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Delivery of human NKG2D-IL-15 fusion gene by chitosan nanoparticles to enhance antitumor immunity

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ABSTRACT

Nanoparticles are becoming promising carriers for gene delivery because of their high capacity in gene loading and low cell cytotoxicity. In this study, a chitosan-based nanoparticle encapsulated within a recombinant pcDNA3.1-dsNKG2D-IL-15 plasmid was generated. The fused dsNKG2D-IL-15 gene fragment consisted of double extracellular domains of NKG2D with IL-15 gene at downstream. The average diameter of the gene nanoparticles ranged from 200 nm to 400 nm, with mean zeta potential value of 53.8 ± 6.56 mV. The nanoparticles which were loaded with the dsNKG2D-IL-15 gene were uptaken by tumor cells with low cytotoxicity. Tumor cells pre-transfected by gene nanopartilces stimulated NK and T cells in vitro. Intramuscular injection of gene nanoparticles suppressed tumor growth and prolonged survival of tumor-bearing mice through activation of NK and $CD8^+$ T cells. Thus, chitosan-based nanoparticle delivery of dsNKG2D-IL-15 gene vaccine can be potentially used for tumor therapy.

1. Introduction

Exogenous genes can be delivered into patients to treat genetic diseases or suppress tumor growth [1,2]. Nanoscale-sized particles have been used in diagnosis and treatment of many diseases. The polymeric nanoparticle, nanohydrogel, shows several advantages. Nanohydrogels are physically or chemically cross-linked hydrophilic polymer networks with huge loading capacity of water-soluble compounds. These particles show high biostability in physiological media, with distinct responsiveness to environmental factors. They are safe and can be degenerated without toxicity in hosts [3,4]. Thus, they are versatile tools for the delivery of peptides, carbohydrates, and oligonucleotides.

NKG2D, which is widely expressed by NK, $CD8^+$T, γδ T, and NKT cells, functions as an important activating receptor. NKG2D ligands include retinoic acid early induced transcript-1, H60, and murine ULBP-like transcript 1 in mouse, as well as MICA/B and ULBPs (UL-16 binding proteins) in humans [5,6]. These ligands are preferentially expressed in stress-induced cells or tumor cells and absent in most normal tissues, thereby serving as potential targets in tumor immunotherapy [7,8]. Moreover, serum soluble MICA/B and ULBP concentrations increase in late-stage cancer patients. These soluble ligands suppress antitumor immunity through competitive binding with NKG2D and downregulation of NKG2D expression in lymphocytes [9,10].

IL-15 is an essential cytokine for activation and survival of NK and $CD8^+$T cells [11]. Exogenous IL-15 has been evaluated for tumor therapy in mice and clinical studies [12,13]. We previously generated two recombinant fusion proteins, namely, human dsNKG2D–IL-15 (hdsNKG2D-IL-15) and mouse dsNKG2D-IL-15 (mdsNKG2D-IL-15), in which two identical NKG2D extracellular domains were fused to IL-15 [14]. DsNKG2D-IL-15 binds to NKG2D ligand-positive tumor cells through double NKG2D extracellular domain and activates NK or $CD8^+$ T cells via the IL-15 moiety,

Abbreviations: MICA, major histocompatibility complex class I chain-related protein A; DD, degree of deacetylation; PBS, phosphate buffer saline; NP, nanoparticle; DLS, dynamic light scattering; ELISA, enzyme-linked immunosorbent assay.

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thereby retarding tumor growth and enhancing the survival of tumor-bearing mice.

Chitosan is one of the most promising polymers for drug delivery because of its polycationic, biocompatible, and biodegradable nature [15,16]. In the present study, we aimed to generate chitosan-based nanoparticles to deliver the dsNKG2D-IL-15 fusion gene, which was inserted into a eukaryotic expression vector. Physical and chemical characterizations, biosafety, and transfection efficiency of nanoparticles were evaluated in vitro, and the antitumor activity of nanoparticles in tumor-bearing mice was observed.

2. Materials and methods

2.1. Plasmids, cell lines, and reagents

The pcDNA3.1 (−) plasmid was obtained from Clontech (Mountain View, CA, USA). The recombinant pQE3.1-dsNKG2D-IL-15 plasmid was previously generated [14]. *Taq* DNA polymerase, restriction endonucleases, T4 DNA ligase, PCR product purification, and DNA recovery kits were all obtained from Takara Bio (Dalian, China). Lipofectamine™ 2000 was obtained from Invitrogen (Grand Island, NY, USA). EndoFree Plasmid Maxi Kit was purchased from Qiagen (Düsseldorf, Germany). Chitosan, (molecular weight, 20 kDa) with deacetylation degree (DD) of 75%–85%, was obtained from Yuhuan County Marine Chemical Company (Yuhuan, Zhejiang, China). IL-15 antibody (H-114) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against CD8 (53.67), NK1.1 (PK136), CD69 (LG.3A10), NKG2D (CX5), and CD44 (IM7) were purchased from Biolegend (San Diego, CA, USA). Secondary antibodies conjugated to a fluorophore were obtained from Invitrogen (Grand Island, NY, USA). B16BL6, RAW264.7, and CT-26 cell lines were obtained from ATCC. MICA was expressed ectopically on B16BL6 cells, as previously described [17].

