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Original Article

Dendritic cells modified by tumor associated antigen SMP30 have enhanced antitumor effect against mouse hepatocarcinoma cells in vitro and in vivo

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Abstract: Objectives: Tumor immunotherapy based on dendritic cells (DC) is one of the most promising approaches to treat cancers. This therapy uses an immunogenic tumor antigen to present it to T cells. Senescence marker protein 30 (SMP30) is identified as a tumor associated antigen (TAA) with high immunogenicity and specificity for hepatocellular carcinoma (HCC). DCs are the most potent antigen presenting cells, and can be transduced with tumor antigens to enhance antitumor immune response. The purpose of this study was to investigate the antitumor effect of DCs transduced with a recombinant lentiviral vector (*LV-SMP30*) expressing SMP30. Methods: A recombinant lentiviral vector (*LV-SMP30*) expressing SMP30 was constructed and transduced into DCs. The expression of SMP30 was detected by western blot. Mouse bone marrow-derived DCs were divided into four groups: *LV-SMP30* group (transduced with *LV-SMP30*), *Protein* group (co-cultured with *SMP30* protein), *LV* group (transduced with the empty vector) and *Untreated* group (the normal DCs). The effect of *LV-SMP30* on DCs was detected through surface markers (CD123, CD11c, CD80 and CD86) and cytokine production. The activation and proliferation of $CD3^+CD8^+$ T cells were detected by CCK-8 kit. Flow cytometry was used to detect $CD3^+CD8^+$ T cell-mediated cytotoxicity. After construction of a mouse subcutaneous xenograft model, the volume and growth of tumors in different groups were observed. The changes in serum immune indexes in the treated groups were compared with those in the control group. Results: The *LV-SMP30* recombinant was constructed and transduced into DCs successfully, and *LV-SMP30*-transduced DCs stably expressed SMP30. The percentages of expression in the *LV-SMP30* and *Protein* groups were significantly higher than those in the *LV* or *Untreated* groups *($P<0.05$).* Meanwhile, after the DCs were cultured for 72 hours, the levels of IL-2, IL-6, IL-12, and IFN-γ were significantly higher in the *LV-SMP30* and *Protein* groups than in the *LV* group or *Untreated* group ($P<0.05$). After the DCs were continuously cultured for one week, however, the cytokine levels in the *LV-SMP30* group were significantly higher than those in the *Protein* group ($P<0.05$). In addition, $CD3^+CD8^+$ T cell proliferation and activation levels were substantially higher in the *LV-SMP30* and *Protein* groups than in the *LV* or *Untreated* groups ($P<0.05$). Furthermore, as the ratio of effectors/target cells increasing in the *LV-SMP30* group, $CD3^+CD8^+$ T cell-mediated cytotoxicity in H22 cells became higher (0:1, 10:1; 20:1; 40:1, respectively). In comparison to the control group, the cytotoxicity of the *LV-SMP30* group was considerably increased at the ratios of 10:1, 20:1 and 40:1 ($P<0.05$). However, in the case of Hep1-6 cells, there was no significant difference in $CD3^+CD8^+$ T cell-mediated cytotoxicity among the groups. In addition, when compared with other groups, the mice in the *LV-SMP30* group showed the most volume reduction, the slowest tumor growth, and the highest level of IL-2 and IFN-γ ($P<0.05$). Conclusion: DCs transduced with *LV-SMP30* can dramatically enhance specific $CD3^+CD8^+$ T cell immune responses against mouse hepatocarcinoma cells in vitro and in vivo. These findings lend significant support to the development of the DC-based SMP30 antigen vaccine for hepatocarcinoma immunotherapy.

Keywords: Dendritic cell, senescence marker protein 30, lentivirus vector, immunotherapy

Introduction

Vaccination based on dendritic cells (DCs) is one of the most promising cancer immunotherapy techniques. Many studies have demonstrated that a DC-based vaccination can elicit an efficient antitumor immune response [1, 2]. The primary basis for the immune system to eliminate tumors is the existence of immunogenic tumor antigens recognized by B and T cells [3]. Therefore, screening and identifying appropriate tumor antigens is a precondition for cancer immunotherapy.

In the past few years, more than 2000 tumor antigens, including NY-ESO-1 and MAGE, have been identified by SEREX (serological identification of antigens by recombinant expression cloning) [4, 5]. We previously used SEREX to screen and identify a range of tumor associated antigens, including Senescence marker protein 30 (SMP30), GC-binding factor 2 (GCF2) and Kinectin, from Guangxi human hepatocellular carcinoma (HCC) cDNA library [6, 7]. SMP30 was overexpressed in most human and animal liver cancer tissues and cells, and it was strongly linked to liver cancer development and poor clinical outcome [8, 9]. Most importantly, SMP30 is highly immunogenic and specific for HCC [10, 11]. Hence, SMP30 appears to be a suitable tumor antigen for HCC immunotherapy.

DCs are the most potent antigen-presenting cells according to previous research [12]. DC activation is linked to antigen acquisition and cytokine production [3]. DCs are typically loaded by incubation with tumor peptides, tumor lysates or tumor cells to improve the activation as well as cytotoxicity against cancer [3, 13-15]. DCs pulsed with tumor antigen peptides can elicit a T cell antitumor immune response in vitro [16, 17]. After co-incubation with SMP30 peptide, DCs were activated and then induced the naïve T cell activation, and our previous study showed that the latter mediated specific cytotoxic T cell immune response against HCC cells [10, 11, 18]. However, the limitations of SMP30 peptide, including unstable expression and degradation of SMP30, severely limited its induction of DCs.

Gene modification is an important strategy in gene function research that can overcome the limitation of tumor antigen peptides [19]. In terms of current technology, DCs can be modified in a variety of ways, including gene insertion encoding tumor antigen. Gene insertion can offer multiple antigen epitopes to DCs while increasing immune duration [19, 20]. Lentiviral vector (LV) is another effective tool that is widely used in gene function research. LV can integrate antigenic genes into the target cell genome and sequentially produce a continuous expression of tumor antigen in target cells [19, 20]. Those target cells include dividing and non-dividing cells, such as T cells and DCs [3, 21, 22].

In the current study, we first constructed a SMP30 gene expressed recombinant *LV* (*LV-SMP30)* and sequentially transduced it into DCs derived from mouse bone marrow. We investigated whether *LV-SMP30* could increase DC activation and proliferation, as well as whether *LV-SMP30*-transduced DCs could enhance $CD3^+CD8^+$ T cell-mediated antitumor immune response. Our results revealed that *LV-SMP30* significantly increased DC activation and proliferation, and *LV-SMP30*-transduced DCs intensively enhanced $CD3^+CD8^+$ T cell-mediated cytotoxicity against specific liver cancer cells in mice.

In addition, we successfully constructed a BALB/C nude mouse model to further verify the $CD3^+CD8^+$ T cell-mediated cytotoxicity. In subcutaneous xenograft models, we also assessed whether *LV-SMP30*-transduced DCs could elicite $CD3^+CD8^+$ T cell-mediated antitumor immune response. Our findings revealed that the *LV-SMP30* group showed a better antitumor effect in vivo than the other groups. All these findings encourage the development of a DC-based SMP30 antigen vaccines for future HCC immunotherapy.

Materials and methods

Cell culture

Mouse liver cancer cell lines H22 and Hep1-6 were employed, purchased from iCell Bioscience Inc. In a humidified 5% CO_2 incubator at 37°C, the cells were cultured in RPMI-1640 media (Hyclone, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 200 U/ml penicillin and 100 mg/ml streptomycin.

