Extracellular Vesicles Derived from Cytokine-Activated CD8⁺ T Cell Exhibit Broad Anticancer Activity

Lin Zhang

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Yang An

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Xuena Yang

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Wenwen Yu

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Baihui Li

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Zhenyu Ji

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Shuai Meng

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Quanyong Yang

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Limei Wang

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Feng Wei

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Lili Yang

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

xiubao ren (✉ renxiubao@tjmuch.com)

Tianjin Cancer Institute & Hospital

Research

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Abstract

Background: Extracellular vesicles (EVs) are the nano-sized membrane particles secreted by various cell types, which are involved in many important cellular processes. Recently, EVs originating from immune cells, such as dendritic cells, chimeric antigen receptor T cells (CAR-T) and natural killer cells, have attracted much attention because of their known direct and indirect antitumor activity. Here, we reported another EV released by cytokine-activated CD8⁺ T cells (caCD8-EVs) and its phenotype and function.

Methods: CaCD8-EVs were purified by ultracentrifugation and phenotypically characterized by flow cytometry. The cytotoxicity induced by caCD8-EVs was assessed using the CellTiter-Glo Viability assay *in vitro* and xenograft tumor established *in vivo*. The uptake of caCD8-EVs was detected by a Live Cell Imaging System and flow cytometry. Mass spectrometry and protein bioinformatics analysis, together with western blotting and real-time PCR, were used to investigate the effect of caCD8-EVs on the cancer cells.

Results: CaCD8 cells released EVs following stimulation of CD8⁺ T cells with an anti-CD3 antibody and a cytokines cocktail *ex vivo*. Phenotype analysis showed that caCD8-EVs carried cytotoxic T cell membrane molecules (CD3, CD8 and TCRs) and partially inherited NKG2D molecule which was inductively expressed on the caCD8 T cells. In contrast, the nano-particles were devoid of checkpoint proteins on their surface. Notably, caCD8-EVs displayed extensive cytotoxicity against cancer cells, but limited effects on non-cancerous cell lines. Uptake assessment showed that actively dividing tumor cells were more likely to capture caCD8-EVs compared with resting cells. Mechanism analysis demonstrated for the first time that caCD8-EVs contained not only typical cytotoxic proteins (i.e., granzyme B and perforin) but also IFN γ that participated in EVs-induced cytotoxicity.

Conclusion: Our data reveal the characteristics of caCD8-derived EVs and support the use of these EVs as a novel potential approach for cancer therapy.

Background

Extracellular vesicles (EVs) are the bioactive nanoparticles with lipid bilayer membrane secreted by various cell types, including both normal and tumor cells [1]. A growing number of studies have shown that EVs can deliver proteins, lipids and nucleic acids into the extracellular environment or into biological fluids to mediate intercellular communication[2-4]. Recently, EVs released from immune cells, such as dendritic cells (DCs), T cells and natural killer (NK) cells, have been widely considered as antitumor nanoparticles[5-7]. These immuno-EVs inherit the essential cytotoxic or immunostimulatory components from donor cells. Notably, using EVs for cancer therapy may have distinctive advantages compared with using donor cells. EVs are able to pass through biological barriers for tumor therapy with prolonged circulation time *in vivo* because of their nanoscale size. Furthermore, EVs have intrinsic cell targeting properties and lower toxicity. Finally, EVs are more subject to strictly monitored manufacturing processes and can maintain stability in frozen storage (-80°C) for at least one year[8-10].

Like DCs, DC-derived EVs also have the molecular composition that activates T cell immune response, such as MHC-peptide complexes, costimulatory molecules (CD80 and CD86), and other components (ICAM) that interact with immune cells [11, 12]. DC-EVs stimulate T cell responses against cancers through either a direct interaction (EVs/T cell) or indirect antigen presentation (EVs/bystander APCs/T cell) [11]. In addition, the EVs can lead to NK cell activation through surface expression of NKG2D-L or BAG6 [13]. The potential benefits for immunotherapy has resulted in DC-EVs being developed for clinical applications as cell-free cancer vaccines.

Comparatively, EVs derived from NK cells display direct cytotoxic activity against tumor cells. These nanovesicles contain target receptor NKG2D and killer proteins (e.g., FasL, perforin, granzysin and granzymes A and B), which induce tumor cell apoptosis through the caspase-3, caspase-7 and caspase-9 pathway [14, 15]. Moreover, a novel large-scale, inexpensive procedure to obtain functional NK EVs has been developed, laying the foundation for its use in future anticancer therapeutics [15].

