In vitro induction of anti-lung cancer immune response by the A549 lung cancer stem cell lysate-sensitized dendritic cell vaccine

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Abstract. Lung adenocarcinoma is one of the most fatal types of cancer worldwide, with non-small cell lung cancer being the most common subtype. Therefore, there is need for improved treatment approaches. Tumor growth results from the proliferation of a very small number of tumor stem cells, giving rise to the theory of cancer stem cells (CSCs). Lung CSCs are associated with lung cancer development, and although chemotherapy drugs can inhibit the proliferation of lung cancer cells, they have difficulty acting on lung CSCs. Even if the tumor appears to have disappeared after chemotherapy, the presence of a small number of residual tumor stem cells can lead to cancer recurrence and metastasis. Hence, targeting and eliminating lung CSCs is of significant therapeutic importance. In this study, we cultured A549 cells in sphere-forming conditions using B27, EGF, and bFGF, isolated peripheral blood mononuclear cells (PBMCs), and induced and characterized dendritic cells (DCs). We also isolated and expanded T lymphocytes. DC vaccines were prepared using A549 stem cell lysate or A549 cell lysate for sensitization and compared with non-sensitized DC vaccines. The content of IFN-γ in the supernatant of cultures with vaccines and T cells was measured by ELISA. The cytotoxic effects of the vaccines on A549 cells and stem cells were assessed using the Cytotox96 assay, and the impact of the vaccines on A549 cell migration and apoptosis was evaluated using Transwell assays and flow cytometry. DC vaccines sensitized with human lung CSC lysates induced significant *in vitro* cytotoxic effects on A549 lung cancer cells and CSCs by T lymphocytes, while not producing immune cytotoxic effects on human airway epithelial cells. Moreover, the immune-killing effect induced by DC vaccines sensitized with lung CSC lysates was superior to that of DC vaccines sensitized with lung cancer cells.

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Introduction

Cancer stem cells (CSCs) are a small subset of cells within tumors that possess stem-like properties, including self-renewal capabilities, and serve as a source for both differentiated tumor cells and continued tumor expansion (1). Under different selection pressures, CSCs differentiate into various functional directions, contributing to tumor cell collective migration and heterogeneity. CSCs can enter a quiescent state *in vivo*, exhibiting minimal proliferation (2).

CSCs typically express common stem cell markers, such as CD133, octamer-binding transcription factor 4, sex determining region Y-box 2 (Sox2) and ATP-binding cassette sub-family G member 2 (ABCG2), which regulate self-renewal and differentiation (3), potentially influencing their biological characteristics, including interactions within the immune system (4). Additionally, CSCs often exhibit low levels of major histocompatibility complex (MHC) class I molecules, reducing their recognition by $CD8^+$ T cells (5). CSCs also possess immunoregulatory functions, modulating the activities of T cells, B cells and natural killer (NK) cells, thereby suppressing immune responses (6). Collectively, these features enable CSCs to evade immune surveillance, maintaining their presence and functionality within the body.

CSCs interact with epithelial-mesenchymal transition (EMT), IL-4 signaling, drug efflux proteins and aldehyde dehydrogenase (ALDH), which collectively contribute to CSC maintenance and drug resistance, impacting tumor development and treatment efficacy (7). The EMT is a biological process through which tumor cells transition from epithelial to mesenchymal states, enhancing migration, invasion and drug resistance (7). This transformation provides tumor cells with stem cell-like characteristics, increasing resistance to therapy (7,8). IL-4 signaling promotes CSC proliferation and survival, reducing sensitivity to therapeutic drugs (9). The IL-4 signaling pathway serves a critical role in modulating the tumor microenvironment, influencing tumor cell survival and treatment responses (9). Drug efflux proteins, including

membrane transporters, actively pump drugs out of cells, reducing drug accumulation and efficacy, therefore contributing to tumor cell resistance (10). The elevated activity of ALDH is also associated with CSC drug resistance, as ALDH enzymes facilitate the detoxification of chemotherapeutic agents within tumor cells, thereby diminishing their cytotoxic effects (11). Thus, these factors collectively contribute to CSC resistance to conventional therapies, posing significant challenges in treatment. Eliminating CSCs is crucial for complete eradication of tumors, making them a promising target for cancer therapy (12,13).

Targeting and eliminating lung CSCs holds therapeutic promise. The immune system can recognize tumor-specific peptides or neoantigen fragments, inducing cytotoxic responses against malignant cells (14). In a preclinical model of lung cancer, researchers at the University of Cincinnati Cancer Centre (Cincinnati, OH, USA) isolated and cultured lung CSCs, facilitating the development of immune-based therapies targeting these cells (15).

Cancer immunotherapy aims to elicit or reinvigorate cellular immune responses, particularly T cell-mediated cytotoxicity against tumor-specific antigens and tumor-associated antigens (TAAs), selectively targeting and destroying tumors (16). Among immunotherapies, immune checkpoint inhibitors (ICIs) have revolutionized the treatment landscape for non-small cell lung cancer (NSCLC); however, only 20% of patients with NSCLC exhibit durable responses to ICIs, highlighting the need for alternative approaches (17).

Dendritic cell (DC) vaccines for treating tumors have emerged as promising biological therapies (18). DCs are the most potent known antigen-presenting cells (APCs) capable of activating naïve T lymphocytes, which have a central role in initiating and regulating innate and adaptive immune responses (18). As specialized APCs, DCs serve crucial roles in initiating and regulating both cellular and humoral immune responses, interacting with various cells of the innate immune system, including NK cells, macrophages and mast cells (19). Furthermore, DCs interact with B lymphocytes to indirectly promote the proliferation and differentiation of $CD4^+$ T helper cells, playing a significant role in regulating humoral immunity (20). Due to their pivotal role in initiating immune responses, DCs are essential for antigen presentation and vaccine strategies in cancer treatment.

Evidence has indicated that DC vaccines exhibit efficacy against various types of cancer, such as gallbladder (21), prostate (22), gastrointestinal (23), oral (24), pancreatic (25) and breast cancer (26), malignant glioma (27) and ovarian cancer (28), although complete tumor eradication remains elusive, partly due to immune evasion mechanisms involving CSCs (6). Therefore, strategies targeting CSCs with DC vaccines to reduce immune evasion have practical significance. Traditional DC vaccines often co-culture DCs with autologous or allogeneic tumor cell lysates, potentially leaving stem cells untouched, thereby posing the risk of recurrence (21). Hence, there is an urgent need for novel therapeutic approaches with sustained responses. The present study explored the induction of anti-lung cancer immune responses through A549 lung CSC lysate-sensitized DC vaccines *in vitro*, aiming to advance vaccine-based therapies for lung cancer.

Materials and methods

Cell culture. The A549 (cat. no. CL-0016) and 16HBE cell lines (cat. no. CL-0249) were purchased from Procell Life Science & Technology Co., Ltd. A549 lung cancer and 16HBE cells were routinely cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; both Gibco; Thermo Fisher Scientific, Inc.). 100 μg/ml streptomycin, and 100 U/ml penicillin sodium (Beijing Solarbio Science & Technology Co., Ltd.). The cells were cultured in an incubator at 37˚C and 5% CO_2.Upon reaching confluence (100%), the culture medium was discarded, and the cells were washed twice with PBS and then dissociated with trypsin. The trypsin digestion was terminated by adding an equal volume of complete culture medium. After centrifugation (10,000 x g for 10 min at 4˚C), cells were washed once with PBS, the supernatant was discarded and cells were resuspended in 5 ml culture medium. Gentle pipetting was used to obtain a single-cell suspension, which was then seeded at a density of 20,000 cells/well in low-attachment culture dishes. The culture medium (serum-free medium) used was DMEM/F12 supplemented with B27 [Absin (Shanghai) Biotechnology Co., Ltd.], EGF [Epidermal Growth Factor; Absin (Shanghai) Biotechnology Co., Ltd.] and bFGF [basic Fibroblast Growth Factor; Absin (Shanghai) Biotechnology Co., Ltd.]. Third-generation cell spheres (spherical structures formed spontaneously in a growth factor-containing medium). were collected and SP (Side Population) cell subpopulations were sorted using a flow cytometer (CytoFLEX SRT; Beckman Coulter International Trading Co., Ltd.). In the CytExpert for CytoFLEX SRT software (version CytExpert SRT 1.0; Beckman Coulter). A single-cell suspension was prepared at a concentration of $1x10^6$ cells/l. Hoechst 33342 dye was added at a concentration of 5 μg/ml (Beijing Solarbio Science & Technology Co., Ltd.). The cells were incubated for 90 min at 37˚C in the dark. For the control group, verapamil was added at a concentration of 100 mM (Beijing Solarbio Science & Technology Co., Ltd.). A flow cytometer was used to sort SP (side population) cell subpopulations and non-SP cell subpopulations at an excitation wavelength of 355 nm. The sorted SP cell subpopulation was prepared as a $1x10^6$ cells/ml single-cell suspension and was cultured in serum-free conditions to form spheres (29).