2.2. Preparation of chitosan-plasmid nanoparticles

Human dsNKG2D-IL-15 fusion gene fragment was cut from pQE31-dsNKG2D-IL-15 plasmid and inserted into pcDNA3.1 (−) in accordance with usual practice. DNA fragment of signal peptides from β2-microglobulin was also added at the upstream of NKG2D gene. Large amounts of pcDNA3.1-dsNKG2D-IL-15 plasmid were extracted and purified using plasmid extraction columns. Chitosan was chemically modified with N-(2-hydroxyl) propyl-3-trimethyl ammonium [18]. Chitosan and plasmids were separately dissolved in ultrapure water to obtain 1 mg/mL solutions. The plasmid solution was added dropwise into chitosan solutions and mixed at weight ratios of 1:1, 2:1, or 1:2. Mixed liquids were shaken at 300 rpm for 30 min. The mixture was centrifuged to form sediments. Sediments were collected and dissolved with phosphate-buffered saline for future use.

2.3. Physicochemical characterization of chitosan-plasmid nanoparticles

The amount of plasmid present in the nanoparticles was determined by the difference of total amount used and the amount present on the supernatant, which was detected using an ultraviolet spectrophotometer. Free plasmids in the supernatants were verified via agarose gel electrophoresis. Particle size and zeta potential of the chitosan/DNA complexes were detected by dynamic light scattering and zeta potential measurements in aqueous solutions on a Zetasizer Nano ZS 90 (Malvern, UK). The sizes of nanoparticles were confirmed using an electron microscope.

2.4. Transfection with chitosan-plasmid nanoparticles

B16BL6 (a murine melanoma cell line) or RAW264.7 (a murine macrophage cell line) cells were previously plated onto a 24-well plate with 10^5 cells. At the time of transfection, the culture medium of each well was replaced with serum-free DMEM medium. Chitosan-plasmid nanoparticles were added into plates at final dilutions of 10% or 20% by DMEM. No-load chitosan nanoparticle was used as negative control. After 6 h, the serum-free culture medium was removed, and a complete medium was added. Following 48 h cultures, cell supernatants were collected for assessment of dsNKG2D-IL-15 secretion by B16BL6 or RAW264.7 cells. ELISA kit from R&D Systems (Boston, MA, USA) was used to determine IL-15 concentration in accordance with the manufacturer's protocol. The viability of both cell lines cultured with chitosan-plasmid nanoparticles was assessed with an MTS/PMS kit from Promega (Madison, WI, USA). After adding various doses of no-load chitosan particles (1.25, 2.5, and 5 μg) and chitosan-plasmid particles (1.25, 2.5, and 5 μg) into cell wells for 24 h, the MTS/PMS solution was added to each well. After 4 h, absorption data at 490 nm were detected to evaluate the viable cell numbers.

