NKG2D-IL-15 fusion protein encapsulated in N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride retards melanoma growth in mice

Rong Chen[1#], Yimei Ding[1#], Juqun Xi[1], Guotao Lu[2], Weiming Xiao[2,3], Yanbing Ding[2], Li Qian[1,4], Zhijie Lin[1,3,4,5], Weijuan Gong[1,2,3,4,5]

[1]Department of Immunology, Institute of Translational Medicine, Medical College, [2]Department of Gastroenterology, Affiliated Hospital, [3]Jiangsu Key Laboratory of Integrated Traditional Chinese and Western Medicine for Prevention and Treatment of Senile Diseases, [4]Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, [5]Jiangsu Key Laboratory of Zoonosis, Yangzhou University, Yangzhou 225009, China

Contributions: (I) Conception and design: W Gong; (II) Administrative support: W Xiao, Y Ding, L Qian; (III) Provision of study materials or patients: J Xi, G Lu; (IV) Collection and assembly of data: Z Lin, R Chen, Y Ding; (V) Data analysis and interpretation: Z Lin; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Weijuan Gong; Zhijie Lin. Department of Immunology, Institute of Translational Medicine, Medical College, Yangzhou University, Yangzhou 225009, China. Email: wjgong@yzu.edu.cn; zjielin@yzu.edu.cn.

Background: Chitosan can be modified to increase the efficiency of the delivery of chemical drugs, nucleic acids, or proteins. Sodium tripolyphosphate (TPP) is a noncytotoxic and polyanionic crosslinker that binds with the positively charged ions of chitosan. DsNKG2D-IL-15 is a fusion protein that exerts promising antitumor effects via lymphocyte activation. The extracellular domains of double NKG2D is linked to IL-15.

Methods: To increase the stability and efficiency of dsNKG2D-IL-15 protein, the fusion protein was encapsulated in nanoparticles based on chitosan pre-modified with N-(2-hydroxy) propyl-3-trimethyl ammonium (HTCC). Moreover, the biological activity of protein nanoparticle was evaluated on the mouse lymphocyte *ex vivo* and mouse tumor model *in vivo*.

Results: TPP sharply promoted the HTCC chitosan encapsulating efficiency (85–95%) with dsNKG2D-IL-15. The protein nanoparticle displayed a spherical shape with a diameter of 200–400 nm and zeta-potential value of 15.6±4.82 mV. DsNKG2D-IL-15 could be released from the nanogel within 72 h. In addition, the protein biological activity for lymphocyte activation was maintained. Natural killer (NK) and CD8^{+} T cells increased the activity of IFN-γ production and degranulation after incubation with the dsNKG2D-IL-15-HTCC-TPP nanoparticle *ex vivo*. Treatment with dsNKG2D-IL-15 nanoparticles exhibited better effects of inhibiting tumor growth and prolonging the life span of B16BL6-MICA tumor-bearing mice *in vivo* than by using the dsNKG2D-IL-15 protein alone.

Conclusions: The dsNKG2D-IL-15 protein nanoparticle exhibited notable effects of lymphocyte activation and tumor inhibition. The protein nanoparticle could be developed further for tumor therapy in clinical practice.

Keywords: Nanoparticle; chitosan; NKG2D; IL-15; melanoma

Introduction

Chitosan has good biological activity and biocompatibility and can be absorbed by the body. Chitosan has been widely used as a carrier for the delivery of biological substances due to its natural features of linear and cationic polymer of glucosamine and N-acetyl-glucosamine. However, chitosan is only soluble in acidic medium because of its pKa value of approximately 6.5. The amino groups of chitosan are only partially protonated in neutral and physiological environments, thereby leading to nonsufficient binding with most physiological proteins. N-(2-hydroxy) propyl-3-trimethyl ammonium chitosan chloride (HTCC) is a quaternary chitosan that has been constructed to increase the positive charge and solubility (1,2).

The recombinant fusion protein of NKG2D-IL-15 had been previously generated in our laboratory (3,4). NKG2D binds to major histocompatibility complex (MHC) I chain-associated antigens A and B (MICA/MICB) or UL-16 binding proteins (ULBP) in humans. The NKG2D ligands of mouse consist of retinoic acid early inducible-1 gene (RAE-1), H60, and murine UL16-binding protein-like transcript (MULT)-1. The NKG2D ligands are generally expressed by tumor or stress-associated cells (5,6). IL-15 is an important cytokine for activation, proliferation, and survival of natural killer (NK) and CD8$^+$ T cells (7,8). The double gene fragments of extracellular domains of NKG2D were connected with the interleukin (IL)-15 gene and inserted into a prokaryotic expression vector. The fusion protein, called dsNKG2D-IL-15, was expressed at a large quantity and isolated for bioactivity evaluation. Both human and mouse dsNKG2D-IL-15 displayed higher activity against tumor growth than IL-15. DsNKG2D-IL-15 not only was efficiently distributed in tumor tissues but also able to promote several NK and CD8$^+$ T cells to inhibit tumor growth (3,4).

We also constructed the recombinant eukaryotic expression vectors of pcDNA3.1(-)-dsNKG2D-IL-15 (9) and pcDNA3.1(-)-dsNKG2D-IL-21 (10). These DNA sequences were delivered by nanoparticles based on chitosan. Two nanoparticle gene vaccines showed favorable effects on tumor growth and prolonged survival of tumor-bearing mice. The dsNKG2D-IL-21 gene nanoparticle-vaccine had been shown to have a tumor-targeted distribution due to the enhanced permeability and retention (EPR) effect of nanoparticle in tumors (10). However, when the gene nanoparticle vaccine is used, the nanoparticle must be taken up first, and the phagocytosed gene should efficiently encode the corresponding protein in the cytoplasm. Here, we generated an HTCC-sodium tripolyphosphate (TPP) nanoparticle for delivery of the dsNKG2D-IL-15 fusion protein. The physical, chemical, and release features and also the antitumor activity *in vivo* of this protein nanoparticle were also evaluated.

