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Suppressor of cytokine signaling 1 (SOCS1) silencing and Hep-2 sensitizing dendritic cell vaccine in laryngocarcinoma immunotherapy

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Abstract. – OBJECTIVE: Many studies have recently suggested that dendritic cell (DC) vaccine contributes to the immunotherapy of various types of human tumors. It has been proved that the tumor antigen sensitizing and the gene silencing are effective methods for the preparation of the DC vaccines. The aim of this study is to investigate the specific anti-laryngocarcinoma immune response for the suppression of cytokine signaling1 (SOCS1) silencing and Hep-2 sensitizing DC.

MATERIALS AND METHODS: The dendritic cells derived from peripheral blood mononuclear cells were induced by cytokines GM-CSF, IL-4, and TNF-α in vitro, and the morphological characteristics of dendritic cells were observed under a microscope, indicating that they successfully differentiated into dendritic cells. The RNA interference vector was used to transfect dendritic cells. The expression of SOCS1 was detected by Western blot and the effective target sequence for inhibiting the expression of SOCS1 was screened. The expressions of CD83, CD86, and HLA-DR on dendritic cells were detected by flow cytometry. The content of IFN-γ in the supernatant was analyzed by enzyme-linked immunosorbent assay (ELISA). Methyl thiazolyl tetrazolium (MTT) was used to evaluate the ability of dendritic cells to stimulate T cell proliferation and induce the killing activity of cytotoxic T cells.

RESULTS: The result of PCR and Western blot analysis shows that the expression of SOCS1 significantly decreased under the influence of the 5th interference sequence. The flow cytometric analysis results show that SOCS1 silencing and Hep-2 sensitizing dendritic cells had high expressions of CD83 (85.61±0.96)%, CD86 (96.86±1.20)%, and HLA-DR (98.02±0.94)%. The DC vaccine could increase the production of IFN-γ according to the ELISA assay results. The MTT assay results show that the DC vaccine could also stimulate the proliferation of the T cells and effectively and eventually enhance the specific killing effect of CTL.

CONCLUSIONS: SOCS1 silencing and Hep-2 sensitizing DC vaccine could induce an effective and specific anti-laryngocarcinoma immune response.

Key Words:
Suppressor of cytokine signaling 1, Dendritic cell, Laryngocarcinoma, Immunotherapy.

Introduction

Laryngocarcinoma, a common malignance[1], has been arousing increasing morbidity around the world recently. Among all the pathogenic factors, the squamous cell carcinoma is the most common type, accounting for 95% to 99% of all laryngeal malignance[2]. More than 500,000 patients in the world develop head and neck squamous cell carcinoma (HNSCC) every year and more than half a million new cases of HNSCC were projected to occur in 2020. On the one hand, the current clinical treatment of the laryngocarcinoma is still surgery combined with radiotherapy and chemotherapy[3-5]. But it has two weaknesses, the easy recurrence or metastasis that cause radiotherapy and chemotherapy ineffectiveness, and the damage of the immune system and organs function in the long term[6,7]. On the other hand, due to its atypical early manifestations, laryngo-

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carcinoma used to be diagnosed at an advanced stage with evident symptoms, such as enlarged cervical lymph nodes, dyspnea and hemoptysis, which brought great difficulties for clinical treatments[8,9]. In conclusion, it is necessary to develop a new treatment to improve long-term survival for patients with laryngeal squamous cell carcinoma (LSCC)[10].

The dendritic cell (DC) functions as the most potent professional antigen-presenting cell, and displays an extraordinary capacity to induce, sustain, and regulate the immune responses. The DC vaccine is considered as the most potential tumor immunotherapy[11,12]. It plays a pivotal role in the induction of both immunity and tolerance, which is specialized in antigen-presenting. The DC's ability to initiate the immune responses or to induce the immune tolerance is strictly dependent on its maturation state, which is considered to be a key determinant for the outcome of the T cell activation[13,14]. The mature DCs, which express high levels of major histocompatibility complex (MHC) and costimulatory molecules on their surface, can induce an immune response. Many methods of the DC vaccine preparation have tried to enhance its antitumor capacity, such as DC fused with tumor cells, DC pulsed by tumor RNA, DC sensitized with tumor antigen, and gene transfection[15]. But most attempts failed because its negative feedback mechanism limits its antigen-presenting capacity[16].

