ORIGINAL RESEARCH

Chitosan Nanoparticles Strengthen Vγ9Vδ2 T-Cell Cytotoxicity Through Upregulation Of Killing Molecules And Cytoskeleton Polarization

Li Lin[1,2,*]
Junyi He[2,*]
Jiawei Li[2]
Yan Xu[2]
Jingxia Li[2]
Yangzhe Wu[1,2]

[1]Zhuhai Precision Medical Center, Zhuhai People's Hospital, Zhuhai Hospital Affiliated with Jinan University, Jinan University, Zhuhai, Guangdong 519000, People's Republic of China; [2]The Biomedical Translational Research Institute, Faculty of Medical Science, Jinan University, Guangzhou, Guangdong 510632, People's Republic of China

*These authors contributed equally to this work

Background: During the past few years, immune cell therapy for malignant cancer has benefited a considerable amount of patients worldwide. As one of several promising candidates for immunotherapy, Vγ9Vδ2 γδ T cells have many unique biological advantages, such as non-MHC restriction and have been noted as the earliest source of IFN-γ. However, potentiating anti-tumor functions of γδ T cells has become of particular interest to researchers studying γδ T cell applications.

Purpose: In this study, we proposed a nanotechnology-based methodology for strengthening γδ T cell functions.

Methods: As a type of reliable, biocompatible material, chitosan nanoparticles (CSNPs) were used to enhance anti-tumor immunity of γδ T cells.

Results: First, we found that the size of prepared CSNPs distributed 50 to 100 nm, and that CSNPs had optimal immunocompatibility. Then, we observed that CSNPs could induce α-tubulin cytoskeleton polarization and rearrangement, correlating with a higher killing ability of γδ T cells. Furthermore, we revealed that CSNPs could enhance Vγ9Vδ2 T cell anti-tumor functions by upregulating killing of related receptors, including NKG2D, CD56, FasL, and perforin secretion.

Conclusion: Our work provided evidence of application for CSNPs based bio-carrier in immunotherapy. More importantly, we proposed a new strategy for enhancing γδ T cell anti-tumor activity using nanobiomaterial, which could benefit future clinical applications of γδ T cells.

Keywords: chitosan nanoparticles, Vγ9Vδ2 γδ T cell, cytotoxicity, anti-tumor activity

Introduction

During the past few years, immune cell therapy has been highlighted as a new strategy for treating malignant cancers, particularly after the success of CD19 Car-T. Among a few candidates that could be promising options for immunotherapy, γδ T cells have shown great potential for development as a new alternative immune cell therapeutic methodology. γδ T cells (specifically Vγ9Vδ2 subset) innate-like T lymphocytes distinguished by T-cell receptors (TCRs) consist of γ and δ chains that are mainly distributed in peripheral blood.[1] Scientific literature articles now report that Vγ9Vδ2 T cells can recognize stress-induced phosphonate antigens presented by both cancer cells and pathogen-infected cells in a MHC-independent manner. This is a unique advantage of Vγ9Vδ2 T cells, differing from CD4+ or CD8+ T cells (αβ T cells). It has also been reported that γδ T cells are the earliest source of

Correspondence: Yangzhe Wu
Zhuhai Precision Medical Center, Zhuhai People's Hospital, Zhuhai Hospital Affiliated with Jinan University, Jinan University, Zhuhai, Guangdong 519000, People's Republic of China
Tel +86 020 8522 2787
Email tyzwu@jnu.edu.cn

IFN-γ,[2] and tumor infiltrated γδ T cells could become the best biomarker for tumor prognosis when compared with all other types of immune cells.[3,4] More importantly, for the first time, we announced the application of allogenic Vγ9Vδ2 T cells for treating recurrent liver cancer.[5] This research has opened a new avenue for Vγ9Vδ2 T cell-based cancer immunotherapy in malignant tumors.

One of the major concerns in clinical application of Vγ9Vδ2 T cells is related to obtaining a large number of cells with optimal immune effector functions. Currently, there are reviews[6] explaining expansion methodology; however, minimal research on potentiating Vγ9Vδ2 T cell cytotoxicity has been reported. Therefore, in this work, we proposed a nanobiomaterial-based strategy to strengthen the Vγ9Vδ2 T cell killing ability of cancer cells.

Among considerable amounts of biomaterials, chitosan is a well-known type of macromolecules with high biological activity.[7] Chitosan and its derivatives have been used as nanocarriers, attributed to their unique properties such as biocompatibility, biodegradability, antimicrobial activity, adjuvant nature, and non-immunogenicity.[8,9] For instance, Rafael de Oliveira Pedro prepared a self-assembled, pH-sensitive drug-delivery system to deliver quercetin to breast cancer cells.[8] Shi and Zhang developed CSNPs modified with mannose (Man-CTS NPs) moieties for specific dendritic cell (DC) targeting, enhancing anti-tumor immunity in tumor cell lysates-based vaccine.[10] The application of chitosan as a carrier in anticancer drugs and vaccines has been intensively investigated. Furthermore, researchers have now begun to evaluate how chitosan itself could affect effector functions of immune cells in circulatory systems and tumor microenvironments. Research have shown that chitosan could be a potential modulator or immune stimulator, capable of driving potent cell-mediated immunity.[11] For example, the chitosan/poly (γ-glutamic acid) nanoparticles (NPs) are capable of modulating macrophage and DC functions, thus enhancing their ability to promote T cell proliferation and reduce the capacity to induce colorectal cancer cell invasion.[12]

In our present work, we used Vγ9Vδ2 T cells, which could directly recognize and kill cancer cells, as shown in our research model, to test how CSNPs modulated their effector functions. More importantly, our research provided a paradigm on using nanotechnology to modulate anti-tumor activity of cytotoxic T cells, rather than gene modification. We found that CSNPs could greatly strengthen Vγ9Vδ2 T cell anti-tumor effector functions by inducing α-tubulin cytoskeleton polarization and rearrangement, and

upregulating expression of killing related receptors, including NKG2D, CD56, FasL, and cytokine perforin secretion. Altogether, our study provided concept-of-proof for application of CSNPs in immune cell modification. Moreover, we proposed a new strategy for enhancing T cell anti-tumor activity using nanobiomaterial, which could benefit future clinical T cell applications.

Materials And Methods
Materials
Chitosan (≥95% deacetylated, 20–500 mPa. S viscosity, MV~600,000) was obtained from Coolaber (Beijing, People's Republic of China), sodium tripolyphosphate, fluorescein isothiocyanate isomer (FITC) and zoledronic acid monohydrate were purchased from Sigma-Aladdin (Shanghai, People's Republic of China), phosphotungstic acid (Leagene, Beijing, People's Republic of China), Ficoll-Paque™ Plus (GE Healthcare, Sweden), recombination human IL-2 (Si Huan Sheng Wu, Beijing, People's Republic of China), Alexa Fluor 647 anti-tubulin-α (Biolegend, San Diego, USA), Oregon Green 488 Phalloidin (Invitrogen, USA). All chemicals and reagents were commercially obtained and used directly without further purification.

CSNPs Preparation And Labeled With FITC
For preparation of CSNPs, 150 mg of chitosan was dissolved in 30 mL 0.4% v/v acetic acid solution, and NaOH was used to adjust the pH of the mixture to 4.6–4.8. Then, 10 mL 0.25% (w/v) sodium tripolyphosphate solution was slowly dropped into the chitosan mixture, while constantly stirring. Next, the CSNPs suspension was centrifuged at 9000 g for 30 mins after stirring for 1 hr and washed three times with water. Finally, CSNPs were re-suspended in PBS solution with the final concentration of 30 mg/mL.

For FITC labeling, FITC-CSNPs were prepared by mixing the 1 mL CSNPs suspension (15 mg/mL) with 20 μL FITC solution ($N_{FITC(-N=C=S)}$: $N_{Chitosan(-NH2)}$=1:4), stirring at room temperature overnight. Then, FITC-CSNPs were collected using centrifugation at 9000 g for 30 mins and washed twice with 70% ethanol solution.

CSNPs Characterization
The synthesized CSNPs were characterized by transmission electron microscopy (TEM) (JEOL 2100F) and dynamic light scattering (DLS). For TEM analysis, the CSNPs suspension was deposited on the carbon grid, followed by

covering with phosphotungstic acid for 2 mins. Then, TEM analysis was performed under vacuum. Additionally, a particle sized distribution and zeta potential of CSNPs were measured by DLS (Malvern, Zetasizer Nano ZS).