Methods

Reagents, antibodies, and cell lines

The human dsNKG2D-IL-15 fusion protein was generated as described before (3) and adjusted to a concentration of 1 mg/mL. Chitosan (MW 20 kDa) with around 85% deacetylation was purchased from Yuhuan County Marine Chemical Company (Yuhuan, Zhejiang, China). TPP was from Sinopharm (Shanghai, China). Antibodies against CD8 (53.67), NK1.1 (PK136), CD69 (LG.3A10), NKG2D (CX5), CD107a (1D4B), and CD44 (IM7) were all purchased from Biolegend (San Diego, CA, USA). YAC-1, RAW264.7 cell lines were obtained from ATCC. B16 cells with stable ectopic expression of MICA were constructed and stored regularly.

Generation of HTCC

HTCC was prepared according to previous method (11,12). Briefly, 2 g of chitosan powder was suspended in deionized water containing 2% HAc, and then the mixture was stirred for 30 min prior to dropwise addition of glycidyltrimethylammonium chloride (GTMAC) with continuous stirring. The reaction mixture was then stirred at 80 ℃ for 6 h, and the product was collected by filtration after being precipitated and washed by hot alcohol. The obtained product was dissolved in distilled water, dialyzed against distilled water for 5 days, and lyophilized. The final polymer was HTCC.

Preparation of HTCC-dsNKG2D-IL-15 nanoparticles

TPP was first added into the HTCC solution (1 mg/mL) at a mass ratio of 1:10 (13). The recombinant dsNKG2D-IL-15 protein was put into the solution at a mass ratio (chitosan:protein) of 1:1, 1:2, or 2:1, and the mixed solution was completely shaken at room temperature for 1 h. Afterward, the solution was centrifuged for 30 min at the speed of 12,000 g. The precipitate and supernatant were separated. The supernatant was collected for the detection of the content of free protein by a spectrophotometer. The

free protein was also confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The pellet was re-suspended with sterile PBS buffer for future use. The encapsulation efficiency was calculated by the following formula: encapsulation rate = (total amount of recombinant protein – free protein content)/total amount of recombinant protein × 100%.

Characterization of HTCC-dsNKG2D-IL-15 nanoparticles

The sizes of the nanoparticles were measured by an electron microscope (Tecnai 12, Phillip, The Netherlands). Aqueous solutions of HTCC/protein nanoparticles were analyzed by dynamic light scattering and zeta potential measurements (Zetasizer Nano ZS 90, Malvern, UK) to determine their particle size and zeta potential.

Protein release from nanoparticles

The HTCC nanoparticles loaded with dsNKG2D-IL-15 protein were put into a small dialysis bag with 220 µm filter and then placed in a sterile phosphate buffer saline (PBS). After gentle stirring, buffer outside the dialysis bag was collected at 1, 2, 4, 8, 24, 48, and 72 h. After being centrifuged with tubes (30 kD aperture), the dialyzed buffers were concentrated and analyzed by SDS-PAGE.

Cell cytotoxicity

RAW264.7 and B16BL6 cells in the logarithmic phase were divided into seven groups, which consisted of culture medium, vacant HTCC (1.25, 2.5, and 5 µg), and HTCC protein (HTCC =1.25, 2.5, and 5 µg, chitosan: protein =1:1). Nanoparticles were incubated with RAW264.7 or B16BL6 cells for 5 d. Then, the MTS reagent from Promega (Madison, WI, USA) was mixed with PMS solution with a volume ratio of 20:1 and added into cell culture system. The color change was detected within 1–4 h, and OD_{490} value was determined by a microplate reader.

Activation of NK and CD8+T cells by nanoparticles

Mouse mononuclear cells (1×10^6) of spleen were incubated with vacant HTCC (1.25, 2.5, and 5 µg) or HTCC nanoparticles encapsulated with dsNKG2D-IL-15 protein (HTCC =1.25, 2.5, and 5 µg, chitosan: protein =1:1) for

24 h. The recombinant dsNKG2D-IL-15 protein was also used to stimulate splenocytes as a positive control. Proliferation of splenocytes was determined by the cell numbers of culture systems and assayed by the MTS/PMS kit described previously. The IFN-γ production of NK ($CD3^-NK1.1^+$) or $CD8^+$ T ($CD3^+CD8^+$) cells was detected by flow-cytometry intracellular staining with its monoclonal antibody. For the analysis of degranulation activity of NK or $CD8^+$ T cells, YAC-1 or B16BL6-MICA cells as target cells were mixed with NK or $CD8^+$ T cells at a ratio of 1:3. After 2 h, monensin (10 µm, GolgiStop; BD Biosciences, San Jose, CA, USA) was added for another 2 h. Then, cells were collected to beco-stained with NK1.1 or CD8 antibody and CD107a antibody and analyzed by flow cytometry.

Mouse tumor models

B16BL6-MICA cells (2×10^6) in log phase were subcutaneously injected into the back of C57BL/6 mice. After 5 days of inoculation, mice were injected intraperitoneally with PBS, vacant HTCC (50 µg), HTCC-dsNKG2D-IL-15 nanoparticle (HTCC =50 µg, chitosan :protein =1:1), or dsNKG2D-IL-15 protein (50 µg) every 3 days. The volume of tumor was measured according to the following formula: $V = 1/2ab^2$ (The longest diameter of the mouse subcutaneous tumor is a, and the vertical diameter is b) every 2 days. The survival of each mouse was also observed and recorded. Some tumor-bearing mice were sacrificed to analyze the phenotypic changes of spleen NK ($CD3^-NK1.1^+$) or $CD8^+$ T ($CD3^+CD8^+$) cells after different treatments.

Statistical analysis

ANOVA was used to compare the differences among more than two groups. The differences of two groups were analyzed by group Student's t-test. Survival curves were plotted by Kaplan-Meier analysis. All analyses were carried out with the GraphPad Prism software. $P<0.05$ was considered statistically significant.