The suppressor of cytokine signaling 1 (SOCS1) serves as negatively regulated signaling of various cytokines, such as IFN-γ, IL-2, IL-6, IL-12, and IL-15, by inhibiting the Janus kinases (JAKs) in the T cells and other immune cells[15]. It influences the regulation of the DC activation, development, and differentiation and plays a pivotal role in the regulation of the functions[17]. Moreover, SOCS1 has been shown to be a regulator of cytokine signal transduction in various cell types and a feedback inhibitor of the JAK/STAT signaling pathway to modulate the antigen presentation of DCs[18,19]. The SOCS1-modified DC could up-regulate the intensity and duration of the antigen-specific T cell immune response. It has been demonstrated that SOCS1 plays a pivotal role in the regulation of the activation, development, and differentiation of DC, by participating in the regulation of DC maturation and function. It is a genetic engineering target to manipulate DC[16,20]. Therefore, silencing SOCS1 in DC is beneficial for tumor immunotherapy.

In this study, we silenced SOCS1 with the RNA interference (RNAi) technique and sensitized DC with Hep-2 antigen to induce highly effective and specific anti-laryngocarcinoma immune responses. It may be proved to be an innovative tumor vaccine and a novel idea for the clinical applications of laryngocarcinoma therapy.

Materials and Methods

Experimental Materials

Restriction enzyme Age I, EcoR I, and ELISA kit were purchased from New England BioLabs (BioLabs Inc., Ipswich, MA, USA). Recombinant human granulocyte macrophage colony stimulating factor (GMCSF), IL-4, and TNF-α were purchased from PeproTech (PeproTech EC Ltd., London, UK). The rabbit polyclonal to SOCS1 was purchased from Abcam (Abcam Ltd., Cambridge, UK). FITC anti-human CD83, PE anti-human CD86, and PE anti-human HLA-DR were purchased from BD Bioscience (Franklin Lakes, NJ, USA). The Methyl Thiazolyl Tetrazolium (MTT) Cell Cytotoxicity assay kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The laryngeal squamous carcinoma cell line (Hep-2) was kindly provided by the Chinese PLA General Hospital. The peripheral blood was provided by the Weifang Central Blood Bank (Weifang, China).

DCs Differentiation In Vitro

The peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by Ficoll-Hypaque density gradient centrifugation. The cells were seeded into 12-well plates at a density of 1×10^7 cells/mL and cultured in Roswell Park Memorial Institute-1640 (RPMI1640) containing 5% fetal bovine serum (FBS). The cells were incubated at 37°C for 2 h, and the suspended cells were kept for subsequent T cells isolation. Then, the adherent cells were cultured with DC medium (RPMI1640 containing 10% FBS, 50 ng/mL of GM-CSF, and 20 ng/mL of IL4). The non-adherent PBMC fractions were harvested and frozen at –80°C. On the 5th day, other 100 ng of TNFα was added to DCs to induce maturation. Finally, the morphological feature of DCs was observed with an inverted microscope every day. The DCs were harvested on the 7th day.

Design of SiRNA and Construction of RNAi Vector

The selection of siRNA sequences against human SOCS1 (RefSeq ID: NM_003745) was ob-

tained by the web-based program and was optimized by referring to published articles[21,22]. The specific targeted sequences include the following: sequence 1, CTCGCACCTCCTACCTCTT; sequence 2, CCTCCTACCTCTTCATGTT; sequence 3, CCTCTTCATGTTTACATAT; sequence 4, GGTTGTTGTAGCAGCTTAA; sequence 5, CCTTCCTCCTCTTCCTCCT; sequence 6, GGCCAGAACCTTCCTCCTCTT, and a negative control (NC) sequence, TTCTCCGAACGTGTCAC. All those sequences were analyzed by the Basic Local Alignment Search Tool (BLAST) to ensure that there would be no significant homologies with other genes. Two complementary oligonucleotides were synthesized by DENECHEM, among which the annealed siRNA was ligated into the enzyme cutting sites of the RNAi vector. The plasmid was identified by PCR.

