Acta Pharmaceutica Sinica B 2021;11(2):476–487

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

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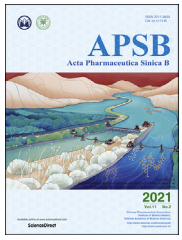

ORIGINAL ARTICLE

Generated SecPen_NY-ESO-1_ubiquitin-pulsed dendritic cell cancer vaccine elicits stronger and specific T cell immune responses

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Received 3 March 2020; received in revised form 2 July 2020; accepted 2 July 2020

KEY WORDS

Dendritic cells;
Cancer vaccine;
NY-ESO-1;
SecPen;
Ubiquitin

Abstract Dendritic cell-based cancer vaccines (DC vaccines) have been proved efficient and safe in immunotherapy of various cancers, including melanoma, ovarian and prostate cancer. However, the clinical responses were not always satisfied. Here we proposed a novel strategy to prepare DC vaccines. In the present study, a fusion protein SNU containing a secretin-penetratin (SecPen) peptide, NY-ESO-1 and ubiquitin was designed and expressed. To establish the DC vaccine (DC-SNU), the mouse bone marrow-derived DCs (BMDCs) were isolated, pulsed with SNU and maturated with cytokine cocktail. Then peripheral blood mononuclear cells (PBMCs) from C57BL/6 mice inoculated intraperitoneally with DC-SNU were separated and cocultured with MC38/MC38$^{NY\text{-}ESO\text{-}1}$ tumor cells or DC vaccines. The results show that SNU was successfully expressed. This strategy made NY-ESO-1 entering cytoplasm of BMDCs more efficiently and degraded mainly by proteasome. As we expected, mature BMDCs expressed higher CD40, CD80 and CD86 than immature BMDCs. Thus, the PBMCs released more IFN-γ and TNF-α when stimulated with DC-SNU *in vitro* again. What's more, the PBMCs induced stronger and specific cytotoxicity towards MC38$^{NY\text{-}ESO\text{-}1}$ tumor cells. Given the above, it demonstrated that DC-SNU loaded

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Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2020.08.004

with SecPen and ubiquitin-fused NY-ESO-1 could elicit stronger and specific T cell immune responses. This strategy can be used as a platform for DC vaccine preparation and applied to various cancers treatment.

1. Introduction

Dendritic cells (DCs), originally discovered in lymphoid tissues by Ralph Steinman and Zanvil Cohn in 1973[1], are considered as the most powerful antigen-presenting cells (APCs) and called "nature's adjuvant" because of its pivotal role in innate and adaptive immune response. DC-based cancer immunotherapy has been proved safe and effective in clinical trials (more than 200 cases)[2−5]. However, the clinical response is barely satisfactory with classic objective tumor response rates rarely exceeding 15%[6]. Therefore, an additional method for DC vaccine preparation may be needed.

Cancer/testis antigens (CTAs) have been regarded as promising targets for cancer immunotherapy because of their restricted expression in adult somatic tissues, high immunogenicity and re-expression in many cancers[7−9]. NY-ESO-1, also known as cancer/testis antigen 1, is a protein with 18 kDa molecular weight and its expression has been detected in extensive types of cancers with expression frequencies ranging from 20% to 100%[10]. NY-ESO-1-based DC vaccines have already been tested in clinical and proved low-grade toxicity[11]. NY-ESO-1 vaccination together with poly-ICLC and montanide enhanced T cell immune responses in melanoma patients[12]. Injection of NY-ESO-1 SPEAR T cells was associated with systemic immunity in synovial sarcoma[13]. Anti-NY-ESO-1 specific antibodies were also detectable in serum of cancer patients but not in healthy individuals[14]. However, the clinical outcome was not always satisfied, which might be correlated with low percentage of patients who achieved specific $CD8^+$ T cell immune responses[12,15].

$CD8^+$ T cells exert an important function in antitumor immunity as they can directly recognize plenty of antigens that are expressed by tumor cells. However, induction of $CD8^+$ T cells responses during DC vaccination requires MHC I molecule-presented epitopes of intracellular antigens degraded by proteasome, which depends on cross-presentation of exogenous antigens by APCs, especially DCs[16,17]. Application of RNA as antigen source is considered as a solution and has been proved to be more effective than whole tumor lysates in animal models[18]. Nonetheless, antigen RNA needs to be transfected into primary DCs, which may cause unavoidable damage to cells. The optimal approach for intracellular delivery of antigens to produce the strongest $CD8^+$ T cell responses has yet to be identified[19].

Cell penetrating peptides (CPPs) are a group of short peptides with 5−30 amino acids, that are able to penetrate cell membrane in many cell types[20]. These peptides have been investigated as a delivery tool to transport cargoes (such as proteins, peptides, DNAs, siRNAs and small drugs) into cells for many years[21,22]. Though none of the CPPs was approved by U.S. Food and Drug Administration (FDA), there is evidence showing that CPP-antigen conjugation-loaded DC vaccines boost more intense immune responses than antigen alone in mouse models[23−25]. Penetratin, one of the most commonly used CPPs, was able to deliver cargoes into different cell types. And combination with secretin peptide, the SecPen was proved to be able to penetrate membrane of cells with tight junction[26]. Because of low effectiveness of DC vaccines, SecPen might be a promising material for antigen pulsing to elicit stronger $CD8^+$ T cell responses in future clinical studies.

In this work, we expressed a fusion protein termed as SNU containing a SecPen domain, NY-ESO-1 and ubiquitin with *Escherichia coli* expression system. This novel chimeric recombinant protein was delivered into mouse bone marrow-derived DCs to develop a next generation of DC vaccine. This DC vaccine could induce stronger and specific immune responses as expected against $MC38^{NY\text{-}ESO\text{-}1}$ tumor cells *ex vivo*.

2. Materials and methods

2.1. Cell lines and reagents

Murine colon carcinoma cell line MC38 was ordered from Suzhou LongMab Biosciences, Suzhou, China. BMDCs were separated from female C57BL/6 mice and sorted using EasySep™ Mouse CD11c Positive Selection Kit II (STEMCELL, Vancouver, Canada) according to the manufacturer's instructions. Anti-mouse CD16/32 antibody and mouse antibodies of FITC-H-2Kb/H-2Db, FITC-I-A/I-E, PE/APC-CD11c, FITC-CD40, FITC-CD80, FITC-CD86 and PE-CCR7 were all purchased from BioLegend, San Diego, CA, USA. All cytokines used in this work were kindly provided by Novoprotein, Shanghai, China. Primary anti-NY-ESO-1 and anti-His rabbit antibodies are CST products (Danvers, MA, USA), and secondary goat anti-rabbit Alexa fluor 488 antibody is a product of Invitrogen, Carlsbad, CA, USA. The proteasome inhibitor lactacystin was bought from APExBIO, Houston, TX, USA. SYBR GREEN qPCR mix was purchased from Vazyme Biotech, Nanjing, China. All ELISA kits and the mouse IFN-γ ELISPOT kit were obtained from DAKEWE Biotech, Beijing, China. Mouse MIP-3β (CCL19) was purchased from Peprotech, Cranbury, NJ, USA.