Results

Characterization of HTCC-based nanoparticles

The HTCC molecular structure is shown in *Figure 1A*. After HTCC was synthesized from the original chitosan, we used HTCC to encapsulate dsNKG2D-IL-15 protein at the serial different ratios. Free proteins of supernatants
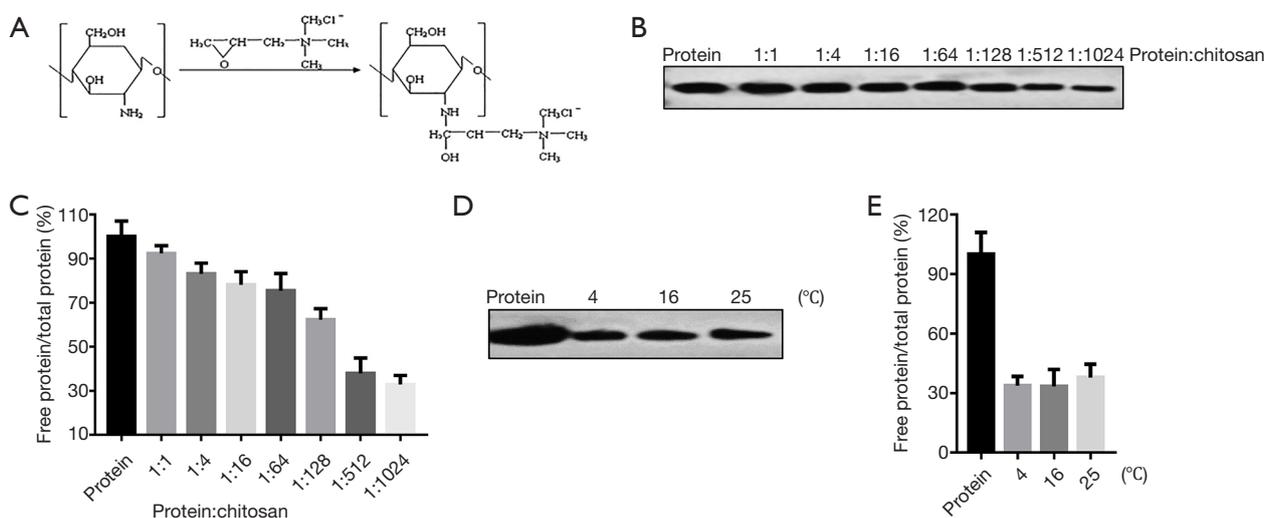
Figure 1 Preparation of protein chitosan nanoparticle based on HTCC. (A) Molecular structure of HTCC; (B) SDS-PAGE analysis of free proteins in supernatants after nanoparticles were prepared at the indicated mass ratios of protein to chitosan; (C) relative density of free proteins; (D) SDS-PAGE analysis of free proteins in supernatants after nanoparticles were prepared at the mass ratio of 1,024:1 (HTCC: protein) under different temperatures; (E) the relative density of free proteins. The protein supernatants were separated by SDS-PAGE and transferred to PVDF membranes, and the protein bands were visualized by ponceau staining. HTCC, N-(2-hydroxy) propyl-3-trimethyl ammonium; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride.

from different encapsulation systems were run through sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As shown in *Figure 1B,C*, when the mass ratio of protein to HTCC was 1:1, no clear protein encapsulation was found, because free dsNKG2D-IL-15's quantity was similar to that of the protein alone. If the ratio reached 1:512, the encapsulation rate increased to approximately 70%. Therefore, when we use 1 µg protein, the HTCC mass should be at least 512 µg. This condition is not feasible in protein encapsulation based on the HTCC itself. The effects of temperature on the encapsulation rate were also compared at 4, 16, or 25 ℃. We did not observe any significant changes of encapsulation rate under different temperatures (*Figure 1D,E*).

TPP facilitates the encapsulation of HTCC nanoparticles

TPP can bind with the positively charged ions in chitosan, which significantly promotes chitosan encapsulating with RNA (14). During TPP addition to prepare the nanoparticles, the encapsulation rate sharply increased to more than 80%, and the best encapsulation was obtained at the ratio (chitosan to protein) of 1:1 (*Figure 2A,B*). The size of protein nanoparticle was measured by both electric microscopy and dynamic light scattering. The protein nanoparticle displayed

spherical shape with a diameter from 200 to 400 nm (*Figure 2C,D*). The zeta-potential value was 15.6±4.82 mV (*Figure 2E*). Thus, TPP could promote efficiency to generate the dsNKG2D-IL-15 protein nanoparticle based on HTCC.