Western blotting. The induced mature A549 lung CSCs were removed from the incubator and the cell culture suspension was aspirated into a 15-ml centrifuge tube. The cell suspension was washed twice with PBS and centrifuged at 4˚C and 10,000 x g for 10 min. The supernatant was discarded and A549 CSCs and A549 cells were lysed with RIPA lysis buffer (Shanghai Beibo Biotechnology Co., Ltd.). Total protein concentration was quantified using a BCA protein assay kit (Beyotime Institute of Biotechnology). Protein samples (20 μg/lane) underwent SDS-PAGE on a 10% gel) and the transfer of separated proteins to nitrocellulose membranes. The membranes were then blocked with Protein-Free Rapid Blocking Buffer (1x) (Shanghai Yamei Biotechnology Co., Ltd.) for 20 min at 4˚C, then incubated overnight at 4˚C with primary monoclonal antibodies against CD133 (1:1,000; cat. no. 48082S; CST Biological Reagents Co., Ltd.), ABCG2 (1:1,000; cat. no. 42078T; CST Biological Reagents Co., Ltd.), Sox2 (1:1,000; cat. no. 5067S; CST Biological Reagents Co., Ltd.) and β-actin (1:3,000; cat. no. ab8226; Abcam). The

membranes were washed three times with TBS-0.1% Tween 20 (TBST) for 10 min each and were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000; cat. nos. SA00001-1 and SA00001-2; Proteintech Group, Inc.) at room temperature for 1 h. The membranes were then washed with TBST and incubated with ECL reagent (Beyotime Institute of Biotechnology). The protein bands were analyzed using Image Lab analysis software (version 4.0; Bio-Rad Laboratories, Inc.). The experiments were performed in triplicate.

In vitro induction and culture of human DCs. The use of all patient samples in the present study was approved by The Ethics Committee of The Second Affiliated Hospital of Nanchang University [Nanchang, China; approval no. O-Medical Research Ethics Approval (2023) no. 89], and all participants provided written consent to participate.

A total of nine healthy volunteers (age, 18 and 60 years, with an average age of 36.6. The sex ratio is 5:4 (male to female) were recruited. Peripheral blood was collected with fresh anticoagulant at Department of Pulmonary and Critical Care Medicine, The Second Affiliated Hospital of Nanchang University, and peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte separation fluid (Cytiva). Blood collection occurred in December 2023, January 2024 and February 2024. The specific steps are as follows: Peripheral blood was divided equally into 50 ml centrifuge tubes, with 15 ml blood/tube. PBS was added (15 ml, and the contents were mixed well. The diluted blood sample was then transferred, in a volume of 15 ml, on top of 15 ml of lymphocyte separation fluid in four additional 50 ml centrifuge tubes. The tubes were centrifuged at 1,200 g for 30 min at 25˚C. The white mist-like membrane from the second layer was extracted and transferred into a 15 ml centrifuge tube. Three times the volume of PBS was added to the tube, and the contents were mixed well before centrifuging at 500 g for 10 min at 25˚C. Supernatant was removed, the white precipitate was collected, and it was washed twice with PBS. The cell concentration was then adjusted to 1x10^7 cells/ml using RPMI-1640 medium supplemented with 10% inactivated fetal bovine serum. PBMCs were cultured in an incubator for 2 h at 37˚C and 5% CO_2, non-adherent cells were removed through aspiration and adherent cells were gently washed once with culture medium. At this stage, adherent PBMCs were obtained. The cells were cultured in an incubator at 37˚C and 5% CO_2, Each well was supplemented with RPMI-1640 cell culture medium (Gibco; Thermo Fisher Scientific, Inc.), containing recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; Gibco; Thermo Fisher Scientific, Inc.) and recombinant human interleukin-4 (IL-4; Gibco; Thermo Fisher Scientific, Inc.). Starting from day 6, recombinant human tumor necrosis factor-α (TNF-α; Gibco; Thermo Fisher Scientific, Inc.) was added and cells were harvested on day 8 of DC culture at 37˚C and 5% CO_2). On day 8 of the DC culture, mouse anti-human CD80 (cat. no. FHP080-025; 1:40;), CD83 (FHP083-025; 1:40; Beijing Sizhengbo Biotechnology Co., Ltd.), CD86 (FHP086-100; 1:40; Beijing Sizhengbo Biotechnology Co., Ltd.) and HLA-DR (FHPDR-025; 1:40; all Beijing Sizhengbo Biotechnology Co., Ltd.) fluorescent antibodies were added separately, with mouse anti-human IgG (410707; 1:40; BioLegend, Inc.) serving as a blank control. After mixing antibodies with cells, the mixture was incubated at 4˚C in the dark for 30 min, followed by washing with PBS. Cells were fixed with paraformaldehyde solution (4%) for 30 min at 4˚C and analyzed using flow cytometry (FACScan; BD Biosciences) in the Cell Quest software (version 6.0.1; Becton, Dickinson and Company) (30).

Preparation of human T-lymphocyte suspension (effector cells). Peripheral blood from the nine healthy volunteers was anticoagulated with heparin and lymphocyte separation solution was used to obtain PBMCs. After a 2-h incubation in a cell culture incubator (at 37˚C, non-adherent cells were aspirated, T lymphocytes were separated using nylon wool (Polysciences, Inc.) and phytohemagglutinin (Add 100 μl to each culture flask) was added. After 3 days of culture, IL-2 (30 U/ml; CST Biological Reagents Co., Ltd.) was used to induce expansion, at 37˚C and 5% CO_2, with medium changes every 2 days (31).

Preparation of lung CSC lysates. Lung CSCs were collected, suspended in RPMI-1640 complete culture medium, transferred to cryotubes, immersed in liquid nitrogen for 5 min and then placed in a 37˚C water bath for 5 min. This process was repeated three times. After centrifugation (4˚C; 1,000 x g for 10 min), the supernatant was collected, transferred to a new cryotube and stored at -80˚C. A549 lung cancer cell lysates were prepared using the same method.

Effects of two different sources of cell lysates on DCs. To verify the effects of cell lysates from two different sources on DC maturation and antigen presentation, on day 6, $1x10^5$ immature DCs not yet induced with TNF-α were incubated (Incubated at 37˚C and 5% CO_2 for 48 h) with sorted A549 lung CSC and A549 lung cancer cell lysates (at a 5:1 ratio). Cells were harvested 2 days later and mouse anti-human MHC-II [abs1840769; 1:40; Absin (Shanghai) Biotechnology Co., Ltd.] fluorescent antibodies were added. Mouse anti-human IgG (410707; 1:40; BioLegend, Inc.) was used as a negative control. After mixing antibodies with cells, the mixture was incubated at 4˚C in the dark for 30 min, followed by washing with PBS. Cells were fixed with paraformaldehyde solution (4%) for 30 min at 4˚C and analyzed using flow cytometry (FACScan; BD Biosciences) in the CellQuest software (version 6.0.1; Becton, Dickinson and Company) environment (30).