Cytotoxic T lymphocytes (CTLs) play vital roles in cell-mediated immunity. CTL-EVs have been shown to cause unidirectional lethal hits to targeted tumor cells [16, 17]. These particles contain CTL surface molecules (TCR/CD3 ζ complex, CD8) and impact target cells using a TCR/antigen-MHC combination. Lethal chemical compounds in CTL-EVs, such as granzymes, lysosomal enzymes and perforin, are delivered to the target cells and induce cell death. It has also been reported that CTL-EVs prevent tumour progression by depleting tumoural mesenchymal cells [7]. CAR-T cells are genetically engineered CTLs expressing a chimeric antigen receptor (CAR). Notably, EVs released from these cells carry CAR on their surface and directly inhibit tumor growth through high levels of granzyme B and perforin [18]. Compared with CAR-T cells, CAR-T EVs do not express programmed cell death protein 1 (PD1), and their antitumor effect cannot be weakened by PD-L1 expression on tumors [18]. Applying CAR-T EVs appropriately may be a promising option for targeted cancer treatment.

Previous studies have reported that CTL-EVs are highly produced by CD8⁺ T cells upon TCR activation with anti-CD3 antibodies, anti-CD3/CD28 antibodies or specific antigens [7, 19, 20]. Here, we gain insight into another extracellular vesicle, which is derived from cytokine-activated CD8⁺ T cells (caCD8). CaCD8 cells can be obtained when CD8⁺ T cells are stimulated with anti-CD3 monoclonal antibodies (mAb) and with a combination of cytokines, including IFN γ , IL-2 and IL-1, which is obviously different from conventional CTL expansion. After stimulation, caCD8 cells significantly expand and acquire phenotypic markers of NK cells (such as CD56⁺) [21, 22]. CD8⁺CD56⁺ cells are terminally differentiated T lymphocytes expressing high levels of the killing receptor NKG2D and exhibit MHC-unrestricted antitumor activity *in vitro* and *in vivo* [21]. These cells constitute the core of cytokine-induced killer (CIK) cells that are widely used in clinical cancer therapy [23]. Although the function of caCD8 cells has been fully established, the characteristics of caCD8 EVs (caCD8-EVs) are still unknown.

In the present study, we found that caCD8-EVs are released through the differentiation process from CD8⁺CD56⁻ to CD8⁺CD56⁺ cells. The nanoparticles inherit some specific membrane molecules from parental cells but lack checkpoint molecules. CaCD8-EVs caused significant inhibition of tumor growth *in*

vitro and *in vivo*. Dynamic detection showed that actively dividing tumor cells were more likely to capture EVs compared with resting cells. Mechanically, caCD8-EVs contained not only typical cytotoxic proteins (i.e., granzyme B and perforin) but also IFN γ that partially induced the cytotoxicity of cancer cells. To our knowledge, this is the first report on the phenotype and functional characteristics of human caCD8 EVs, as well as EVs enriched with IFN γ . Therefore, our data suggest that caCD8-EVs could be a novel approach for cancer therapy.

Materials And Methods

1.1 Cell lines and antibodies

All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). For routine growth, cell culture media contained 10% (vol/vol) fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin (Invitrogen). Human MDA-MB-468 and SK-BR-3 cells were grown in DMEM media (Invitrogen). Other cells were grown in RPMI-1640 media (Invitrogen), such as MCF-7, MDA-MB-231, SK-BR-3, T47D, MDA-MB-453, MDA-MB-435, BT-474, H520, H446, GLC-82, H1975, H1299, SPC-A-1, A549, LTEP-A2 and CALU-6 cells. MCF-10A and BEAS-2B cells were cultured in media and supplements according to previous reports [24, 25]. For CaCD8-EVs treatment, the cells were maintained in corresponding medium supplemented with 10% EV-depleted FBS (SBI System Biosciences) for a specified time. Information about the primary antibodies used in this study is listed in Supplementary Table 1.