Protein releasing from nanoparticles

dsNKG2D-IL-15 protein released from the HTCC-TPP nanoparticle was analyzed. Dialysis buffers were collected at indicated time courses. The results of electrophoresis showed that the protein release peaked after the protein nanoparticles were placed into the phosphate-buffered saline (PBS) for 72 h (*Figure 3A,B*). The nanoparticles were mixed with the mononuclear cells of the spleen from normal mice to ensure that the dsNKG2D-IL-15 released from nanoparticles exhibited biological activity. As IL-15 promotes proliferation and survival of CD8+ T and NK cells, the number of changes of splenocytes treated by the protein nanoparticle reflected the function of the released protein. Expectedly, splenocytes treated with the protein nanoparticles at each dose for 72 h obtained higher cell numbers compared with vacant HTCC (*Figure 3C*). The apoptosis of splenocytes was also evaluated after the protein nanoparticle treatment. As shown in *Figure 3D,E*, low apoptosis was observed in splenocytes treated with 5 µg nanoparticles (*Figure 3D,E*). Therefore, dsNKG2D-
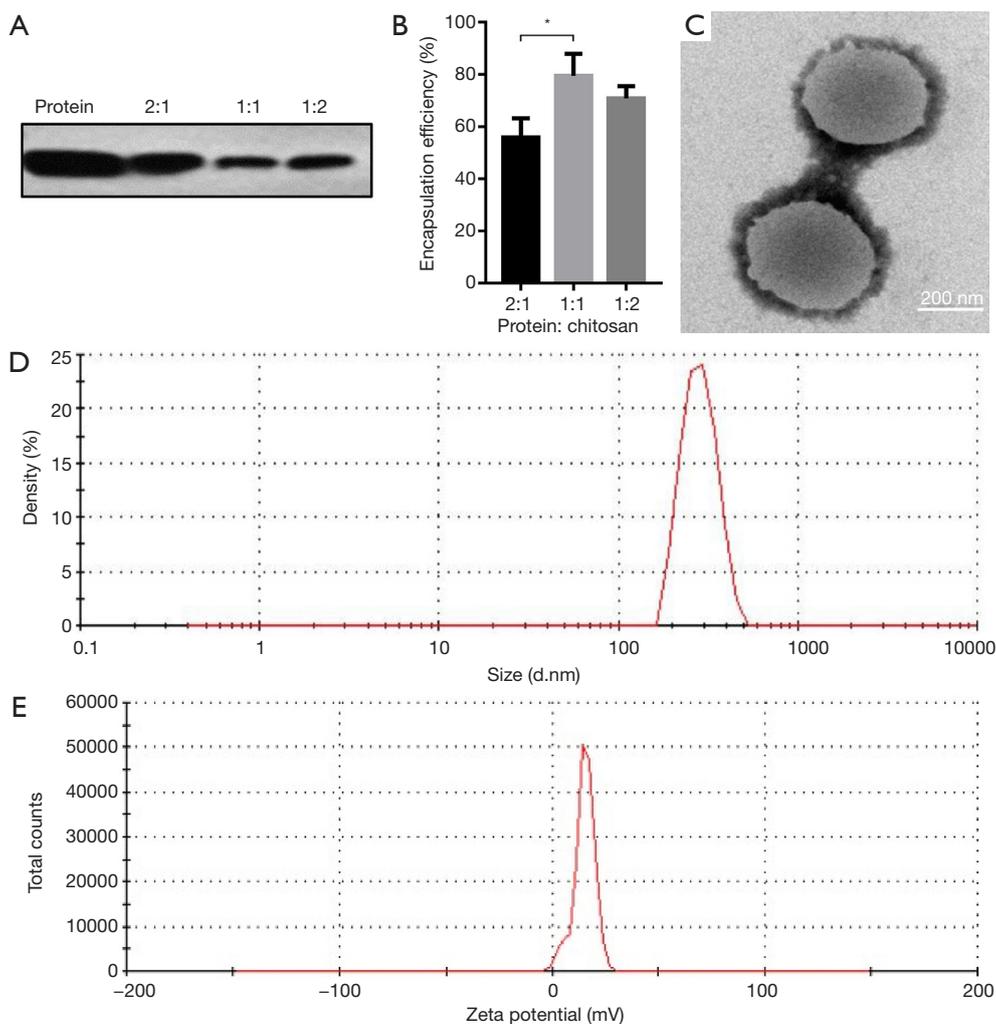
Figure 2 Characterization of dsNKG2D-IL-15 protein-nanoparticle based on HTCC and TPP. Adding TPP into the reactive system at the ratio of 10:1 (TPP:HTCC). (A) SDS-PAGE analysis of free proteins in supernatants after nanoparticles were generated; (B) the encapsulation rates of indicated mass ratios of HTCC to protein. n=3, *, P<0.05; (C) morphology and size detected by transmission electronic microscope; (D) size; (E) zeta-potential distribution. HTCC, N-(2-hydroxy) propyl-3-trimethyl ammonium; TPP, tripolyphosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

IL15 could not only be easily released from the HTCC-TPP nanoparticle, but its activity on the lymphocytes could be retained.

Cytotoxic effects of HTCC nanoparticles ex vivo

The cytotoxicity of nanoparticles on RAW264.7 or B16-MICA cells was detected by MTS/PMS method. No notable change in the chitosan nanoparticle was found between 1.25 and 2.5 μg doses and culture medium. However, the 5.0 μg dose exerted toxic effects on B16BL6-
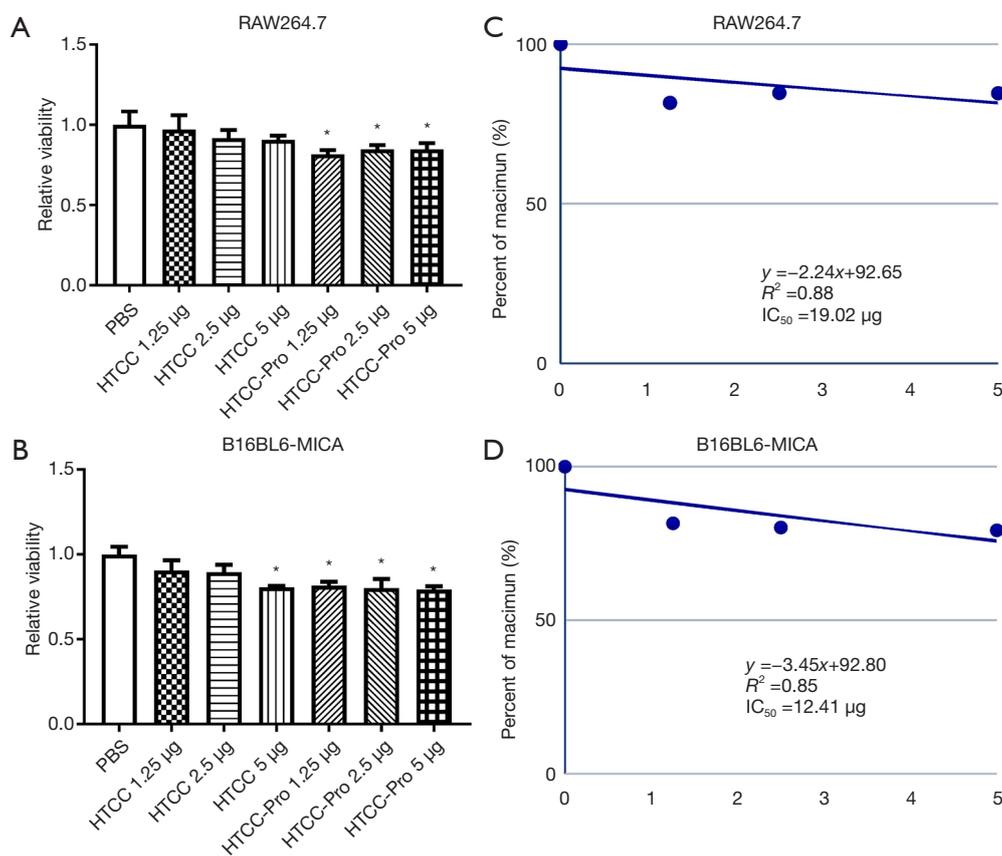
MICA but not on RAW264.7 cells (*Figure 4A,B*). The linear relationship between the nanoparticle quantity and inhibition rate and also IC$_{50}$ of nanoparticles of each cell type are shown in *Figure 4C,D*. Thus, low-dose HTCC nanoparticle is biologically safe, but high-dose HTCC nanoparticle has some cytotoxic effects.