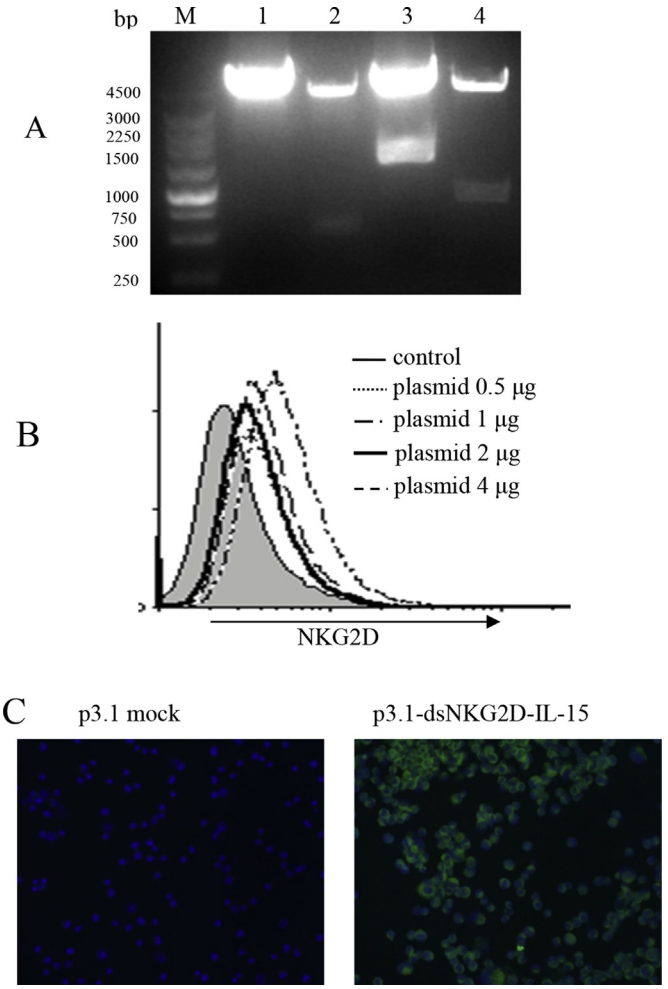

Fig. 1. Identification of the recombinant p3.1-dsNKG2D-IL-15 plasmid. The fusion gene fragment of dsNKG2D-IL-15 was inserted into pcDNA3.1 vector by DNA recombination technique. (A) The p3.1-dsNKG2D-IL-15 plasmid was digested by restrictive enzymes (M. DNA ladder; 1. Complete p3.1-dsNKG2D-IL-15 plasmid; 2. Digestion by BamH CT-26 cells pre-transfected by liposome and p3.1-dsNKG2D-IL-15 plasmid complex was detected with NKG2D staining by flow cytometry (B) IL-15 staining (C) immunofluorescence (100×). Each experiment was repeated thrice.