In order to verify the presence of LPS contamination, we performed LAL gel detection on the CSNPs suspension. 0.125 EU/mL endotoxin standard (Xiamen Bioendo Technology Co., Ltd.) was used as a positive control and TAL reagent water (TRW, Xiamen Bioendo Technology Co., Ltd.) was used as a negative control. Then, 200 µL of 50 µg/mL CSNPs solution was added to the ToxinSensor™ Single Test Kit (GenScript, sensitivity is 0.06 EU/mL) and incubated at 37°C for 1 hr to check whether a gel had formed or not.

Human PBMCs Isolation And Vγ9Vδ2 T Cell Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy donors by following routine Ficoll-Paque protocol. For Vγ9Vδ2 T cell culture, 3.5×10^6 PBMCs per 24-well plate were cultured in complete RPMI1640 medium, which was supplemented with 10% fetal bovine serum (FBS), 40 ng/mL recombination human IL-2, and 50 µM zoledronic acid monohydrate for 72 hrs. Then, the culture medium was replaced with new complete medium, supplemented with only FBS and IL-2 every 2 or 3 days. Since this culture protocol selectively expanded Vγ9Vδ2 T cells, the purity of Vγ9Vδ2 T cells could achieve over 90% at 10 days of in vitro culturing with the same medium.

Cell Viability Measurements

Vγ9Vδ2 T cell viability was detected using Annexin V-FITC Apoptosis Detection Kit after co-incubating with different CSNPs and CS molecular (0.4% v/v acetic acid) concentrations for 6 hrs. Briefly, Vγ9Vδ2 T cells were collected and washed with PBS, then re-suspended in 1× binding buffer, followed by staining Annexin V-FITC and propidium iodide (PI) for 10 mins at room temperature. Then, cell viability of stained Vγ9Vδ2 T cells was measured by flow cytometry. Results were analyzed using FlowJo software version 10.

Cytoskeleton Detection Of Vγ9Vδ2 T Cells

The cytoskeleton of Vγ9Vδ2 T cells was analyzed by detecting cytoskeleton proteins (β-actin and α-tubulin) using Confocal Microscopy and Flow Cytometry. Vγ9Vδ2 T cells

were collected and 1×PBS washed after chitosan or CSNPs (0, 50 µg/mL) treatment. Then, cells were stained with Alexa Fluor 647 anti-tubulin-α (Ex: 650 nm, Em: 668nm), Oregon Green 488 Phalloidin (Ex: 495 nm, Em: 518 nm) for actin and Hoechst 33342 (Ex: 346 nm, Em: 460 nm) for cell nuclear according to reagent provider's protocol, after which cell samples were used for confocal imaging. For flow cytometry analysis, cells were first stained with anti-human antibodies including Vδ2-PercP and CD3-APC-H7, and subsequently stained with Oregon Green 488 Phalloidin and Alexa Fluor 647 anti-tubulin-α by following standard protocols.

AFM Measurements Of Cell Morphology

The topographic properties of Vγ9Vδ2 T cells after chitosan and CSNPs (0, 50 µg/mL) treatments were detected using AFM (Atomic Force Microscope). The methodology for AFM imaging has been described in detail in our previous publications.[13–15] In our measurements, tapping mode AFM (Bioscope Catalyst, Bruker) was used to image cells at room temperature. The spring constant of the cantilever was calibrated at ~0.6 N/m. Obtained images were analyzed using instrument equipped analysis software.

Absorption Of CSNPs By Vγ9Vδ2 T Cells

To determine the absorption and endocytosis of CSNPs by Vγ9Vδ2 T cells, the cells were first pretreated with FITC-labeled CSNPs, followed by Vδ2-PercP staining for 30 mins at 4°C. Cells were finally stained with DAPI prior to image by Leica laser scanning confocal microscopy (SP8). 63× oil objective and 405 nm and 488 nm laser lines were used during images acquiring.

Cytokine Analysis

To analyze whether or not CSNPs could stimulate surface molecular expression or cytokine secretion, Vγ9Vδ2 T cells were co-incubated with CSNPs (0, 50 µg/mL) for 6 hrs. Then, cells were collected and washed once in cold PBS, subsequently stained using various fluorochrome-conjugated monoclonal antibodies in PBS (4°C, 30 mins). After washing with PBS, cells were detected on a FACSVerse (BD Biosciences) and analyzed using FlowJo software version 10. The following Abs were used: Vδ2-PercP, Fas-L-PE, PD-1-Pacific Blue, NKG2D-PE/Cy7, TNF-α-FITC, CD107a-APC, Ki-67-APC, Perforin-Brilliant Violet421, IL-17A-FITC, CD3-APC-H7, CD3-V500, CCR7-Alexa Fluor 647, CD45RA-FITC, IFN-γ-PE-Cy7, Granzyme B-BV510, and IL-4-PE. For intracellular cytokine staining,

CSNPs pre-treated cells were first stimulated with 50 ng/mL PMA, 1 μg/mL ionomycin and blocked with GolgiStop (1:1000), followed by fixation/permeabilization treatment according to manufacturer's protocol. For Ki67 staining, eBioscience™ Foxp3/Transcription was used accordingly.

Samples For mTOR Signaling Detection Of Vγ9Vδ2 T Cells

mTOR signaling downstream proteins of Vγ9Vδ2 T cells in the presence of CSNPs were detected by Western blot and flow cytometry. CSNPs (0, 50 μg/mL) pre-treated Vγ9Vδ2 T cells were collected and lysed in RIPA Lysis Buffer. For Western blot, primary antibodies including mTOR, p-mTOR, p-P70S6K (Thr389), p-P70S6K (Ser371) and β-tubulin were used, and then followed by established routine protocol to accomplish experiments. For P-4E-BP1 detection, CSNPs (0, 50 μg/mL) pre-treated Vγ9Vδ2 T cells were collected and stimulated with anti-CD3 (1 μg/mL) and anti-CD28 for 30 min (4°C). Then, cells were stained for Vδ2-PercP and CD3-APC-H7 (15 mins, 4°C). Finally, Vγ9Vδ2 T cells were stained with Phospho-4E-BP1 (Thr37/46) (Alexa Fluor 647 Conjugate) (30 mins, 4°C) and detected by flow cytometry.

Vγ9Vδ2 T Cell Killing Ability Of Cancer Cells In Vitro

In our research, HL60 and K562 tumor cell lines (purchased from Jiniou Company, Guangzhou, People's Republic of China) were used as model cell lines to test Vγ9Vδ2 T cell killing ability. Briefly, fluorescent dye CFSE stained cancer cells were used as target cells (T), and Vγ9Vδ2 T cells were used as effector cells (E). For killing assays, effector cells versus target cells (E:T) were set as 5:1, 10:1, and 20:1. After 6 hrs of co-incubation of E and T cells, the percentage of dead cancer cells was determined using propidium iodide (PI) staining and flow cytometry analysis. For confocal imaging, after 6 hrs of co-incubation of Vγ9Vδ2 T cells (fluorescent dye Dil pre-stained) and cancer cells (CFSE pre-stained), the samples were then visualized by Leica SP8 confocal system.

Statistical Analysis

All graphs and statistical analyses were performed using Gradpad (Prism) 7.0 and SPSS Statistics 17.0, respectively. Data presented as mean±standard error of mean (SEM, error bars in figures). The differences between groups were determined by one-way ANOVA, and

statistical significances between the control group and experimental groups were assessed using unpaired t-tests when data were consistent with normal distribution or non-parametric tests. Statistical significance was achieved when *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Results

Characterization And Stability Of CSNPs

Prepared CSNPs were characterized by transmission electron microscopy (TEM, Figure 1A). The TEM image showed the diameter of CSNPs was approximately 50–100 nm, which was consistent with the hydrodynamic diameter detected by Malvern Zetasizer Nano ZS (113±10.5 nm, PDI=0.295) (Figure 1B). The surface zeta potential of CSNPs was measured at 54.63±2.38 mV. Such data implicated with optimal uniformity of prepared CSNPs.