2.2. Expression and purification of recombinant protein SNU and SN

The recombinant fusion proteins SNU and Sumo_NY-ESO-1 (SN) were expressed and purified by Shanghai Novoprotein Biotechnology Company, Shanghai, China.

2.3. Preparation of dendritic cell-based cancer vaccine

BMDCs were isolated from C57BL/6 mice as previously described with several modifications[23]. After the red blood cells were lysed, the remaining cells were cultured with RPMI-1640 medium containing 10% fetal bovine serum (FBS), 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10 ng/mL interleukin (IL)-4 for 6 days with culture medium replacement every 2 days. Then the cells were collected, pulsed with 100 μg SNU or SN for 24 h and maturated with a cytokine cocktail (10 ng/mL IL-1β, 1000 U/mL IL-6, 100 ng/mL IFN-γ, 10 ng/mL TNF-α and 1 μg/mL prostaglandin E2 (PGE2)) for 24 h. After centrifugation at 1000 rpm (HERAEUS Multifuge X1R, Thermo Fisher Scientific, Waltham, MA, USA) for 5 min, the cells were harvested as DC vaccine (DC-SNU or DC-SN) and the supernatant were used to detect IL-12 titer by ELISA according to the manufacturer's instructions (DAKEWE).

2.4. Flow cytometry

BMDCs were collected and washed with PBS. After blocked by anti-CD16/32 antibody (Biolegend). Cells were double labeled with CD11c and H-2Kb/H-2Db, I-A/I-E, CD40, CD80, CD86 or CCR7 at 4 °C for 30 min. After washing step with cold PBS, cells were analyzed by CytoFlex S Flow Cytometry (Beckman Coulter, Brea, CA, USA).

2.5. Laser scanning confocal microscopy (LSCM)

To confirm the cytoplasmic antigen of BMDCs, an immunofluorescence assay was performed. Firstly, the confocal dishes were treated with 0.01% poly-D-lysine for 10 min at room temperature and dried for 1 h at 60 °C. Then the BMDCs were counted, planted in the dishes at 1.5×10^5 cells/mL and cultured at 37 °C overnight. At the second day, unattached cells were removed and fresh medium containing 5 μg/mL SNU/SN were added. Under incubated with or without 3 μmol/L lactacystin (APExBIO), the cells were collected in indicated times and stained with primary antibody and secondary Alexa fluor 488 antibody at 4 °C in dark. After washed with PBS for 3 times, cells were dyed with DAPI and analyzed by a Zeiss confocal machine (Oberkochen, Germany).

2.6. Transwell assay

Transwell model was applied to evaluate the cell migration capacity as previously described[27]. Briefly, BMDCs were provoked with or without cytokine cocktail for 24 h and then seeded in the upper chambers of a 24-well Transwell plate (1×10^5 cells/chamber). The same culture medium that contained indicated concentrations of MIP-3β were added into the lower chambers. Twenty-four hours later, the cells were washed with PBS and stained with 1% crystal violet. After that, cells of the lower layer of upper chambers were scraped off and the upper layer cells were washed with PBS for 3 times. Images of the upper layer cells were acquired using an inverted microscope (Nikon, Shinagawa, Tokyo, Japan). The migrated cells were collected and counted.

2.7. Scanning electron microscope

The sorted BMDCs were harvested and washed with ice-cold PBS twice, and then fixed with phosphate-buffered glutaraldehyde at 4 °C overnight after cultivated with or without cytokine cocktail for 24 h. All samples were observed by a Hitachi SU8000 scanning electron microscope (Marunouchi, Japan).

2.8. Coculture of sensitized PBMCs and DC vaccine or NY-ESO-1 protein

To evaluate the immune activation capacity of DC vaccine, 30 female C57BL/6 mice were immunized with 10^6 DC-SNU cells/mouse. Seven days later, PBMCs were isolated from whole blood with peripheral blood monocytes separation solution kit (TBD science, Tianjin, China) according to the manufacturer's instructions, and then restimulated with DC or DC-SNU in different ratios for 24 h, respectively. The comparative mRNA level of *Ifng*, *Tnfa*, *Il2* and *Il12* and protein expression of IFN-γ (IFNG) and IL12 were assessed by qPCR and ELISA, respectively.

To compare the immune activation capacity of DC-SN and DC-SNU, PBMCs from C57BL/6 mice were isolated and cocultured with DC-SN or DC-SNU *in vitro*. The comparative mRNA and protein level of IFNG, TNF-α (TNFA) and IL2 were detected.

We also cultivated allergic PBMCs with NY-ESO-1 protein (RayBiotech, Norcross, GA, USA) for 24 h. And the IFNG expression was evaluated.

2.9. Construction of stable NY-ESO-1 expressing MC38 cells, $MC38^{NY\text{-}ESO\text{-}1}$

The full length mRNA of *NYESO1* (Accession number: NM_001327) was amplified from cDNA of a human breast cancer cell line SK-BR-3 by PCR using paired primers (forward primer: CCGGAATTCATGCAGGCCGAAGGCCGGGGCACA; reverse primer: CGCGGATCCTTAGCGCCTCTGCCCTGAGGGAG) with restriction enzymes EcoRI and BamHI. After gel extraction and double enzyme digestion, the product was cloned into a lentivector pCDH-CMV-MCS-EF1-copGFP-T2A-Puro and sequenced with universal primers. The recombinant lentivector, pCDH-NYESO1, was subsequently co-transfected with packaging plasmids pMD2. G and psPAX2 into HEK293T cells. After 24 h, the supernatant was discarded and fresh medium was added. Another 48 h later, the supernatant was collected and added into MC38 cells. The cells were screened by 2 μg/mL puromycin and the living attached cells were expanded and sorted by flow cytometry. To identify the sorted cells, qPCR was applied to evaluate the mRNA level of *NYESO1* and Western blot was used to determine the NY-ESO-1 protein expression.

2.10. Coculture of sensitized PBMCs and $MC38^{NY\text{-}ESO\text{-}1}$ cells

The experimental protocol was consistent with that mentioned above. Briefly, the isolated PBMCs were cocultured with 5×10^5 $MC38^{NY\text{-}ESO\text{-}1}$ cells for 12 h. Then the total mRNA of suspended cells was extracted to detect the transcriptional level of *Ifng*, *Tnfa*,

Il2, perforin and granzyme B. The protein expression of IFNG, TNFA and IL2 in the supernatant were analyzed by ELISA (DAKEWE).

2.11. IFN-γ ELISPOT assay

IFN-γ released by PBMCs was captured with mouse IFN-γ pre-coated ELISPOT kit (DAKEWE) according to the manufacturer's instructions. Briefly, the plate was firstly activated by RPMI-1640 medium and then 1×10^6 sensitized PBMCs were inoculated alone or with 1×10^4 DC-SN or DC-SNU cells and 1×10^5 MC38 or $MC38^{NY\text{-}ESO\text{-}1}$ cells. Positive, negative and blank control wells were arranged. The plate was cultured at 37 °C for 21 h and the spots were calculated by Bioreader 4000 ELISPOT analyzer (Biosys, Germany).