Detection of T-lymphocyte proliferation using the Cell Counting Kit-8 (CCK-8) assay. After activating homologous T lymphocytes with the aforementioned sensitized DCs, cells were mixed and 100 μl of each sample was transferred into separate wells of a 96-well plate (3,000 cells/well). Each group had three replicate wells. Cell proliferation rates were measured using the CCK-8 assay (Dojindo Laboratories, Inc.) at 0, 24, 48 and 72 h (incubated at 37˚C for 2 h), and cell proliferation was assessed using the optical density (OD) 450 nm) values.

Preparation of DC vaccines sensitized with lung CSC and lung cancer cell lysates. The pre-prepared cell lysates with mature DCs induced by TNF-α at a ratio of 5:1. The primary cells extracted from blood in the previous groups were further

divided into three subgroups. These subgroups were sensitized with either stem cell lysates, A549 cell lysates, or were not sensitized with any cell lysates). GM-CSF and IL-4 cytokines were added (Incubated at 37˚C and 5% CO_2) and DCs were collected the following day. The control vaccine (DC vaccine sensitized with A549 lung cancer cell lysates) was prepared using the same method as described for preparing the DC vaccine sensitized with A549 lung CSC lysate (32).

Detection of IFN-γ content in supernatant after co-culture of A549 lung CSC lysate-sensitized DC vaccines and T lymphocytes. A total of three types of DC vaccines (A549 lung CSC lysate-sensitized DC vaccine, A549 lung cancer cell lysate-sensitized DC vaccine and DC vaccine without lysate sensitization) were mixed with prepared homologous T lymphocytes at a 1:10 ratio. The cells were cultured in RPMI-1640 medium containing 10% FBS, IL-12 and IL-2 for 8 days at 37˚C and 5% CO_2, and the supernatants were collected daily. Human IFN-γ ELISA KIT (CHE0017; Beijing Sizhengbo Biotechnology Co.) was used to detect the IFN-γ content in the supernatant according to the manufacturer's protocol (32).

In vitro induction of anti-lung cancer immune cytotoxic effects by DC vaccines. The specific cytotoxicity of cytotoxic T lymphocytes (CTLs) stimulated by the aforementioned three groups of DC vaccines against A549 cells and A549 lung CSCs was assessed *in vitro* using the lactate dehydrogenase release method with the Cytotox96 Non-Radioactive Cytotoxicity Assay kit (G1780; Promega Corporation). In the experiment, the specific steps were as follows: The target cells used were A549 lung cancer cells and A549 lung CSCs. The effector cells were CTLs stimulated by the respective groups of DC vaccines after being cultured for 8 days at 37˚C and 5% CO_2. The effector cells were then mixed with the target cells at different ratios (60:1, 30:1 and 10:1) and added to U-bottomed 96-well culture plates. Each sample had three replicate wells. Control groups of target cells with spontaneous (baseline level of marker or enzyme released into the culture medium by cells under normal conditions, without any treatment or stimulation) and maximum release (Refers to the total amount of marker or enzyme released into the culture medium when cells are completely lysed or destroyed. This indicates the maximum potential release of substances from cells under experimental conditions) were also included. The plates were then incubated at 37˚C with 5% CO_2 for 4 h. After incubation, the plates were centrifuged at 250 x g for 4 min at 4˚C. A total of 50 μl supernatant from each well of the centrifuged plates was transferred to a new 96-well ELISA plate. Subsequently, 50 μl substrate mixture was added to each well and incubated at room temperature in the dark for 30 min. The reaction was stopped with 50 μl stop solution per well in a dark room and the OD at 490 nm) were promptly measured using a microplate reader. The cytotoxicity percentage was calculated using the formula: Cytotoxicity (%)=[(OD value of experimental group-OD value of spontaneous release)/(OD value of maximum release-OD value of spontaneous release)] x100. Normal human airway epithelial cells (16HBE cells) were used as negative controls for this experiment (33).

Transwell migration assay. A549 lung cancer cells were used as target cells and CTLs stimulated with three groups of DC vaccines cultured for 8 days were used as effector cells. Effector and target cells were mixed at a ratio of 3:1. Transwell inserts with a pore size of 8 μm were used. Each upper chamber was loaded with 200 μl cell suspension (The medium in the upper chamber was serum-free DMEM, with 3×10^4 target cells added to each well) and the lower chamber was filled with 500 μl complete medium containing 15% FBS. Cells were cultured in a 5% CO_2, 37˚C incubator for 24 h. Cells that had migrated to the lower chamber were treated with 3% paraformaldehyde at 25˚C for 30 min, followed by staining with crystal violet at 25˚C for 15 min. The cells were then imaged and counted using an inverted microscope (Light microscope) and ImageJ (version 1.8.0; National Institutes of Health, USA).

Flow cytometric apoptosis assay. Effector CTLs stimulated with three groups of DC vaccines cultured for 8 days were used as effector cells) and target cells (A549 lung cancer cells) were mixed at a ratio of 3:1 and cultured in a 5% CO_2, 37˚C incubator for 24 h. Cells were digested with trypsin solution without EDTA and adjusted to a concentration of $1x10^6$ cells/ml. Staining was performed using FITC-Annexin V/PI apoptosis detection kit (Suzhou UELandy Biotechnology Co., Ltd.), with 5 μl Annexin V-FITC and 5 μl PI, followed by incubation in the dark at room temperature for 15 min. Finally, flow cytometry (FACScan; BD Biosciences) was used to detect cell apoptosis and the results were analyzed using FlowJo (version 10; FlowJo LLC).

Statistical analysis. Data analysis was performed using GraphPad Prism v10.2.3 software (Dotmatics). Differences between the two groups were analyzed by unpaired Student's t-test. One-way analysis of variance followed by Dunnett's post hoc test were used for comparisons between multiple groups. All quantitative experiments were repeated three times and data are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Relative expression of CD133, ABCG2 and Sox2 in A549 lung CSCs. CD133, ABCG2 and Sox2 are widely recognized as markers of lung CSCs, closely associated with the stemness and drug resistance properties of lung CSCs (3). Therefore, CD133, ABCG2 and Sox2 were used to validate the formation of lung cancer A549 stem cell spheres. Western blot analysis revealed that the expression of CD133, ABCG2 and Sox2 in lung cancer A549 stem cells was significantly higher compared with that in A549 lung cancer cells (Fig. 1A-D).

Flow cytometric identification of DCs. CD80, CD83, CD86 and HLA-DR are typical markers used to identify DCs, as they serve crucial roles in the function and maturation status of DCs (34). Therefore, fluorescent-labeled monoclonal antibodies against CD80, CD83, CD86 and HLA-DR were used for flow cytometric analysis of induced mature DCs. The expression levels of fluorescent-labeled monoclonal antibodies on DCs were IgG, 0.3% (Fig. 1E); CD80, 94.66% (Fig. 1F); CD83, 95.35% (Fig. 1G); CD86, 96.79% (Fig. 1H); and HLA-DR, 98.35% (Fig. 1I). These