Stimulation of protein nanoparticles on NK and CD8$^+$ T cells ex vivo

The stimulatory effects of dsNKG2D-IL-15 nanoparticle
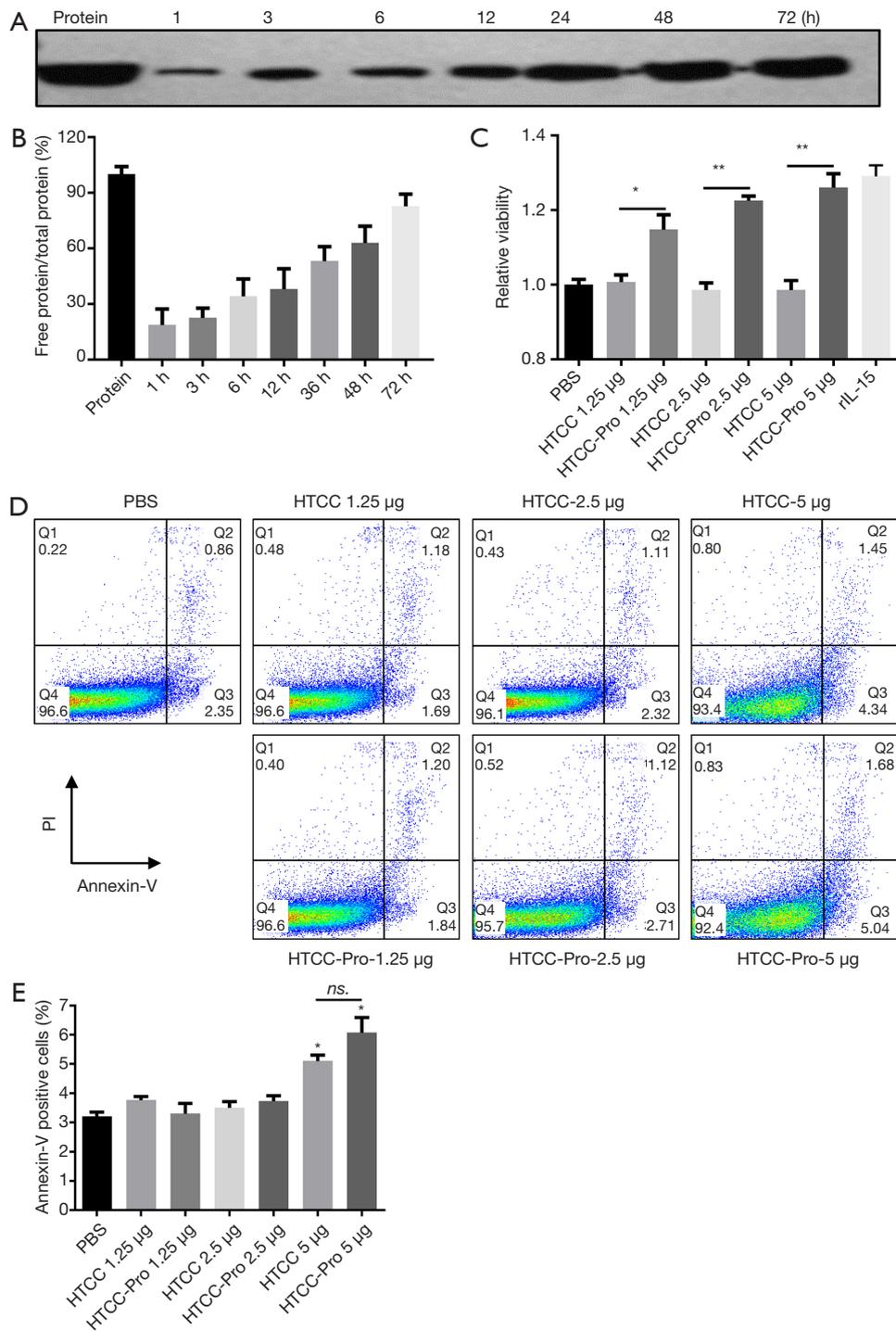
Figure 3 Release features of dsNKG2D-IL-15 protein–nanoparticle. (A) SDS-PAGE analysis of free proteins in dialysates after nanoparticles were added into PBS at the indicated time points; (B) relative density of free proteins, n=3; (C) relative viability measured by MTS/PMS kit after splenocytes were mixed with protein-HTCC nanoparticle. n=6, *, P<0.05; **, P<0.01; (D) cells apoptosis measured by Annexin-V-PI stain kit; (E) statistical analysis of rate of Annexin-V positive cell. n=6, Each experiment was performed thrice. *, P<0.05; ns, no significance. SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]; PMS, phenazine methosulfate; HTCC, N-(2-hydroxy) propyl-3-trimethyl ammonium.