1.2 CaCD8 cell preparation

This study was approved by the Ethical Committee of Cancer Hospital of Tianjin Medical University (Approved No., E2016055). Informed consent was obtained from all subjects. CaCD8 cells were prepared as described in previous studies [26, 27]. Briefly, peripheral blood mononuclear cells (PBMCs) from donors were collected using Ficoll (Merck) and T lymphocytes were negatively selected by a Pan T Cell Isolation Kit (Miltenyi Biotec 130-096-535). CD8 $^{+}$ T cells were subsequently isolated using CD4 $^{+}$ T cells depletion microbeads (Miltenyi Biotec 130-045-101). Isolated primary human CD8 $^{+}$ T cells were then cultured in fresh serum-free AIM-V media (Invitrogen) containing 50 ng/ml of anti-CD3 antibody (eBioscience) at 37°C with 5% CO $_2$ for 24 h. Thereafter, 300 U/ml of recombinant human IL-2 (rhIL-2, Proleukin), 100 U/ml of recombinant human IL-1 α (eBioscience) and 1,000 U/ml of IFN γ (PeproTech) were added to the media. Media was replaced with fresh medium every five days. At days 12–15, caCD8 cells were harvested and used for detection.

1.3 Isolation and characterization of EVs

Preparation of EVs was performed using standard protocols as previously described [28]. CaCD8 cells were culture in AIM-V medium without exogenous EVs. The culture media was collected and pre-cleared by differential centrifugation at 750 $\times g$ for 5 min, 1500 $\times g$ for 15 min and 14,000 $\times g$ for 35 min. Finally the supernatants were filtered through a 0.22 μm filter (Millipore, Billerica, MA) and ultracentrifuged at

100,000xg for 90 min at 4°C to pellet EVs. The resulting pellet was washed in DPBS (Dulbecco's Phosphate Buffered Saline) and ultracentrifuged again. EVs were stored at -80°C. The protein concentration of vesicles was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific). The size of vesicles was measured using a Nanoparticle Tracking Analysis (NTA) (NS300, Malvern Instruments, UK) instrument and their morphology was detected using a transmission electron microscope. The EVs' related markers Alix, TSG101, HRS and CD63 were detected using western blotting.

1.4 Construction of control liposomes

Construction of liposomes was performed as previously described [29, 30]. A lipid commixture including 64.89 mol% lecithin, 32.08 mol% cholesterol, 3.02 mol% phosphatidylethanolamine, was solubilized in chloroform. After dissolution, the liposomal system was transferred to a round-bottom flask attaching to a rotary vacuum evaporator at 55°C for removing the organic solvent until a thin film was obtained. Then the film was rehydrated with 40 ml media (0.01 M phosphate buffer, 150 mM NaCl, pH 7.4) under vortexing. The obtained liposome solutions were extruded 20 times through a 0.1 µm polycarbonate filter with the Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL).

1.5 Flow cytometry analysis

Purified EVs (10µg) were incubated with 4 µm diameter aldehyde/sulfate latex beads (10µl) (Interfacial Dynamics) in DPBS overnight at 4°C with gentle agitation. After vesicles adhered to the beads, samples were washed with DPBS, and then resuspended in PBS/0.5% BSA and incubated with corresponding antibodies. Finally, samples were analyzed using a FACSCanto II (BD Biosciences) and data was analyzed using Flowjo Software.

1.6 Cell viability assay

Cells were seeded into 96-well plates at 2,000–3,000 cells per well. After cell adhesion to the plate, caCD8-EVs were added to cells to a final concentration of 0–200 µg/ml. Cells were then incubated for 96 h and cell viability was measured using the CellTiter-Glo Viability assay (Promega) following the manufacturer's instructions. Relative cell viability in the presence of EVs was normalized to the vehicle-treated controls after background subtraction. Specifically, the percentage of viability was calculated as follows: (sample counts – spontaneous counts) / (controls counts – spontaneous counts) × 100%. GraphPad Prism software was used to determine a viability curve.

1.7 Xenograft tumor models

All experiments involving mice were approved by the Animal Ethical and Welfare Committee of Tianjin Medical University Cancer Institute and Hospital (Approved No., AE2018121). Athymic nude mice (BalB/C, female) were used for *in vivo* experiments and housed in a specific pathogen-free barrier facility. For the pre-treatment model, tumor cells (2×10^5) were pre-treated with caCD8-EVs (200 µg/ml) or liposomes *in vitro* for 48 h and then inoculated subcutaneously in bilateral flanks, respectively. Following injections,

mice were permitted to recover and were monitored twice a week. Tumor sizes were measured using calipers. If humane endpoint criteria were met, mice were euthanized with CO₂ asphyxiation and tumor weights were measured.