2.12. LDH cytotoxicity assay

The 96-well plate was seeded with 1×10^4 MC38 or $MC38^{NY\text{-}ESO\text{-}1}$ cells and cultured at 37 °C overnight for cell attaching. Afterwards, separated PBMCs sensitized by DC-SN or DC-SNU were added to each well in indicated proportions. The released lactate dehydrogenase (LDH) was detected by LDH cytotoxicity assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions.

2.13. Statistical method

All data are presented as the mean ± standard deviation (SD). Significance analyses were performed by two-tailed Student's *t* test. A $P < 0.05$ was defined as significance.

3. Results

3.1. Design, expression and purification of NY-ESO-1-based fusion protein SNU

The cancer/testis antigen NY-ESO-1 is thought to be an appropriate candidate for DC vaccine on account of its immunogenicity and existence of variable MHC restricted epitopes (Fig. 1A). Therefore, we designed a fusion protein SNU which contains a N-terminal SecPen, full length NY-ESO-1and C-terminal ubiquitin to be the tumor antigen for DC vaccine preparation instead of orphan NY-ESO-1 antigen (Fig. 1B). And we employed *E. coli* expression system to express the SNU protein. As shown in Fig. 1C, the SNU was successfully expressed and verified by SDS-PAGE and Western blot after denaturation and renaturation.

3.2. Preparation of DC vaccine pulsed with fusion protein SNU (DC-SNU)

BMDCs were isolated from C57BL/6 mice and cultured with GM-CSF and IL-4 for 6 days. The cells appeared to grow in clumps (Fig. 2A) and displayed an immature state that the cells expressed high levels of MHC molecules and low levels of co-stimulatory molecules such as CD40, CD80 and CD86 after stimulated with GM-CSF and IL-4 for 6 days (Fig. 2B). To ensure the purity of DCs, magnetic beads were used for CD11c positive selection to sort the cultured cells and yielded the purity of DCs to >99% (Fig. 2C). The SNU protein was designed to be capable of entering the cytoplasm of DCs and be degraded through proteasome. After coculture of DCs and SNU, it was found that SNU had entered the cytoplasm since 1 h and yielded to the maximum at 6 h (Fig. 2D). When proteasome was inhibited by lactacystin (APExBIO), more

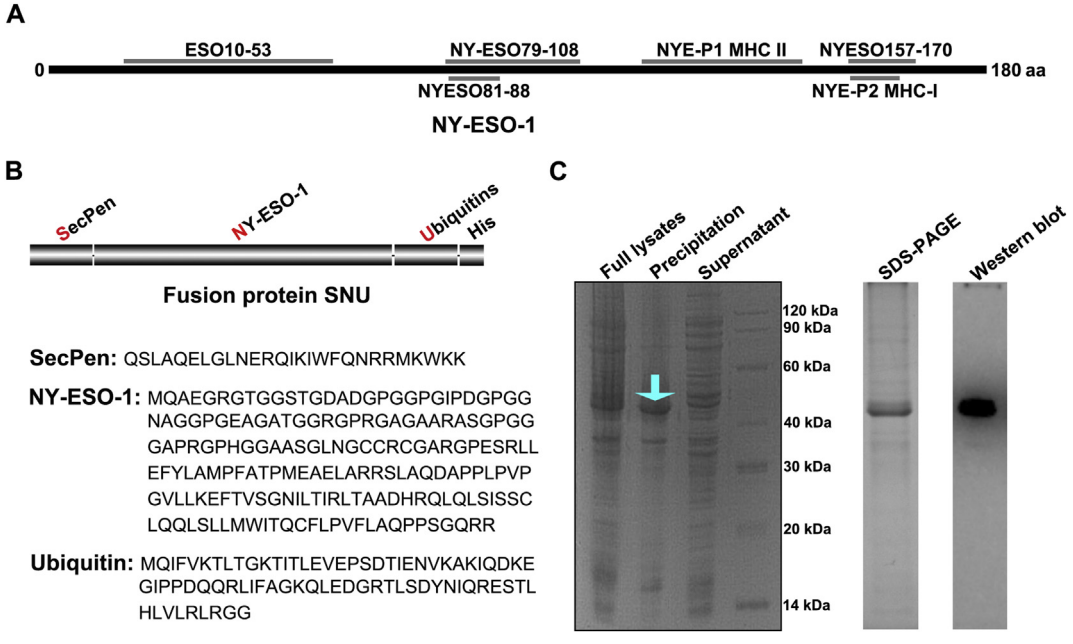

Figure 1 Design, expression and purification of recombinant fusion protein SNU. (A) Reported MHC epitopes located in NY-ESO-1. (B) Diagram of fusion protein SNU and its sequence. (C) The fusion protein SNU was expressed using *E. coli* expression system. After purified, SNU was detected by Western blot using anti-his mAb.

