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Harnessing Natural Killer Cell-Derived Exosomes for Cancer Therapy

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Abstract. NK cells (natural killer cells) represent a critical component of innate immune effectors, capable of directly eliminating malignant cells without requiring prior sensitization. Extracellular vesicles (EVs), particularly exosomes, have emerged as essential mediators of intercellular signaling and have also gained considerable attention as cell-free therapeutic agents in immunotherapy. Nevertheless, the mechanisms underlying the anti-tumor efficacy of NK cell-derived exosomes in the background of lung cancer remain insufficiently characterized. First of all, NK-92 cells were cultured and then their exosomes were collected from culture supernatants through successive ultracentrifugation. As for size distribution, it was found that most particles had an average diameter of around 104.76 nanometers, while the zeta potential was about minus twenty-three millivolts. By means of western blotting analysis, it was shown that some NK-specific molecules, such as CD56 and NKG2D, were present in the NK-Exo group. Some functional tests showed that labeled exosomes attached to A549 cells very well, and part of them eventually entered A549 cells. What is more, treatment with the NK-92-derived exosomes reduced A549 cell viability quite efficiently and induced tumor-cell death in vitro compared with control groups. Therefore, it is reasonable to conclude that NK-cell-derived exosomes may develop into a cell-free therapy platform which would have great clinical application value in future lung cancer treatment.

Keywords: exosome, natural killer cells, cancer immunotherapy, lung cancer, cytotoxicity

1. Introduction

The landscape of modern oncology has been fundamentally transformed by cancer immunotherapy. This strategy operates through the activation of the host's innate immune defenses, enabling the recognition and destruction of malignant cells [1]. Currently, a range of therapeutic strategies have been developed, including checkpoint-based immunotherapy, tumor vaccination, and cell-based adoptive transfer strategies [2]. Within the immune effector cell population, NK cells constitute a pivotal element of innate immune defense. This is because that their ability to mount rapid responses against aberrant or pathogen-infected cells without the need for prior activation [3]. Consequently, NK cells serve as a critical mediator in tumor monitoring [4,5]. Their anti-tumor effects are mainly achieved through cytokine release, particularly interferon gamma, and direct killing via FAS ligand, TNF-related apoptosis-inducing ligand, perforin, and granzyme [6-8].

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Extracellular vesicles (EVs) are membrane-bound nanoparticles encapsulated by a lipid bilayer structure, which are actively secreted by cells into the surrounding extracellular space [9,10]. Exosomes and microvesicles, two pivotal subclasses of EVs, have been extensively implicated in mediating intercellular communication [11,12]. A defining characteristic of EVs lies in their capacity to transport biologically active molecules — encompassing proteins, nucleic acids, and lipids — into target cells, consequently influencing their physiological behavior and functional properties [10,13-16]. Therefore, EVs have captured more and more attention because of these characteristics as a cell-free therapeutic method, as well as for their use as nature's own drug delivery system, a predictive and diagnostic biomarker, and a component of translational biomedical strategies [17].

Immune-cell-derived EVs play a regulatory role across innate as well as adaptive immune regulation, including anti-tumor responses [10,13,18-20]. Tumor peptide-pulsed dendritic cells release EVs that can stimulate T-cell responses and inhibit tumor growth [21]. Likewise, CD8+ T-cell-derived EVs suppress tumor progression by targeting tumor-associated stromal or lesional cells and by transferring regulatory cargo [22-24]. CD4+ T cells also secrete distinct EV populations with immunomodulatory functions relevant to anti-tumor immunity [25,26]. In contrast, NK-cell-derived EVs, although reported to possess cytotoxic properties, remain less well characterized. In the present study, exosomes were isolated from the human NK-cell line NK-92 and their interaction with A549 lung cancer cells was evaluated. The results showed that NK-92-derived exosomes retained NK-cell-associated markers and induced tumor-cell death, supporting their potential as cell-free anti-cancer effectors.