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2236

Chen et al. NKG2D-IL-15 nanoparticle retards tumor growth

Figure 4 Cytotoxic effects of nanoparticle. (A) Variations in RAW264.7 or (B) B16BL6-MICA cell relative viability determined by MTS/PMS kit after cell incubation with indicated quantities of protein-HTCC nanoparticles for 5 days. numbers of cells treated by nanoparticles were assayed by the absorbance at OD490 nm. Linear relationship of nanoparticle quantity with inhibition rate of each cell type (C,D). n=6, *, P<0.05. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]; PMS, phenazine methosulfate; HTCC, N-(2-hydroxy) propyl-3-trimethyl ammonium.

on NK and CD8^{+} T cells were then analyzed. When the protein nanoparticle was incubated with lymphocytes for 72 h, NK cell showed enhanced CD69 expression (a surface marker of activation) and IFN-γ production compared with cells treated with PBS or vacant HTCC. The cytotoxicity of NK cell was also significantly higher in the protein nanoparticle treatment than that of the PBS or vacant HTCC group. This result was evaluated by the degranulation activity based on CD107a staining (*Figure 5A*). Similar changes of CD8^{+} T cells were found after the treatments, as shown in *Figure 5B*. Thus, the dsNKG2D-IL-15-nanoparticle treatment promoted the functions of NK and CD8^{+}T cells *ex vivo*.

Antitumor effects mediated by protein nanoparticles in vivo

We analyzed whether the treatment of dsNKG2D-IL-15

nanoparticle could suppress tumor growth *in vivo*. After B16BL6-MICA cells were subcutaneously transplanted into the back of mice for 5 days, PBS, vacant HTCC, dsNKG2D-IL-15-HTCC nanoparticle, or dsNKG2D-IL-15 alone was peritoneally injected into the mice twice per week. We did not observe clear symptoms, including diarrhea, hair loss, or irritation, among all mice. DsNKG2D-IL-15-HTCC-TPP nanoparticle treatment obtained the highest tumor growth inhibition (*Figure 6A*). The tumor-bearing mice treated by the dsNKG2D-IL-15 nanoparticle had the longest period of survival (*Figure 6B*). Given that melanoma does not form solid tumors in the skin, we can only analyze the functional changes of the NK and CD8^{+} T cells of the spleens. Treatment with dsNKG2D-IL-15 nanoparticle increased the frequency of NK1.1^{+}CD69^{+} and NK1.1^{+}NKG2D^{+} cells compared with PBS or vacant HTCC (*Figure 6C*). CD44 is a cell surface
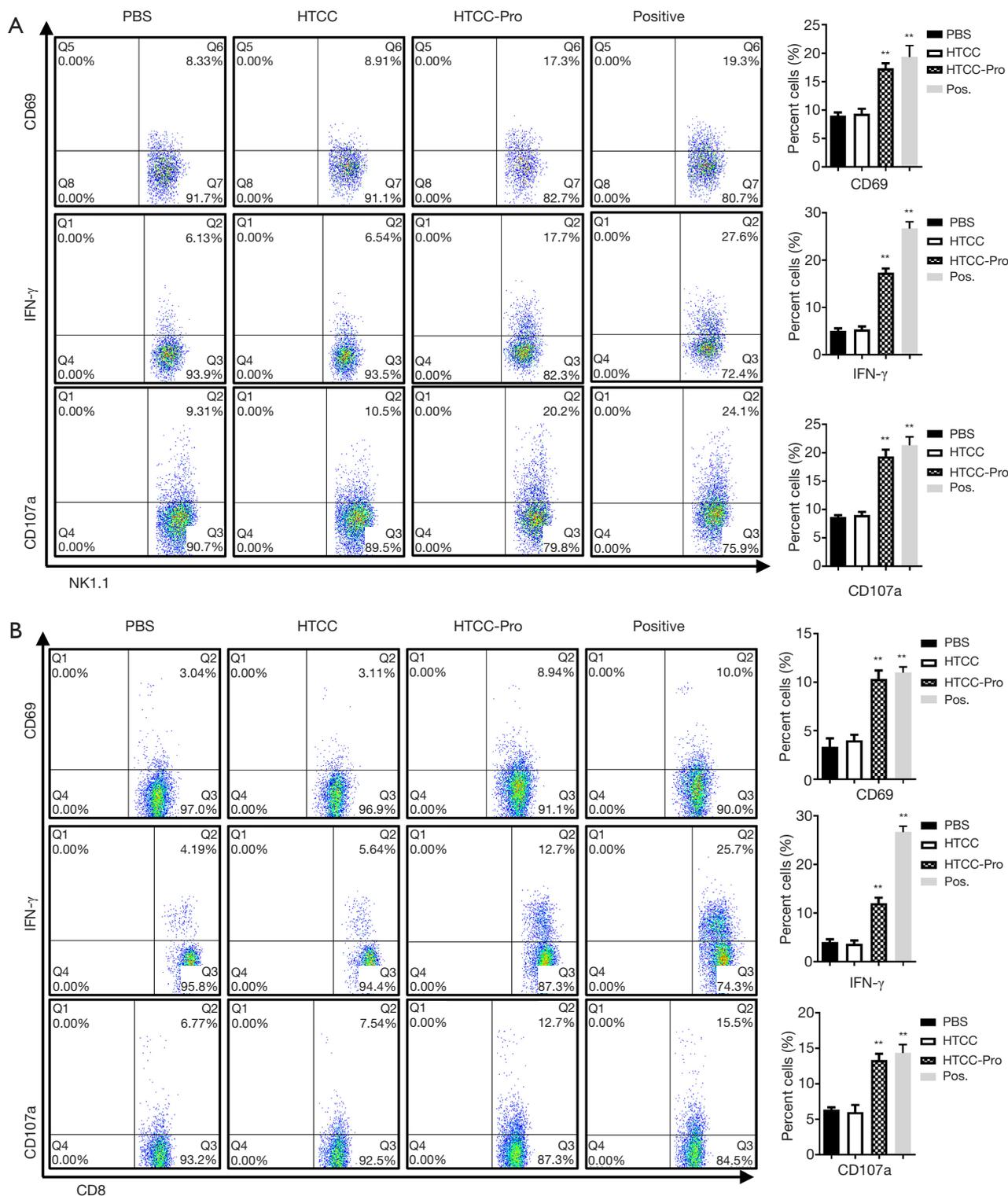
Figure 5 Stimulation of NK and CD8⁺T cells by nanoparticle. The CD69 and CD107a expression levels of splenic (A) NK1.1⁺ or (B) CD8⁺ T cells after co-cultured with nanoparticle (2.5 µg) were detected by flow cytometry. IFN-γ production was measured by flow-cytometry intracellular staining. Eecombinant IL-15 (25 ng/mL) used as positive control for CD69 and CD107a assay. Cell Stimulation Cocktail (eBioscience) as positive control used in IFN-γ detection. Each experiment was performed thrice. n=6, **, P<0.01. NK, natural killer.