Animals

BALB/c mice (6-8 weeks old) and BALB/c nude mice (4-6 weeks old) were purchased from Beijing Huafukang Biotechnology, China, and kept at the animal experiment center of Guangxi Medical University. The housing conditions were an SPF level environment with 4-6 mice per cage with a laboratory temperature of 20°C-25°C and a humidity of 50%. All animal breeding was in compliance with the principles and procedures prescribed by the Animal Management and Use Committee of Guangxi Medical University.

Construction of recombinant lentivirus vector (LV-SMP30) expressing SMP30

RT-PCR was used to amplify the SMP30 gene from mouse cells. The following primers were used for PCR amplification of a 900 bp-long SMP30 gene: 5'-AGG GTT CCA AGC TTA AGC GGC CGC GCC ACC (forward); 5'-GAT CCA TCC CTA GGT AGA TGC ATT CAC CCT GCA TAG GAA TAT GGG GCA ATT CCT TTG ACT-3' (reverse). The PCR product was inserted into *NotI* sites of *LV*5 Shuttle Plasmid (Genepharma, Suzhou, China). *LV*5 Shuttle Plasmid encoded a Green Fluorescent Protein (GFP) to track transduction efficiency. Direct sequencing was used to confirm the DNA sequence of the positive clone.

Preparation of DCs and transduction with LV-SMP30

Mouse DCs (i.e., mononuclear cells derived from bone marrow of BALB/c mice) were donated by Guangxi Key Laboratory of Biological Targeted Diagnosis and Treatment. DCs were incubated in RPMI 1640 medium with 10% FBS (Gibco) for 3-4 hours. Then, they were adhered to the wall by inducing granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) for 7 days and stimulated with Lipopolysaccharide (LPS) for 24 hours.

After that, the DCs were transduced with *LV-SMP30*. On the day before infection, DCs were seeded in 24-well plates at a density of 0.5×10^5 cells per well with 0.5 ml of complete culture media overnight at 37°C with 5% CO_2. After 12-24 hours, DCs were cultured overnight at 37°C in a 5% CO_2 incubator using RPMI-1640 media supplemented with 10% FBS, 0.5 ml of 10-fold diluted lentivirus solution and polybrene infection promoter at a final concentration of 5 μg/ml. The supernatants were discarded 24 hours after infection, and DCs were incubated in fresh medium for another 72 hours. GFP expression was observed using a fluorescence microscope to assess efficiency. In this study, the group of DCs transduced with *LV-SMP30* was named as *LV-SMP30* group. The group with positive control of DCs co-cultured with SMP30 protein (100 ng/ml) was named the *Protein* group. The control groups were designed as follows as a parallel control: DCs transduced with the empty vector (named as *LV* group) and the normal DCs (named as *Untreated* group).

Western blot assay for measuring SMP30 protein expression

Total proteins were extracted from DCs in each group using RIPA buffer (Solarbio, Beijing, China), separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a PVDF membrane. After incubation with 5% nonfat milk for 2 hours at room temperature, the membrane was treated with anti-SMP30 antibody (Fitzgerald, USA). After being washed, the membranes were incubated with an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Beyotime, Shanghai, China) for one hour at room temperature. Electrochemiluminescence (ECL) was used to detect immunoreactive bands. The manufacturer's instructions were followed during the western blot assay.

Flow cytometry (FCM) analysis for determining LV-SMP30-transduced DC surface markers

DC surface staining utilized direct immunofluorescence and was analyzed by FCM (BD/Biometra). The following fluorescent mAbs were used to identify surface markers: anti-CD123 (5B11), anti-CD11c (N418), anti-CD8a (53-6.7), anti-CD80 (1G10), anti-CD86 (GL1) and anti-CD3 (17A2). All of the Abs mentioned above were purchased from eBioscience Inc. To analyze the phenotypes of the *LV-SMP30*-transduced DCs, 1×10^6 DCs per tube were collected and the supernatant was removed after centrifuging. Then, the cells were washed once with precooled PBS and incubated with the fluorescent mAbs (CD123, CD11c, CD80, CD86, CD3 and CD8a, respectively) for 30 minutes at room temperature in the dark. FCM was used

to perform fluorescence-activated cell sorting and analysis after the cells were washed and resuspended in PBS.

Determination of cytokines by ELISA

ELISA kits (Cusabio, Wuhan, China) were used to measure the concentrations of IL-2, IL-6, IL-12 and interferon-γ (IFN-γ) in the supernatant of culture media according to the manufacturer's instructions. DCs were cultured for 72 hours and 7 days in 24-well plates, then adjusted to 5×10^5 cells/ml. After 48 hours, the supernatant from each group was collected to measure IL-12 and IFN-γ. Likewise, the concentrations of IL-2, IL-6, IL-12, and IFN-γ in the supernatant of DCs co-cultured with mice peripheral blood mononuclear cells (PBMC) for 72 hours and 7 days were also measured.

Isolation and purification of $CD3^+CD8^+$ T cells

PBMCs were first isolated from six BALB/c mice (6-8 weeks old) using a lymphocyte isolation method. Then, PBMCs were induced for 2 days with 500 U/ml IL-2 (Novartis, Camberley, UK), 0.5 μg/ml anti-CD3 (BD Pharmingen, Cowley, UK) and 0.5 μg/ml anti-CD28 (BD Pharmingen, Cowley, UK). Following that, PBMCs werediffer-entiated into T cells using only IL-2 to maintain cell culture for 7 days. Finally, $CD8^+$ T cells were isolated using $CD8^+$ T magnetic beads separation kit (Miltenyi Biotech, Gladbach, Germany) according to the manufacturer's protocol. The purity of $CD3^+CD8^+$ T cells was examined by FCM.

Detection of $CD3^+CD8^+$ T cells proliferation by CCK-8

DCs were co-cultured with mice peripheral blood mononuclear cells (PBMC). After acquiring DC-presented antigens, the proliferation of naive T cells during the activation of $CD3^+CD8^+$ T cells was detected by CCK-8 kit (Abcam, Shanghai, China) according to manufacturer's protocol.

Detection of $CD3^+CD8^+$ T cell-mediated cytotoxicity in vitro by FCM

$CD3^+CD8^+$ T cell-mediated cytotoxicity was measured by FCM and the procedure was performed as before [23]. Briefly, H22 cells and Hep1-6 cells were labeled with green fluorescent dye DIOC18 (Beyotime, Shanghai, China) before co-incubation with $CD3^+CD8^+$ T cells. After co-incubation, the red fluorescent PI (Beyotime, Shanghai, China) was employed to mark dead cells. The cell-mediated cytotoxicity was expressed as percentage. The formula is as follows: Cytotoxicity = [Control-viable cell %]-[Co-incubation-viable cell %]/[Control-viable cell %] [23].

Construction of BALB/c nude mice model

BALB/c nude mice (4-6 weeks old) were divided into 4 groups. H22 cells (1×10^7 H22 cells, 0.1 ml) were injected subcutaneously into the right abdomen of mice. On approximately the seventh day, when the tumor had grown to approximately 100 mm^3, the model was deemed successful, and the mice were randomly assigned to 4 groups: *LV-SMP30* group, *Protein* group, *LV* group, *Untreated* group and *PBS* group. Then, 0.3 ml bulk $CD3^+CD8^+$ T cells (3×10^7 $CD3^+CD8^+$ T cells, 0.1 ml) were injected from the caudal vein every four days until the 13th day, and the mice were sacrificed on the 15th day after vaccination.