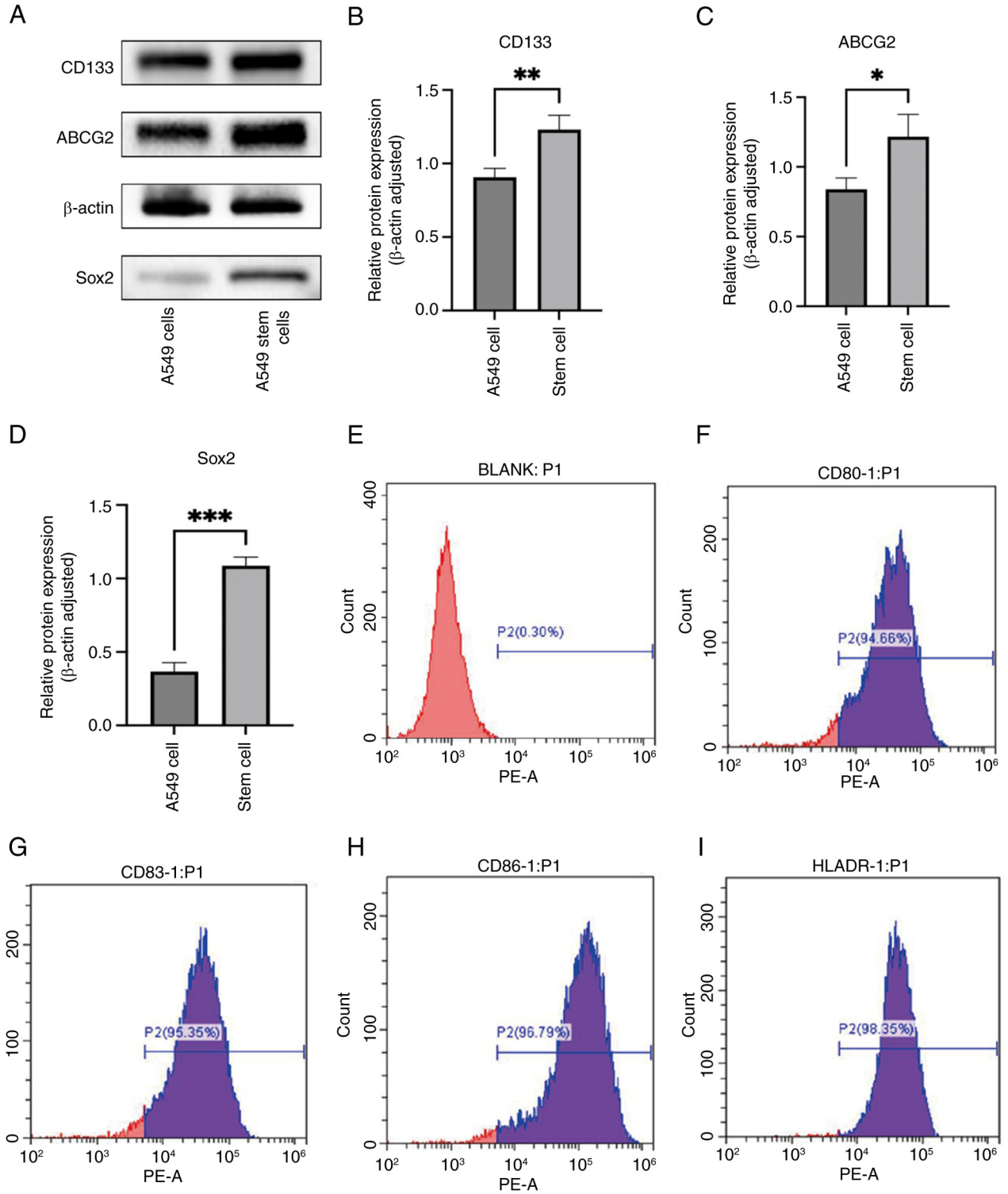

Figure 1. Relative expression of CD133, ABCG2 and Sox2 in A549 lung cancer stem cells and flow cytometric identification results of DCs. (A) Relative expression of (B) CD133, (C) ABCG2 and Sox2 in A549 lung cancer stem cells. (D) Statistical chart of relative expression levels from the Western blot experiment for Sox2. Expression of IgG (E), CD80 (F), CD83 (G), CD86 (H) and HLA-DR (I) in DCs. *P<0.05; **P<0.01; ***P<0.001. DC, dendritic cell; ABCG2, ATP-binding cassette sub-family G member 2; Sox2, sex determining region Y-box 2.

findings indicated that the purity and maturation of DCs were satisfactory and suitable for subsequent experiments.

Impact of two different sources of cell lysates on DCs. MHC-II serves as a key marker for assessing the maturation and antigen-presenting capacity of DCs, providing direct indications of their functional status, this is crucial for evaluating the role of DCs in immune responses and vaccine design (35). Therefore, MHC-II was used to evaluate the ability of lysates from two different cell sources to sensitize immature DCs and promote their maturation (35). The expression of fluorescent-labeled monoclonal antibodies on DCs was

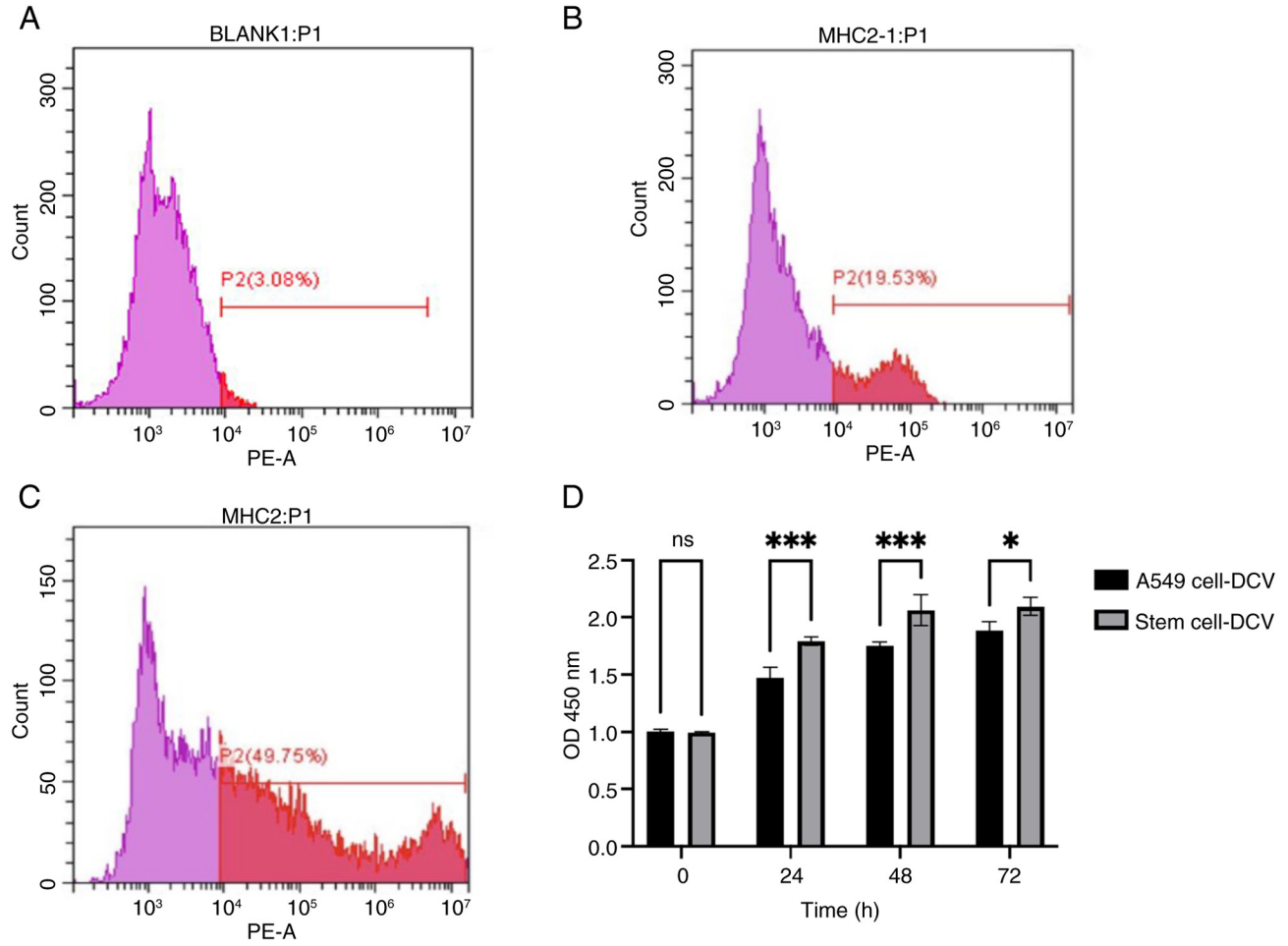

Figure 2. Effect of two types of cell lysates on the maturation induction capacity of immature DCs, and the proliferation rate of T lymphocytes activated by DCs. (A) IgG control for DC. (B) The effect of A549 cell lysate on MHC-II expression in immature dendritic cells. (C) The effect of A549 stem cell lysate on MHC-II expression in immature dendritic cells. (D) Proliferation rate of T lymphocytes. *P<0.05; ***P<0.001. DC, dendritic cell; MHC-II, major histocompatibility complex-II; ns, not significant; OD, optical density; DCV, DC vaccine.