For the therapeutic model, tumor cells (5×10^5) were inoculated subcutaneously in one flank. When tumor volumes reached an average of approximately 30 mm³ (approximately one week), the mice were randomly divided into groups of 6–8 mice each. The mice were intravenously injected with vehicle (DPBS, 100 µl), liposomes or caCD8-EVs (200 µg, 100 µl) every two days for two weeks. Tumor volumes were monitored twice a week and tumor weights were measured at the end of study. The tumor volumes were calculated using the following formula: $\text{volume} = \text{length} \times (\text{width})^2/2$.

1.8 Dynamic uptake of EVs

To verify the physical interaction between EVs and cancer cells, a dynamic monitor technology was utilized. Purified EVs were labeled with 5 µM CFSE solution (Biolegend) for 15 min at room temperature. The labeled samples were then washed with 30 ml DPBS and pelleted using ultracentrifugation to remove excess fluorescent dye. Then the CFSE-labeled EVs (100 µg/ml) were added into the dish of plated H520 cells and co-cultured for 24–48 h with EVs -free media. During this period, the subsequent uptake of labeled EVs was monitored using a Live Cell Imaging System (Olympus, Japan) and Andor iQ2 software according to the manufacturer's instructions. The fluorescence intensity was calculated using Image J software.

1.9 Western blot analysis

Whole-cell or EVs proteins were extracted in lysis buffer (1% Triton X-100, 0.1% SDS, and 0.1 M Tris-HCl (pH 7.0)) with protease inhibitors (Roche). Subsequently, protein samples were separated on an SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane (Millipore), and blocked with 10% non-fat dry milk at room temperature for 1 h. The membranes were incubated overnight at 4°C with the corresponding primary antibodies at dilutions recommended by the suppliers. Thereafter, the membranes were incubated with the secondary antibodies for 1 h. Signals were visualized using ECL detection reagents (Pierce), and images of the blots were analyzed using ImageJ software (NIH, Bethesda, MD, USA). For EVs identification, CD63, HRS, Alix, and TSG101 were used as EVs markers. b-actin was used as a loading control. For apoptosis, cleaved caspase-3/ caspase-9/PARP were used as indicators.

1.10 Cell Apoptosis Assay

After treatment, single-cell suspensions were washed with PBS. They were then resuspended in binding buffer containing Annexin V and propidium iodide and stained according to the manufacturer's instructions (BD). After incubation for 20 min at room temperature in the dark, samples were analyzed using a FACSCanto II flow cytometer and data was analyzed using Flowjo software.

1.11 Quantitative real-time PCR

Cancer cells were either treated with 200 µg/ml caCD8-EVs or left untreated for 48 h. For the IFNγ pathway inhibition assay, an IFNγ blocking antibody (10 µg/ml) (Biolegend) or 0.6 µM of the JAK1/2 inhibitor Ruxolitinib (Selleckchem) was added to the caCD8-EVs treatment cultures. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's specifications. Reverse transcription was performed on 1 µg of total RNA using the MML-V reverse transcriptase (Invitrogen). Quantitative PCR (qPCR) was performed by the Platinum SYBR Green SuperMix kit (Invitrogen) and analyzed using an ABI Prism 7500 Real-Time PCR machine (Applied Biosystems) with the SDS v2.3 software (Applied Biosystems). b-actin was used as an internal standard for data calibration. The $2^{-\Delta\Delta Ct}$ formula was used for calculation of differential gene expression. Information about primers is included in Supplementary Table 2.

1.12 Label-free quantification (LFQ) with LC-MS/MS analysis

For the identification of the precise mechanism of caCD8-EVs cytotoxicity, MCF-7 cells were treated with 200 µg/ml EVs or liposomes. After 48 h of incubation, the cells were harvested and suspended in up to 30 µl of urea buffer (50 mM Tris-HCl pH 7.5, 8 M urea, 2 M thiourea, and 1 mM DTT) for protein extraction. The cell protein content was analyzed by mass spectrometry. For the identification of the caCD8-EVs component, caCD8-EVs and caCD8 cells were harvested and digested as described above.