It has been reported that pH values in blood circulation, endosomes, and lysosomes are approximately 7.4, 6.5, and 4.5, respectively. Therefore, we determined the stability of CSNPs in different pH buffer solutions by detecting size and zeta potential. The results (Supplementary Figure 1) show that the size of CSNPs increased in an acidic environment of pH 4.5, and the zeta potential also increased from 5.1 at pH 7.4 to 15.2. However, the particle size and potential in the pH 6.5 solution did not change significantly. This may be due to the difference in ionic strength between solutions at different pH that cause the particles to agglomerate or adsorb certain ions. The specific reasons need to be further studied.

In order to verify the presence of LPS contamination (an immunostimulator), we performed LAL gel detection on the CSNPs suspension. The result is shown in Supplementary Figure 2: a positive reaction (0.125 EU/mL standard endotoxin) is characterized by the formation of a firm gel and a negative reaction (TRW) is characterized by the absence of a solid clot. Although the lysate of CSNPs suspension and chitosan molecular solution showed an increased turbidity, no solid gel formed. It
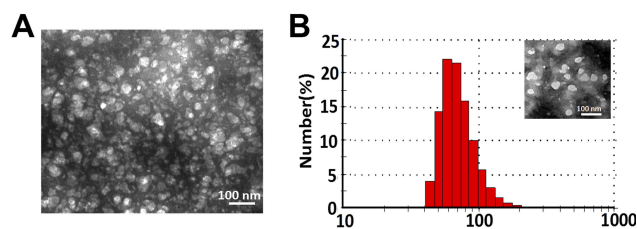

Figure 1 The characterization of CSNPs. (**A**) TEM image of CSNPs. (**B**) DLS measurements of CSNPs.

indicated that the CSNPs suspension in the experiment was not contaminated with LPS.

CSNPs Possess Promising Immunocompatibility

Currently, pieces of literature have claimed that chitosan possesses ideal biocompatibility; however, little is known about its interactions with immune cells. We, therefore, first tested the immunocompatibility of CSNPs to help us fully understand how CSNPs affect immune cells as a drug-delivery system or bio-carrier. Then, we checked cell viability in the absence and presence of CSNPs and found that CSNPs had no toxic effects on cells; in contrast, chitosan induced cell death in a dose-dependent manner (Figure 2A). We further tested whether or not CSNPs could induce cell apoptosis using Annexin V/PI kit and observed that CSNPs induced neither early apoptosis (Figure 2B) nor late apoptosis (Figure 2C) of Vγ9Vδ2 T cells, even at a concentration of 200 μg/mL. On the contrary, chitosan could significantly induce early apoptosis of cells at the concentration of 100 μg/mL and higher (Figure 2B and C; Supplementary Figure 3A). These results

clearly indicated that CSNPs had optimal immunocompatibility, rather than free chitosan molecules.

It is known that immune cells also express negative immune inhibitory molecules like PD-1 to avoid excess activation. However, in anti-tumor immunity, PD-1 plays negative roles including promotion of immune escape of cancer cells;[16] thus it needs to be downregulated or blocked as best as possible. Therefore, we further analyzed how CSNPs could affect PD-1 expression of Vγ9Vδ2 T cells. We surprisingly found that free chitosan could dramatically upregulate PD-1 expression even at low concentrations (12.5 μg/mL), suggesting immune inhibitory effects of chitosan (Figure 2D). However, CSNPs only induced a slight increase in PD-1 expression (no significant difference) even at the concentration of 50 μg/mL and had no effect at lower concentrations (Figure 2D and E; Supplementary Figure 3B). It should be noted that when the concentration reached 100 μg/mL and higher, CSNPs could significantly upregulate PD-1 expression as well (Figure 2E). In our following experiments, we chose 50 μg/mL CSNPs to perform analyses in consideration of results of killing molecule NKG2D expression (refer to Figure 3).
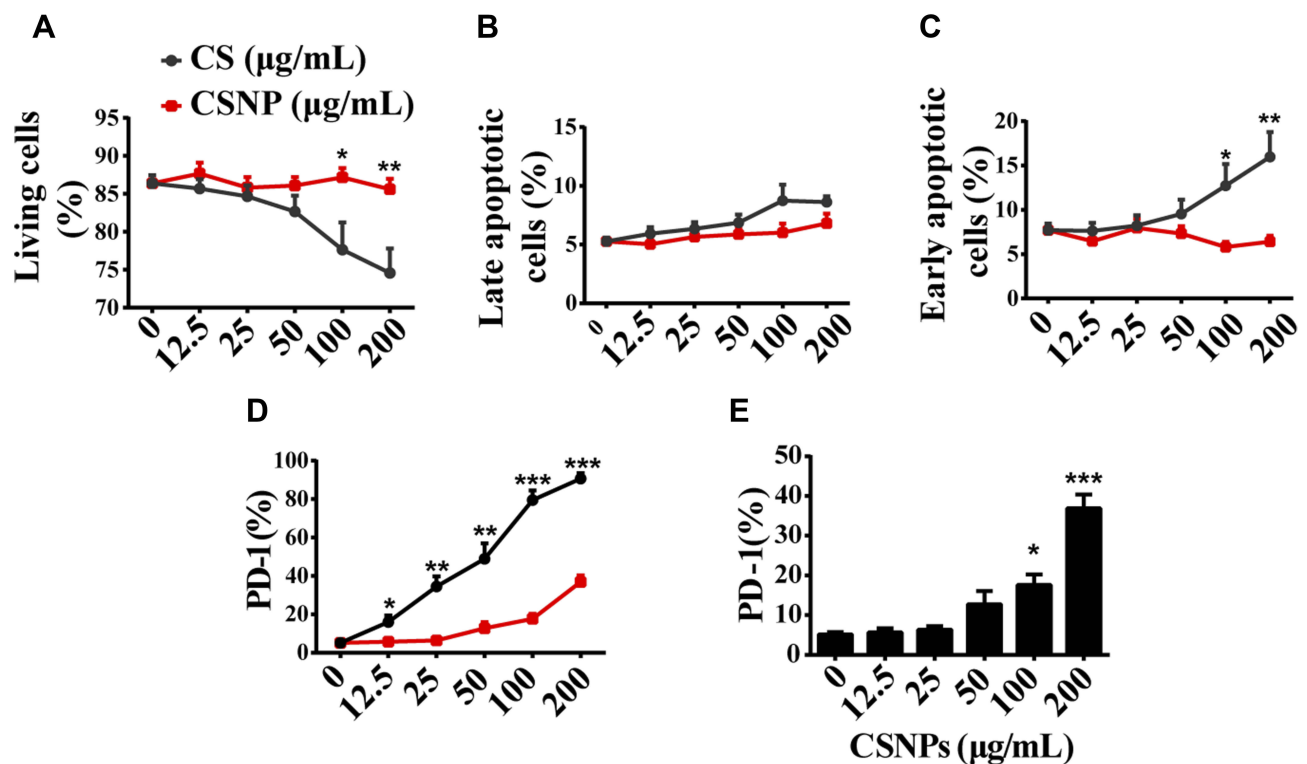