3.08% for IgG (Fig. 2A), DCs sensitized with stem cell lysates exhibited markedly higher surface expression of MHC-II compared with those sensitized with A549 lung cancer cell lysates (Fig. 2B and C). These findings indicated that stem cell lysate could more effectively activate and mature DCs, enhancing their antigen-presenting capability and immunogenicity.

Detection of T-lymphocyte proliferation using the CCK-8 method. Using the CCK-8 assay, cell proliferation rates (OD values) were measured at 0, 24, 48 and 72 h. The results showed that the proliferation rate of T lymphocytes activated by DCs sensitized with ALDH1^{+} stem cell lysates was significantly higher than those activated by DCs sensitized with A549 lung cancer cell lysates (Fig. 2D).

Detection of IFN-γ levels in the supernatant after co-culture of A549 lung CSC lysate-sensitized DC vaccine with T lymphocytes. From day 1-8, significant differences in IFN-γ concentrations were observed in the supernatants after co-culturing T lymphocytes with three different vaccines daily (Fig. 3A-H). IFN-γ production by T lymphocytes stimulated with DC vaccines sensitized with A549 lung cancer cell lysates declined starting from day 2, with the difference from non-sensitized DC vaccines diminishing after day 2. T lymphocytes stimulated with DC vaccines sensitized with A549 lung CSC lysates showed no marked decline in IFN-γ production in the first 5 days and exhibited significant differences compared with the other two vaccine groups throughout the 8 days (Fig. 3A-H).

In vitro induction of anti-lung cancer immune cytotoxic effects by DC vaccines. The results showed that DC vaccines sensitized with stem cell lysates had the highest cytotoxicity against A549 cells at an effector-to-target (E:T) ratio of 60:1, with cytotoxicity gradually decreasing as the E:T ratio decreased (Fig. 4A). Compared with DC vaccines sensitized with stem cell lysate, the DC vaccines sensitized with A549 cell lysates exhibited slightly lower cytotoxicity against stem cells, with cytotoxicity also decreasing as the E:T ratio decreased (Fig. 4B). Non-sensitized DC vaccines at different E:T ratios showed no significant differences in cytotoxicity against stem cells, Compared with DC vaccines sensitized with cell lysates, non-sensitized DC vaccines exhibited lower overall cytotoxicity (Fig. 4C).

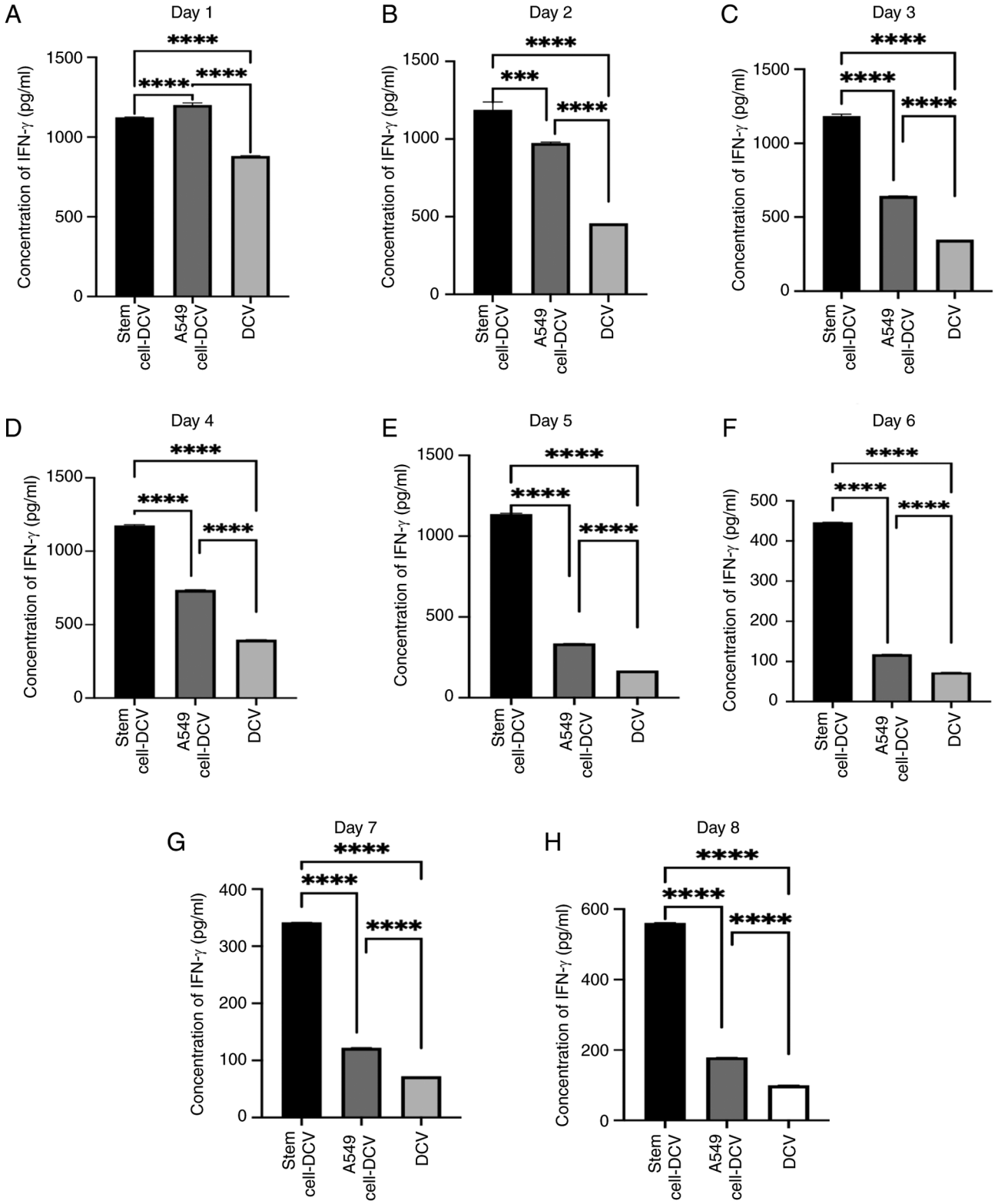

Figure 3. Detection of IFN-γ levels in supernatants of DC vaccine and T lymphocyte co-cultures. Comparison of IFN-γ concentrations in supernatants collected from co-cultures of three types of DCVs with T lymphocytes on days (A) Day 1, (B) Day 2, (C) Day 3, (D) Day 4, (E) Day 5, (F) Day 6, (G) Day 7, (H) Day 8. ***P<0.001; ****P<0.0001. DC, dendritic cell; DCV, DC vaccine.

The DC vaccines sensitized with stem cell lysates also showed the highest cytotoxicity against A549 cells at an E:T ratio of 60:1 (Fig. 4D). Similarly, DC vaccines sensitized with A549 cell lysates exhibited relatively high cytotoxicity against A549 cells at an E:T ratio of 60:1 (Fig. 4E). Non-sensitized DC vaccines at different E:T ratios showed no significant differences in cytotoxicity against A549 cells, Compared with the other two groups of DC vaccines sensitized with cell lysate, the non-sensitized DC vaccines exhibited lower overall cytotoxicity (Fig. 4F).