Western Blot Analysis

According to different processing factors, the DCs were divided into nine groups as following: SOCS1-siRNA-1, SOCS1-siRNA-2, SOCS1-siRNA-3, SOCS1-siRNA-4, SOCS1-siRNA-5, SOCS1-siRNA-6, NC-siRNA, Mock, and Blank. The cell transfection was in accordance with the manufacturer's instructions. The DCs were collected and washed twice with ice-cold PBS 72 h later. The protein concentrations were determined via Bicinchoninic Acid Assay (BCA). The primary antibody was diluted to 1:2000 and the secondary antibody was diluted to 1:5000. A human β-actin probe was used as an internal control. The antibodies were visualized using the enhanced enhanced chemiluminescence (ECL) kit.

Preparation for Hep-2 Antigen

The LSCC cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% FBS and collected at logarithmic period. The concentration of the cells was adjusted to 1×10^7 cells/mL. The freeze-thaw (–80°C/37°C) was repeated three times. After centrifugation for 30 min, the tumor lysate was harvested as Hep-2 antigen.

Grouping

The DCs were not cultured until the 3rd day according to the above process and were into three groups. Group A was transfected with SOCS1-siRNA-5 and sensitized with Hep-2 antigen on day 5. Group B was sensitized with Hep-2 antigen on the 5th day. Group C was the control group which was untreated. All those groups were harvested 48 h later.

Flow Cytometric Analysis

To assess the surface phenotypes of the three groups, all cells were harvested on the 7th day and incubated with the following antibodies: FITC-CD83, PE-CD86, and PE-HLA-DR, respectively. The appropriate isotype controls and all these three groups were stained for 30 min at 4°C, washed twice in phosphate-buffered saline (PBS), and then resuspended in 1% paraformaldehyde. All samples were kept at 4°C before the flow cytometric analysis.

Enzyme-Linked Immunosorbent Assay

The standard wells, the blank wells, and the testing sample wells were set. The IFN-γ levels were assayed using enzyme-linked immunosorbent assay (ELISA) kits according to the instructions and we took the blank well as zero. The absorbance at 450 nm of each well was measured. The standard curve was drawn according to the sample absorbance value, and the straight-line regression equation was calculated by excel. The IFN-γ levels of the different groups were calculated.

Proliferation Assay

We recovered the freeze-stored cells and separated the T cells as effect cells (E) by Nylon column. The three group cells were harvested on the 7th day and processed with mitomycin C as stimulating cells (S). S:E=1:10. The cells were incubated at 37°C for 72 h. The MTT 10 µl and Formazan 100 µl were added to each well, and the absorbance at 570 nm was measured by the ELISA Microplate Reader 8 h later.

Cytotoxicity Detection

The CTLs induced by the DCs were used as effector cells (E) and LSCC cells as target cells (T). The effector cells and target cells were mixed at ratios of 50:1, 25:1, and 10:1, respectively. The MTT Cell Cytotoxicity assay kit was used to measure the cytotoxicity of CTL. The formula was as follows: the killing rate = [(E+T-Mix) / T] ×100%.

Statistical Analysis

The Statistical Product and Service Solutions SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was applied for data analysis. All results were expressed as mean ± SEM. The differences among the groups were analyzed with One-way analysis of variance (ANOVA) followed by the Least Significant Difference (LSD). A value of $p<0.05$ was considered significantly different.

Results

Morphologic Appearance

The morphologic appearance of the cells was round and scattered. On the 3rd day, the DC colony emerged, and the volume of the cells enlarged. On the 5th day, DC's surface arose many protuberant *microvilli*. The dendritic synapses of some cells were bigger on the 7th day, while the DC volume further increased and became irregular with the slender synapses on their surface (Figure 1).