Recording the changes in tumor volume for each group

Tumor size was assessed with a caliper before $CD3^+CD8^+$ T cells were injected into BALB/c nude mice in each group. Tumor size was measured every 3 days after $CD3^+CD8^+$ T cell injection until the mice were euthanized, and tumor volume was calculated.

Calculation of tumor inhibition rate

The mice in each group were euthanized on the 15th day after $CD3^+CD8^+$ T cell injection, and then the tumor tissues were completely stripped and weighed. Tumor inhibition rate was calculated according to the following formula. Tumor inhibition rate (%) = (average tumor weight of *PBS* group-average tumor weight of the experimental group)/average tumor weight of *PBS* group * 100%.

Measurement of cytokines by ELISA

On the 15th day following injection of $CD3^+CD8^+$ T cells, the levels of cytokine INF-γ and IL-2 in

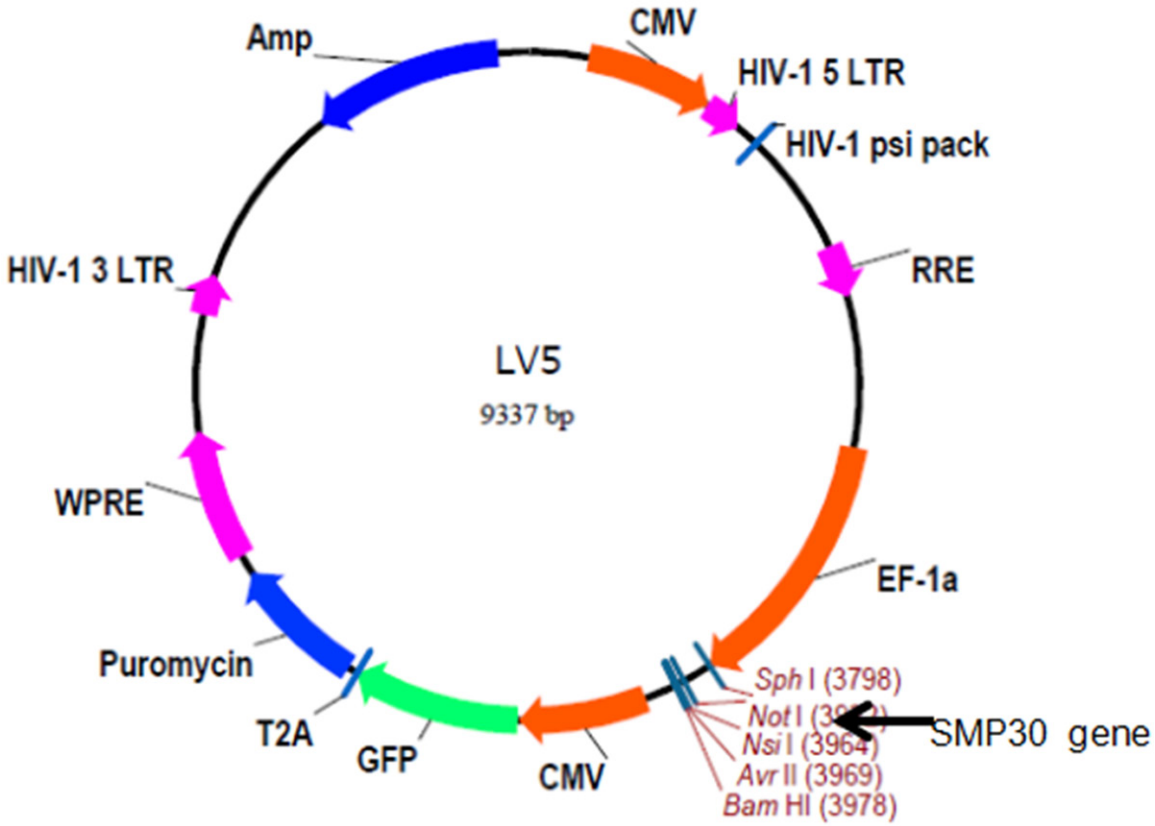

Figure 1. Construction of recombinant *LV*5 expressing SMP30 gene.

the serum of mice in each group were measured by ELISA kit (Cusabio, Wuhan, China) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis used Statistica 7.0 (Dell, Austin, TX, USA). All data are presented as the Mean ± SD. The t-test was used to evaluate statistical significance for two-group comparisons, and one-way analysis of variance (ANOVA) was used for three-group comparisons. Statistical significance was defined as $P<0.05$.

Results

Construction of the recombinant LV-SMP30 expressing SMP30

To construct the recombinant *LV-SMP30* expressing SMP30, the 900 bp-length SPM30 gene derived from mice was amplified, purified and cloned into *NotI* sites of a *LV*5 (**Figure 1**). The *LV*5 containing SMP30 insertion was confirmed by direct sequencing.

Stable expression of SMP30 protein in DCs transfected with LV-SMP30

Mouse DCs were transfected with *LV-SMP30.* GFP expression was used to test the transduction efficiency of DCs under a fluorescence microscope (**Figure 2A**). Transduction efficiency in the *LV-SMP30* group was nearly 70% 72 hours after transfection.

SMP30 protein was examined in DCs by western blot to establish the ability of *LV-SMP30* on protein expression. The results revealed that SMP30 protein increased more significantly in the *LV-SMP30* group than in the *LV* group or *Untreated* group (**Figure 2B**). The results suggested that *LV-SMP30* is an effective recombinant that can express SMP30 protein in a stable manner.

Surface marker identification of LV-SMP30-transduced DCs

CD123 and CD11c are commonly employed to identify markers of DCs [3], while the upregula-