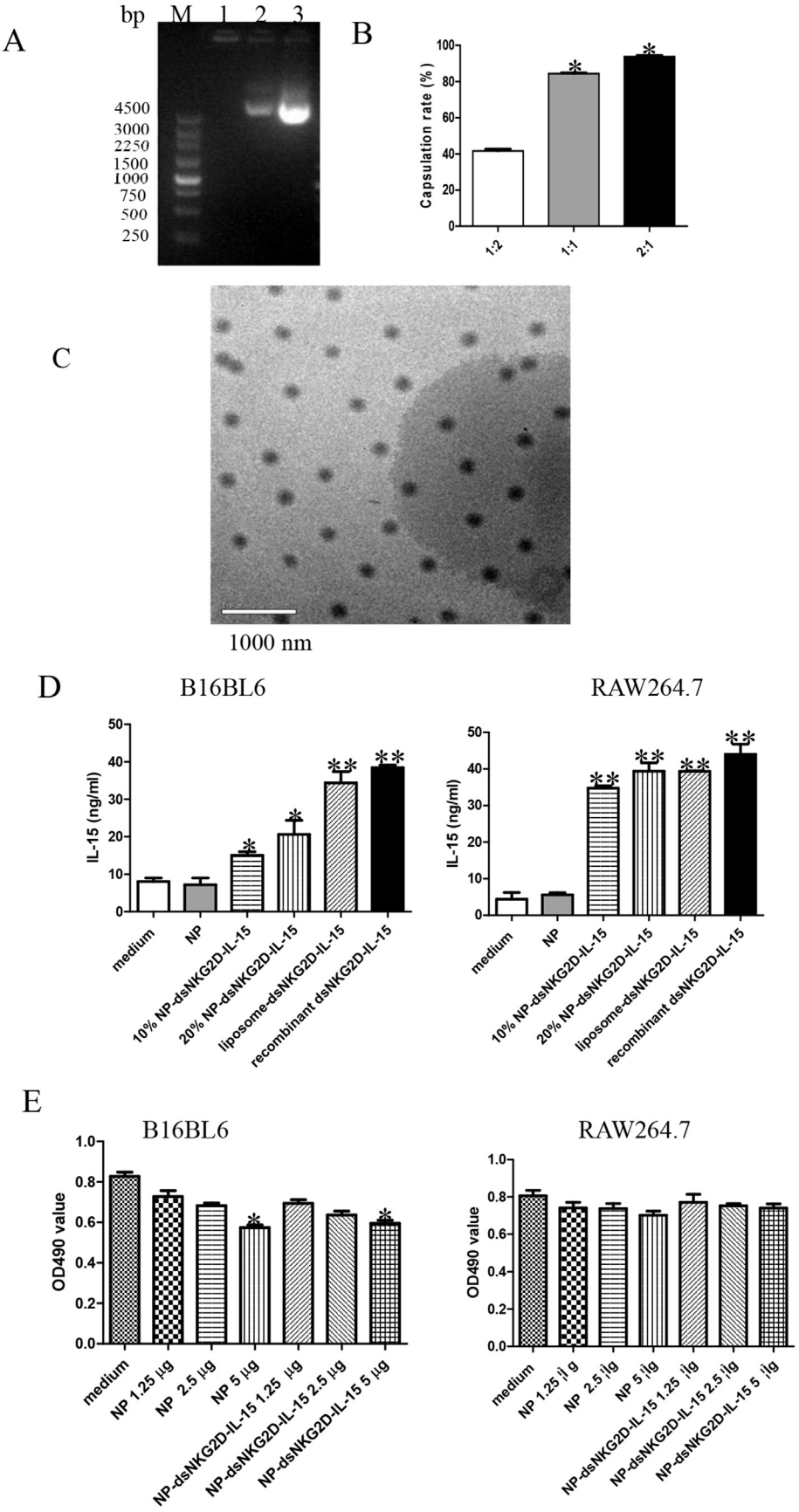

Fig. 2. Generation and identification of chitosan nanoparticles encapsulated with dsNKG2D-IL-15 fusion gene. (A) Agarose gel electrophoresis of supernatant solutions after nanoparticles were centrifuged with different ratios of pDNA to chitosan (weight:weight). Lane M (DNA ladder); Lane 1 (1:2); Lane 2 (1:1); Lane 3 (2:1). (B) Encapsulation rate

2.5. Immunofluorescent staining

CT-26 cells (murine colon cancer cell line), which were pre-transfected with pcDNA3.1-dsNKG2D-IL-15 or pcDNA3.1 by liposome, were added into the sections. After fixing with cold acetone, the sections were blocked with donkey serum and stained with IL-15 antibody and fluorescent-labeled goat anti-rabbit IgG secondary antibody. With complete wash, the sections were covered with 30 μL of mounting medium with DAPI (Vector Labs). Fluorescence was detected using Eclipse E600 (Nikon).

2.6. Mouse tumor models

B16BL6-MICA cells ($n = 6$; 2×10^6 cells) were implanted subcutaneously into the rear of C57BL/6 mice. After 7 d, the mice were treated daily with chitosan-dsNKG2D-IL-15 gene nanoparticles (100 μg) via intratumoral or intramuscular injection. Recombinant human dsNKG2D-IL-15 protein (60 μg) was used as positive control. Tumor growth was measured with digital calipers, and tumor volume was calculated as $V = (\text{width})^2 \times \text{length}/2$. All the mice were killed on day 21, and their spleens were removed. Splenocytes were isolated, and CD69 on NK and NKG2D on $CD8^+$T cells were detected via flow cytometry. To evaluate the tumor-bearing mice survival, mouse viability was recorded daily. All the experiments were conducted in accordance with protocols approved by the Yangzhou University's Institutional Animal Care and Use Committee.

2.7. Statistical analysis

Differences between groups were analyzed by Student's *t*-test. Values of $P < 0.05$ were considered significant. Kaplan–Meier survival curves were plotted and analyzed with GraphPad Prism software.

3. Results

3.1. Identification of recombinant pcDNA3.1-dsNKG2D-IL-15 plasmid

Recombinant eukaryotic expression vector, pcDNA3.1-dsNKG2D-IL-15, was identified by digestion of double restrictive enzymes. With digestions by BamH I/Kpn I, BamH I/Hind III, or Hind III/Kpn I gene fragments, which contained β2m signal peptides fused with the first NKG2D domain, the second NKG2D domain and IL-15 were inserted into pcDNA3.1 sequentially (Fig. 1A). The accuracy of fused gene sequences was confirmed by gene sequencing (100% identity with expected sequence). After cells were transfected with various liposome–plasmid complexes, the intracellular expression of dsNKG2D-IL-15 protein by CT-26 cells was observed via flow cytometry with intracellular anti-NKG2D antibody staining (Fig. 1B). DsNKG2D-IL-15 protein expression was also confirmed by anti-IL-15 antibody staining after transfection by immunofluorescence (Fig. 1C).

3.2. Physicochemical features of chitosan-plasmid nanoparticles

After encapsulation of plasmids by chitosan nanoparticles, free DNA plasmid was detected via agarose gel electrophoresis. Almost no excess DNA was found in supernatants with mass ratios of chitosan to plasmid at 2:1 (Fig. 2A). The highest encapsulation rate was obtained (92.8 ± 0.37%) at this ratio (Fig. 2B). The nanoparticles exhibited mean zeta potential values of 53.8 ± 6.56, and the nanoparticle diameters ranged from 200 nm to 400 nm. The morphology of chitosan-plasmid nanoparticles was confirmed using an electronic microscope (Fig. 2C). The particles showed even sizes with diameters around 300 nm.