2238

Chen et al. NKG2D-IL-15 nanoparticle retards tumor growth
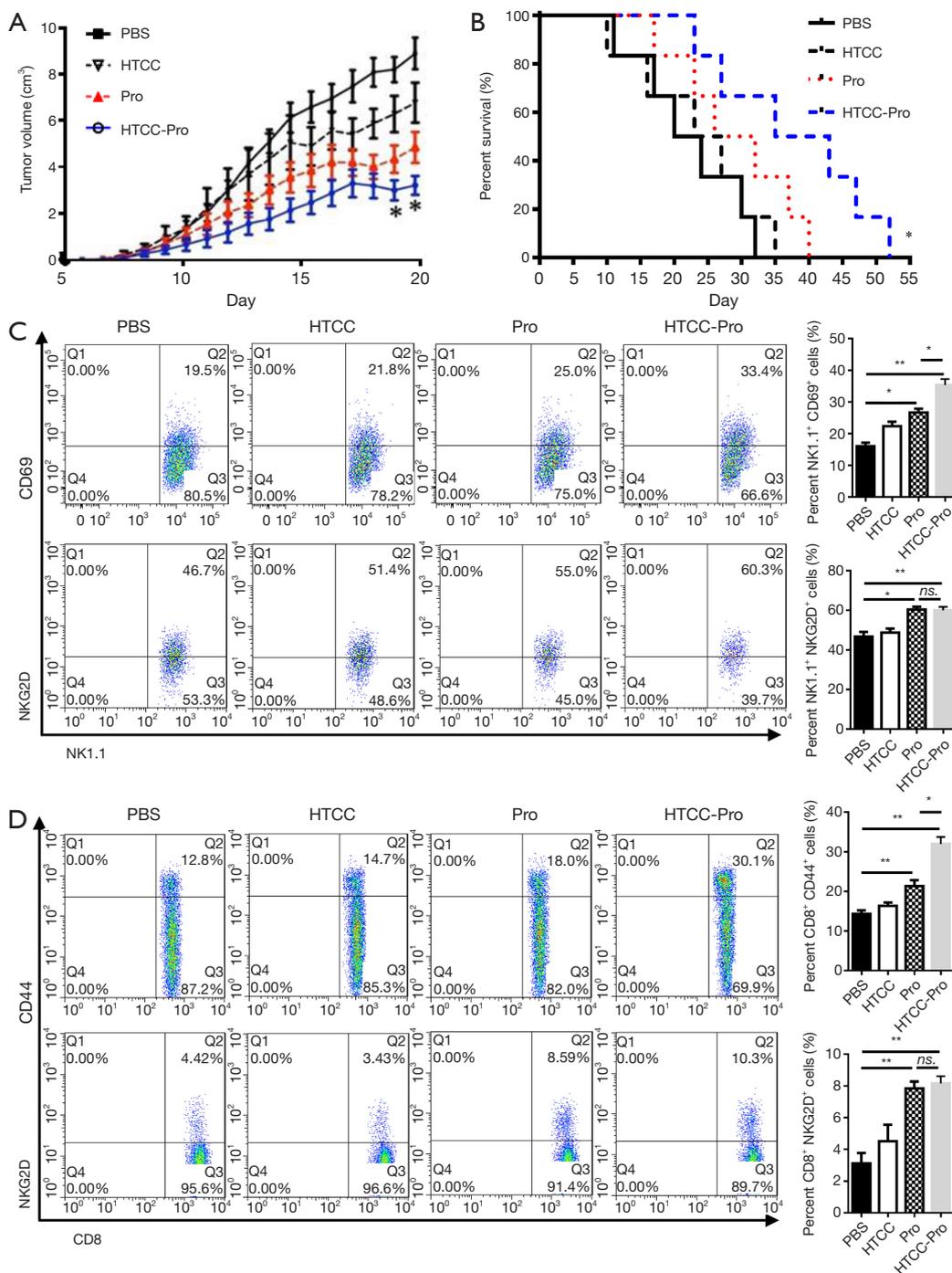
Figure 6 Anti-tumor activity mediated by dsNKG2D-IL-15 protein-HTCC nanoparticle. After the B16BL6-MICA cells were injected subcutaneously into the back of C57BL/6 mice for 5 days, protein-HTCC nanoparticles were peritoneally injected into mice every 3 days. Tumor growth was measured daily and expressed as the mean standard deviation (A); mouse deaths were documented over time. Data were presented in Kaplan-Meier survival curves (B). Some mice were sacrificed on day 20, and their spleens were collected. The frequencies of NK1.1$^+$CD69$^+$ and NK1.1$^+$NKG2D$^+$ (C), CD8$^+$CD44$^+$, and CD8$^+$NKG2D$^+$ cells (D) in spleens were detected by flow cytometry. The left panel shows the representative results of flow cytometry. The right panel shows the statistical data. The experiment was conducted twice. n=6, *, P<0.05, **, P<0.01. HTCC, N-(2-hydroxy) propyl-3-trimethyl ammonium; NK, natural killer; ns, no significance.