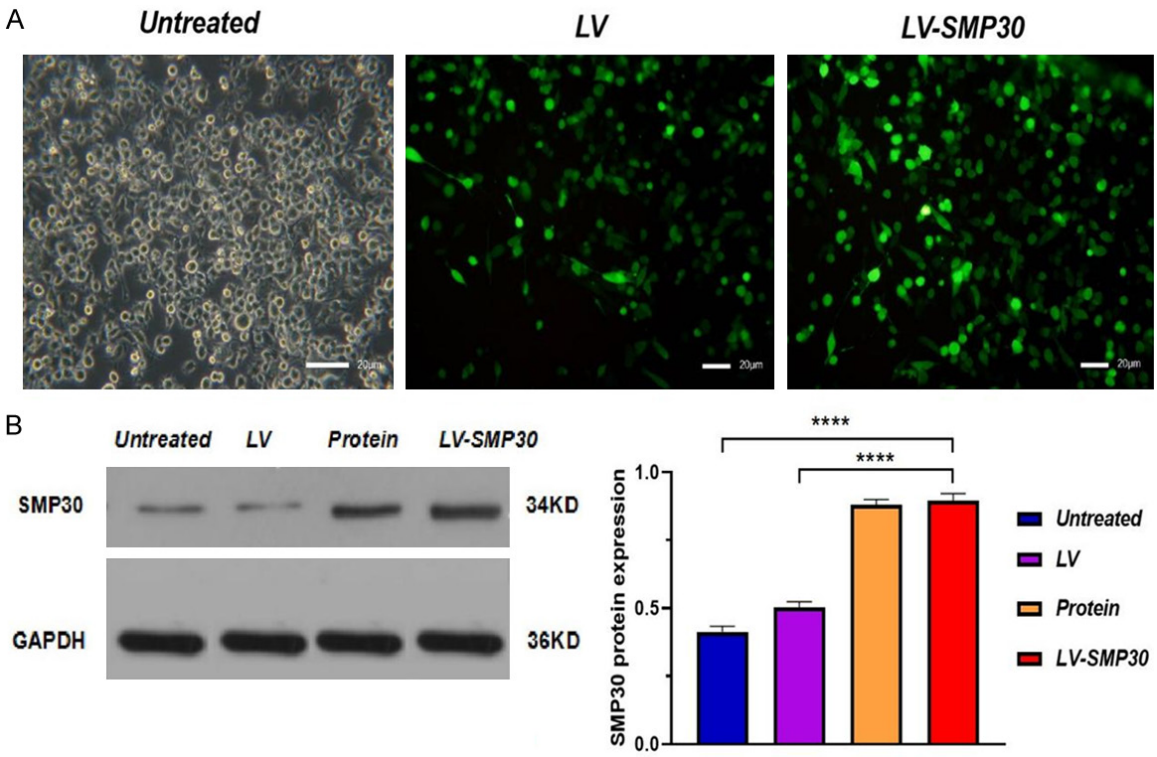

Figure 2. DCs were transfected with *LV-SMP30* or *LV*. A. GFP expression was used to assess transduction efficiency under a fluorescence microscope (× 200). 72 hours after transfection, the transduction efficiency in the *LV-SMP30* group was nearly 70%. B. Western blot was used to examine SMP30 protein expression. The results showed that SMP30 protein increased more significantly in the *LV-SMP30* group than in the *LV*-group or *Untreated* group. Overall, the results confirmed that *LV-SMP30* was an effective recombinant that could express SMP30 protein in a stable manner.

tion of CD80 and CD86 are used as the activation markers of DCs [3]. As illustrated in **Figure 3A**, CD123 had a substantially larger cell percentage than CD11c [(64.9±0.06)% *vs.* (0.29±0.04)%] (P<0.05), indicating that DC123 was the main subtype of DCs in mice. Additionally, the cell percentages of CD80 and CD86 were (86.3±0.30)% and (72±13.12)%, respectively, in the *LV-SMP30* group. Both percentages were considerably higher in the *LV-SMP30* group than in the *LV* group or *Untreated* group (P< 0.05) (**Figure 3B**, **3C**). The results above suggested that *LV-SMP30* enhanced DC activation.

LV-SMP30-transduced DCs induce specific cytokine production

Cytokines released during DC activation can strongly influence the immune responses through IL-2, IL-6, IL-12 and INF-γ [3, 4]. These cytokines are mainly secreted by Th1 cells. After 72 hours of culture of DCs, the levels of IL-2, IL-6, IL-12, and IFN-γ in the *LV-SMP30* group and *Protein* group were substantially higher than those of the *LV* group or *Untreated* group (P<0.05) (**Figure 4A**). After one week of continuous culture of DCs, the levels of IL-2, IL-6, IL-12, and IFN-γ in the *LV-SMP30* group were significantly higher than those of the *Protein* group (P<0.05) (**Figure 4A**).

The results suggest that *LV-SMP30* promotes the production of IL-2, IL-6, IL-12, and IFN-γ during DC activation.

After *LV-SMP30*-transduced DCs were co-cultured for 72 hours with mice PBMC, the levels of IL-2, IL-6, IL-12 and IFN-γ in the supernatant were measured. As shown in **Figure 4B**, both IL-12 and IFN-γ in the supernatant increased more in the *LV-SMP30* group and *Protein* group than in the *LV* group or *Untreated* group (P<0.05). However, after one week of continuous co-culture of DCs and mice PBMC, the levels of IL-2, IL-6, IL-12, and IFN-γ in the *LV-SMP30*

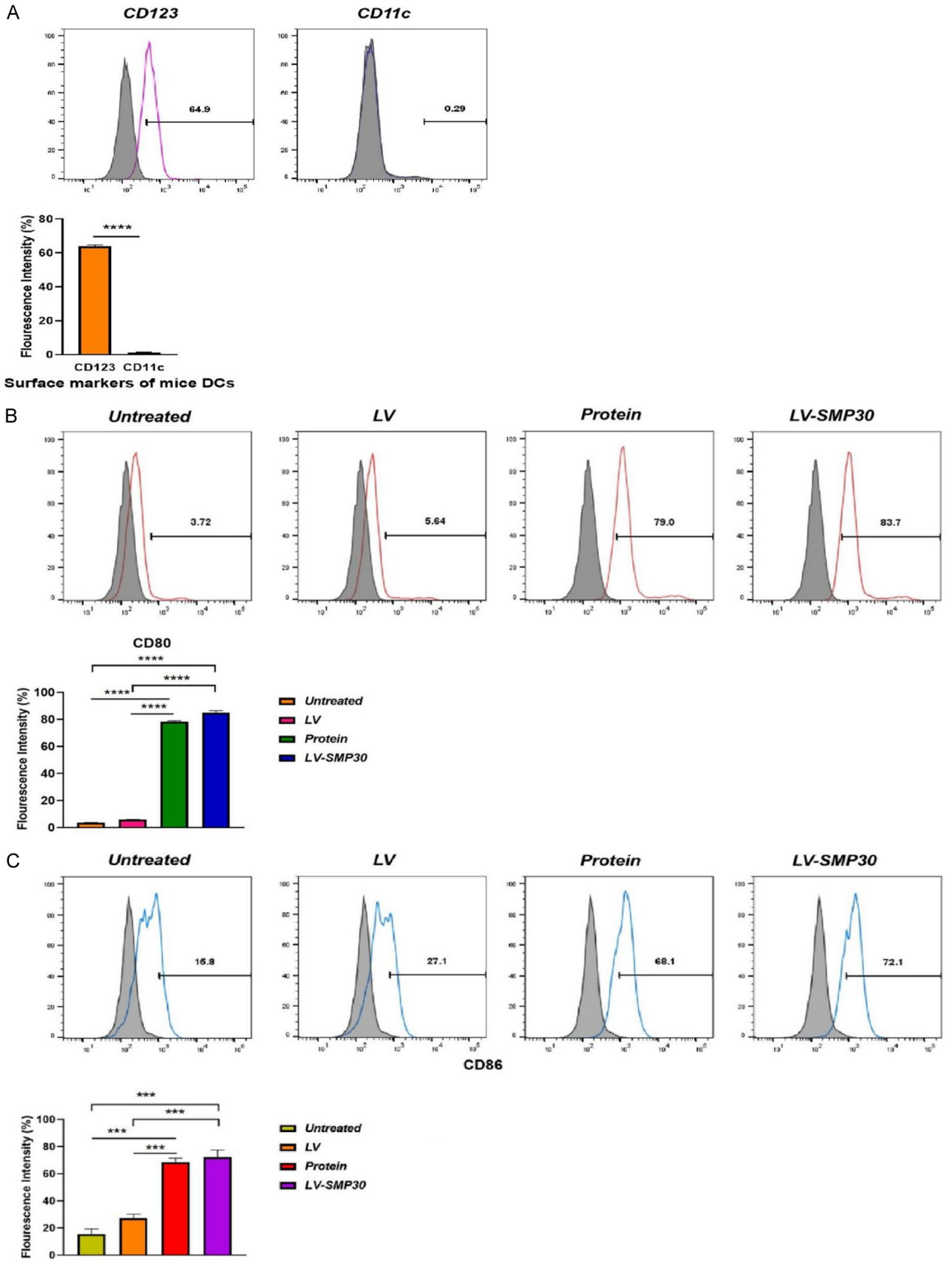

Figure 3. Phenotype identification of DCs by surface markers. A. The percentage of CD123 was substantially higher in comparison to CD11c [(64.9±0.06)% *vs*. (0.29±0.04)%, $P<0.05$], indicating that DC123 was the main type of DCs in mice. B. The cell percentage of CD80 increased more significantly in the *LV-SMP30* group and *Protein* group than in the *LV* group or *Untreated* group ($P<0.05$). C. Similarly, the cell percentage of CD86 increased more significantly than in the *LV* group or *Untreated* group ($P<0.05$). Overall, the results indicated that *LV-SMP30* improved DC maturation. A bar graph shows statistical significance for group comparisons.