Identification of RNAi vector

The RNAi vector was digested by restriction endonucleases Age I and EcoR I and confirmed

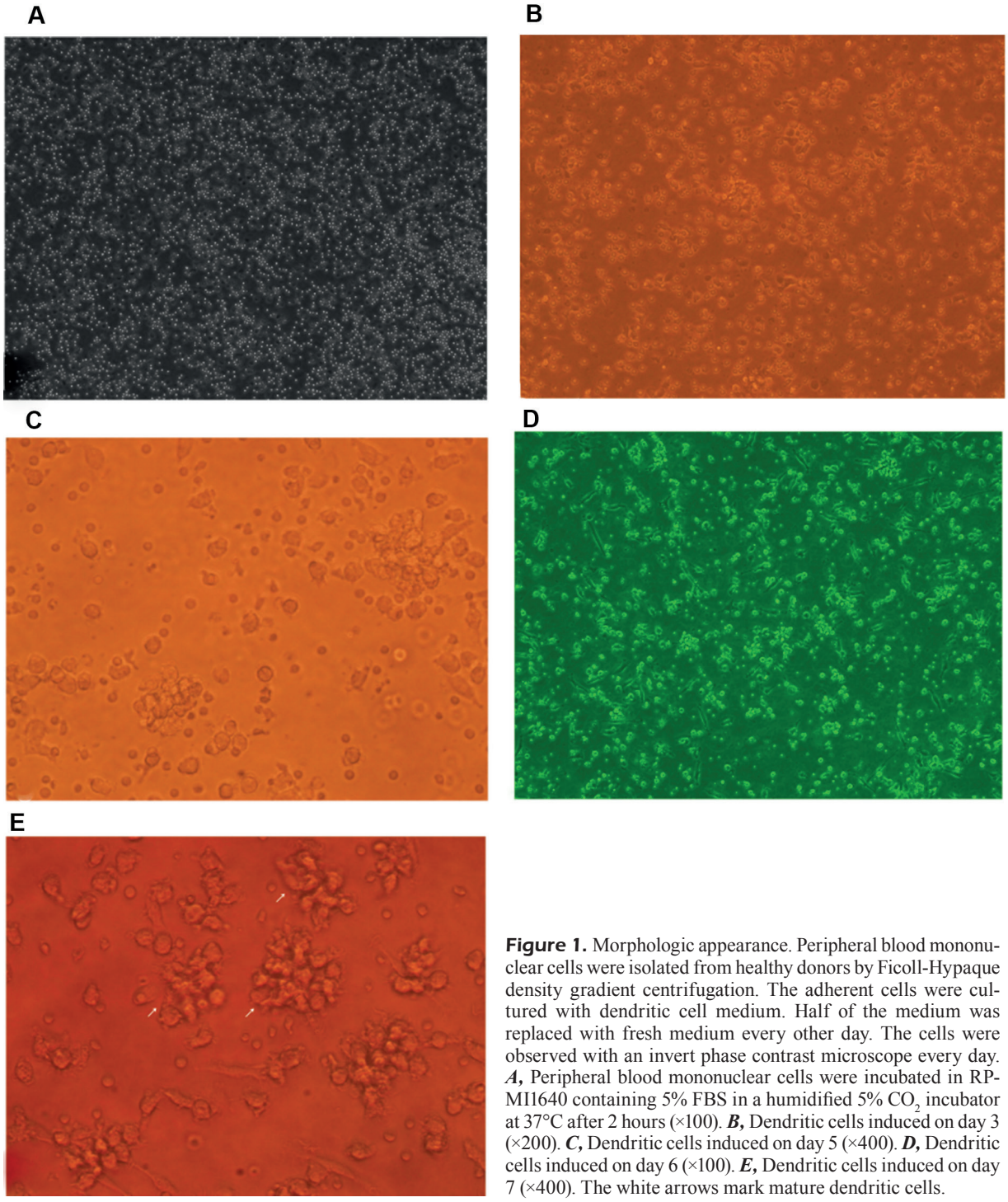

Figure 1. Morphologic appearance. Peripheral blood mononuclear cells were isolated from healthy donors by Ficoll-Hypaque density gradient centrifugation. The adherent cells were cultured with dendritic cell medium. Half of the medium was replaced with fresh medium every other day. The cells were observed with an invert phase contrast microscope every day. ***A,*** Peripheral blood mononuclear cells were incubated in RPMI1640 containing 5% FBS in a humidified 5% CO_2 incubator at 37°C after 2 hours (×100). ***B,*** Dendritic cells induced on day 3 (×200). ***C,*** Dendritic cells induced on day 5 (×400). ***D,*** Dendritic cells induced on day 6 (×100). ***E,*** Dendritic cells induced on day 7 (×400). The white arrows mark mature dendritic cells.

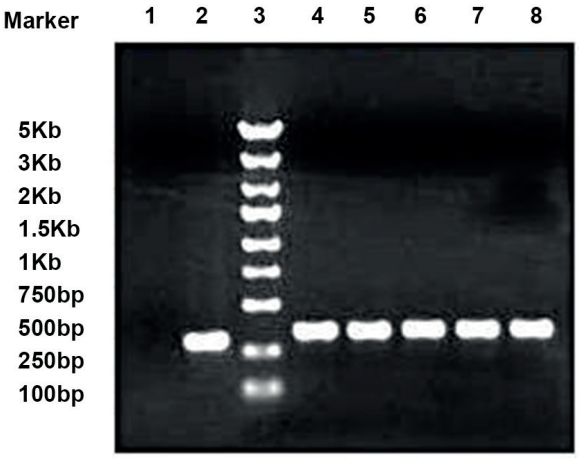

Figure 2. PCR result of RNA interference vector. The selections of siRNA sequences against human SOCS1 were analyzed by BLAST to ensure that there were no significant homologies with other genes. RNA interference vector was digested with restriction endonucleases Age I and EcoR I. Then, The RNA interference vector was identified by PCR. Line 1: Negative control (ddH_2O); line 2: Negative control (mock) was located in 307 bp; line 3: Marker; line 4 - 8: SOCS1-siRNA vectors were located in 366 bp, which were upper than the mock and between 250 bp to 500 bp.