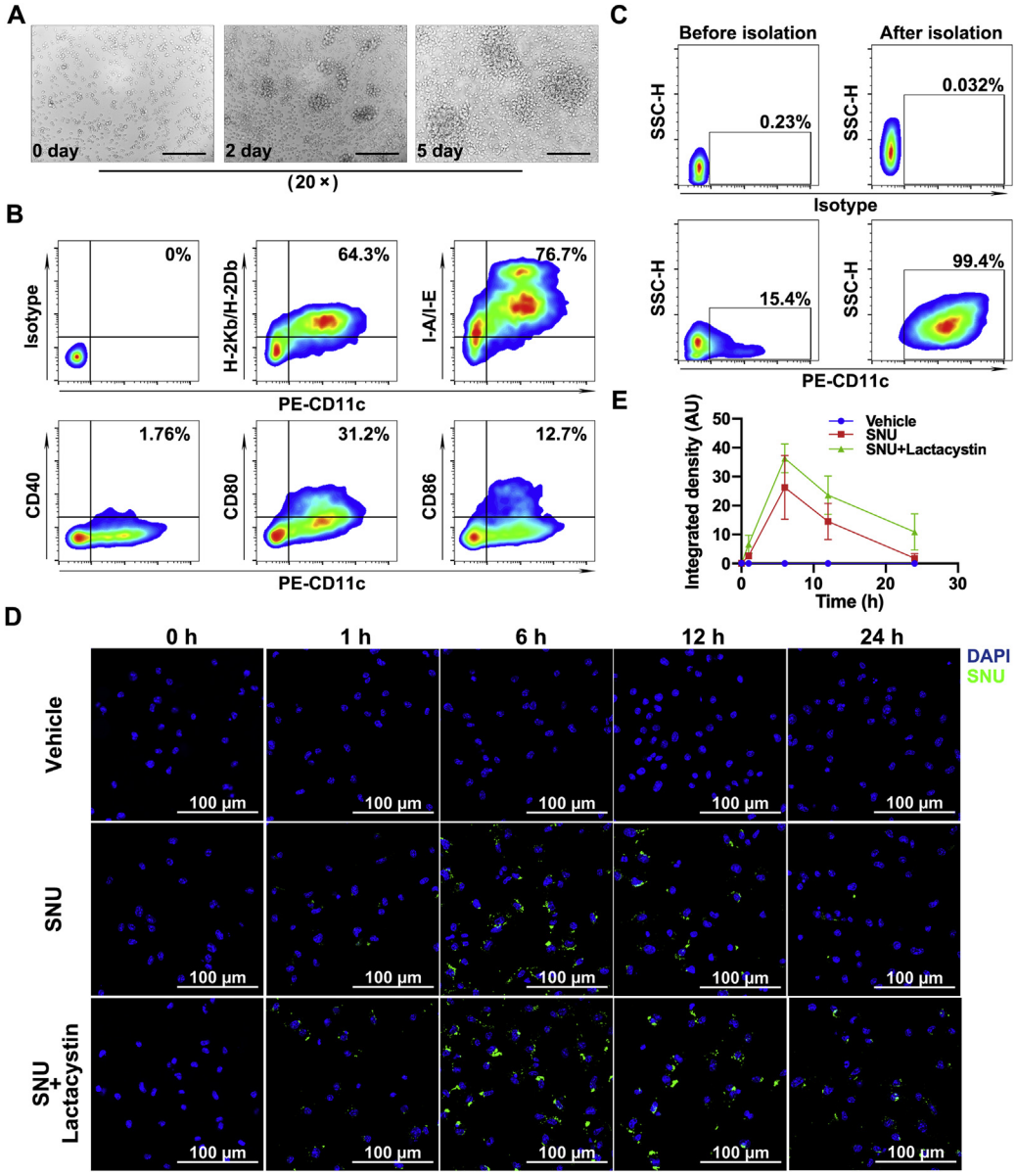

Figure 2 Isolation, sorting and SNU loading of BMDCs. (A) The BMDCs were flushed from femurs and tibias of C57BL/6 mice and cultured in 1640 complete medium containing GM-CSF and IL-4. Images at Days 0, two and five were taken with optical microscope. Scale = 100 μm. (B) After 6 days, the expression of MHC molecules (H-2Kb/H-2Db and I-A/I-E) and costimulatory molecules (CD40, CD80 and CD86) were analyzed by flow cytometry. (C) The BMDCs were positively selected using CD11c positive selection kit. The selected and unselected cells were stained with PE-CD11c and detected by flow cytometry. (D) The isolated cells were incubated with or without SNU under stimulation with or without lactacystin in indicated times. The intracellular SNU were observed through LSCM at 0, 1, 6, 12 and 24 h. Scale = 100 μm. (E) The fluorescence value was calculated by ImageJ software and shown as mean ± SD, $n = 4$.

SNU were observed in the cytoplasm of DCs at all time points (Fig. 2D and E).

Maturation of DCs is an unavoidable step to generate functional DC vaccines. Thus, in this work, we applied a cytokine cocktail including IL-1β, IL-6, IFN-γ, TNF-α and PGE2 to maturate DCs. Once DCs were stimulated by cytokine cocktail, the expressions of both MHC and co-stimulatory molecules (CD40, CD80, and CD86) and IL12 were elevated (Fig. 3A and B). Additionally, the chemokine CCR7 which is involved in homing of T cells to the spleen as well as trafficking of T cells within the spleen was also upregulated under the impact of cytokine cocktail (Fig. 3C). Mature DCs have a stronger migration capacity than immature DCs. In this study, more DCs migrated through the Transwell membrane after maturation (Fig. 3D and Supporting Information Fig. S1). As shown in Fig. S1, mature BMDCs migrated towards MIP-3β (CCR7 ligand) in a dose-dependent manner. However, a majority of untreated DCs remained in the upper layer of the chambers and a small number of cells migrated even though the concentration of MIP-3β reached 100 ng/mL (Fig. 3D and Fig. S1). Through the scanning electron microscope, DCs treated with cytokine cocktail performed a mature state showing rough surface and more tentacles like branches than that without treatment (Fig. 3E).

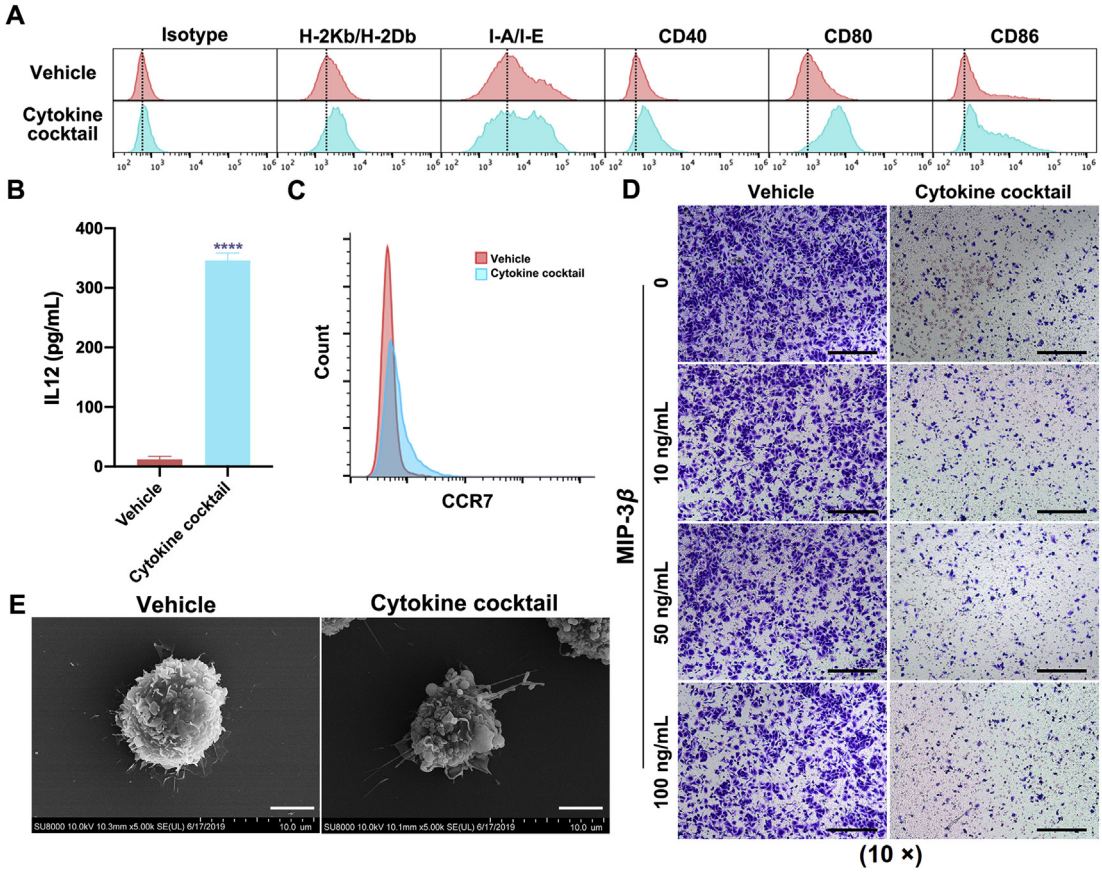

Figure 3 Maturation of BMDCs. The SNU-pulsed DCs were matured by cytokine cocktail (IL-1β, IL-6, TNF-α, IFN-γ and PGE2) for 24 h and the MHC molecules (A), costimulatory molecules (A) and CCR7 (C) expression were analyzed by flow cytometry. Also, the secretion of IL12 in the supernatant was measured by ELISA (B). (D) The migration to MIP-3β of DCs stimulated with or without cytokine cocktail was performed with a Transwell model. After 24 h, the unmigrated cells in the upper chamber were stained with crystal violet and imaged by optical microscope. Scale = 200 μm. (E) The morphological characteristics of DCs stimulated with or without cytokine cocktail were analyzed by scanning electron microscope. Scale = 10 μm.