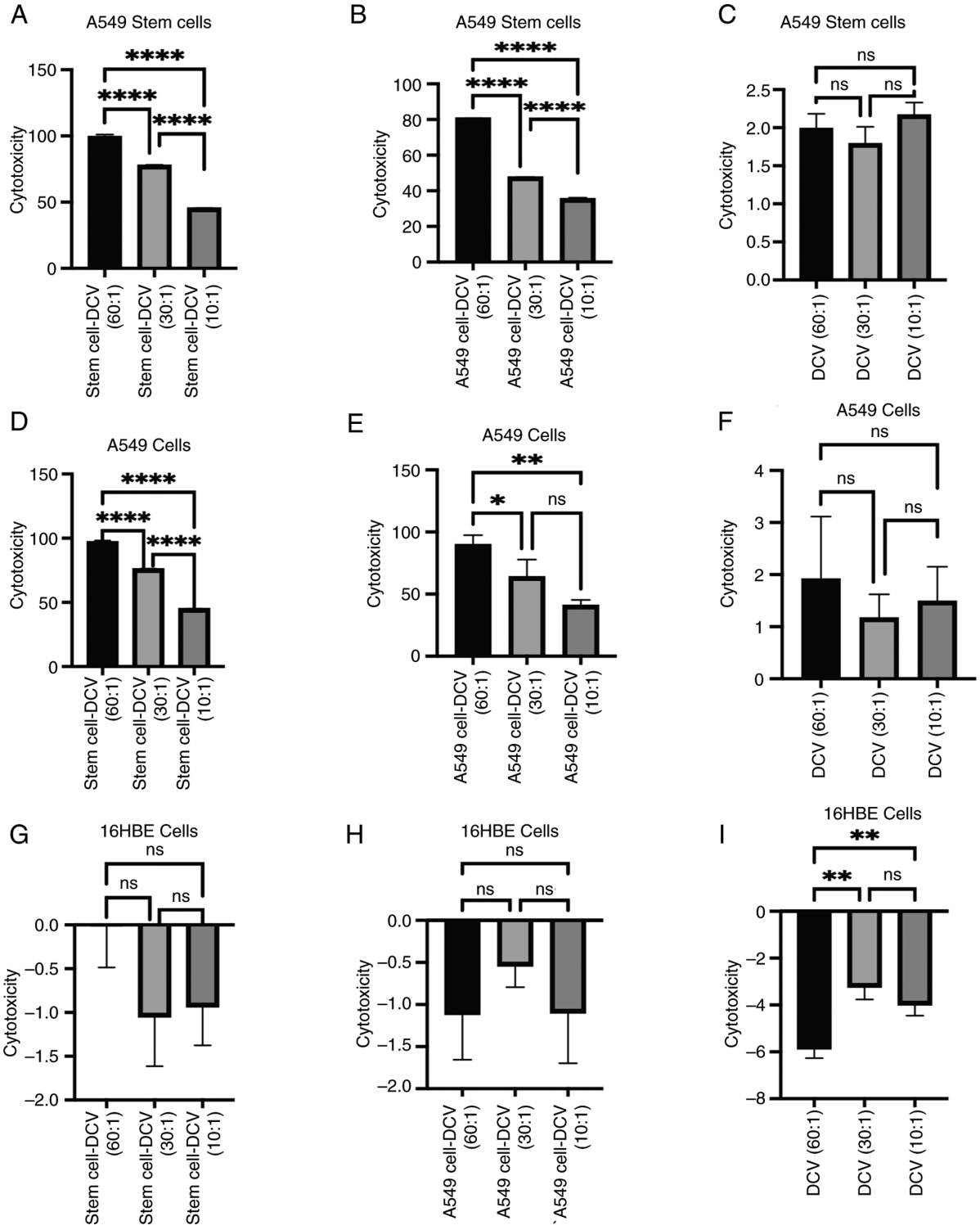

Figure 4. Tumor cell-specific cytotoxicity assay. (A) Stem Cell-DCV. (B) A549 Cell-DCV, (C) DCV; Cultured three types of DCV and T lymphocytes with A549 cells at three different effector-to-target cell ratios to assess toxicity to A549 cells: (D) Stem Cell-DCV, (E) A549 Cell-DCV, (F) DCV; Cultured three types of DCV and T lymphocytes with 16HBE cells at three different effector-to-target cell ratios to assess their toxicity to 16HBE cells: (G) Stem Cell-DCV, (H) A549 Cell-DCV, (I) DCV. *P<0.05; **P<0.01; ****P<0.0001. DC, dendritic cell; ns, not significant; DCV, DC vaccine.

All three vaccines showed no cytotoxic effects against 16HBE cells (Fig. 4G-I). However, non-sensitized DC vaccines at an E:T ratio of 60:1 exhibited a larger negative cytotoxicity value against 16HBE cells. This may be due to non-specific immune responses triggered by a large number of non-sensitized DCs in the cell culture environment, potentially promoting cell survival or proliferation upon contact with 16HBE cells (Fig. 4I).

The same vaccines exhibited varying cytotoxicity against different target cells. DC vaccines sensitized with A549 stem cell lysates exhibited varying cytotoxicity against A549 stem cells and A549 cells. At E:T ratios of 60:1 and 10:1, there was no significant difference in cytotoxicity between A549 stem and A549 cells. However, at an E:T ratio of 30:1, the cytotoxicity against stem cells was slightly higher than that against A549 cells (Fig. 5A-C). DC