Figure 2 Immunocompatibility assays of CSNPs. (**A–C**) Viability of Vγ9Vδ2 T cells determined by Annexin V-FITC/PI. (**D–E**) The immune checkpoint molecule PD-1 expression of Vγ9Vδ2 T cells in the presence of chitosan or CSNPs. Black line represents the chitosan group, whereas red lines in (**A–D**) represent CSNPs group. Data were collected from at least three independent experiments. Co-incubation time between CSNPs or chitosan and cells was 6 hrs. The statistical differences were calculated by parallel comparisons of data at specific concentration points of chitosan and CSNPs (**A–D**); however, in (**E**), experimental group was, respectively, compared with control group ("0"). *p<0.05, **p<0.01, ***p<0.001, n=3.
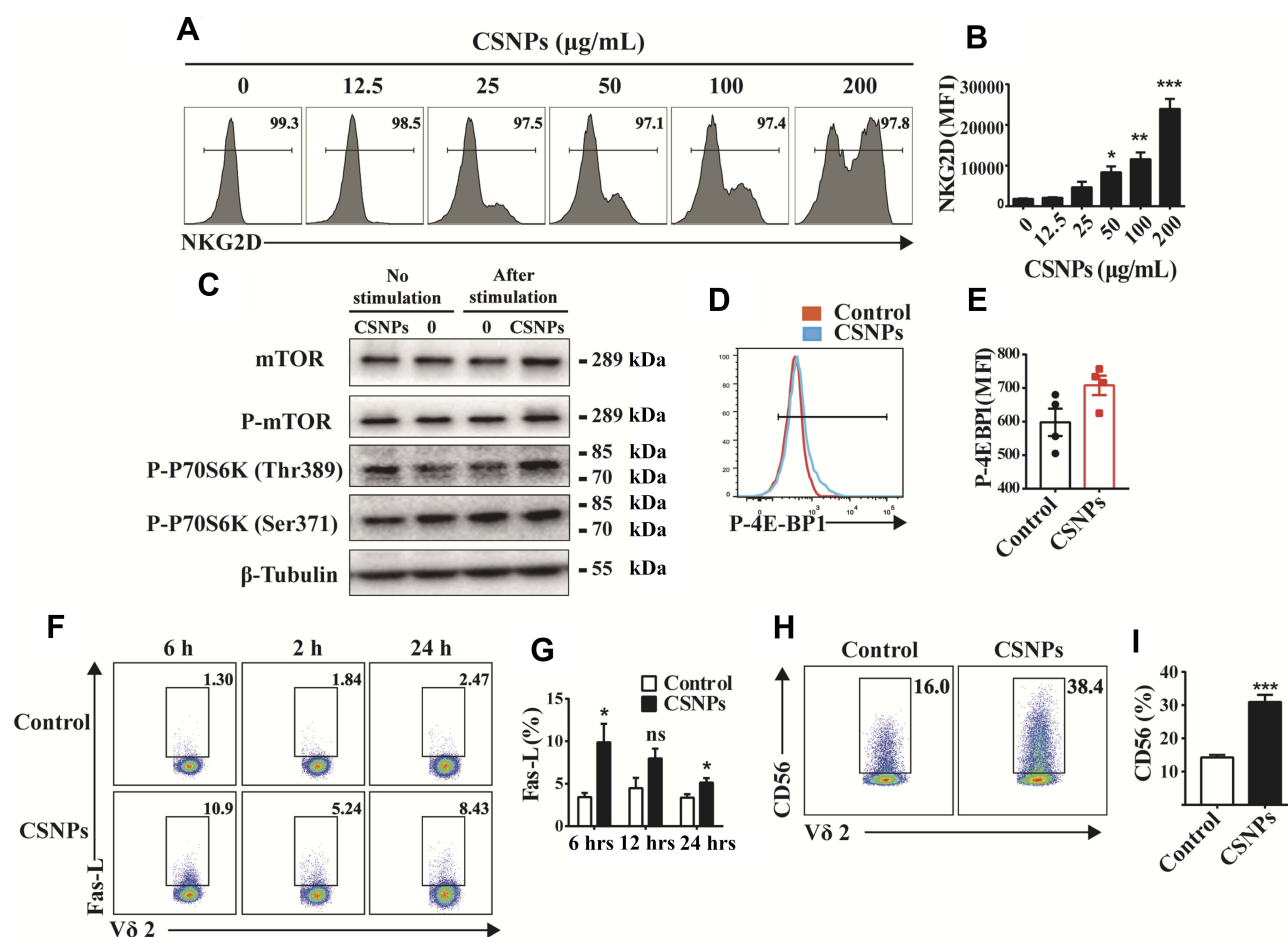
Figure 3 CSNPs promote killing associated receptor expression of Vγ9Vδ2 T cells. (**A**) Fluorescence profiles and mean fluorescence intensity (MFI) (**B**) of NKG2D expression of Vγ9Vδ2 T cells treated with CSNPs for 6 hrs. (**C**) The Western blot of mTOR signal pathway-related protein of Vγ9Vδ2 T cells was treated with CSNPs only or stimulated with anti-CD3, anti-CD28. (**D, E**) Mean fluorescence intensity (MFI) of P-4E-BP1 expression of Vγ9Vδ2 T cells. (**F, G**) Flow cytometry plots and Fas-L expression statistics of Vγ9Vδ2 T cells treated with CSNPs for 6, 12, and 24 hrs. (**H, I**) Flow cytometry plots and CD56 expression of Vγ9Vδ2 T cells stimulated with CSNPs for 6 hrs. Data were collected from at least three independent repetitions and analyzed using one-way ANOVA test or t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$, n=3).

Together, our results demonstrated that CSNPs could neutralize inhibitory effects of free chitosan on anti-tumor activity of Vγ9Vδ2 T cells, and that only nanoforms of chitosan (chitosan nanoparticles) could be potentially developed into drug-delivery carriers; thus, negative immune regulatory effects of the chitosan molecule could not be neglected. Additionally, we also tested the solvent (0.4% acetic acid) of chitosan and found that it had little effect on both apoptosis and PD-1 expression of Vγ9Vδ2 T cells (Supplementary Figure 3A–F).

CSNPs Induced α-Tubulin Polarization And Altered Membrane Surface Nanostructure Of Vγ9Vδ2 T Cells

Since cytoskeleton plays prominent roles in physiological processes of cells, including morphology, activation, proliferation, differentiation, mobility, apoptosis, signal transduction, and other immune response-related functions,[17–20] it was important to evaluate how CSNPs would interact with cytoskeletal structures of Vγ9Vδ2 T cells. We, therefore, investigated α-tubulin and β-actin expression of Vγ9Vδ2 T cells in the presence and absence of chitosan or CSNPs by using confocal microscopy and flow cytometry. As shown in Figure 4A–D, chitosan could slightly reduce β-actin content, and at the same time, very slightly elevate α-tubulin quantity. Similarly, CSNPs pretreatment did not alter either α-tubulin or β-actin quantity. However, it should be noted that CSNPs induced apparent polarization of α-tubulin Vγ9Vδ2 T cells. Since dynamic polarization of the microtubule of T cells was required to form immune synapse,[21,22] our observation suggested that CSNPs could promote the formation of immune synapse of Vγ9Vδ2 T cells and possibly potentiate its killing ability against cancer cells.
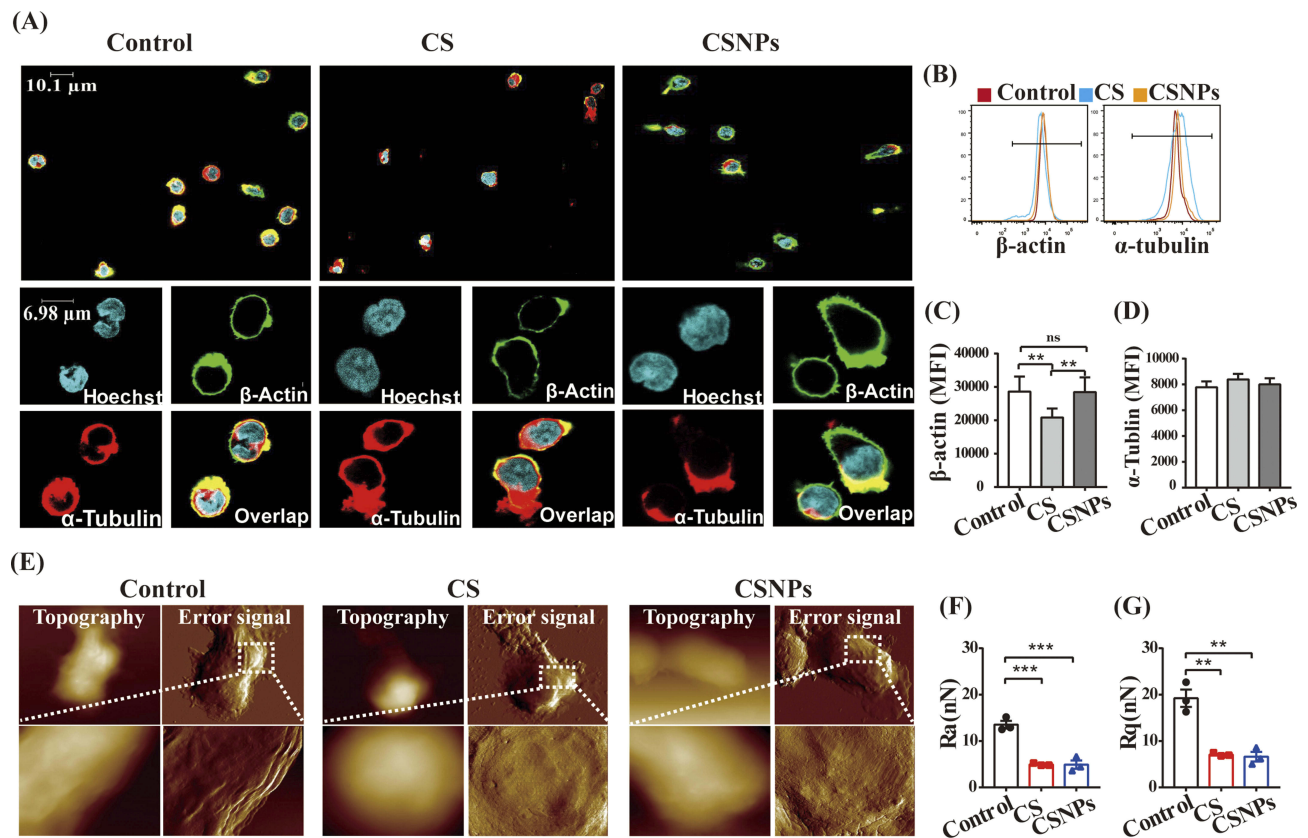
Figure 4 CSNPs induced polarization of α-tubulin and changed membrane surface nanostructure of Vγ9Vδ2 T cells. (**A**) Cytoskeleton (β-actin, α-tubulin) visualizations by confocal laser scanning microscopy. (**B–D**) Quantified analyses of α-tubulin and β-actin expression of Vγ9Vδ2 T cells by flow cytometry, showing as fluorescence intensity profiles (**B**) and histograms of mean fluorescence intensity (MFI) (**C**, **D**). (**E**) The topography and membrane surface nanostructure images of Vγ9Vδ2 T cells acquired by AFM (atomic force microscope). Upper: topography and corresponding error signal mode images, scanning size: 15 or 20 μm. Bottom: membrane surface nanostructure images, scanning size: 4 μm. (**F, G**) Membrane surface roughness (Ra, Rq) at the detection scale of 4 μm. Data were collected from at least three independent experiments. Statistical differences between experimental groups and the control group were analyzed using one-way ANOVA test (**p<0.01, ***p<0.001, n=3).