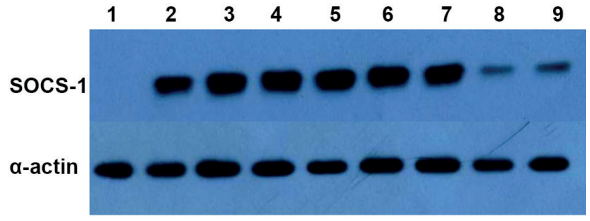

Figure 3. Expression of SOCS1 in dendritic cells. The protein levels of SOCS1 and β-actin were determined by Western blot. Marker band in protein of 24KD corresponded to SOCS1 band. Band 1: blank; band 2: mock; band 3: negative control-siRNA; band 4: SOCS1-siRNA-1; band 5: SOCS1-siRNA-2; band 6: SOCS1-siRNA-3; band 7: SOCS1-siRNA-4; band 8: SOCS1-siRNA-5; band 9: SOCS1-siRNA-6. The experimental results revealed that the 5th interference sequence (band 8) could effectively down-regulate the SOCS1 expression.

to be corrected by PCR. The results matched with expectations (Figure 2).

Expression of SOCS1 in DCs

The marker band in the protein of 24KD corresponded to SOCS1 band (Figure 3). The expression of SOCS1 significantly decreased compared with those in the negative controls (band 2 and band 3), especially under the influence of the 5th interference sequence (band 8).

Expression of Cell Surface Markers on DC

The analysis of the characteristic molecules on the surface of DC shows a high expression of HLA-DR (80.54±0.82)%, as well as the expression of mature mark molecules CD83 (13.00±1.55)%, and costimulatory molecules CD86 (78.71±1.36)%. The DC sensitized with Hep-2 antigen shows an increase of all test surface molecules [CD83: (29.43±0.87)%; CD86: (80.54±0.54)%; HLA-DR: (94.06±0.65)%]. The expressions of CD83, CD86, and HLA-DR on the mature DCs significantly increased after silencing of SOCS1 by siRNA, compared with those sensitized only with Hep-2 antigen (Figure 4).