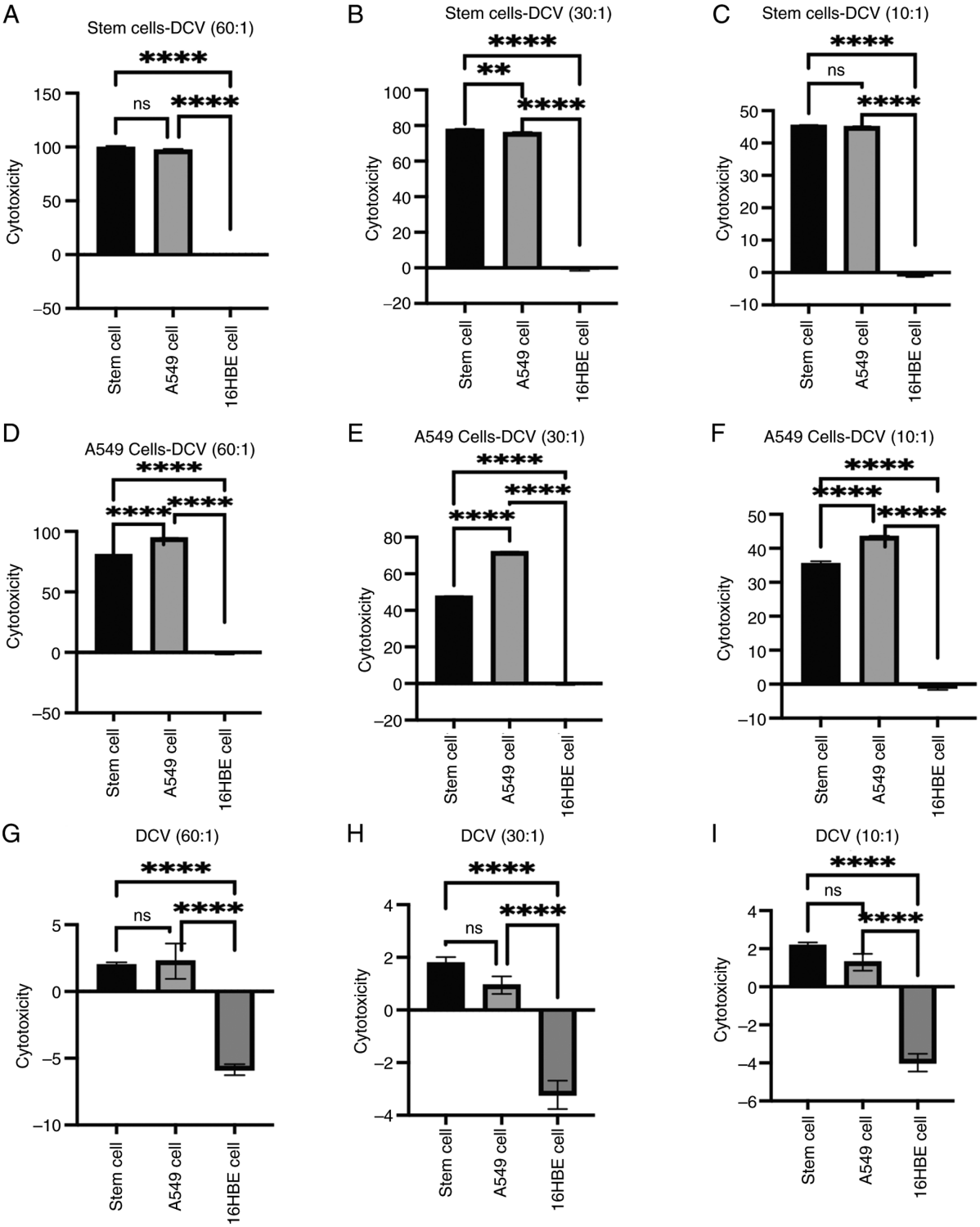

Figure 5. Tumor cell-specific cytotoxicity assay. Differential cytotoxicity exhibited by DC vaccines sensitized with stem cells at effector-to-target ratios: (A) 60:1, (B) 30:1, (C) 10:1; The differential cytotoxicity exhibited by DC vaccines sensitized with A549 cells at various effector-to-target ratios: (D) 60:1, (E) 30:1, (F) 10:1; The differential cytotoxicity exhibited by unsensitized DC vaccines at various effector-to-target ratios: (G) 60:1, (H) 30:1, (I) 10:1. **P<0.01; ****P<0.0001. DC, dendritic cell; ns, not significant; DCV, DC vaccine.

vaccines sensitized with A549 cell lysates demonstrated significantly higher cytotoxicity against A549 cells compared with A549 stem cells (Fig. 5D-F). Non-sensitized DC vaccines showed no significant difference in cytotoxicity against A549 stem cells and A549 cells but the results indicated negative cytotoxicity against 16HBE cells (Fig. 5G-I). 16HBE cells may lack the expression of specific antigens or co-stimulatory molecules, which could lead to the difficulty of DC vaccines sensitized with lysates in effectively recognizing these cells and activating T lymphocytes, thereby weakening the overall immune response (36), while 16HBE cells also exhibited natural proliferation during the assay period.

Impact of DC vaccines on A549 lung cancer cells. DC vaccines sensitized with low doses of A549 stem cell lysates significantly

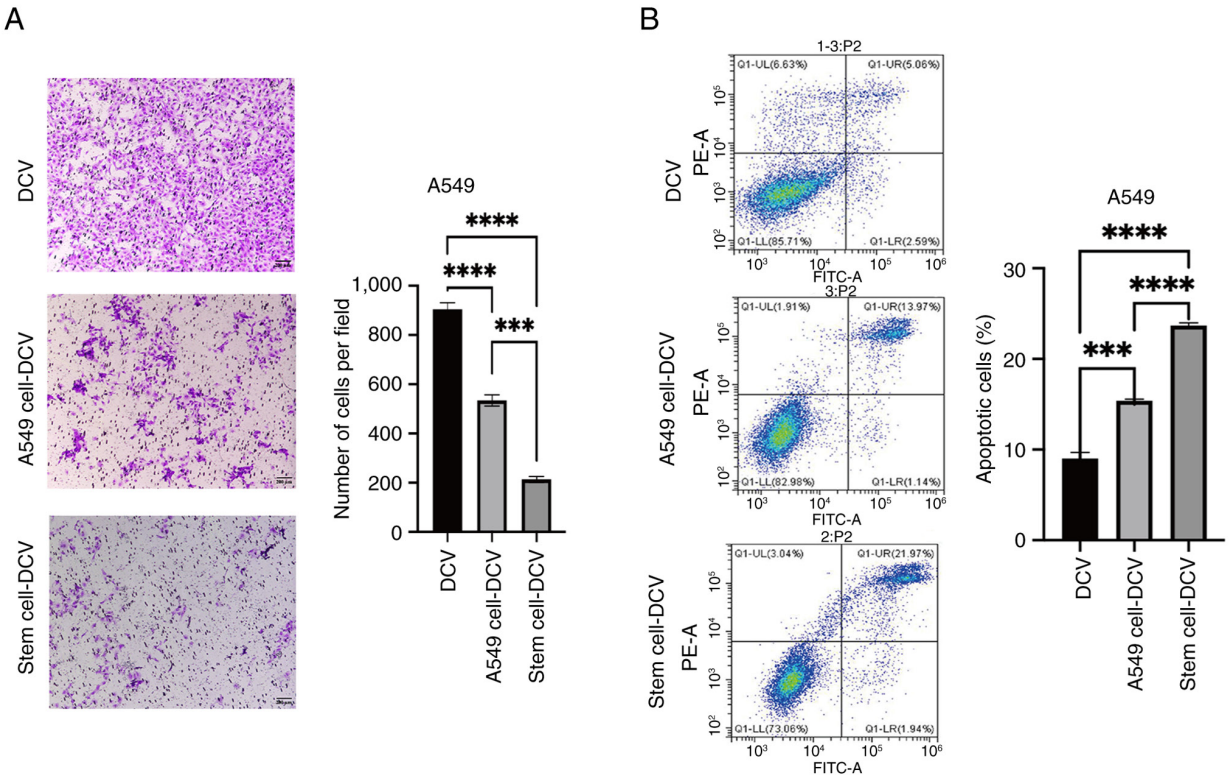

Figure 6. Impact of DCVs on A549 lung cancer cell. (A) Transwell assay evaluating the effect of DCVs on the migration of lung cancer cells. DCVs sensitized with low doses of A549 cancer stem cell or A549 cell lysates reduced the migration of A549 lung cancer cells (magnification, x100). (B) Flow cytometric apoptosis assay was performed to detect changes in cell apoptosis. ***P<0.001; ****P<0.0001. DC, dendritic cell; DCV, DC vaccine.

reduced the migration of A549 cells, Compared with vaccines sensitized with stem cell lysates, the dendritic cell vaccines sensitized with low doses of A549 cell lysates also induced a moderate decrease in A549 cell migration, but the effect was not as pronounced as that of the stem cell lysate-sensitized vaccines. Compared with the other vaccine groups, non-sensitized DC vaccines had a significantly reduced effect on the migration of A549 cells (Fig. 6A). Flow cytometric apoptosis assays demonstrated that, compared with other vaccines, DC vaccines sensitized with low doses of A549 stem cell lysates notably promoted apoptosis in A549 lung cancer cells while the effect of DC vaccines sensitized with A549 cell lysates on apoptosis in A549 lung cancer cells was less pronounced, compared with other vaccines. Compared with the other two vaccine groups, non-sensitized DC vaccines had a significantly reduced effect on apoptosis in A549 cells (Fig. 6B).

Discussion

Tumor cell immune escape is a critical issue hindering the efficacy of tumor vaccines (6). Tumor stem cells effectively evade immune system by reducing MHC-I levels, escaping T cell-mediated death, inhibiting T cell anti-tumor functions, and directly interfering with T cell effector functions through their secreted factors, that enable them to evade recognition and attack by the immune system (6). As seen in most tumors, the onset and maintenance of lung cancer is associated with lung CSCs, which correspond to the malignant transformation of respective lung stem cells (37). Therefore, targeting and eliminating lung CSCs holds significant therapeutic promise. The present study, to find a more effective way to eradicate tumor stem cells, aimed to investigate the *in vitro* induction of anti-lung cancer immune responses by DC vaccines sensitized with A549 lung CSC lysates.

Firstly, A549 lung CSCs were successfully induced and it was observed that the expression of CD133, ABCG2 and Sox2 in A549 lung CSCs was significantly higher compared with that in A549 cells. Additionally, DCs and T lymphocytes were successfully induced. Subsequently, using immature DCs sensitized with A549 stem cell lysates and A549 lung cancer cell lysates, it was found that A549 stem cell lysates were more effective in promoting DC maturation and antigen presentation capability, and could significantly enhance T-lymphocyte proliferation. These findings indicated that stem cell lysates could more effectively activate and mature DCs, enhancing their antigen presentation capability and immunogenicity.

In subsequent experiments, three types of DC vaccines were prepared (DC vaccines sensitized with A549 lung CSC lysates, DC vaccines sensitized with A549 lung cancer cell lysates and DC vaccines without sensitization) and their induction of T-cell responses and antitumor activity were compared *in vitro*. It was revealed that DC vaccines sensitized with A549 lung CSC lysate significantly stimulated the release of IFN-γ from homologous T lymphocytes, with levels significantly higher than those released from the other two groups.