The label-free quantification (LFQ) experiment was conducted at Shanghai Applied Protein Technology Co. Ltd in China. LC-MS/MS was performed using a Q-Ex active mass spectrometer (Thermo Scientific) coupled to an Easy nLC 1200 system (Thermo Fisher Scientific) with an analysis time of 60 min. The MS raw data for each sample were combined and searched using the MaxQuant 1.5.3.17 software for identification and quantitation analysis. Hierarchical clustering analysis of proteins was performed using the log₂-transformed expression values and a cutoff of $p < 0.05$. The protein-protein interaction analysis was conducted using STRING software (<http://string-db.org/>). The biological process of the identified proteins was analyzed in Gene Ontology (GO).

1.13 IFNγ release and sharing assay

For the cell-to-cell IFNγ sharing experiments, cancer cells were treated with 200 µg/ml caCD8-EVs for 24 h, and then washed three times with warm RPMI. A total of 2×10^5 EVs-pulsed cells were co-cultured with 1×10^5 CFSE-labeled cancer cells (un-pulsed, receptor cells). After 24 h, phosphorylation of STAT1 in receptor cells was measured by flow cytometry. For the IFNγ pathway inhibition assay, an IFNγ blocking antibody (10 µg/ml) or JAK1/2 inhibitor Ruxolitinib (0.6 µM) was added to the cell-to-cell co-culture system. For intracellular phospho-flow cytometry, cells were fixed and permeabilized with 1.6% paraformaldehyde for 10 min on ice, followed by incubation with 90% methanol for at least 20 min on ice. Cells were then stained with p-STAT1 for flow cytometry analysis.

1.14 ELISA

ELISA plates (96- well) (Corning) were coated with 0.3 µg per well (100 µl) of capture antibodies (IFNg #502401, HLA #311402 or CD3 #317301, BioLegend) overnight at 4 °C. BSA (3%) was used to block free binding sites for 1 h at room temperature. Then, EVs samples with serial dilution were added to each well and incubated 2 h at room temperature for capturing. After washing, test biotinylated antibodies (IFNg Ab#502503, HLA #311434 or CD3 #300303, BioLegend) was added to each well and incubated for 1 h at room temperature. A total of 100 µl per well of horseradish peroxidase-conjugated avidin (#405103, BioLegend) diluted in PBS containing 0.1% BSA was then added and incubated for 20 min at room temperature. The plates were developed with tetramethylbenzidine (Pierce), and the reactions were stopped with 0.5 N H₂SO₄. The plates were read at 450 nm using a BioTek plate reader.

1.15 Statistical analysis

Results are presented as the mean ± SD (shown as error bars) from at least three independent experiments. Statistical analyses were performed using unpaired two-tailed Student's t-test in GraphPad Prism 8 software. P < 0.05 was considered statistically significant (*p < 0.05, **p < 0.01, and ***p < 0.001).

Results

2.1 Characterization of phenotypes of caCD8-derived EVs

Previous studies have established that CD8+ T cells can be expanded and differentiated into CD8+CD56+ cells when they are stimulated with anti-CD3 mAb and a combination of cytokines, including IFNg, IL-2 and IL-1 [21, 22]. When caCD8 cells continue expanding for 12–15 days, the supernatant can be collected for EVs generation using well-established ultracentrifugation protocols [31] we have applied before [28] (Figure 1A). The yield of EVs in our study was 5-8 µg/million cells and they showed a typical round or disk-like morphology under the electron microscopy (Figure 1B). NTA analysis revealed that most of the nanoparticles were physically homogeneous with a size distribution in the range of 30–200 nm, peaking at an 88 nm diameter (Figure 1B). In addition, western blot analysis further confirmed the presence of typical EVs-related proteins (CD63, HRS, Alix, TSG101) in the nano-vesicles and the exclusion of the cells marker (GM130) (Figure 1C). Collectively, these results indicate the successful separation of caCD8-EVs from culture medium.

The phenotypes of the EVs were analyzed by flow cytometry after EVs were coated onto 4 µm diameter aldehyde/sulfate latex beads. CaCD8-EVs inherited T cell markers, such as CD3, TCR, HLA and CD8, as well as the EVs-related marker CD63 (Figure 1D). NKG2D was inductively expressed on caCD8 cells and also expressed on the EVs surface with approximately 50% positive rate. This protein is known to interact with its ligand on tumor cells (such as MIC A/B and ULBPs), and mainly mediates tumor-killing activity of caCD8 cells [32]. Unlike NKG2D, CD56 expression is appreciably low on caCD8-EVs (Figure 1D). Furthermore, caCD8 cells acquired checkpoint phenotype (i.e., PD-L1+ TIM3+ LAG3+ CEACAM+) as we

have previously reported [27], however, these inhibitory proteins were not expressed on the EVs (Figure E). These results indicate that caCD8-EVs selectively inherit molecular markers of the parental cells.