Then, we further used atomic force microscope (AFM) to observe the morphology and nanostructure of Vγ9Vδ2 T cells. AFM image revealed no apparent changes in cell morphology of Vγ9Vδ2 T cells (Figure 4E). For membrane nanostructure, surface average roughness (Ra) and root mean square roughness (Rq) were measured at 13.5±1.5 nm and 19.2±3.2 nm, respectively. After chitosan treatment, Ra and Rq decreased to 4.9±1.6 nN and 6.6±1.8 nN, respectively; similarly, CSNPs decreased Ra and Rq to 5.0±0.3 nN and 7.1±0.4 nN, respectively (Figure 4F–G). These results indicated that either chitosan or CSNPs could reduce surface roughness of Vγ9Vδ2 T cells, which implicates with smoother membrane surface that might promote cell mobility.

CSNPs Accumulated On The Membrane Of Vγ9Vδ2 T Cells Within 6 H

Finally, in order to determine whether or not CSNPs were up-taken before regulating T cells, the localization of FITC-labeled CSNPs (FITC-CSNPs) on Vγ9Vδ2 T cells were observed after co-culturing for 6 hrs by confocal laser scanning microscopy. As shown in Supplementary Figure 4, the cytomembrane is represented in red (PercP fluorescence for Vδ2); thus, each red circle represents a Vγ9Vδ2 T cell. In addition, nucleus was stained with DAPI in blue, while green fluorescence for FITC-CSNPs was seen around the cell membrane. Altogether, the merge image (scale bars, 13.2 μm) visually demonstrated CSNPs primarily binding on the cell membrane of Vγ9Vδ2 T cells after 6 hrs co-culture; this affected the physiological functions of Vγ9Vδ2 T cells via direct contact with each other. However, we also found that a few CSNPs actually tended to internalize. The results suggested that CSNPs interacted with Vγ9Vδ2 T cells mainly by means of surface binding, and the specific mechanisms needed further investigation.

CSNPs Could Not Affect Proliferation And Differentiation Of Vγ9Vδ2 T Cells

To examine whether or not CSNPs could affect cell proliferation and differentiation, we first detected proliferation marker Ki67 protein expression and found that CSNPs had no effect on Ki67 expression (Figure 5A). Furthermore, we analyzed cell differentiation by detecting expressions of CD45RA and CCR7 using flow cytometry. We found that CSNPs could selectively augment naïve subsets (CD45RA$^+$, CCR7$^+$), but had no effect on the other three: (EM, CM, EMRA) subset cells (Figure 5B and C).

CSNPs Promoted Killing Related Receptor Expression Of Vγ9Vδ2 T Cells

To assess the effects of CSNPs on antitumor activity of Vγ9Vδ2 T cells, we analyzed expressions of related surface receptors with cytotoxicity by using flow cytometry. Since receptor NKG2D of Vγ9Vδ2 T cells is directly involved in recognizing ligand MICA/B or ULBPs of cancer cells, we proceeded to detect how CSNPs regulated NKG2D expression. The results showed that CSNPs could upregulate NKG2D expression in a dose-dependent manner (Figure 3A and B).

Since NKG2D expression is regulated by the mTOR signal pathway,[23] we checked whether or not CSNPs also altered expression of mTOR-related proteins. We found that CSNPs could facilitate mTOR signaling by upregulating P-mTOR, P-P70S6K (Thr389), and P-4E-BP1 expressions (Figure 3C–E). Moreover, we revealed that CSNPs apparently elevated Fas ligand (FasL, CD95L) expression, a receptor expressed on cytotoxic T lymphocytes,[24] and implicated that CSNPs strengthened cytotoxicity of Vγ9Vδ2 T cells against cancer cells (high expression of Fas receptor)