Furthermore, in terms of cytotoxic effects on A549 cells and A549 CSCs, DC vaccines sensitized with A549 lung CSC lysates showed efficacy in A549 stem cell cytotoxicity and demonstrated strong cytotoxic effects on A549 cells. The cytotoxic effect was significantly superior to the other two

vaccine groups, and all three vaccine groups did not induce immune cytotoxic effects on 16HBE human airway epithelial cells. However, they exhibited negative cytotoxicity on 16HBE cells. This may be due to 16HBE cells not expressing specific antigens required by DC vaccines or related co-stimulatory molecules, rendering DC vaccines ineffective in activating T lymphocytes to attack them. Meanwhile, during the testing period, 16HBE cells underwent natural proliferation and the aforementioned result may also be due to a large number of unsensitized DCs in the *in vitro* cell culture environment that triggered nonspecific immune responses upon contact with 16HBE cells, thereby increasing cell survival rates or even promoting cell proliferation.

Additionally, it was observed that low-dose DC vaccines sensitized with A549 lung CSC lysates exhibited significant anti-migratory and pro-apoptotic effects on A549 lung cancer cells. Their effects were superior to those of DC vaccines sensitized with A549 lung cancer cell lysates. This may be attributed to low-dose DC vaccines not only directly killing a small number of tumor cells but also inhibiting their activity; however, the specific mechanisms involved require further investigation for clarification.

Notably, neither A549 lung CSC lysate-sensitized DC vaccines nor A549 lung cancer cell lysate-sensitized DC vaccines achieved a 100% killing efficiency against A549 lung CSCs and A549 cells. This may be related to the use of vaccines on day 8 after co-cultivation. The experimental results from testing the IFN-γ levels in the supernatant after mixing three types of DC vaccines with T lymphocytes showed that compared with the higher IFN-γ levels observed in the first five days, the IFN-γ levels for A549 lung CSC and A549 cell-sensitized dendritic cell vaccines were relatively lower on day 8. Additionally, this could be due to functional defects in DCs and effector cells, such as inadequate antigen presentation and cytokine release, which prevent them from overcoming immune suppression that limits DC and effector cell function (38).

Despite the potential of DC vaccines in cancer immunotherapy, they have not demonstrated notable superiority over traditional treatment methods, such as chemotherapy, targeted therapy or ICIs in terms of treatment efficacy and patient survival rates (39). DCs possess unique biological characteristics, making the design and production of long-lasting effective vaccines challenging (40). *Ex vivo*-induced differentiated DCs exhibit tolerance to immune suppression, and have a relatively weak and limited lifespan, thereby restricting their ability to induce sustained immune responses (41,42). Increasingly, studies have indicated that genetically modified DC vaccines can significantly enhance antitumor efficacy (43,44). Researchers have optimized DC vaccines by introducing methods such as mRNA, small interfering RNA, viral gene transduction, and fusion with tumor cells, the results indicate that combining these approaches can significantly enhance the clinical effectiveness of dendritic cell vaccines (45,46). Previous research has demonstrated that elevated expression of origin Recognition Complex Subunit 6) may impact DC activity, exacerbating immune evasion by tumor cells, thereby contributing to tumor initiation, progression and metastasis (47). Further investigation is needed to determine whether downregulating ORC6 in DCs can enhance their activity and the stimulation of T cells, thereby augmenting the antitumor immune response of T cells.

In addition to modifying DCs, introducing mRNA encoding multiple antigen epitopes can enhance the breadth and depth of immune responses by simultaneously activating multiple antigen-specific T-cell responses (48), mRNA can be used in conjunction with stimuli, such as CD40L or cytokines, to enhance DC activation and antigen epitope presentation (49). Adjuvants are compounds that enhance the immunogenicity of vaccines by promoting antigen uptake and processing, and activation of immune cells, thereby boosting immune responses, and effectively stimulating function of DCs (50). Combination therapy aims to enhance the effectiveness of DC vaccines, Studies have shown that combining PD-1 blockade with DC vaccine administration can prolong the survival of treated Mice, whereas monotherapy with any single agent does not significantly impact the survival of animals with established tumors (51,52). DC vaccine administration significantly increases tumor-infiltrating lymphocytes (TILs), and the increase in PD-1 expression is associated with elevated TILs post-vaccination (51). Chemotherapy may weaken the immune response, so patients who have undergone chemotherapy may have a reduced response to subsequent immunotherapy, even though the combination of immunotherapy and chemotherapy may seem unconventional, several clinical trials have explored this approach, demonstrating potential synergistic effects that could improve treatment outcomes and survival rates for patients (53,54). Recent research has indicated that combining DC vaccines with cytokine-induced killer (CIK) cell therapy in patients with cancer has had a significant positive impact on treatment (55). This combination therapy leverages the synergistic effects of DCs and CIK cells, as DCs effectively present tumor antigens, compensating for the limited tumor antigen specificity of CIK cells, thus offering promising clinical prospects for enhancing the immune system ability to combat tumors (56). Radiotherapy can induce immunogenic cell death of tumor cells, releasing damage-associated molecular patterns and TAAs, thereby activating DCs and promoting their migration to lymph nodes, which in turn induces systemic antitumor immune responses; therefore, injecting exogenously prepared unloaded DCs into tumors followed by radiotherapy may offer additional benefits (57).

Notably, the production process of DC vaccines is complex, requiring multiple technical barriers (such as insufficient antigen presentation, migratory capacity, and cytokine release) to be overcome to enhance the effectiveness of the treatment (43). Compared with other treatment modalities, such as chemotherapy and radiotherapy, the safety of DC vaccines is predominantly reflected in their lower rates of non-specific toxic side effects and more personalized therapeutic approaches (58). However, due to the current stage of research and clinical trials, the long-term safety and efficacy of DC vaccines still require further evaluation and confirmation through additional clinical studies and practical experience.

In conclusion, DC vaccines sensitized with A549 lung CSC lysates can induce more effective antitumor immune responses in T cells. However, these experimental results have not yet been validated *in vivo*. Therefore, it is necessary to establish a mouse model of lung cancer and validate the antitumor efficacy of DC vaccines. Additionally, the lack of testing on the

effects of different concentrations of DC vaccines on T-cell proliferation and IFN-γ production limits the accurate determination of the optimal dose. Hence, further validation of the optimal dose of DC vaccines in T-cell function and immune response is needed. Moreover, the lack of in-depth exploration into the mechanisms of interaction between DC vaccines sensitized with stem cell lysates and tumor stem cells highlights the need for advanced genomic sequencing techniques, such as single-cell transcriptomics or proteomics analysis, to be performed during co-culture, to improve understanding of how DC vaccines influence the molecular mechanisms of tumor stem cells and validate these biological interactions in preclinical experiments. Furthermore, during the cultivation and co-cultivation of DCs, the growth factors IL-2 and TNF-α were added; therefore, after co-culturing DC vaccines with T lymphocytes, these growth factors were not detected. These limitations underscore the necessity for further research to improve the understanding of how DC vaccines sensitized with A549 lung CSC lysates can effectively induce antitumor immune responses and their potential impact in disease treatment.

With the continuous emergence of new technologies, it is possible to reduce manufacturing and production costs associated with DC vaccines, thereby enhancing overall practicality. A deeper understanding of DC biology and immune resistance mechanisms in the tumor microenvironment holds promise for designing more optimized DC vaccines to meet the demands of personalized therapy.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

Study design was performed by JX and LC. Data collection and analysis were performed by WR and YC. LC, WR and YC contributed to data interpretation, and to the writing and review of the manuscript. JX and LC confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of The Second Affiliated Hospital of Nanchang University [Nanchang, China; approval no. O-Medical Research Ethics Approval (2023) No. 89], and all participants provided written consent to participate in this study. All experiments in this study comply with the ethical standards of the World Medical Association (Declaration of Helsinki).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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