2. Results and discussion

2.1. Isolation and characterization of NK-92-derived exosomes

Natural killer (NK) cells have been well-established as pivotal participants in natural defense against cancer, though it is uncertain whether some of their functions can be fulfilled via the secretion of exosomes. Therefore, this possibility was examined. First, NK-92 cells were cultivated and analyzed before exosome extraction. Fluorescence microscopy of DAPI- and AF647-stained NK-92 cells illustrated the typical cellular morphology, as shown in Figure 1a. Subsequently, flow cytometry showed a high level of CD56, which is one type of NK cell marker, as shown in Figure 1b. Moreover, besides confirming the presence of NKG2D, whose function is to recognize certain kinds of stress-induced ligands presented on targets in an antigen-independent way [27,28], PD-1 was also examined. Since PD-1 has been reported to affect NK cell activity [29], its effect was also evaluated here, as shown in Figure 1c. Then, exosomes were purified from NK-92 conditioned medium using sequential differential centrifugation. According to the Zetasizer results, the ζ potential was nearly equal to minus twenty-three mV, as shown in Figure 1d, while dynamic light scattering measured the average size to be about 104.76 nm, as shown in Figure 1e. Finally, western blotting experiments showed that some proteins could still be detected in both NK-92 cell lysate and the exosome-containing fraction, including CD56 and NKG2D. To avoid false-positive results caused by non-specific binding between primary antibodies and protein samples, β-actin served as a loading control in the WB experiments, as shown in Figure 1f. Therefore, these findings suggested that NK-92-derived exosomes carrying specific NK-cell markers were successfully obtained.

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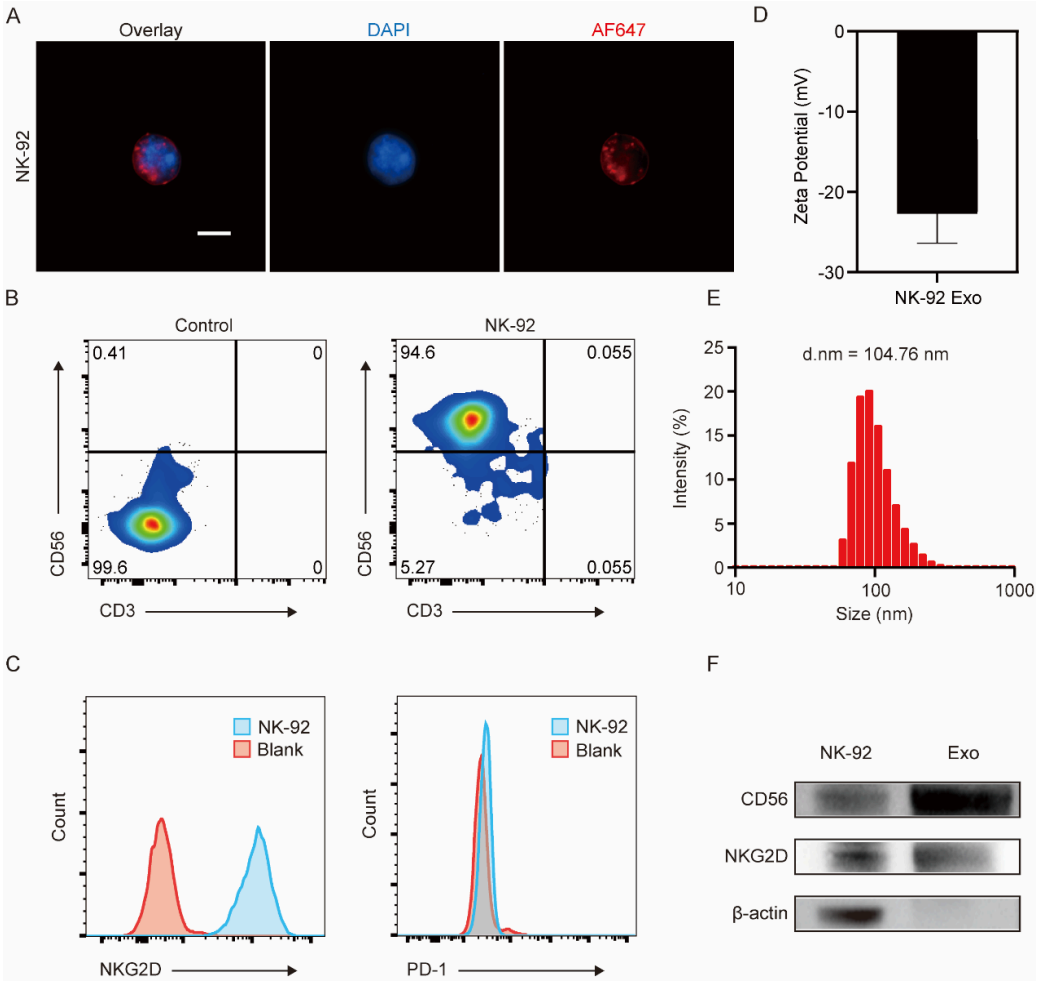

Figure 1. Characterization of NK-92 cells and their derived exosomes: a, fluorescence microscopy images of NK-92 cells stained with DAPI and AF647, scale bar, 10 μm; b and c, flow-cytometric analysis of NK-92 cells; d, zeta-potential distribution of NK-92-derived exosomes; e, size distribution of NK-92-derived exosomes measured by dynamic light scattering; f, western blot analysis of NK-92 cells and NK-92-derived exosomes