IFN-γ Quantitative Detection

The standard curve (Figure 5) was drawn according to the sample absorbance value. Then, we calculated the IFN-γ concentration of different groups. The numerical value was as follows: group A: 259.448±23.146 ng/L; group B: 107.261±11.781 ng/L; group C: 56.599±2.964 ng/L. The IFN-γ secretion levels of cells in group A significantly rose. There was a statistical difference between these four groups ($p<0.01$).

The Proliferation of T Cells Stimulated by DC

The capacity of stimulating the T cell proliferation by DC in group A is the strongest (70.5%±4.8%), followed by group B (42.7%±4.8%), and group C (16.0%±4.1%). There was a statistical difference between these three groups ($p<0.01$) (Figure 6A).

The Cytotoxic Effect of CTL Induced by DC

The CTLs induced by DCs in group A expressed significantly higher specific killing effects than others when E:T is at the ratio of 50:1 ($p<0.01$). According to the results, the killing rate increased with the increase of the effect cells (Figure 6B).

Discussion

RNAi was discovered in the late 20th century[23]. It could efficiently block the gene expression by specifically inducing homologous complementary mRNA degradation in the cell through double-stranded RNA, therefore causing gene silence[24]. This technique is supplied to the functional analysis of the endogenous genes and cure of intractable genetic diseases by sequence-specific

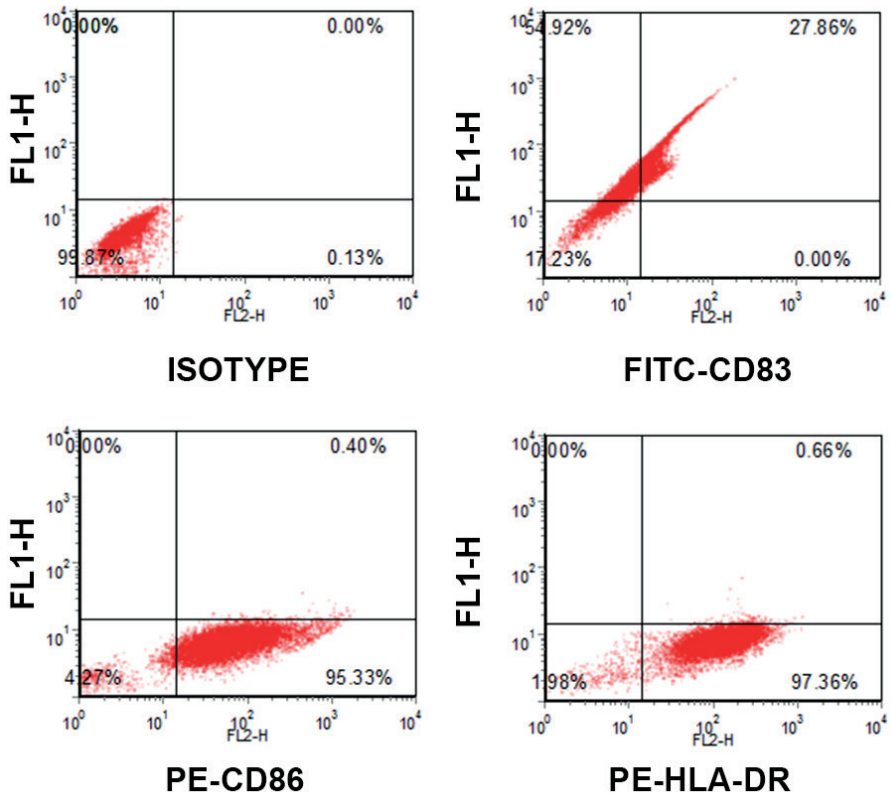

Figure 4. Expression of cell surface markers on dendritic cell. The above figure shows the percentage of cell surface antigen expression tested via flow cytometric analysis. Expressions of FITC-CD83 (85.61±0.96), PE-CD86 (96.86±1.20), and PE-HLA-DR (98.02±0.94) on mature dendritic cells significantly increased after SOCS1 silencing by siRNA. This finding demonstrated that SOCS1 silencing greatly promoted the dendritic cells maturation.