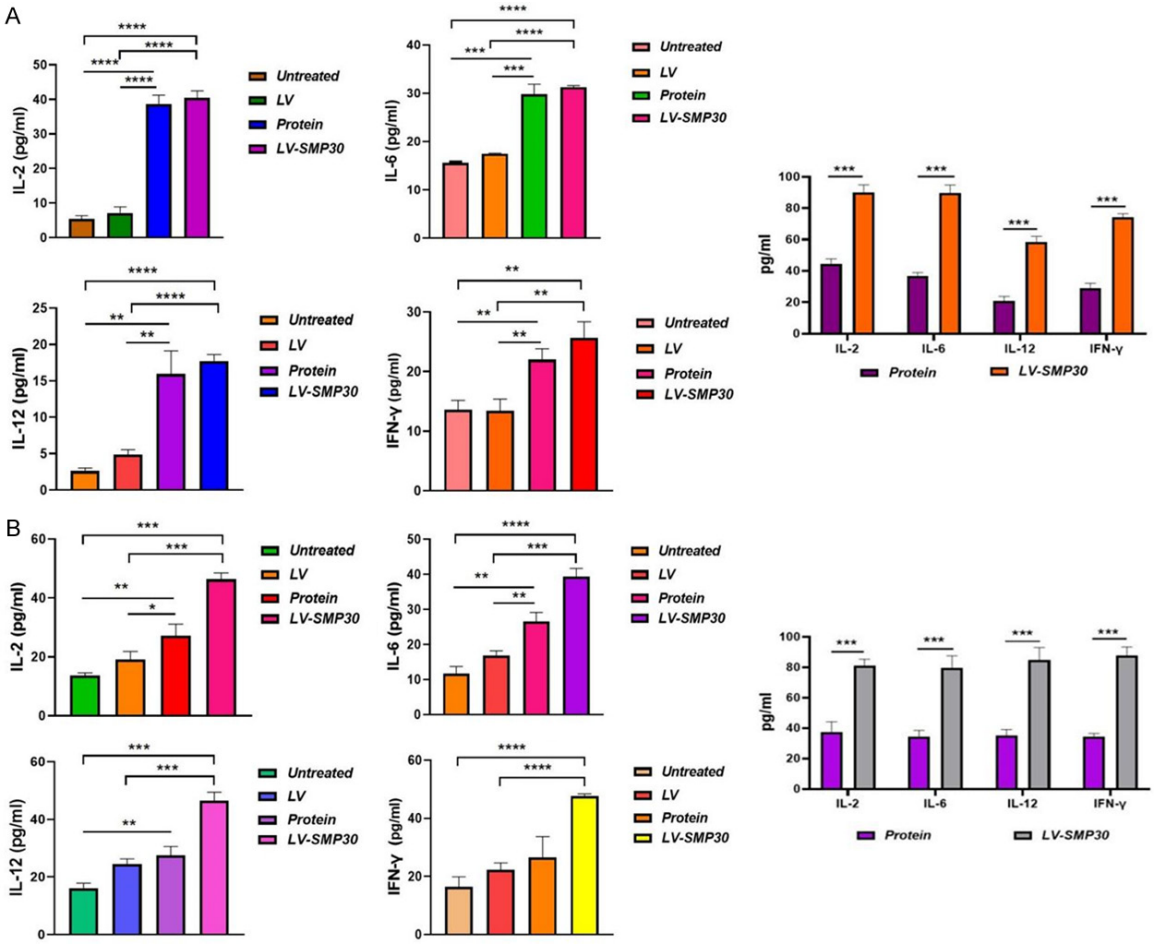

Figure 4. IL-2, IL-6, IL-12 and IFN-γ concentrations in supernatant were analyzed by ELISA. A. At 72 hours, IL-2, IL-6, IL-12 and IFN-γ in *LV-SMP30* group and *Protein* group were increased significantly in comparison with those of the *LV*-group or *Untreated* group ($P<0.05$). At one week, the levels of IL-2, IL-6, IL-12 and INF-γ in the *LV-SMP30* group were significantly higher than those in the *Protein* group ($P<0.05$). The results suggest that *LV-SMP30* possesses a strong capability to induce the production of IL-2, IL-6, IL-12, and IFN-γ during DC activation. B. Significant increase in IL-2, IL-6, IL-12, and IFN-γ in the *LV-SMP30* group and *Protein* group after co-incubation with the isolate mice PBMC, compared to the *LV* group or *Untreated* group ($P<0.05$). Furthermore, at one week, the levels of IL-2, IL-6, IL-12, and INF-γ in the *LV-SMP30* group were significantly higher than those in the *Protein* group ($P<0.05$). The results suggest that *LV-SMP30* enhances the production of IL-2, IL-6, IL-12 and IFN-γ.

group were significantly higher than those in the *Protein* group ($P<0.05$) (**Figure 4B**). These results suggest that *LV-SMP30*-transduced DCs promote T cell differentiation and cytokine production.

LV-SMP30-transduced DCs induce $CD3^+CD8^+$ T cell activation and proliferation

Since PBMC can differentiate into T cells when stimulated with IL-2, anti-CD3, and anti-CD28, we assessed whether *LV-SMP30*-transduced DCs could induce the differentiation of PBMCs. We firstly isolated $CD8^+$ T cells using $CD8^+$ a magnetic beads separation kit, then used FCM to determine the $CD3^+CD8^+$ T cell counts. As seen in **Figure 5**, $CD3^+CD8^+$ T cells were the most abundant in the isolate (98.55%).

In addition, *LV-SMP30*-transduced DCs were more successful than other control groups at inducing $CD3^+CD8^+$ T cell proliferation based on the CCK-8 kit (**Figure 6**).

$CD3^+CD8^+$ T cell-mediated cytotoxicity

As shown in **Figure 7A**, for H22 cells, the cytotoxicity in the *LV-SMP30* group increased as