3.3. DC-SNU induced stronger and specific T cell immune responses ex vivo

To test the immune activation capacity of DC-SNU, DC-SNU was injected into female C57BL/6 mice intraperitoneally, which was considered as an appropriate injection route[28,29]. Seven days after immunization, PBMCs were isolated and cocultured with DC or DC-SNU. Our data show that DC-SNU significantly upregulated the mRNA and protein level of IFNG and IL12 in PBMCs (Fig. 4A and B). The comparative mRNA level of *Tnfa* and *Il2* in each group was also detected and the results were similar (Supporting Information Fig. S2). Furthermore, PBMCs sensitized by DC-SNU always released more IFN-γ than that by DC in different DC:PBMC ratios (Fig. 4C). To verify the results above, we conducted the IFN-γ ELISPOT assay. The results reveal that more IFN-γ spots were observed when sensitized PBMCs were retreated with DC-SNU than other two groups, and the exact number of spots in each well was counted and analyzed (Fig. 4D). When the PBMCs were stimulated with NY-ESO-1 protein again, the PBMCs from DC-SNU immunized mice could release more IFN-γ than that of vehicle or DC (Fig. 4E). All these results were consistent revealing that DC-SNU immunization induced T cell immune responses in mice.

The SNU was designed to prove that DC-SNU could induce stronger T cell immune responses than NY-ESO-1-pulsed DC vaccine. To affirm this thought, we expressed another fusion protein SN which contained a SUMO tag and full-length NY-ESO-1 (Fig. 5A). After purification, the protein was identified at nearly 40 kDa by SDS-PAGE and Western blot using anti-NY-ESO-1 rabbit mAb (Fig. 5B). Specific anti-NY-ESO-1 antibody was detected in the serum of SN or SNU immunized mice, which clarified that addition of CPP and ubiquitin did not change the immunogenicity of NY-ESO-1 (Supporting Information Fig. S3). Without fusion of SecPen, the cytoplasm SN significantly decreased compared with SNU at six and 12 h illustrating that fusion of SecPen could mediate entering of tumor antigen into DCs more efficiently (Fig. 5C and D). During 12−24 h, SNU was comparatively degraded more efficiently than SN according to the gradient of curves (Fig. 5D). Then the PBMCs were separated as described above. After stimulated by DC-SN or DC-SNU *in vitro*, the PBMCs boosted with DC-SNU secreted higher titer of IFN-γ and IL-2 at both mRNA and protein level. However, elevated TNF-α was only

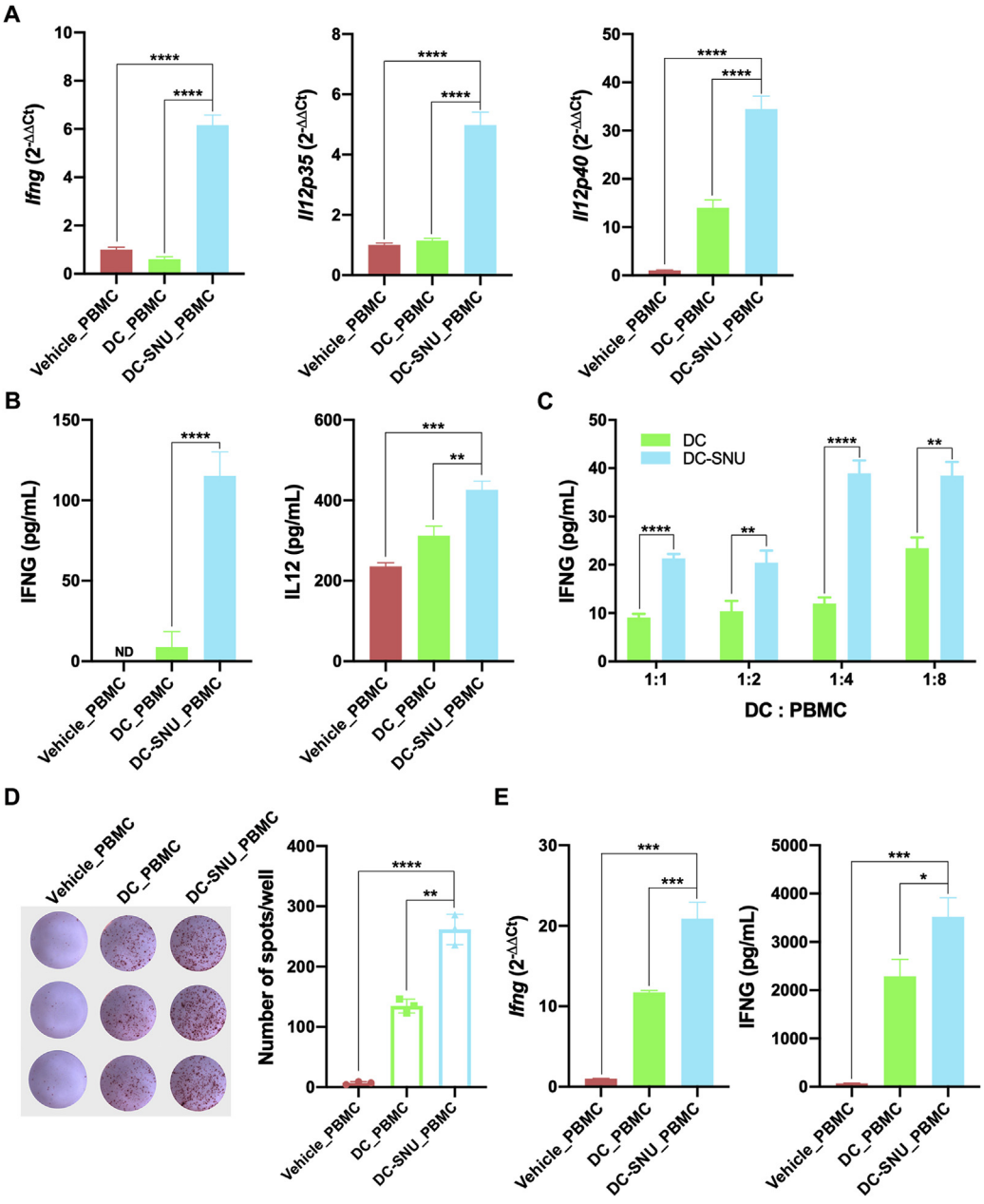

Figure 4 DC-SNU could elicit T cell immune responses *ex vivo*. PBMCs isolated from DC-SNU immunized mice were cocultured with DC/DC-SNU, respectively. The comparative mRNA (A) and protein (B) expression of IFNG and IL12 were quantified by qPCR and ELISA, respectively. (C) The IFN-γ release of DC-PBMC coculture system in different ratio was detected by ELISA. (D) The IFN-γ spots of PBMCs restimulated with DC/DC-SNU were counted by ELISPOT assay. (E) The PBMCs isolated from DC/DC-SNU immunized mice were stimulated with NY-ESO-1 protein and the mRNA and protein level of IFNG were quantified by qPCR and ELISA, respectively. All the data were represented as mean ± SD, $n = 3$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

detected by qPCR (Fig. 5E and F). In line with the above results, DC-SNU boosted PBMCs released more IFN-γ than DC or DC-SN. As shown in Fig. 5G, more spots were observed in DC-SNU_PBMC group but fewer spots in DC-SN_PBMC group. And PBMCs without any stimulation barely secreted IFN-γ.