To confirm whether chitosan nanoparticles can deliver the dsNKG2D-IL-15 fusion gene to tumor cells, supernatants from B16BL6 and RAW264.7 cells pre-transfected with 10% or 20% nanoparticles were collected to determine dsNKG2D-IL-15 production. Liposome-pcDNA3.1-dsNKG2D-IL-15 complex and recombinant dsNKG2D-IL-15 protein were both used as positive controls. As expected, 10% and 20% nanoparticle transfections efficiently secreted dsNKG2D-IL-15 protein, which was identified using IL-15 antibody (Fig. 2D). No significant difference existed between the transfections with 10% and 20% nanoparticles, inferring that 10% nanoparticles can generate good efficiency during transfection. The biosafety of chitosan-plasmid nanoparticles was evaluated among B16BL6 and CT26 cell lines. Nanoparticles at high dosages (5 μg) showed toxicities to B16BL6 cells compared with culture medium alone, and the inhibition rates were <25%. No nanoparticle cytotoxicities existed at all doses in RAW264.7 cells. Therefore, chitosan nanoparticles were generated for safe delivery of dsNKG2D-IL-15 fusion gene to tumor cells.

3.3. Activation of lymphocytes by tumor cells pre-transfected with nanoparticles

To confirm lymphocyte activation by tumor cells, which were pre-transfected by chitosan-dsNKG2D-IL-15 nanoparticles, CD69 expression on NK cells, as well as NKG2D and CD44 expression on $CD8^+$T cells, was detected after mouse splenocytes were cultured with transfected tumor cells. Both B16BL6 and RAW264.7 cells transfected with 10% or 20% dsNKG2D-IL-15 nanoparticles significantly stimulated NK cells to express CD69 (Fig. 3). The stimulations generated by dsNKG2D-IL-15 nanoparticles were similar to those produced by dsNKG2D-IL-15 liposome and recombinant dsNKG2D-IL-15 protein. NKG2D is only expressed in activated mouse $CD8^+$T cells, different from its constitutive expression in human $CD8^+$T cells. The DsNKG2D-IL-15 nanoparticles in tumor cells also promoted $CD8^+$T cells to express NKG2D and CD44.

3.4. Inhibition of tumor growth by nanoparticles in tumor-bearing mice

To verify the activation of NK and $CD8^+$T cells in vivo, chitosan-dsNKG2D-IL-15 gene nanoparticles were injected intramuscularly into normal mice. After 72 h, the mice were killed to analyze the splenocytes' serum IL-15 levels and expression of CD69 in NK cells, as well as NKG2D in $CD8^+$T cells. No significant variations existed in the serum IL-15 levels (data not shown) in mice. We speculated that secreted dsNKG2D-IL-15 protein may be trans-captured by IL-15 receptor α of macrophages and dendritic cells in mice. However, compared with no-load chitosan nanoparticles, chitosan nanoparticles encapsulated within dsNKG2D-IL-15 fusion gene significantly stimulated CD69 expression in NK cells and NKG2D expression in $CD8^+$T cells (Fig. 4A). Therefore, these chitosan-DNA

was calculated by ratio of the amount of DNA encapsulated into nanoparticles to total DNA quantities. (C) Transmission electron microscopy displayed morphology and size of nanoparticle. (D) Supernatants of B16BL6 or RAW264.7 cells pre-transfected with 10% or 20% chitosan-dsNKG2D-IL-15 gene nanoparticles were collected to determine dsNKG2D-IL-15 concentration by ELISA. * indicates $P < 0.05$ versus medium or NP, ** indicate $P < 0.01$ versus medium or NP. (E) Variations of B16BL6 or RAW264.7 cell numbers were checked by an MTS/PMS kit after these cells were in co-culture with chitosan-plasmid nanoparticles for 72 h * indicates $P < 0.05$ versus medium.