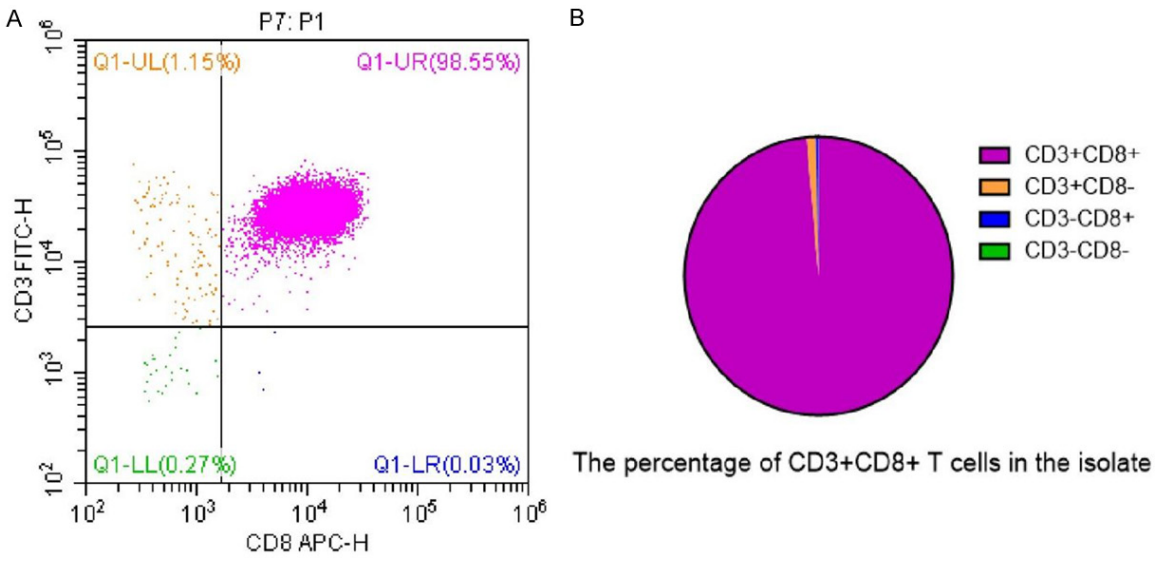

Figure 5. The components of the isolate were assessed by flow cytometry. A. Q1-UR: $CD3^+CD8^+$ T cells; Q1-UL: $CD3^+CD8^-$ T cells; Q1-LL: $CD3^-CD8^-$ T cells; Q1-LR: $CD3^-CD8^+$ T cells. B. A significant difference between $CD3^-CD8^+$ T cells and other cells. The result indicated that $CD3^+CD8^+$ T cells had the highest content in the isolate.

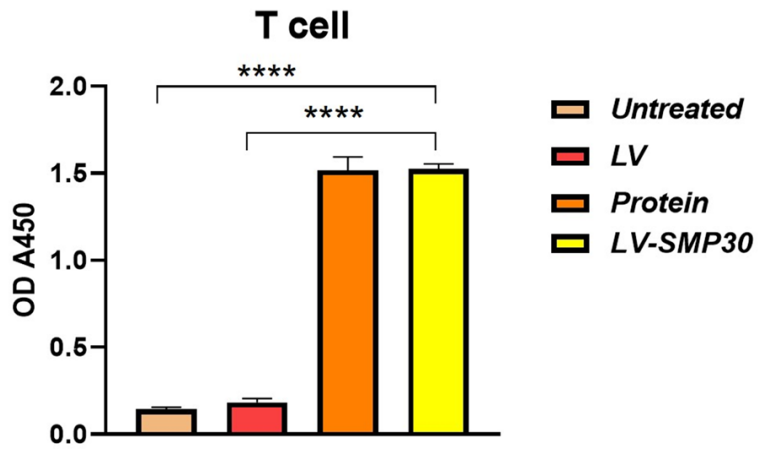

Figure 6. Proliferation of $CD3^+CD8^+$ T cells was assessed by the CCK-8 kit. After the mononuclear cells were isolated, stimulatory antibodies and cytokines were added to differentiate into T cells. T cells screened with $CD8^+$ immunomagnetic beads can have a purity of up to 98%. After co-culture with DCs in each group, they can be induced into $CD3^+CD8^+$ T cells. Compared to the *Untreated* or *LV* groups, the *LV-SMP30* group could significantly proliferate T cells [(1.53.30±0.02)% vs. (0.18±0.02)% vs. (0.14±0.01)%, respectively, $P<0.05$].

These results indicate the *LV-SMP30*-transduced DCs enhanced the $CD3^+CD8^+$ T cell-medicated specific antitumor immune response against H22 cells.

Construction of a liver cancer model and comparison of tumor volume

The BALB/c nude mouse model of subcutaneous xenografts was successfully constructed (**Figure 8A**, **8B**). By analyzing the changes in tumor volume in each group, it was found that the volume in *LV-SMP30* group dropped significantly with the prolongation of survival time compared to those in other control groups ($P<0.05$) (**Figure 8C-E**).

the ratio of effectors/target cells increased (0:1, 10:1; 20:1; 40:1). The cytotoxicity in the *LV-SMP30* group was considerably higher than that of the control groups at ratios of 10:1, 20:1 or 40:1 ($P<0.05$) (**Figure 7B**). We found no substantial cytotoxicity in each group of Hep1-6 cells, as shown in **Figure 7C**. The cytotoxicity of the *LV-SMP30* group was not higher than that of control groups ($P>0.05$) (**Figure 7D**).

In vivo killing mechanism of $CD3^+CD8^+$ T cells

The killing mechanism in vivo can be analyzed by measuring the secretion levels of cytokines INF-γ and IL-2 in mice serum and calculating tumor inhibition rate. Compared to the other control groups, IL-2 and IFN-γ in the *LV-SMP30* group were considerably higher ($P<0.05$) (**Figure 9A**). Furthermore, the *LV-SMP30* group