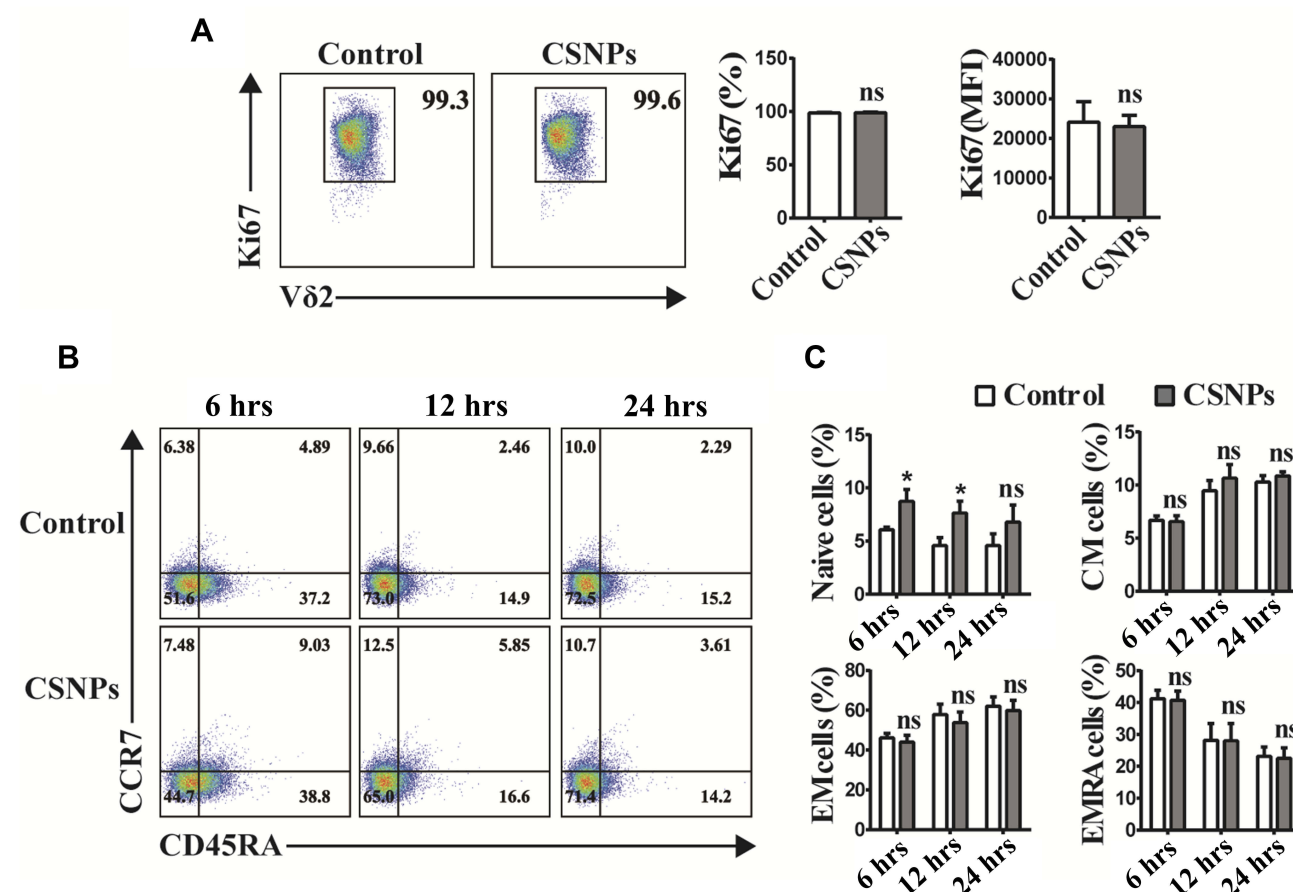**Figure 5** CSNPs had no impact on proliferation and differentiation of Vγ9Vδ2 T cells. Vγ9Vδ2 T cells were treated with 50 μg/mL CSNPs. (**A**) Percentage (%) and mean fluorescence intensity (MFI) of Ki67 (cell proliferation marker) expression of Vγ9Vδ2 T cells after CSNPs treatment. (**B**) Cell differentiation phenotype of Vγ9Vδ2 T cells after CSNPs treatment. Four cell subsets could be analyzed by gating CD45RA and CCR7 expression: γδ Tnaïve (CD45RA+, CCR7+), γδ TCM (CD45RA-, CCR7+), γδ TEM (CD45RA-, CCR7-), γδ TEMRA (CD45RA+, CCR7-). (**C**) Statistical analysis of cell differentiation phenotype, showing that CSNPs only enhanced the percentage (%) of naïve Vγ9Vδ2 T cells. Data were collected from at least three independent repetitions. *p<0.05, n=3.
Abbreviation: ns, no significant difference.

([Figure 3F and G). Furthermore, we detected CD56 expression of Vγ9Vδ2 T cells before and after CSNPs treatment. Results indicated that CSNPs dramatically upregulated CD56 expression by 2 folds, which implicated with enhanced cell adhesion, synaptic plasticity, and cytotoxicity (Figure 3H and I).[4]

Additionally, we also analyzed expressions of CD16 and CD86 of Vγ9Vδ2 T cells. Specifically, CD16 was involved in antibody-dependent-cell-mediated cytotoxicity (ADCC) immune response;[4] whereas, CD86 played a role in antigen-presenting cell (APC) functions as a co-stimulatory molecule.[25,26] However, our results demonstrated that expressions of both CD16 and CD86 were not significantly upregulated by CSNPs, as shown in Supplementary Figure 5A–C.

Therefore, these results suggested that CSNPs promoted Vγ9Vδ2 T cell cytotoxicity by upregulating NKG2D, FasL, and CD56, rather than CD16 or CD86. Particularly, CSNPs could activate mTOR signaling and then promote antitumor immune responses of Vγ9Vδ2 T cells by upregulating NKG2D.

CSNPs Specifically Promoted Perforin Secretion Of Vγ9Vδ2 T Cells

Since cytokines are the critical soluble factors that exert anti-tumor immunity, we proceeded to evaluate how CSNPs could affect cytokine production of Vγ9Vδ2 T cells. After assaying different types of cytokines, including perforin, IFN-γ, TNF-α, granzyme B, and CD107a (a protein found in the membrane of perforin-containing cytolytic granules),[27] we were surprised to find that only perforin expression was upregulated (Figure 6A and B) and all other assayed cytokines were not affected (Figure 6A and C–F). It is known that perforin can generate pores on target cell membranes, which facilitates cytotoxicity T cells to deliver cytotoxic cytokines like granzyme B to target cells via immune synapse. Therefore, perforin plays very important roles in anti-tumor immunity of cytotoxicity T cells. Our results indicated that CSNPs could specifically enhance perforin secretion, which was evidenced by the role of CSNPs in enhancing Vγ9Vδ2 T cell cytotoxicity.
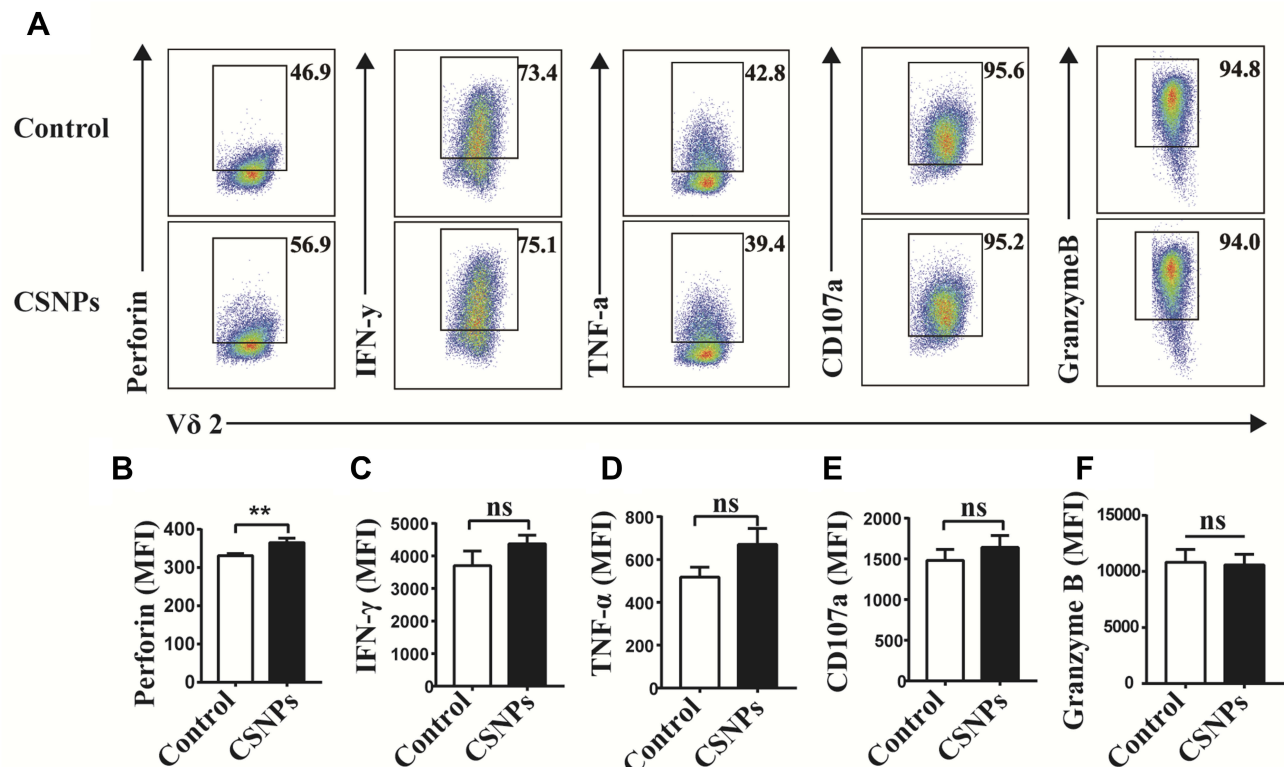

Figure 6 CSNPs specifically promoted perforin secretion of Vγ9Vδ2 T cells. (**A–F**) Flow cytometry plots and perforin, IFN-γ, TNF-α, CD107a, and granzyme B expression statistics of Vγ9Vδ2 T cells treated with CSNPs for 6 hrs. Data were collected from at least three independent repetitions and analyzed using T-test (*p<0.05, **p<0.01, ***p<0.001, n=3).