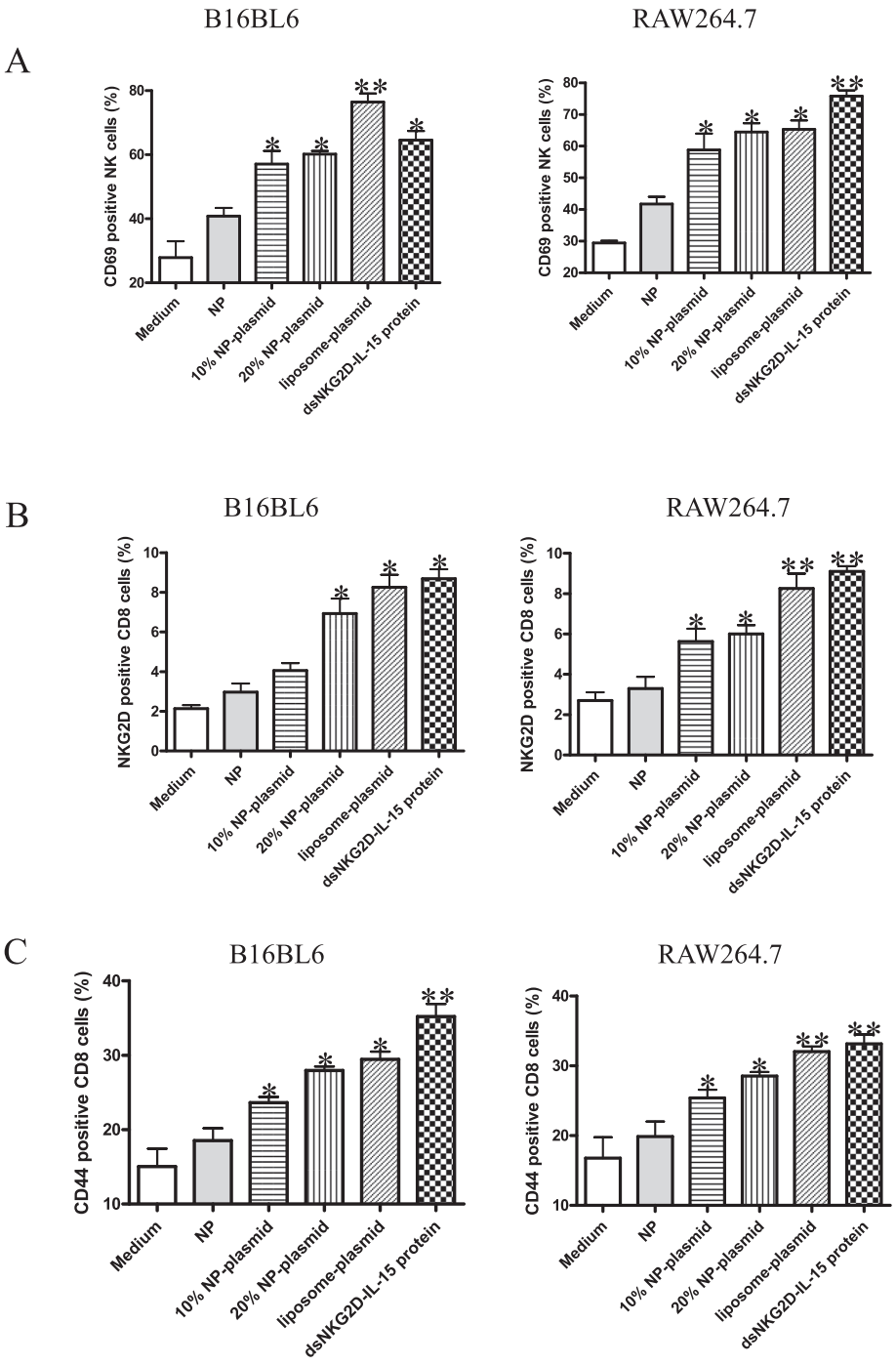

Fig. 3. Stimulation of NK and CD8$^+$T cells by tumor cells pre-transfected with chitosan-dsNKG2D-IL-15 gene nanoparticles in vitro. B16BL6 or RAW264.7 cells pre-transfected with 10% or 20% chitosan-plasmid nanoparticles, cultured with mouse splenocytes overnight. Frequencies of NK1.1$^+$CD69$^+$ (A), CD8$^+$NKG2D$^+$ (B), and CD8$^+$CD44$^+$ (C) cells, were detected by flow cytometry. * indicates $P < 0.05$ versus medium or NP, ** indicates $P < 0.01$ versus medium or NP.

nanoparticles may be endocytosed and expressed efficiently by cells in vivo.

B16BL6-MICA cells, which were ectopically expressed by human NKG2D ligand (MICA) on B16BL6 cells, were transplanted into C57BL/6 mice. Intramuscular treatment with chitosan-dsNKG2D-IL-15 gene nanoparticles suppressed tumor growth and prolonged survival of tumor-bearing mice, similar to the treatment with recombinant dsNKG2D-IL-16 protein (Fig. 4B, C). The use of nanoparticles by intratumoral injection did not show significant antitumor activity, which reflected different expression efficiencies of nanoparticles through distinct injection pathways. The tumor-bearing mice treated with dsNKG2D–IL-15 nanoparticles also showed no signs of inflammatory damage (e.g., diarrhea, skin rash, and ruffled hair), suggesting absence of toxicity.