2.2 Cytotoxic activity of caCD8-derived EVs *in vitro* and *in vivo*

CaCD8 cells present extensive antitumor activity [22]. We therefore further investigated whether the produced nanoparticles have the same antitumor effects. Breast cancer and lung cancer were chosen as experimental models. A cell viability (CellTiter-Glo Luminescent) assay was conducted on cell lines after treatment with caCD8-EVs (200 µg/ml) for 96 h. As shown in Figure 2A, the two non-cancerous cell lines, MCF-10A and BEAS-2B, were much less sensitive to caCD8-EVs. However, 5/8 breast cancer cell lines and 6/9 lung cancer cell lines were more sensitive to the nanovesicles, as demonstrated by a significant reduction in viability (> 50%). The sensitive lung cancer cells included cells of various histological types, such as squamous cell carcinoma (H520), small cell lung cancer cell (H446), anaplastic carcinoma (CALU-6) and adenocarcinoma (A549, GLC-82 and H1975), indicating that caCD8-EVs had a broad spectrum of anticancer activity. Moreover, the cytotoxicity of caCD8-EVs was a time- and dose-dependent manner (Figure 2B, S1B) and determined by IC₅₀ values (Figure S1B). The change in cell morphology induced by caCD8-EVs is shown in the Figure S1A.

To further confirm the inhibitory effect of caCD8-EVs on tumor cells, we treated cancer cells with caCD8-EVs purified from six donors. Meanwhile, EVs derived from different kinds of cancer cells (MCF-7, H520 and HH (cutaneous T cell lymphoma)) were chosen as controls. EVs derived from resting CD8 cells was an optimal control, however, these cells could only grow for 4-6 days under the maintenance of IL-2 and the amount of secreted EVs was very low (0.1-0.3 µg /million cells). In contrast, EVs secreted by caCD8 cells reached a substantially high level (5-8µg/million cells). Since it was difficult to collect enough unstimulated CD8 EVs, we used synthetic liposomes as an alternative. Liposomes are organic nanovesicles consist of single or multiple lipid bilayers encapsulating an aqueous compartment, which is similar to the cell membrane [33]. The mean size of constructed liposomes was approximately 150.5nm (Figure 2D). As Figure 2C shown, caCD8-EVs from different donors revealed similar cytotoxic potency against tumor cells (MCF-7, MDA-MB-468, H520 and H446). In contrast, control EVs displayed limited effect on the cells viability. Taken together, these data demonstrate that caCD8-EVs can exert specific cytotoxic activity against cancer cells *in vitro*.

To evaluate whether caCD8-EVs can affect tumor growth *in vivo*, we utilized athymic nude mice (BalB/C) bearing subcutaneous MCF-7 and GLC-82 xenografts. The GLC-82 model was chosen because of the ease of xenograft engraftment compared to other lung cancer cell lines. A pre-treatment model was first created (Figure 2E). Tumor cells were pre-treated with caCD8-EVs *in vitro* for 48 h and then inoculated subcutaneously into the left flank of the mice. An identical amount of liposomes-treated control cells were loaded in the right flank. Tumor growth curves demonstrated that tumor development on left flank was dramatically slower than on the contralateral flank (Figure 2E). At the end of the study 2/5 MCF-7 tumor-bearing mice and 1/5 GLC-82 tumor-bearing mice did not have tumors form on the left flanks (Figure S2A, S2B).

We then performed studies with a therapeutic model (Figure 2F). Tumor cells were inoculated subcutaneously on one flank. Then mice were treated with vehicle, liposomes or caCD8-EVs, respectively. It was found that tumor growth was significantly suppressed in the mice with caCD8-EVs treatment (Figure 2F) compared with the other two groups. At the end of the study, tumor weights from the caCD8-EVs group were significantly lower than either vehicle group or liposomes (Figure S2C). Collectively, these data demonstrate that caCD8-EVs exert significant antitumor effects *in vivo*.

