1-DCCIKs lymphocytes significantly regressed the tumor volume compared with CIKs and PBS controls in which progressive tumor growth was observed. The experiments were repeated, producing a significant statistical difference. Data of the tumor volume after inoculation of SiHa cells were analyzed using Student's t-test. *P<0.01 (Ad-shSOCS1-DCCIKs vs PBS or CIKs or Ad-shRNAControl-DCCIKs); P<0.05 (Ad-shControl-DCCIKs vs PBS or CIKs).

or PBS. As shown in **Figure 5A**, the mice treated with Ad-shSOCS1-DCCIKs exhibited the greatest survival days of 60 (40%) compared with the mice treated with Ad-shControl-DCCIKs (0%), CIKs (0%) and PBS (0%). Tumor volume experiment demonstrated that Ad-shSOCS1-DCCIKs lymphocytes significantly regressed the tumor volume compared with CIKs and PBS controls in which progressive tumor growth was observed. The experiments were repeated, producing a significant statistical difference (P< 0.01) (**Figure 5B**). Although therapeutic Ad-shSOCS1-DCCIKs could not always eradicate the tumor completely, the survival time was significantly prolonged more than 60 days in 40% of the Ad-shSOCS1-DCCIKs treated mice in respect to the mice in PBS control group which were all dead in 40 days. These results indicated the therapeutic potential of Ad-shSOCS1-DCCIKs against HPV16 $E7^+$ tumor *in vivo*.

*In vivo metastasis assay*

To observe the antimetastatic potential of HPVm16E7-pulsed Ad-shSOCS1-DCCIKs on SiHa cells, the subcutaneous formed SiHa tumor cubes at 1-2 $mm^3$ were implanted into the left liver lobes of nude mice, as the tumor cube implantation in the liver has advantages over injecting cells to ensure tumor formation in liver. **Figure 6B** showed normal lung tissue against **Figure 6A** in which a representative metastic lesion was demonstrated. As demonstrated in **Figure 6C**, Ad-shSOCS1-DCCIKs and Ad-shControl-DCCIKs lymphocytes decreased the tumor metastasis from the liver to the lung in the heterotopic liver implantation models (20%), whereas a high metastasis rate (70%) was observed in the PBS control group.

## Discussion

Stimulated with IFN-γ, IL-2, and CD3 monoclonal antibody (OKT3), CIKs expressing the CD3/CD56 surface markers of T and NK cells are a cell population obtained from peripheral blood mononuclear cells [16]. Possessing the strongest tumor cytotoxicity with an extensive range of tumor killing, CIKs can both directly suppress tumor cells and regulate the body immune system against tumor cells indirectly. Therefore, CIKs can inhibit the tumor's growth and recurrence by immediate cytotoxicity on tumor cells and improve the patients' immunity for long-term effect.

As the antigen-presenting DCs were able to induce robust cell-mediated immunity capable of attacking and eliminating abnormal antigen-bearing cells, allergization for CIKs with tumor antigen-pulsed DCs can both exert non-MHC restrictive cytotoxicity of CIKs and acti-

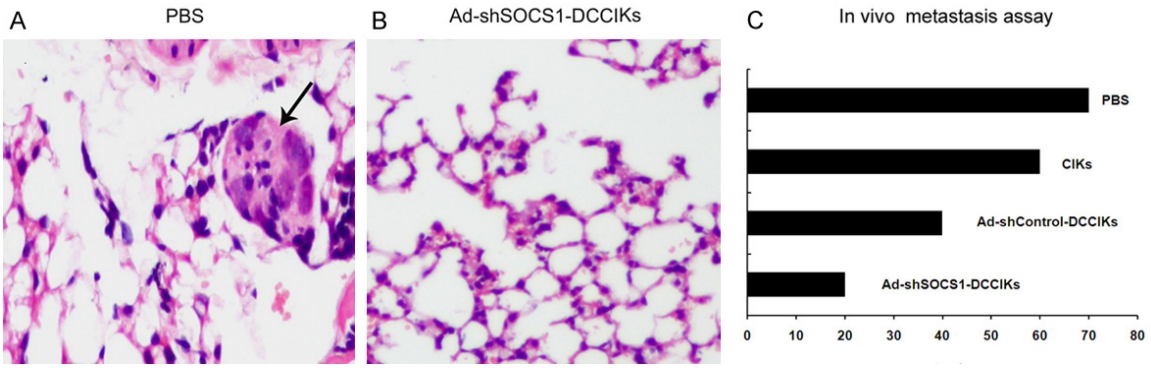


**Figure 6.** *In vivo* metastasis assay by heterotopic liver implantation: a subcutaneously formed tumor in 1-2 $mm^3$ cubes generated from SiHa cells was implanted into the left liver lobes in nude mice. After 9 days of tumor cube implantation, therapeutic treatments of PBS, CIKs, Ad-shSOCS1-DCCIKs, and Ad-shControl-DCCIKs with a total of $1.0\times10^7$ cells were administered intravenously into the tail of the mice, PBS served as control (10 mice in each group). A total of 4 consecutive injections every 5 days were administered with $2.5\times10^6$ cells (per dose). After 6 weeks, the mice were sacrificed for lung metastasis assay examined by sectioning and microscopy. A. Representative tumor lesion in the lung, indicated by an arrow. B. No tumor lesion in the lung. C. Reversion of lung metastasis. Ad-shSOCS1-DCCIKs and Ad-shControl-DCCIKs lymphocytes decreased the tumor metastasis from the liver to the lung in the heterotopic liver implantation model (20% and 40%, respectively), whereas a high metastasis rate (70%) was observed in the PBS control group. *P<0.01, analyzed by Student's t-test.

vate MHC restrictive cytotoxicity reinforced by antigen-pulsed DCs on targeted tumor cells.