The frequencies of NK1.1$^+$, CD69$^+$NK1.1$^+$ (Fig. 4D), CD8$^+$NKG2D$^+$ T, and CD8$^+$CD44$^+$ T cells (Fig. 4E) in the spleen were significantly higher with chitosan-dsNKG2D–IL-15 nanoparticle treatment via intramuscular injection than those with no-load chitosan treatment or chitosan-dsNKG2D–IL-15 nanoparticle treatment via intratumoral injection in B16BL6–MICA bearing mice. Thus, in vivo

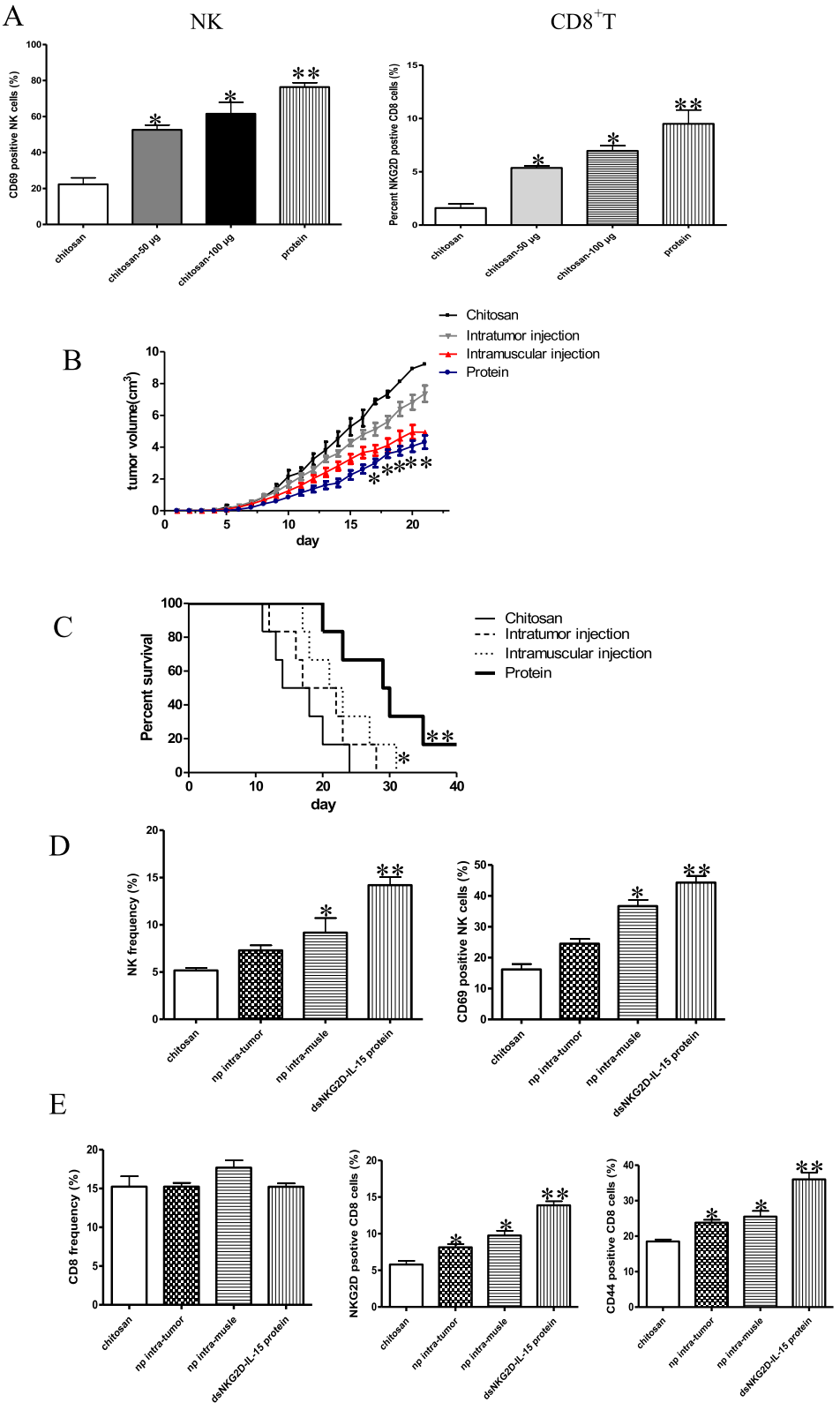

Fig. 4. Antitumor activity of chitosan-dsNKG2D-IL-15 gene nanoparticles in vivo. (A) Nanoparticles encapsulated within the dsNKG2D-IL-15 gene were intramuscularly injected into normal mice for 3 d. The mice were sacrificed to analyze frequencies of $NK1.1^+CD69^+$ and $CD8^+NKG2D^+$ in spleen. (B) B16BL6–MICA were injected into the C57BL/6 mice ($n = 6$, 2×10^6 cells). On day 7, chitosan-dsNKG2D-IL-15 gene nanoparticles (100 μg), were injected daily via intratumoral or intramuscular route. Tumor growth was measured daily and is shown as means ± SD. (C) Mice deaths were documented over time. Data are presented in Kaplan–Meier survival curves. All mice were sacrificed on day 22, and their spleens were collected. Frequencies of $NK1.1^+NKG2D^+$ cells (D), $CD8^+NKG2D^+$ T cells, and $CD8^+CD44^+$ T cells (E) of splenocytes were detected by flow cytometry. * indicates $P < 0.05$ versus chitosan, **indicates $P < 0.01$ versus chitosan. Experiments were performed twice.