To test the antitumor effect of DC-SNU sensitized PBMCs, we constructed a stable NY-ESO-1 expressing cell line MC38$^{NY\text{-}ESO\text{-}1}$ based on mouse colon carcinoma MC38 cells using lentivirus transfection method. The puromycin-resistant cells were sorted with flow cytometry due to GFP expressed by MC38$^{NY\text{-}ESO\text{-}1}$ cells (Fig. 6A). The sorted cells were then detected by two different experiments. The MC38$^{NY\text{-}ESO\text{-}1}$ cells expressed high level of *NYESO1* mRNA than MC38 cells through qPCR (Fig. 6B). At the protein level, expression of NY-ESO-1 was found in MC38$^{NY\text{-}ESO\text{-}1}$ lane while no band was detected in MC38 lane (Fig. 6C). As we expected, PBMCs allergized by DC-SNU expressed higher levels of IFNG, TNFA and IL2 both in the aspects of transcription and translation than that by DC-SN when facing MC38$^{NY\text{-}ESO\text{-}1}$ cells (Fig. 6D and E). And the PBMCs isolated from DC-SNU injected mice also transcribed more mRNAs of perforin and granzyme B

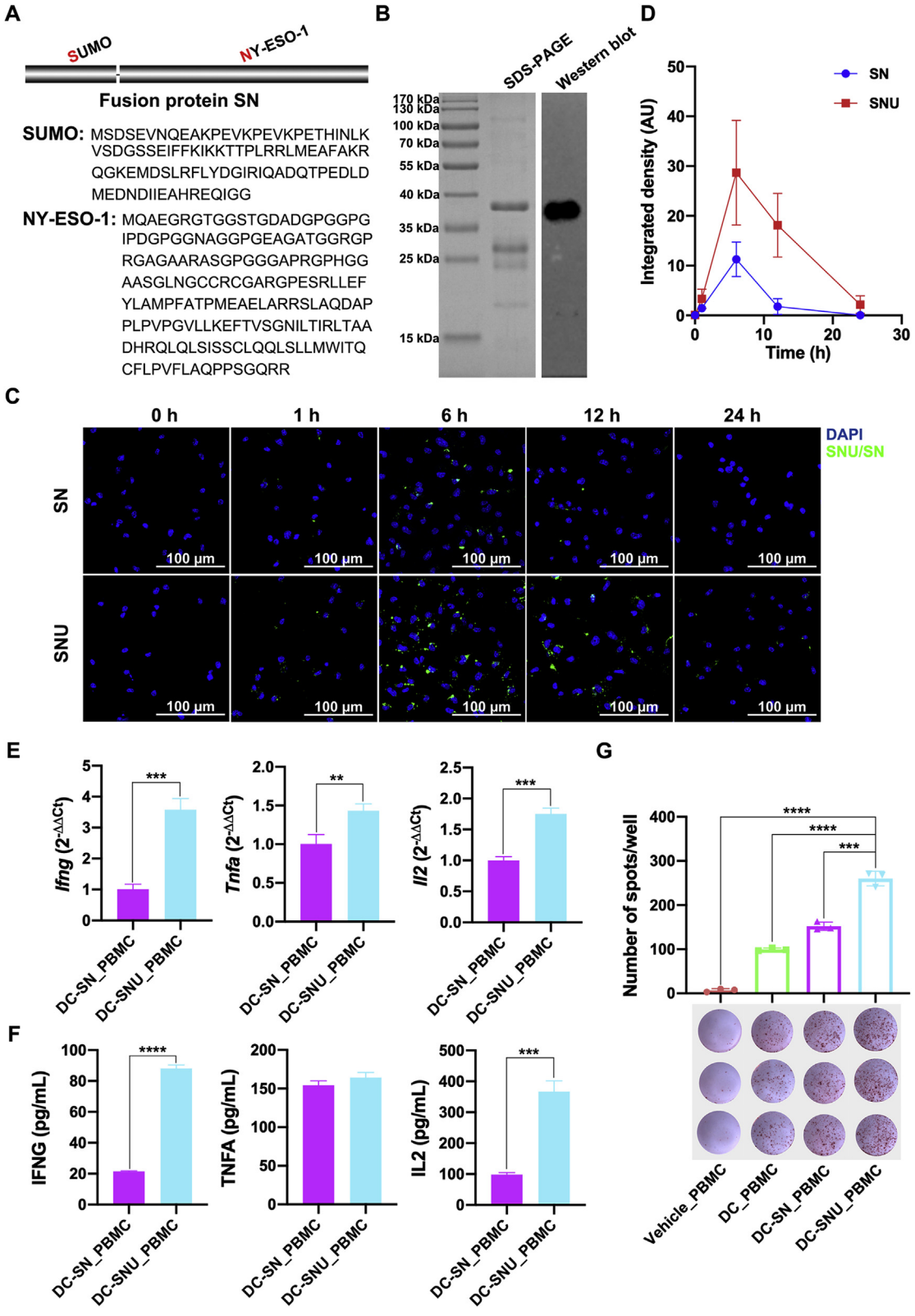

Figure 5 DC-SNU elicited stronger T cell immune responses than DC-SN *ex vivo*. (A) Diagram of fusion protein SN and its sequence. (B) The fusion protein SN was expressed using *E. coli* expression system. After purified, SN was detected by Western blot using anti-NY-ESO-1 mAb. (C) The isolated cells were incubated with SNU or SN in indicated times. The intracellular SNU or SN were observed through LSCM at 0, 1, 6, 12 and 24 h. Scale = 100 μm. (D) The fluorescence value was calculated by ImageJ software and shown as mean ± SD, $n = 4$. PBMCs isolated from C57BL/6 mice were cocultured with DC-SN/DC-SNU, respectively. The comparative mRNA (E) and protein (F) expression of IFNG, TNFA and IL2 were quantified by qPCR and ELISA, respectively. (G) The IFN-γ spots of PBMCs stimulated with DC-SN/DC-SNU were counted by ELISPOT assay. All the data were represented as mean ± SD, $n = 3$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