2.2. Tumor-targeting and cytotoxic activity of NK-92-derived exosomes against A549 cells

Aiming at evaluating the tumor-targeting capacity of NK-cell-derived exosomes, A549 cells were selected to serve as a human lung cancer model in this study. This choice was justified by the fact that lung cancer remains the foremost cause of cancer-related mortality on a global scale [30]. Given this, NK-92-derived exosomes were labeled with AF488 and then incubated with A549 cells for 10 min before confocal imaging, as shown in Figure 2a. These labeled exosomes could bind very well to the surfaces of A549 cells, and some of them also went into the cytoplasm, according to the AF488 fluorescence signals. All the evidence above may show that there is a certain specific interaction between these two types of cells.

Afterwards, NK-92-derived exosomes were examined for their cytotoxic effects on A549 cells. According to the CCK-8 results, cell activity obviously decreased after exosome treatment, as shown in Figure 2b. Meanwhile, calcein-AM and propidium iodide staining showed more tumor cell death after exposure to NK-92-derived exosomes. In this case, higher exosome concentrations had a more significant effect, as shown in Figure 2c. Based on these outcomes, it can be concluded that

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NK-cell-derived exosomes inhibit A549 cell proliferation. Therefore, they may be applied as a cell-free immunotherapy approach.

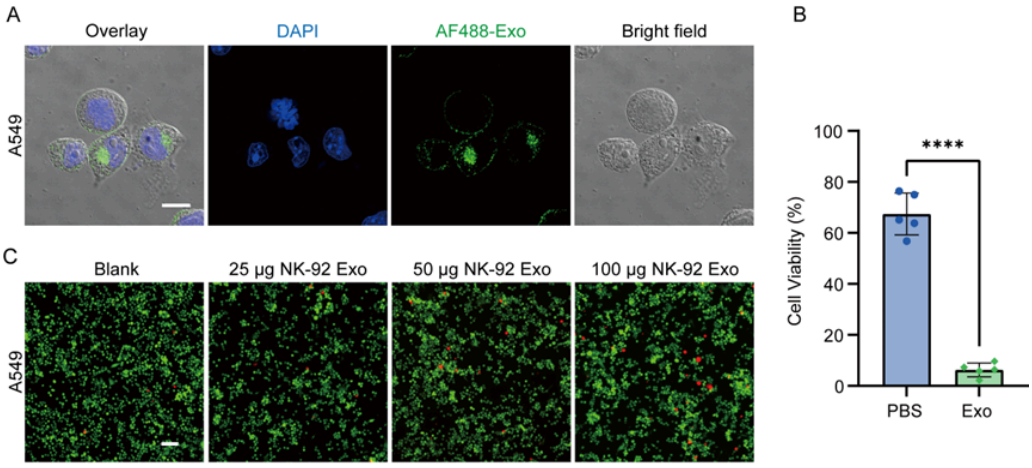

Figure 2. Anti-tumor effects of NK-92-derived exosomes in A549 cells: a, confocal images of A549 cells after incubation with AF488-labeled NK-92-derived exosomes and DAPI nuclear staining, scale bar, 10 μm; b, CCK-8 assay of A549 cell viability after treatment with NK-92-derived exosomes; c, fluorescence microscopy images of A549 cells stained with Calcein-AM and PI after treatment with NK-92-derived exosomes, scale bar, 10 μm

3. Materials and methods

3.1. Materials

β-actin, NKG2D, and CD56 antibodies were sourced from ABMART, Thermo Fisher Scientific, and Abcam, respectively.

3.2. Cell culture

Both the human NK-cell line NK-92 and the lung cancer cell line A549, originally obtained from the National Collection of Authenticated Cell Cultures, were employed in the present study. Both cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 1% penicillin-streptomycin, and 100 IU/mL of recombinant human interleukin-2 (IL-2).