cleavage of mRNA[25-27]. Its advantages include high specificity, high efficiency, high stability, and low cytotoxicity, which makes it a very promising supplement to tumor biotherapy. It has successfully blocked a variety of gene expressions and reversed the progress of many diseases so far. It is necessary to verify the silencing effect of all designed siRNA sequences to get the effective siRNA sequence because of the complex secondary structure and protein binding domain of mRNA[28]. This is an extremely important step. In this research, the selection of siRNA sequences against human SOCS1 (RefSeq ID: NM_003745) refers to design the principles of RNAi[29]. We finally designed six target sequences and a negative sequence as the control group. All sequences were analyzed by BLAST to ensure that there was no significant homology with other genes.

It is proved that DC sensitized with tumor antigen could stimulate the T cell proliferation and activation, as well as induce effective and specific CTL, thus inhibiting the occurrence of tumors[15,30]. It cannot obtain and identify all laryngocarcinoma antigens. Therefore, we used the whole-cell antigen in order to maximize the use of all Hep-2 antigens. The LSCC cells were cultured in DMEM containing 5% FBS and collected in the logarithmic phase. The freeze-thaw (–80°C/37°C) was repeated for three times. This method effectively avoided the difficulty of identifying the specific tumor antigen. Also, it could stimulate cellular immunity against multiple tumor-associated antigens and induce multiple antitumor immune responses[31]. Our results suggested that sensitizing DC with tumor lysate of laryngocarcinoma cells could improve DC maturity, enhance the efficiency of presenting antigen, and led to a significantly increased induction of CTLs against laryngocarcinoma cells.

The general idea of the DC vaccine design was to enhance the specific anti-tumor capacity of the immune system, improve immune microenvironment, induce a strong specific anti-tumor cellular immune response, and finally cure tu-

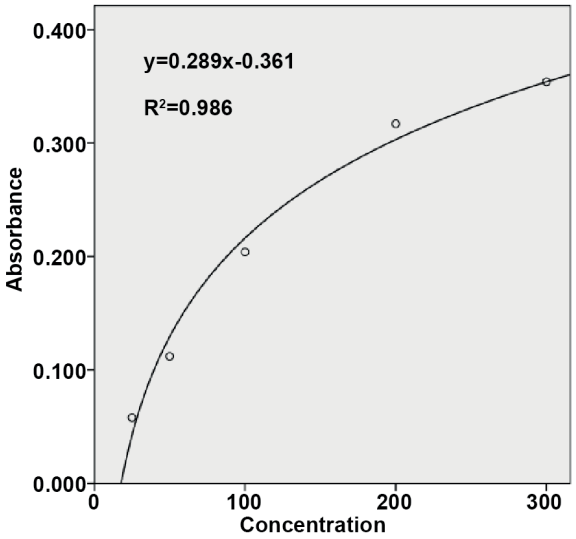

Figure 5. The standard curve of IFN-γ. Taken the standard density as the horizontal (standard density: 300 ng/L, 200 ng/L, 100 ng/L, 50 ng/L, 25 ng/L), the OD value for the vertical (OD value: 0.354, 0.317. 0.204, 0.112, 0.058), drew the standard curve on the paper. A curve was drawn based on the measured absorbance of the standard. The concentration and the OD value (absorbance value) were used to obtain a linear regression equation of the curve using Excel software to calculate the amount of IFN-γ expressed in each group of cells. The sample concentration = the sample OD value × the dilution coefficient.