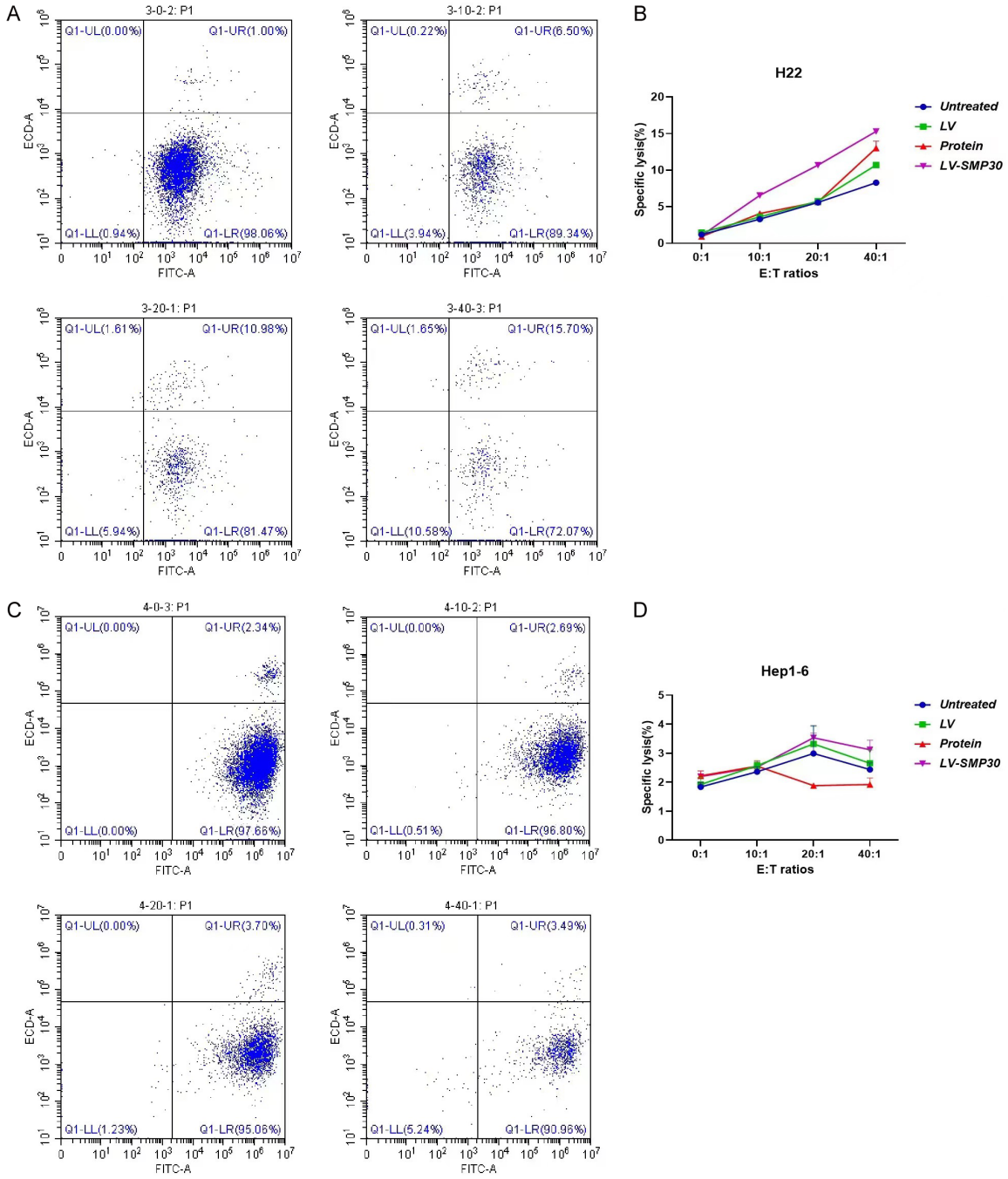

Figure 7. $CD3^+CD8^+$ T cell-mediated cytotoxicity was measured by flow cytometry. A. For H22 cells, the cytotoxicity in the *LV-SMP30* group at the ratio of 10:1, 20:1 or 40:1. B. Statistical chart of target cell H22 specific lysis (%) in each group under different effector-target ratios. C. For Hep1-6 cells, the cytotoxicity in *LV-SMP30* group at the ratio of 10:1, 20:1 or 40:1. D. Statistical chart of target cell Hep1-6 specific lysis (%) in each group under different effector-target ratios. These results indicate that *LV-SMP30* transduced DCs enhances the $CD3^+CD8^+$ T cell-mediated specific cytotoxicity against H22 cells.

had a greater tumor inhibitory impact than other control groups by comparing the tumor inhibition rates of each group (**Figure 9B**). The results suggeste that the *LV-SMP30* group has a better killing effect.

Discussion

Biology and immunology, particularly for tumor targets, are rapidly evolving in response to the changing time. Biological therapy represented

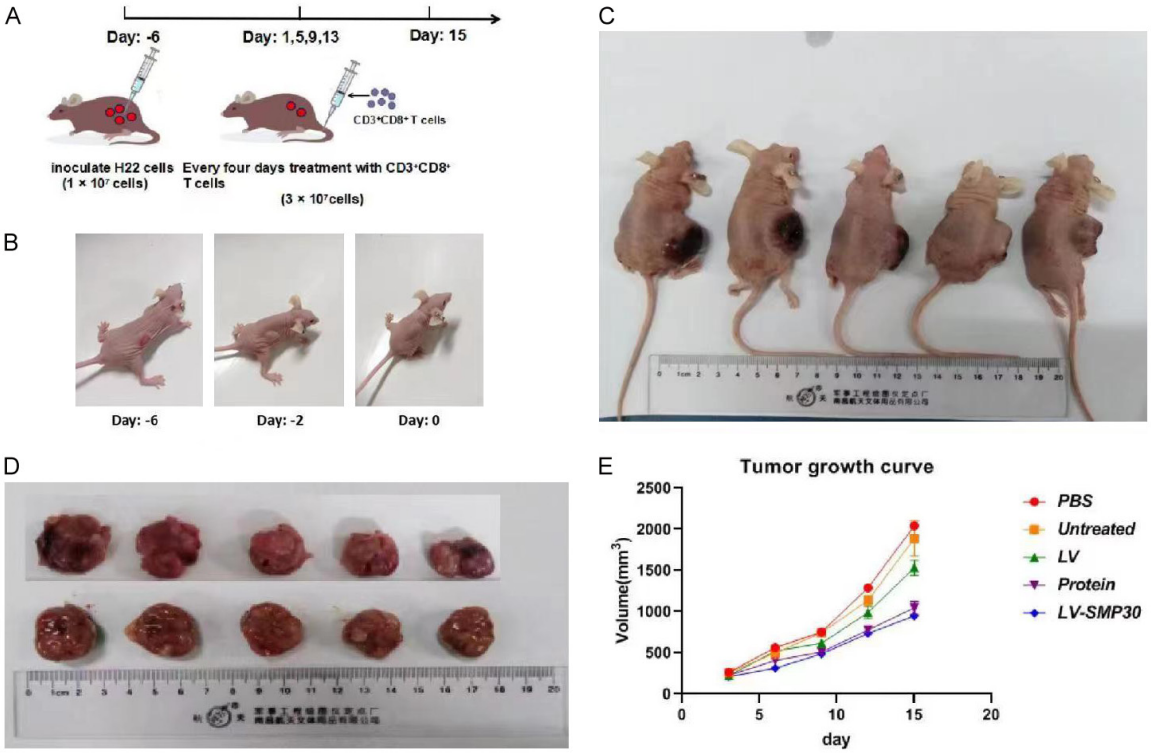

Figure 8. Construction of BALB/c nude mouse models of subcutaneous xenografts. A. BALB/c nude mice with equal-sized (~100 mm^3) single H22 tumors were treated with $CD3^+CD8^+$ T cells. B. Recording chart of tumor growth process. C. Tumor volume on day 15: *PBS* group, *Untreated* group, *LV* group, *Protein* group, *LV-SMP30* group (from left to right). D. Tumors harvested from mice on day 15: *PBS* group, *Untreated* group, *LV* group, *Protein* group, *LV-SMP30* group (from left to right). E. Tumor growth curve. These results demonstrated that the tumor volume in the *LV-SMP30* group was smaller than that of other control groups ($P<0.05$).

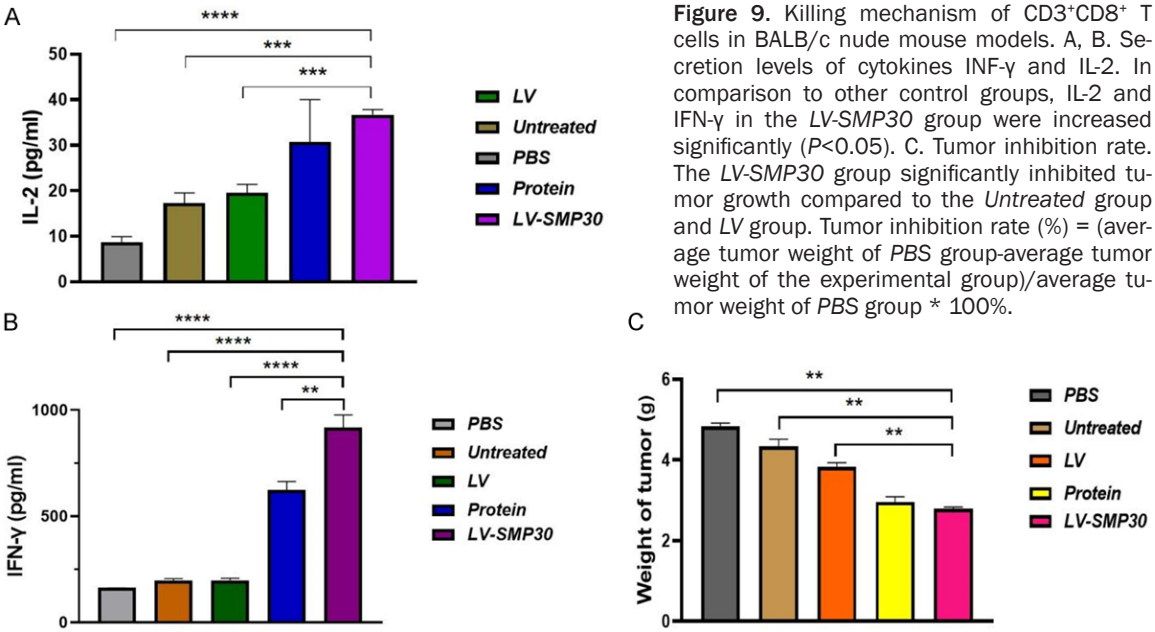

Figure 9. Killing mechanism of $CD3^+CD8^+$ T cells in BALB/c nude mouse models. A, B. Secretion levels of cytokines INF-γ and IL-2. In comparison to other control groups, IL-2 and IFN-γ in the *LV-SMP30* group were increased significantly ($P<0.05$). C. Tumor inhibition rate. The *LV-SMP30* group significantly inhibited tumor growth compared to the *Untreated* group and *LV* group. Tumor inhibition rate (%) = (average tumor weight of *PBS* group-average tumor weight of the experimental group)/average tumor weight of *PBS* group * 100%.