3.3. Characterization of NK-92 cells

Aiming at characterizing NK-92 cells via immunofluorescence, cells were seeded onto confocal dishes. Then, they were maintained at 37°C for as long as 3 hours. Following this incubation, DAPI (Beyotime) and AF647 were applied for a 10-minute staining period. Fluorescence imaging was then performed using a Zeiss confocal laser-scanning microscope

3.4. Isolation of NK-cell-derived extracellular vesicles

The culture supernatants of NK-92 cells served as the source from which extracellular vesicles were isolated. Initially, serum-derived vesicle-free FBS was obtained by ultracentrifugation of serum overnight at 100,000 × g to exclude serum-derived vesicles; then, NK-92 cells were cultured in standard medium as previously outlined. Once approximately 60-70% confluence was reached, the

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cells were harvested by centrifugation at 300 × g and rinsed with PBS. They were subsequently transferred into exosome-depleted medium, where they remained for 72 hours. The conditioned medium was subsequently collected and subjected to differential centrifugation at 4 °C: the supernatant was centrifuged at 800 × g for 10 minutes and then at 2,000 × g for 30 minutes to eliminate cells and cellular debris. An additional centrifugation step at 10,000 × g for 30 minutes was then applied to pellet larger vesicles. Finally, the exosome-containing fraction was obtained by ultracentrifugation at 100,000 × g for a minimum of 1 hour. The resulting pellets were then washed with PBS, resuspended in PBS, and kept at −80°C prior to further use.

3.5. Western blotting

To obtain complete protein extracts, cells and NK-92-derived vesicles were lysed using RIPA buffer (Beyotime). The resulting lysates were combined with loading buffer (Beyotime), heat-treated, and resolved by SDS-PAGE on 10% or 15% gels, after which proteins were transferred onto a PVDF membrane. Blocking was carried out in 5% skimmed milk powder dissolved in TBST at room temperature for a 2-hour blocking period. Primary antibodies against NKG2D, CD56, and β-actin were diluted at a ratio of 1:1000. The membrane was subsequently left to incubate overnight at 4°C with the prepared antibody solution. After thorough rinsing with TBST, HRP-linked secondary antibodies (anti-mouse IgG and anti-rabbit IgG at a 1:5000 dilution in TBST) were introduced and left to react for 1 hour at room temperature. The resulting protein bands were then visualized by means of an enhanced chemiluminescence-based substrate.

3.6. Flow cytometry

Antibodies directed against NKG2D, CD56, PD-1, and CD3 were used to immunolabel NK-92 cells. Flow cytometric acquisition was conducted using a CytoFLEX LX apparatus from Beckman, USA, and subsequent data analysis was carried out using FlowJo software.

3.7. Characterization of exosomes

The size distribution of NK-92-derived exosomes was determined through dynamic light scattering (DLS) analysis. Both zeta potential and particle size measurements were conducted using a Zetasizer Nano ZS90 instrument (Malvern Panalytical, UK).

3.8. In vitro binding assay

DAPI and AF488 fluorescent dyes were used to label A549 cells and NK-92-derived exosomes, respectively. A549 cells were first seeded onto confocal dishes and maintained at 37°C for 3 hours, after which the fluorescently labeled exosomes were introduced for a 10-minute co-incubation period. The samples were then visualized under a Zeiss confocal laser-scanning microscope.

3.9. Cytotoxicity assay

The cytotoxic impact of NK-92-derived exosomes on A549 cells was assessed utilizing the Cell Counting Kit-8 test (CCK-8) from Selleck. A549 cells were inoculated onto 96-well plates and incubated overnight at 37 °C. On the subsequent day, cells were administered 50 μg per mL of NK-92-derived exosomes for a duration of 24 hours. The culture media was subsequently substituted with fresh medium including 10% by volume CCK-8 solution. Following a 2-hour incubation

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period, absorbance at 450 nm was measured through a microplate reader, FLX800, USA. Cells treated with PBS functioned as controls. All exosome concentrations utilized in this investigation were quantified based on total protein content. For fluorescence imaging of cellular apoptosis, A549 cells subjected to different doses of NK-92-derived exosomes were stained with Calcein-AM and propidium iodide (PI) from Beyotime and subsequently observed using a Zeiss fluorescence microscope.

3.10. Statistical analysis

All values are expressed in the form of mean ± s.e.m. except when explicitly specified. When comparing two groups, a two-tailed Student's t-test served as the statistical tool for comparison. In the same time, multi-group comparisons were assessed through one-way ANOVA incorporating Tukey's post hoc test. Statistical significance was established at a p-value of less than 0.05. GraphPad Prism (GraphPad Software, USA) served as the software platform for all statistical analyses.

4. Conclusion

To sum up, NK-92-derived exosomes were isolated and shown to have an average size of 104.76 nm and a zeta potential of about minus twenty-three mV. These exosomes kept NK-cell-related markers and adhered to A549 lung tumor cells. They also decreased tumor-cell viability in vitro. Therefore, according to these results, NK-cell-derived exosomes may represent a novel cell-free immunotherapeutic platform for the treatment of lung cancer. However, future work still needs to clarify what molecular cargo they carry, how they work, and what their therapeutic value will be in vivo.

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