marker of activated and memory cells (15). NKG2D is only expressed by activated CD8$^+$ T cells in mice (16). Similarly, higher frequencies of CD8$^+$CD44$^+$ and CD8$^+$NKG2D$^+$ cells treated by dsNKG2D-IL-15-HTCC nanoparticle were found in tumor-bearing mice compared with PBS or vacant HTCC (*Figure 6D*). However, no significant changes of NK1.1$^+$NKG2D$^+$ and CD8$^+$NKG2D$^+$ cell frequencies were found between the treatments of dsNKG2D-IL-15-HTCC nanoparticle and dsNKG2D-IL-15 protein alone. On the contrary, the frequencies of NK1.1$^+$CD69$^+$ and CD8$^+$CD44$^+$cells were higher in the treatment of dsNKG2D-IL-15-HTCC nanoparticle than that of dsNKG2D-IL-15 alone. Thus, dsNKG2D-IL-15-HTCC nanoparticle could exert significant antitumor effects by activating the function of NK and CD8$^+$T cells.

Discussion

In this study, the NKG2D-IL-15 fusion protein, which can bind with the NKG2D ligands (e.g., MICA) of tumor cells and activate lymphocytes simultaneously, was encapsulated into HTCC–TPP-based nanoparticles. Although chitosan was modified to have quaternary ammonium in the backbone, the encapsulation efficiency was very low without the TPP addition. The protein nanoparticle was positively charged with the size of approximately 200–400 nm. NKG2D-IL-15 could be efficiently released from the nanoparticle within 72 h, and the released protein maintained its biological activity for stimulating lymphocytes. Protein nanoparticle exerted better effects on the inhibition of tumor growth and prolonged the survival of tumor-bearing mice *in vivo* than the NKG2D-IL-15 fusion protein alone. This nanoparticle could be potentially used as a new reagent for tumor therapy.

Several factors are involved in nanoparticle construction, including concentration and molecular weight of chitosan, encapsulant concentration, polymer pH, anionic crosslinker solutions, ratio chitosan/anionic crosslinker, speed, and duration of agitation or mixing (2,17,18). TPP addition before ionic gelation facilitated the encapsulation of both hydrophilic and hydrophobic drugs with excellent drug-loading capacity and encapsulation efficiency (14). We confirmed that the encapsulation efficiency of HTCC-TPP nanoparticles was significantly higher (85–95%) than that of native chitosan nanoparticles (10–20%). In addition, the dsNKG2D-IL-15-HTCC-TPP nanoparticle obtained good protein release *in vitro*. The nanoparticle's stability is generally affected by pH value, temperature, and storage

duration (19,20).

DsNKG2D-IL-15 fusion protein showed more efficiency in suppressing the growth of colon and gastric cancers than soluble IL-15 (3,4). Here, dsNKG2D-IL-15 protein nanoparticle displayed stronger antitumor effects *in vivo* compared with dsNKG2D-IL-15 protein alone. We did not observe significant changes of splenic NK1.1$^+$ NKG2D$^+$ and CD8$^+$NKG2D$^+$ T cells between the treatments of dsNKG2D-IL-15 nanoparticle and protein. This finding inferred that dsNKG2D-IL-15-HTCC nanoparticle mediated the antitumor effects dependent on similar molecular mechanisms with dsNKG2D-IL-15 protein *in vivo*. However, dsNKG2D-IL-15-HTCC nanoparticle was potentially distributed in the target tumor tissues and played a lasting effect (e.g., promoting the generation of memory CD8$^+$ T cells) by slow release *in vivo* (3,6).

A dsNKG2D-IL-15 gene nanoparticle was developed to antagonize the growth of colon cancer in mice. dsNKG2D-IL-15 protein nanoparticle has some advantages compared with the gene nanoparticle. A prerequisite for transporting genes based on nanomaterials is that the nanoparticles are phagocytosed by the cells in the body. In general, the phagocytosis efficiency *in vivo* is hard to predict (21). The genes in the intracellular nanoparticles must be released into the cytoplasm and efficiently expressed in the cells. If the genes encode soluble proteins, then these proteins are expected to be released to the context of cells (22). Finally, when the protein nanoparticle enters the body, it preferentially migrates to and is kept in tumor tissues due to the EPR effect (10). Even if the nanoparticle releases some quantities of dsNKG2D-IL-15 in the peripheral blood, this protein tends to be distributed in tumor tissues, as shown in our study (3).

If the clinical use of this new protein nanoparticle is considered, several questions need to be considered. To avoid *in vivo* toxicities mediated by chitosan, relatively low dose should be used in tumor-bearing mice. We wondered whether we can obtain better antitumor effects at high dose. Meanwhile, the stringent toxic effects of the nanoparticle *in vivo* should be further clarified (23,24). Whether the dsNKG2D-IL-15 nanoparticle plays an important role against other tumor types also requires further analysis.

Conclusions

DsNKG2D-IL-15 fusion protein could be encapsulated into HTCC–TPP nanoparticles. The protein can be efficiently released from the nanoparticle while retaining

its biological activity. The dsNKG2D-IL-15 protein nanoparticle exhibited notable effects on lymphocyte activation and tumor inhibition, thereby indicating that the protein nanoparticle can be developed for tumor therapy in clinical practice.

Acknowledgments

Footnote

Conflicts of Interest: The authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.09.36). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All applicable international and national guidelines for the care and ethics of animals were followed. All animal experimental protocols were approved by the Animal Care and Ethics Committee of Yangzhou University, Yangzhou, China {Approval ID: SYXK [Su] 2017-0044}.

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