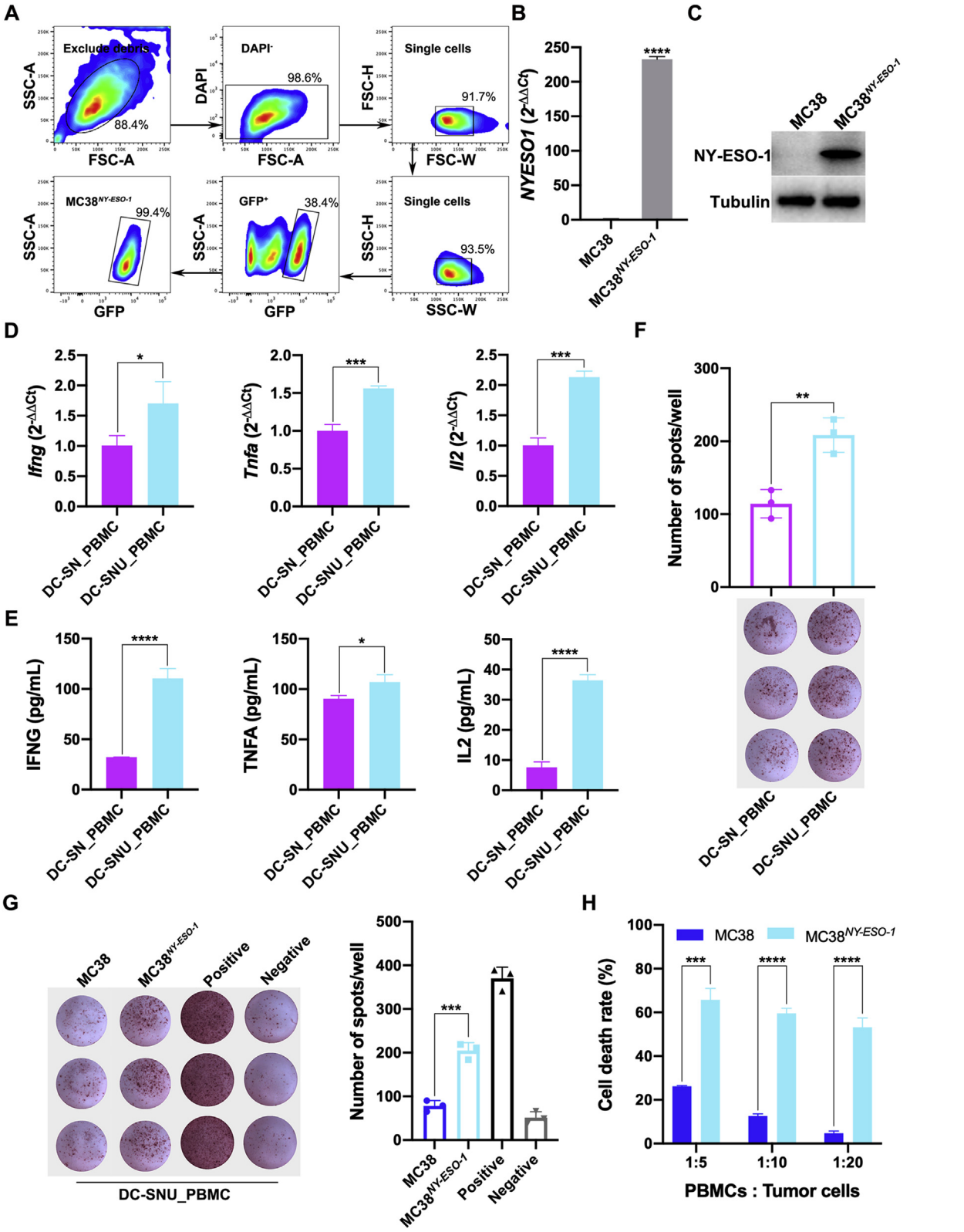

Figure 6 DC-SNU induced stronger and specific T cell immune responses against MC38$^{NY\text{-}ESO\text{-}1}$ *ex vivo*. (A) The MC38$^{NY\text{-}ESO\text{-}1}$ cells was sorted by flow cytometry. The sorted cells were then confirmed by qPCR (B) and Western blot (C). PBMCs isolated from DC-SN/DC-SNU immunized mice were cocultured with MC38$^{NY\text{-}ESO\text{-}1}$. The comparative mRNA (D) and protein (E) expression level of IFNG, TNFA and IL2 were quantified by qPCR and ELISA, respectively. (F) and (G) The IFN-γ spots of PBMCs restimulated with MC38 or MC38$^{NY\text{-}ESO\text{-}1}$ cells were counted by ELISPOT assay. (H) PBMCs isolated from DC-SNU immunized mice were cocultured with different amounts of MC38$^{NY\text{-}ESO\text{-}1}$ cells. The LDH release of supernatant was measured by a microplate reader. And the death rates of tumor cells were calculated. All the data were represented as mean ± SD, $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

when cocultured with MC38$^{NY\text{-}ESO\text{-}1}$ cells (Supporting Information Fig. S4). Same result was obtained by IFN-γ ELISPOT assay (Fig. 6F). Whereas, fewer spots were counted when PBMCs from DC-SNU immunized mice were stimulated with MC38 cells (Fig. 6G) declaring that the cancer vaccine DC-SNU could elicit specific T cell immune responses against NY-ESO-1 tumor antigen. The LDH assay elucidated consistent result that the DC-SNU activated PBMCs exerted specific cytotoxicity to MC38$^{NY\text{-}ESO\text{-}1}$ than MC38 cells, which also confirmed the conclusion (Fig. 6H).

To verify that the fusion strategy for NY-ESO-1 is the best for vaccine preparation, another two fusion proteins, NY-ESO-1_ubiquitin (NU) and SecPen_NY-ESO-1 (SecN), were expressed (Supporting Information Fig. S5A and S5B. Compared with SN, more green fluorescence was detected in BMDC after fused with SecPen in indicated times (Fig. S5C). NY-ESO-1 fused with ubiquitin was less detected than SN at 10 h illustrating that ubiquitin could mediate effective degradation (Fig. S5C). Not only that, the transcription level of *Ifng* in PBMCs isolated from DC-NU, DC-SecN or DC-SNU-immunized mice was upregulated compared with DC-SN when PBMCs were cocultured with MC38$^{NY\text{-}ESO\text{-}1}$ (Fig. S5D). As we expected, the DC-SNU induced the strongest T cell immune responses demonstrating that SecPen and ubiquitin-fused NY-ESO-1 was the best for DC vaccine preparation in our study (Fig. S5D).

All these results demonstrate that DC-SNU could induce stronger and specific T cell immune responses in mice.

4. Discussion

Tumors are originated from normal cells because of genetic alterations. And there is strong evidence that specific immune surveillance systems work at the early stage of tumorigenesis[30]. This teaches us that provoking self-immune system against tumors will be a promising way of cancer therapy[31]. DCs, discovered in 1973 by Ralph Steinman and Zanvil Cohn[1], are the strongest APCs mediating both innate and adaptive immune responses, which making DCs an ideal tool for reactivating endogenous tumor specific immune responses to eradicate tumors[32]. Due to the low efficacy in clinical outcomes, investigations of DC vaccines still have a long road to ride.