injection of chitosan-dsNKG2D–IL-15 nanoparticles via intramuscular pathway can efficiently promote activation of NK and $CD8^+$T cells to mediate antitumor immunity.

4. Discussion

A gene-carrier system based on chitosan nanoparticles was developed to deliver fusion gene, dsNKG2D-IL-15, through enhancement of immune function for tumor therapy. These nanoparticles showed efficient encapsulation for DNA and a high capacity to transfect cells with low cytotoxicity. Soluble dsNKG2D-IL-15 protein was produced to activate NK and $CD8^+$ T cells. In vivo delivery of chitosan-dsNKG2D-IL-15 gene nanoparticles also promoted NK and $CD8^+$ T cell functions, thereby efficiently suppressing mice tumor growth. A new gene vaccine, which can enhance immune function delivered by chitosan nanoparticles, was generated with potential use for therapy in $MICA^+$ tumor patients.

Chitosan is a “smart” material for drug delivery. This material has been modified and processed into various nanoparticles with multiple functions to deliver therapeutic agents [19]. Drugs encapsulated in chitosan nanoparticles can be automatically released in tumor tissues because of their acidic environment induced by its hypoxic state [20,21]. As chitosan is extracted from marine organisms, it exhibits good biocompatibility and less toxicity to the human body. Our study confirmed that chitosan with 20 kDa molecular weight and 55.3% degree of deacetylation displayed slight cytotoxicity against human cells. Tumor cells transfected with 10% or 20% chitosan-dsNKG2D-IL-15 gene particles efficiently produced dsNKG2D-IL-15 protein, indicating that chitosan nanoparticles were endocytosed, and dsNKG2D-IL-15 fusion gene was efficiently released in the cytoplasm.

In vivo injection of chitosan-dsNKG2D-IL-15 gene nanoparticles showed significant effects on tumor inhibition and lymphocyte activation. The magnitude of antitumor activity by the gene nanoparticles remained less than that by recombinant dsNKG2D-IL-15 protein. The gene expression rate was the determining factor in gene vaccine therapies. However, some advantages exist in using chitosan-dsNKG2D-IL-15 gene nanoparticles for tumor therapy. Chitosan-gene nanoparticles are easily manufactured. Compared with the production of recombinant dsNKG2D-IL-15 protein, gene nanoparticles do not need isolation, purification, and renaturing of proteins expressed in bacteria. A large amount of protein would be lost in the process of renaturing. Chitosan-gene nanoparticles are also easily preserved and transported.

Human dsNKG2D-IL-15 fusion protein can bind to MICA of B16BL16-MICA cells, through double NKG2D extracellular domains, and stimulate mouse NK and $CD8^+$ T cells through the IL-15 domain as shown previously [14]. Human NKG2D only binds to MICA or other human ligands but not murine ligands [22]. However, human IL-15 (70% identical with mouse IL-15) can bind to mouse receptor. Given the high expression level of MICA on tumor cells, dsNKG2D-IL-15 protein can potentially block NKG2D of NK and $CD8^+$T cells in ligation with MICA of tumor cells. However, this event cannot occur because low NKG2D expression on NK and $CD8^+$T cells was observed in tumor patients, particularly at late stage [23]. During this time, NK and $CD8^+$T cell functions against tumor were significantly decreased. The addition of exogenous dsNKG2D-IL-15 protein did not downregulate functions of NK and $CD8^+$T cells but promoted their activation through IL-15 [24]. Moreover, treatment with recombinant dsNKG2D-IL-15 protein displayed suppression of tumor growth and elongation of survival in tumor-bearing mice [14].

In summary, nanoparticles based on chitosan for delivery of dsNKG2D-IL-15 fusion gene vaccine were successively generated for lymphocyte activation and suppression tumor growth. The safety and biodistribution of the nanoparticles, as well as the detailing variations of immune function in vivo affected by the nanoparticles, require further investigations. The chitosan-dsNKG2D-IL-15 gene nanoparticles can be easily generated and regarded as novel potential reagents for tumor therapy.

Conflict of interest

None.

Acknowledgments

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