mor[32]. In this work, we proposed a new idea in the preparation of the DC vaccine based on the above analysis. We silenced SOCS1 with siRNA to improve the DC's capacity of antigen presentation and then sensitized SOCS1-silencing DC vaccine with Hep-2 antigen to stimulate an effective specific anti-laryngocarcinoma immune response. The experimental results reveal that the 5th interference sequence could effectively down-regulate SOCS1 expression. Therefore, we chose this sequence to conduct the follow-up experiments. The SOCS1-silencing DC shows a high expression of surface markers according to flow cytometry results. This demonstrates that SOCS1 silencing greatly promoted the DC maturation. We used cytotoxicity detection to study the killing efficacy of the DC vaccine against the LSCC cells. The MTT result showed that the cells in group A had the highest killing rate and group C had the lowest. It also indicated that the raise of the killing effect came along with the growing up of E:T. The results showed that our DC vaccine promoted the Th1 development in the T cells polarization and enhanced the specific killing capacity of CTL against the LSCC cells. As Shen et al[31] reported, SOCS1 the modified DC could enhance antigen-specific antineoplastic immunity. Compared with the control, the above treatment could induce CD8^{+}T cells' proliferation, and strengthen the immune response to LPS and IFN-γ.

Conclusions

We showed that SOCS1 silencing significantly promotes the antigen presenting capacity of DC. The sensitization by Hep-2 antigen remarkably enhances the antitumor response of CTL. Thus,

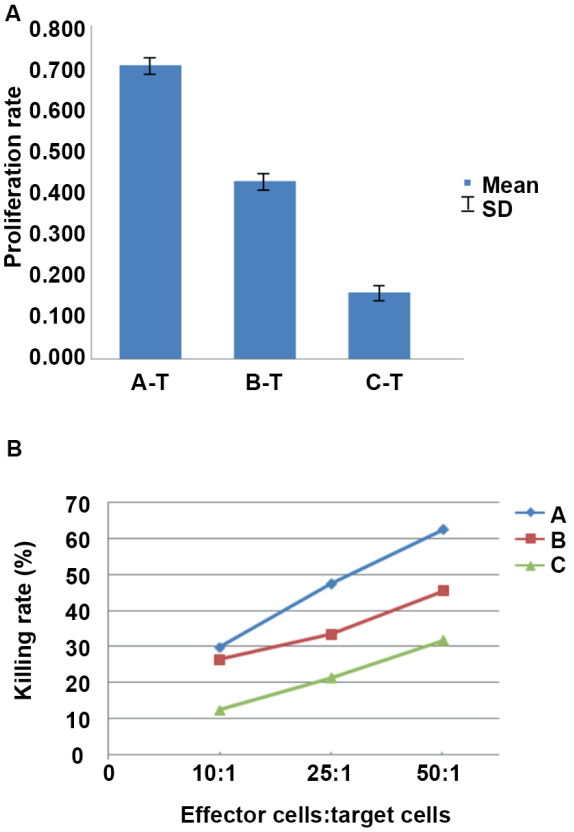

Figure 6. The proliferation of the T cells stimulated by the dendritic cells. ***A,*** Assess the dendritic cells' capacity of stimulating T cells proliferation, and each group of dendritic cells was collected at concentration of 1×10^{5}/ml and co-cultured with T cells at the concentration of 1×10^{6}/ml in 96-well culture plate. The proliferation of T cells was detected by MTT (p <0.01, group A –T *vs.* group C-T, n=6.). ***B,*** Each group of CTLs as effect cells, as well as LSCC cells as target cells, was co-cultured for 72 h at the ratios of 50:1, 25:1, and 10:1. Each group was set to triple wells. This procedure was repeated twice. The cytotoxic effect of CTLs against LSCC cells induced by dendritic cells was measured respectively. (p<0.01, 50:1 *vs.* 25:1, 25:1 *vs.* 10:1, n=6).

an appropriate combination of these two methods is a promising new approach. Our DC vaccine is expected to play an effective role in laryngocarcinoma immunotherapy, thereby reducing the agony caused by surgery, chemotherapy, and radiotherapy for patients and improve the survival rate of patients with advanced laryngocarcinoma.

Conflict of interest

The authors declare no conflicts of interest.

Funding

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