CSNPs Strengthened Vγ9Vδ2 T Cell Killing Ability Of Cancer Cells

After having determined that CSNPs could upregulate cytotoxicity-related molecule expression of Vγ9Vδ2 T cells, such as NKG2D, FasL, CD56, and perforin, it was important to verify whether or not CSNPs could promote the killing ability of γδ T cells against cancer cells. Therefore, we performed in vitro killing assay using CFSE-labeled chronic myeloid leukemia cell line K562 as model cancer cells. After co-culturing leukemia cells (Target) and Vγ9Vδ2 T cells (Effector) for 6 hrs (E:T ratio: 5:1, 10:1), the percentage of dead target cells was detected by flow cytometry. As expected, CSNPs pretreated Vγ9Vδ2 T cells exhibited higher cytotoxicity against K562 than untreated Vγ9Vδ2 T cells (control) at the E:T ratio of 5:1; however, there was no difference at the E:T ratio of 10:1 (Figure 7A and B). Additionally, interaction between tumor cells and Vγ9Vδ2 T cells was visualized using confocal microscopy, and representative images are shown in Figure 7C. Together, our in vitro killing experiments demonstrated that CSNPs could indeed strengthen Vγ9Vδ2 T cell cytotoxicity against cancer cells.

Discussion

Today, chitosan is a type of biocompatible biomaterial that has been developed into attractive drug-delivery carriers.[28] Particularly, chitosan nanoparticle is a promising nanocarrier for anticancer drug encapsulation and delivery,[8,28] and

the nanoparticle itself could also be directly applied in anti-tumor-related development. Previously, Flávia Castro reported that chitosan (CS)/poly (c-glutamic acid) (c-PGA) nanoparticles could affect polarization of macrophages and, thus reverse their promotion of cancer cell invasion.[12] In this work, since Vγ9Vδ2 T cells play crucial roles in anti-tumor immunity, we focused on detecting how CSNPs could affect immune responses of Vγ9Vδ2 T cells.

Discussion of our research mainly centered on immune modulation of CSNPs on Vγ9Vδ2 T cells from activation, proliferation, and differentiation to the effector function. First, we found the CSNPs had promising immune-compatibility and only a slight effect on PD-1 expression when the concentration reached up to 50 μg/mL, after which CSNPs could promote the activation of Vγ9Vδ2 T cells by upregulating the expression of NKG2D and CD56; this contributed to potentiate anti-tumor ability of Vγ9Vδ2 T cells.[4,29] More importantly, we further demonstrated that CSNPs could regulate NKG2D expression of Vγ9Vδ2 T cells through promotion of mTOR signaling pathway; this was due to our discovery that mTOR signaling related proteins were upregulated, which included P-mTOR, P-P70S6K (Thr389), and P-4E-BP1.

In addition, Fas/FasL apoptosis signaling has been considered an important mechanism for Vγ9Vδ2 T cell recognizing and then lysing cancer cells.[24,30] Having observed an upregulation of FasL molecule expression in our results clearly suggested that CSNPs treatment could
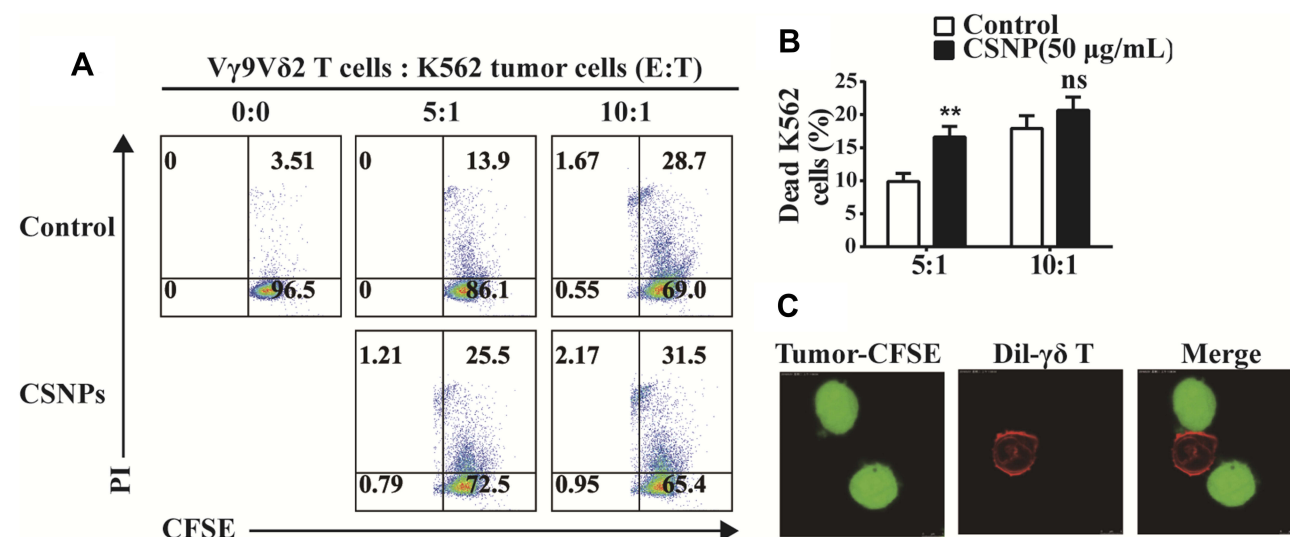

Figure 7 CSNPs strengthened tumor-killing ability of Vγ9Vδ2 T cells in vitro. (**A**) Killing assay of Vγ9Vδ2 T cells against K562 tumor cells using flow cytometry. (**B**) Statistical results based on at least three independent repetitions. (**C**) Representative confocal images of Vγ9Vδ2 T cells (labeled with fluorescence dye Dil) interacting with CFSE-labeled tumor cells. **p<0.01, n=3.
Abbreviation: ns, no significant difference.

enhance Vγ9Vδ2 T cell anti-tumor cytotoxicity. In addition, co-stimulatory molecule CD86 had participated in antigen recognition and antigen-presenting functions;[4] therefore, we observed that CSNPs did not alter CD86 expression, implying CSNPs had no effect on the antigen-presenting functions of Vγ9Vδ2 T cells.

In consideration of the above-noted differentiation, our research team also examined whether CSNPs could specifically impact γδ T subsets. Surprisingly, it showed that the naïve Vγ9Vδ2 T cell population (CD45RA$^+$, CCR7$^+$), which readily proliferated in response to phosphor-antigen stimulation, instead of T$_{EM}$ (CD45RA$^-$, CCR7$^-$) or T$_{EMRA}$ (CD45RA$^+$, CCR7$^-$), increased significantly. Functionally, CSNPs pretreatment could greatly enhance Vγ9Vδ2 T cell killing of K562 cells (Chronic myeloid leukemia cell lines), which was partially attributed to higher perforin secretions induced by CSNPs.

Conversely, the morphological and biophysical alterations of cells are becoming indications of antigen recognition, synapse formation, activation, differentiation, and effector functions.[21] In particular, as morphology and biophysical property regulators, cytoskeleton actin and tubulin have been studied intensively in biological processes, including mitosis, immune synapse formation, cellular polarization, and signaling.[17,31] In our research, we found that chitosan molecules slightly reduced β-actin accumulation, while CSNPs did not influence the quantity of α-tubulin and β-actin. Additionally, it has been reported that rapid dynamic polarization of the microtubule cytoskeleton in T cells occurred during recognition and killing, resulting in microtubule-organizing centre (MTOC) reorientation towards the interfacing of cell–cell interactions.[21,22] As expected, α-tubulin polarization was observed in Vγ9Vδ2 T cells after CSNPs challenge. Such results suggested CSNPs stimulation could induce α-tubulin cytoskeleton rearrangement and thus promote synapse formation of Vγ9Vδ2 T cells correlating with higher cytotoxicity. Furthermore, CSNPs induced smoother membrane surfaces of Vγ9Vδ2 T cells, which might imply a higher cell mobility of Vγ9Vδ2 T cells.

In conclusion, our results demonstrated that CSNPs were capable of functionally regulating Vγ9Vδ2 T cells, inducing activation, and enhancing anti-tumor cytotoxicity. Also, we demonstrated the great potentiality of CSNPs as a type of promising bio-carrier from an immune aspect, evidenced by the fact that CSNPs could strengthen antitumor responses of Vγ9Vδ2 T cells through upregulation of NKG2D, FasL, CD56, perforin secretion, and α-tubulin polarization. Therefore, we believe that chitosan nanoparticles could be developed into promising bio-delivery carriers for anti-tumor drugs, not only because of their biodegradability and biocompatibility, but also because of their capability of immunomodulation on immune cells.