The capacity of DCs to capture, process and present antigens varies in accordance with their differentiation/maturation stage and origin. In contrast to activated mature DCs which can induce protective anti-tumor immunity, immature DCs present self-antigens to T cells leading to immune tolerance by generating suppressor T cells or T cell deletion [14-16]. As activation of the full immunostimulatory talent of DCs to achieve an effective immunity shows potential against tumor, SOCS1, a negative signaling regulator of various cytokines was knocked down in DCs in this study. And the efficiency of SOCS1 silencing was verified. At MOI 100, SOCS1 was significantly knocked down in DCs (**Figure 1B**). The expression levels of the surface markers CD1a, CD80, CD83, CD86, and HLA-DR analyzed by flow cytometry were increased by 2-4 folds during maturation in both Ad-shSOCS1 and Ad-shControl infected and HPV16mE7 pulsed DCs with respect to control, but not in the absence of TNF-α stimulation (**Table 1**). Furthermore, the former increased about 10% more than the latter, indicating an increased maturation by silencing SOCS1. In addition, although TNF-α greatly induced the expression of these CD markers, it was lower than that of the Ad-shSOCS1, which implies that SOCS1 knock down enhances the effect of TNF-α on stimulating DCs maturation. This might be attributed to the increased NF-κB expression by silencing SOCS1, as well as promoting the efficacy of TNF-α on up-regulating NF-κB [23, 24]. SOCS1-restricted DCs also significantly enhanced the secretion of pro-inflammatory cytokines such as IL-12 and IL-6 (**Figure 2**).

The co-culture of CIKs with DCs can increase their proliferation activity and cytotoxicity [20]. As demonstrated in **Figure 3**, TNF-α and IFN-γ in the supernatants of the co-cultured Ad-shSOCS1-DCCIKs and Ad-shControl-DCCIKs were higher than those of CIKs. The $CD3^+CD8^+$ and $CD3^+CD56^+$ double positive cells in Ad-shSOCS1-DCCIKs and Ad-shControl-DCCIKs population were significantly higher than those of the CIKs group. Furthermore, the $CD3^+CD56^+$ double positive cells in the former were improved 42.5% than the latter, which indicates silencing SOCS1 improved the efficacy of DCs on irritating proliferation of CIKs. Moreover, the cytotoxicity of effector cells at 90:1, 30:1 and 10:1 E/T ratios revealed that Ad-shSOCS1-DCCIKs lymphocytes exhibited the highest cytotoxic activity against SiHa cells by CCK-8 assay. The cytotoxic activity against SiHa cells induced by Ad-shSOCS1-DCCIKs and Ad-shControl-DCCIKs was not linear, but it increased with E:T ratio compared to the linear increase of the control group, indicating a dosage-

dependent manner. The strengthened cytotoxic activity on tumor of DCCIKs may be associated with the ascending secretion of IFN-γ and IL-12 as well as increased $CD3^+CD56^+$ double positive cells in DCCIKs. And the increased IFN-γ and IL-12 in co-cultured DCCIKs further stimulated the CIK's proliferation [12-13].

To further investigate the antitumor effects mediated by the Ad-shSOCS1-DCCIKs, BALB/c-nude mice models xenografted with SiHa cells were developed. The tumor-loaded mice received injection of Ad-shSOCS1-DCCIKs and control T cells ($2.5\times10^6$ per dose) for a total of $1\times10^7$. Tumor volume curve demonstrated that Ad-shSOCS1-DCCIKs lymphocytes significantly decreased the tumor volume compared with CIKs and PBS controls in which progressive tumor growth was observed. Statistical analysis of the tumor growth curves revealed significant differences between the Ad-shSOCS1-DCCIKs group and the control group ($P< 0.05$). Although therapeutic Ad-shSOCS1-DCCIKs could not always eradicate the tumor completely, the survival rate was significantly enhanced (40%) at day of 60 compared with the mice treated with Ad-shControl-DCCIKs (0%), CIKs (0%) or PBS (0%). These results indicated the anti-HPV16 $E7^+$ tumor therapeutic potential of the HPV16mE7 pulsed and SOCS1 silenced Ad-shSOCS1-DCCIKs *in vivo*.

As the cure rate in patients with high-grade cervical cancer remains very low in part by the high recurrence rate, the antimetastatic potential of Ad-shSOCS1-DCCIKs on SiHa cells was assessed via heterotopic tumor metastasis assay from the liver to the lung. The result demonstrated Ad-shSOCS1-DCCIKs efficiently inhibited liver tumor metastasis *in vivo* in the mouse models.

In conclusion, compared with Ad-shControl-DCCIKs or CIKs, Ad-shSOCS1-DCCIKs showed prominent oncotherapy effect and powerful antimetastatic efficacy, presenting a favorable application prospect.

## Acknowledgements

This study was supported by the Knowledge Innovation Program of Shenzhen Innovation Committee (No. JCYJ20150402095058890), Health and Family Planning Commission of HuBei Province project (WJ2015XB019 2015-2016) and Research and Development project of Xiangyang City (2014).

## Disclosure of conflict of interest

None.

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