by immunotherapy has become a popular topic in recent studies, particularly in tumor control [23]. Since anti-tumor immunity in the human body is mostly dependent on activation and

proliferation, the initial T lymphocytes have a large number of aggregates in the body, on which their proliferation and killing abilities are based [24]. B cells and macrophages can interact only with memory T cells and activated cells. DCs play a particular role in the immune response because T cells are activated [25]. DCs are highly effective in antigen presentation, as a small amount of DCs activate T cells after antigen presentation [26, 27]. It has been proven that DC vaccination can induce antitumor immunity in a variety of mouse models, so mice were used in related experiments of this study.

DC differentiation includes $CD14^+$ monocyte-derived and $CD34^+$ stem cells *in vitro*, according to the current method for generating DCs. The former is primarily detected in peripheral blood mononuclear cells (PBMCs), whereas the latter was discovered in bone marrow and cord blood [28, 29]. A vast number of studies have shown that $CD34^+$ stem cell-derived DCs can elicit more effective $CD8^+$ T cell response than $CD14^+$ monocyte-derived DCs [28, 30-32]. In the present study, we derived $CD34^+$ DCs from mouse bone marrow and identified *LV-SMP30*-transduced DCs phenotype with surface markers (CD123, CD11c, CD80 and CD86). The CD80, CD86 [33] surface molecules are all markers of DC maturation, while CD123 and CD11c are markers of DC classification in mice. The expression of CD80 and CD86 were significantly up-regulated, implying that the *LV-SMP30*-transfected DCs can increase the expression of mouse DC maturation.

Another essential marker of DC maturation is the secretion of cytokines, such as IL-12. High levels of IL-12 can differentiate Th0 cells into Th1 cells while promoting proliferation of Th1 cells, mediating cellular immune effects, and promoting the activation and proliferation of effector cells (NK cells, lymphokines and macrophages, etc.). They induce a variety of cytokines such as IFN-γ and IL-6 [34, 35]. IL-12 and IFN-γ are both major inflammatory factors. IFN-γ can induce the expression of MHC class II molecules, and the mediation of $CD3^+CD8^+$ T cell responses can also upregulate MHC class I molecules. Our results revealed that *LV-SMP30* improved the production of IL-2, IL-6, IL-12, and INF-γ. The levels of IL-2, IL-6, IL-12, and INF-γ in the *LV-SMP30* group were significantly higher than those in the *Protein* group, especially after the DCs were cultured for continuous days. These results suggest that *LV-SMP30* enhances DC maturation and secretion of Th1 cytokines, and that SMP30 is more effective at sensitizing DCs at the gene level than at the protein level.

$CD3^+CD8^+$ T cells play a crucial role in specific antitumor immunity. The co-incubation of mature DCs with T cells induces the formation and proliferation of tumor-specific $CD3^+CD8^+$ T cells. $CD3^+CD8^+$ T cells mediate the death of target cells mainly by releasing toxic particles and activating death receptor pathways. Perforin and granzymes are major cytotoxic proteins of effector cells [36, 37]. When effector cells come into contact with host cells infected with pathogenic bacteria or cancerous tissues, they produce granzymes and perforin, causing host cells to die [38]. Therefore, the secretion of IL-2 and IFN-γ [39-42] cytokines reflects the activity and cytotoxicity of T cells. The ability of *LV-SMP30*-transduced DCs to induce the activation and proliferation of T cells was then tested. We found that *LV-SMP30*-transduced DCs strongly promoted the differentiation and proliferation of $CD3^+CD8^+$ T cells in mouse PBMCs. The most commonly used assay for $CD3^+CD8^+$ T cells is to detect IFN-γ, a $CD3^+CD8^+$ T cell activation marker, but other cytokines such as IL-2, IL-6, and IL-12 can also be used to differentiate activated T cells. Most importantly, the $CD3^+CD8^+$ T cell-mediated cytotoxicity was considerably higher in the *LV-SMP30* group compared to those in the control groups in H22 cells but not Hep1-6 cells. These findings suggest that *LV-SMP30*-transduced DCs enhanced T cell activation and the specific immune response against specific mouse liver cancer H22 cells.

BALB/c nude mice lack thymus and are unable to differentiate to T cells normally. Literature reports have shown that BALB/C nude mice have lower mortality and are less expensive than SCID mice, so they are more suitable for subcutaneous liver cancer models [43, 44]. Therefore, to attain the desired goal, we chose the BALB/c nude mice to construct a model of liver cancer. Besides, related literature has revealed that mice models can be constructed in a variety of ways, such as intraperitoneal injection, subcutaneous injection, and surgical

transplantation. Axillary, abdominal, and other subcutaneous injection sites are available. We successfully established the BALB/c node mice subcutaneous xenograft model [45].

Next, we evaluated the changes in tumor volume and weight in each group of mice, as well as detected the serum cytokines IL-2 and IFN-γ, all of which demonstrated that the *LV-SMP30* group had a stronger tumor inhibition impact than the other groups. Tumor shrinkage was inversely proportional to the rise in cytokines such as IL-2 and IFN-γ, and directly proportional to the apoptosis of liver cancer cells. The agreement with the FCM killing results *in vitro* showed better antitumor effects both *in vitro* and *in vivo*. Finally, we can confirm the aforesaid findings by evaluating the tumor development curve and calculating the tumor inhibition rate of each group. There are a variety of approaches to evaluate $CD3^+CD8^+$ T cells intervening in tumor growth. At present, we only measured cytokines, which is insufficient to fully comprehend the relevant mechanism. To prove this, more immunohistochemistry and immune infiltration will be studied [46]. In addition, a small animal imaging system can be used to observe the growth of cancer cells in vivo and the process of tumor reduction after intervention, which is more convenient and accurate, and saves the time and effort of killing mice at different stages, which is more ethical and animal friendly.

In conclusion, we successfully constructed and transduced mouse DCs with *LV-SMP30* recombinant expressing SMP30 gene. Experimental results indicated that the *LV-SMP30* group had a higher influence on DC maturation than the *Untreated* group and *LV* group, and that the *LV-SMP30*-transduced DCs induced $CD3^+CD8^+$ T cells to express more IL-2, IL-6, IL-12, and IFN-γ. The *LV-SMP30*-transduced DCs were effective at inducing special T cell cytotoxicity against mice liver cancer cells. Our results revealed that using SMP30-modified DCs could be an immunotherapy strategy for liver cancer. Moreover, we can evaluate the effect of DC-based SMP30 antigen vaccine in a mouse model to verify the previous conclusion. Finally, we will continue to evaluate this approach by constructing a mouse PDX model in a future study.

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Disclosure of conflict of interest

None.

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