Ethics Statement

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

References

1. Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol*. 2010;10(7):467–478. doi:10.1038/nri2781
2. Gao Y, Yang W, Pan M, et al. Gamma delta T cells provide an early source of interferon gamma in tumor immunity. *J Exp Med*. 2003;198 (3):433–442. doi:10.1084/jem.20030584
3. Gentles AJ, Newman AM, Liu CL, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med*. 2015;21(8):938–945. doi:10.1038/nm.3909
4. Wu D, Wu P, Qiu F, Wei Q, Huang J. Human gammadelta T-cell subsets and their involvement in tumor immunity. *Cell Mol Immunol*. 2017;14(3):245–253. doi:10.1038/cmi.2016.55
5. Alnaggar M, Xu Y, Li J, et al. Allogenic Vgamma9Vdelta2 T cell as new potential immunotherapy drug for solid tumor: a case study for cholangiocarcinoma. *J Immunother Cancer*. 2019;7(1):36. doi:10.118 6/s40425-019-0501-8
6. Poggi A, Zocchi MR. Gammadelta T lymphocytes as a first line of immune defense: old and new ways of antigen recognition and implications for cancer immunotherapy. *Front Immunol*. 2014;5:575. doi:10.3389/fimmu.2014.00575
7. Anitha A, Sowmya S, Kumar PTS, et al. Chitin and chitosan in selected biomedical applications. *Prog Polym Sci*. 2014;39(9):1644–1667. doi:10.1016/j.progpolymsci.2014.02.008
8. de Oliveira Pedro R, Hoffmann S, Pereira S, Goycoolea FM, Schmitt CC, Neumann MG. Self-assembled amphiphilic chitosan nanoparticles for quercetin delivery to breast cancer cells. *Eur J Pharm Biopharm*. 2018;131:203–210. doi:10.1016/j.ejpb.2018.08.009
9. Kean T, Thanou M. Biodegradation, biodistribution and toxicity of chitosan. *Adv Drug Deliv Rev*. 2010;62(1):3–11. doi:10.1016/j.addr. 2009.09.004
10. Shi GN, Zhang CN, Xu R, et al. Enhanced antitumor immunity by targeting dendritic cells with tumor cell lysate-loaded chitosan nanoparticles vaccine. *Biomaterials*. 2017;113:191–202. doi:10.1016/j. biomaterials.2016.10.047

11. Carroll EC, Jin L, Mori A, et al. The vaccine adjuvant chitosan promotes cellular immunity via DNA sensor cGAS-STING-dependent induction of Type I interferons. *Immunity*. 2016;44(3):597–608. doi:10.1016/j.immuni.2016.02.004

12. Castro F, Pinto ML, Silva AM, et al. Pro-inflammatory chitosan/poly (gamma-glutamic acid) nanoparticles modulate human antigen-presenting cells phenotype and revert their pro-invasive capacity. *Acta Biomater*. 2017;63:96–109. doi:10.1016/j.actbio.2017.09.016

13. Wu Y, Sims RC, Zhou A. AFM resolves effects of ethambutol on nanomechanics and nanostructures of single dividing mycobacteria in real-time. *Phys Chem Chem Phy*. 2014;16:19156–19164. doi:10.1039/C4CP01317D

14. Wu YZ, McEwen GD, Harihar S, Baker SM, DeWald DB, Zhou AH. BRMS1 expression alters the ultrastructural, biomechanical and biochemical properties of MDA-MB-435 human breast carcinoma cells: an AFM and Raman microspectroscopy study. *Cancer Lett*. 2010;293 (1):82–91. doi:10.1016/j.canlet.2009.12.016

15. McEwen GD, Wu Y, Tang M, et al. Subcellular spectroscopic markers, topography and nanomechanics of human lung cancer and breast cancer cells examined by combined confocal Raman microspectroscopy and atomic force microscopy. *Analyst*. 2013;138:787–797. doi:10.1039/C2AN36359C

16. Meraviglia S, Lo Presti E, Dieli F, Stassi G. Gammadelta T cell-based anticancer immunotherapy: progress and possibilities. *Immunotherapy*. 2015;7(9):949–951. doi:10.2217/imt.15.68

17. Huang X, Guo H, Wang C, et al. Detection of CD28/CD86 co-stimulatory molecules and surface properties of T and dendritic cells: an AFM study. *Scanning*. 2016;38(4):365–375. doi:10.1002/sca.v38.4

18. Hu M, Wang J, Zhao H, Dong S, Cai J. Nanostructure and nanomechanics analysis of lymphocyte using AFM: from resting, activated to apoptosis. *J Biomech*. 2009;42(10):1513–1519. doi:10.1016/j.jbiomech.2009.03.051

19. Wang J, Wan Z, Liu W, et al. Atomic force microscope study of tumor cell membranes following treatment with anti-cancer drugs. *Biosens Bioelectron*. 2009;25(4):721–727. doi:10.1016/j.bios.2009.08.011

20. Cui YH, Hu ZX, Gao ZX, et al. Airborne particulate matter impairs corneal epithelial cells migration via disturbing FAK/RhoA signaling pathway and cytoskeleton organization. *Nanotoxicology*. 2018;12 (4):312–324. doi:10.1080/17435390.2018.1440651

21. Billadeau DD, Nolz JC, Gomez TS. Regulation of T-cell activation by the cytoskeleton. *Nat Rev Immunol*. 2007;7(2):131–143. doi:10.1038/nri2021

22. Kuhn JR, Poenie M. Dynamic polarization of the microtubule cytoskeleton during CTL-mediated killing. *Immunity*. 2002;16(1):111–121. doi:10.1016/S1074-7613(02)00262-5

23. Cao G, Wang Q, Li G, et al. mTOR inhibition potentiates cytotoxicity of Vgamma4 gammadelta T cells via up-regulating NKG2D and TNF-alpha. *J Leukoc Biol*. 2016;100(5):1181–1189. doi:10.1189/jlb.5A0116-053RR

24. Zeng D, Lin J, He H, et al. Therapeutic effect of targeted Fas-expressing adenoviruses method combining gamma delta T cells in a mouse model of human ovarian carcinoma. *Oncol Lett*. 2018;15 (2):2555–2561. doi:10.3892/ol.2017.7599

25. Brandes M, Willimann K, Bioley G, et al. Cross-presenting human T cells induce robust CD8+ T cell responses. *Proc Natl Acad Sci*. 2009;106(7):2307–2312. doi:10.1073/pnas.0810059106

26. Brandes M, Willimann K, Moser B. Professional antigen-presentation function by human gamma delta T Cells. *Science*. 2005;309 (5732):264–268. doi:10.1126/science.1110267

27. Hines MR, Nichols KE. Go with the flow: perforin and CD107a in HLH. *Blood*. 2017;129(22):2954–2955. doi:10.1182/blood-2017-04-773192

28. Sanyakamdhorn S, Agudelo D, Tajmir-Riahi HA. Encapsulation of antitumor drug Doxorubicin and its analogue by chitosan nanoparticles. *Biomacromolecules*. 2013;14(2):557–563. doi:10.1021/bm3018577

29. Alexander AAZ, Maniar A, Cummings JS, et al. Isopentenyl pyrophosphate-activated CD56+ T lymphocytes display potent antitumor activity toward human squamous cell carcinoma. *Clin Cancer Res*. 2008;14(13):4232–4240. doi:10.1158/1078-0432.CCR-07-4912

30. Li Z, Xu Q, Peng H, Cheng R, Sun Z, Ye Z. IFN-gamma enhances HOS and U2OS cell lines susceptibility to gammadelta T cell-mediated killing through the Fas/Fas ligand pathway. *Int Immunopharmacol*. 2011;11 (4):496–503. doi:10.1016/j.intimp.2011.01.001

31. Lafouresse F, Vasconcelos Z, Cotta-de-Almeida V, Dupre L. Actin cytoskeleton control of the comings and goings of T lymphocytes. *Tissue Antigens*. 2013;82(5):301–311. doi:10.1111/tan.2013.82.issue-5