DCs are mainly divided into two subtypes: conventional DCs (cDCs) and plasmacytoid DCs (pDCs), which are both used to generate DC vaccines. cDCs are MHC II$^+$CD11c$^+$ cells and can acquire tumor antigens more efficiently than pDCs and cross-present them to CD4$^+$ and CD8$^+$ T cells[33]. pDCs are MHC II$^+$CD11c$^-$ cells and are main functional cells in immune responses caused by viral and bacterial stimuli because they can produce more IFN-α/β than other immune cells[34]. While pDCs may inhibit anti-tumor immunity because the indoleamine 2,3-dioxygenase (IDO) expressed by accumulated pDCs are inhibitory for T cell proliferation and pDCs play unknown roles in regulation of Treg cells[35]. Hence in this work, we chose to isolate mouse bone marrow-derived CD11c$^+$ DCs to be the candidate for DC vaccine preparation. After sorting, we obtained high purity of DCs (>99%) that displayed an immature state at which MHC molecules were high expressed and costimulatory molecules (CD40, CD80 and CD86) are low expressed.

For the first generation of DC vaccines, immature DCs derived from monocytes or CD34$^+$ cells were used without spare modifications[5,6,36]. In 1995, Mukherji et al.[37] confirmed that tumor antigen-specific cytotoxic T lymphocytes (CTLs) were detectable in the blood and metastatic lesions of patients with advanced metastatic melanoma. After that, many efforts were made to improve the clinical responses of DC vaccines by using additional stimulators such as IL-4[38], Flt3-L[39], TNFα[39] and keyhole limpet hemocyanin (KLH)[38,40]. On the basis of the first generation of DC vaccines, research focus was transferred to DCs maturation for the second generation of DC vaccines[41]. DCs matured by IL-1β, IL-6, TNFα and PGE2 induced a significant CTL response *in vivo* than immature DCs despite both mature and immature DCs being capable of inducing this *in vitro*[42]. Due to the elevated clinical responses of the second generation of DC vaccines, the first cancer vaccine sipuleucel-T, also known as Provenge, was approved by FDA for the treatment of asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer[43–45].

Optimal T cell activation needs production of IL-12 and expression of CCR7 by DCs. IL-12, known as a T cell-stimulating factor or signal three for T cell activation, can stimulate the growth and function of T cells and production of IFN-γ and TNF-α. There is evidence proving that addition of IFN-γ in maturation cocktail could improve the production of IL-12[46,47]. The chemokine receptor CCR7 mediates migration of DCs to lymph nodes or spleens in which T cells can be touched and activated by mature DCs. PGE2 is a key factor for CCR7 surface expression and migration of DCs[47,48]. Based on the information, the CD11c$^+$ DCs we isolated were further maturated by a cytokine cocktail which contains IL-1β, IL-6, IFN-γ, TNF-α and PGE2. After maturated, DCs expressed high CD40, CD80 and CD86 that are considered as signal two for T cell activation. Upregulated CCR7 expression was also found after maturation, but not as high as reported[48]. Nonetheless, the migration capacity of mature DCs were much stronger than DCs without maturation.

The typically antigen loading method is incubation of DCs with tumor peptides, proteins or whole tumor lysates[49]. Because multiple epitopes can be presented on MHC molecules inducing both CD4$^+$ and CD8$^+$ T cell immune responses, proteins or whole tumor cell lysates are often used as tumor antigens for DC vaccine. In the present study, NY-ESO-1, a cancer/testis antigen, was chosen to the tumor antigen for DC vaccine due to its immunogenicity, expression in many cancers but not normal tissues and existing of multiple MHC epitopes. However, the way of DCs dealing with exogenous antigens mainly depends on lysosome-degradation system that presents epitopes on MHC I molecule. Activation of CD8$^+$ T cells depends on an unsettled mechanism called “cross-presentation” with low presentation efficiency[50]. Therefore, the key point is that how to make tumor antigen endogenization and degraded by proteasome.

To achieve this goal, researches invented a microfluidic platform to deliver diverse materials into cytoplasm of different cell types[51]. However, this requires special equipment and is expensive. Thus, we designed a novel strategy to achieve similar effect. We fused a SecPen sequence in the N-terminal of NY-ESO-1 protein, which would make NY-ESO-1 endogenization. CPPs were used in DC vaccines for many years and proved to be efficient in inducing both CD4$^+$ and CD8$^+$ T cell immune responses[25,52,53]. And SecPen was investigated and showed a stronger ability of membrane permeability than other CPPs such as R8 and Tat[54]. Then we fused ubiquitin in the C-terminal of NY-ESO-1 to guide NY-ESO-1 to proteasome and be degraded faster and more efficiently. As we all know, endogenous antigens should be ubiquitinated through ubiquitin activating enzyme E1, ubiquitin conjugating enzyme E2 and

ubiquitin ligating enzyme E3 firstly, and then are degraded by proteasome[55−57]. We compared the immune activation ability of DC vaccines loaded with NY-ESO-1 fused with or without ubiquitin and found DC vaccine loaded with ubiquitin-fused NY-ESO-1 could elicit higher transcriptions of *Ifng*, *Tnfa* and *Il2* in sensitized PBMCs. Based on the fusion strategy, the DC vaccine we prepared could induce stronger T cell immune responses. Also, the DC-SNU activated PBMCs performed a stronger and specific cytotoxicity against $MC38^{NY\text{-}ESO\text{-}1}$ cells *in vitro*, indicating that the DC vaccine might have antitumor capacity.

5. Conclusions

Collecting all data, we proposed a novel thought in DC vaccine preparation. Benefit from the SecPen and ubiquitin, the tumor antigen NY-ESO-1 could enter cytoplasm of DCs and be degraded by proteasome more efficiently. DC vaccine constructed by this method could elicit stronger and specific T cell immune responses in mice. This strategy was easy to handle due to the maturation of experimental methods. The following investigation will be continued to test the therapeutic effect of DC vaccine alone or combination with immune checkpoint therapy in mouse tumor models and human tests if possible. And if the results are positive, we think this method may be applicable for many other tumor antigens and cancer types as a platform of DC vaccine preparation.

Acknowledgments

This study was funded by PDH-SPFDU Joint Research Fund (RHJJ2018-04, China) and Shanghai Science and Technology Committee (No. 19431903000, China). We thank Xuyao Zhang, Ph.D., from Perelman School of Medicine, University of Pennsylvania (USA) for editing the English text of a draft of this manuscript.

Author contributions

Conceived and planned the work, Yunkai Yang and Meiqing Feng; collected the data and performed the analysis, Yunkai Yang, Xiaohan Guo, Bo Hu and Peng He; prepared the manuscript, Yunkai Yang; reviewed and edited the manuscript, Minghua Yu, Lina Hu and Meiqing Feng; provided critical advices in methodology, Xiaowu Jiang, Zuohuan Wang and Huaxing Zhu. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declared no conflicts of interest to this work.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2020.08.004.

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