2.3 CaCD8-derived EVs are preferentially taken up by dividing cells

For caCD8-EVs to exert antitumor effects, they first need to be taken up by tumor cells, such as through a fusion-mediated mechanism [14]. Flow cytometry and confocal microscopy [34] methods only allow for detection of EVs uptake at a static point in time. Instead, we utilized a dynamic technology to monitor EVs uptake in real time. CaCD8-EVs were labeled with CFSE and then added to the culture of adherent H520 cells. During the 24–48 h incubation, the dynamic uptake of EVs was detected using a live cell imaging system (Figure 3A). When tumor cells adhered to the cell culture dish, the uptake of CFSE-labeled EVs was relatively weak. Strikingly, once the cells began to divide and round up, CFSE-caCD8-EVs were rapidly captured by the cancer cells and aggregated on the cell surface, resulting in brightly stained rounded cells. This phenomenon persisted throughout the process of cell division when the cells split into two daughter cells and re-adhered to the cell culture plate (Figure 3A, 3B, supplementary video). These data indicate that the uptake of caCD8-EVs is closely related to cell division.

Cell division causes cell proliferation [35, 36]. To further validate the phenomenon described above, we cultured tumor cells in media with different serum concentrations (high, 10% FBS, low, 0.5% FBS), and then treated the cultures with caCD8-EVs. The large difference in serum concentrations aimed to exacerbate the variable conditions for cell proliferation. After 24 h, flow cytometry was used to detect CD3 molecules on the tumor cell surface (Figure S3A). CD3 is an exclusive marker of T cells [37] and was seen to be abundant on caCD8-EVs (Figure 1D). The CD3 marker was used as an indicator for membrane fusion of tumor cells with captured EVs. With an increase in caCD8-EVs concentration, the cancer cells carrying CD3 molecules also gradually increased. More importantly, in the high serum cultures, the CD3 positive ratio of tumor cells (MCF-7 and H520) was significantly higher than in the low serum cultures (Figure S3B, S3C). In addition, we treated cells with mitomycin C (0.1 μ M, Roche) to induce cell cycle arrested and detected the uptake of caCD8-EVs by cancer cells. Compared with untreated cells, mitomycin-treated cells had lower CD3 positive staining (Figure S3D, S3E). These data suggest cell proliferation facilitates the uptake of caCD8-EVs.

Because tumor cells have faster division and proliferation rates than normal or resting cells, we hypothesized that more EVs would be absorbed by tumor cells. Tumor cells (MCF-7, H520) and non-cancerous cells (MCF-10A, BEAS-2B) were cultured *in vitro* for 48 h with or without caCD8-EVs treatment. Flow cytometry analysis showed that CD3-positive cells were significantly higher in tumor cell cultures than in non-cancerous cell cultures (Figure 3C). Meanwhile the proliferation of tumor cells at 48 h was

approximately 1–2-fold higher than that of non-cancerous cells (Figure 3D). Taken together, these data further testify that caCD8-EVs preferably bind cells with high proliferation rates.

2.4 Apoptosis of cancer cell induced by CaCD8-EVs

To investigate the mechanism of cytotoxic activity of caCD8 EVs, flow cytometry was performed to detect cell apoptosis following caCD8-EVs treatment. Apoptosis of MCF-7 and H520 cells treated with EVs (200 µg/ml) for 96 h increased significantly compared with the liposomes group (Figure 4A). Western blotting showed that the cleavage of Caspase-3, PARP and Caspase-9 increased significantly following caCD8-EVs treatment, further supporting that the cells underwent apoptosis (Figure 4B). Previous studies have shown that EVs secreted by CTL and NK cells induce apoptosis of target cells, which is related to the EVs-contained cytotoxic effectors (such as granzyme B and perforin) [14-17]. We next determined whether caCD8-EVs contained the same cytotoxic composition. Western blot analysis showed that granzyme B and perforin also expressed in the vesicles (Figure 4C). The content of the two proteins was further determined by label-free quantification (LFQ) analysis of mass spectrometry (Figure 4D).

2.5 Protein bioinformatics analysis of cells treated with caCD8-EVs

To explore whether other components of caCD8-EVs, such as miRNAs, are involved in triggering apoptosis, we extracted total RNA from caCD8-EVs and transfected this RNA into cancer cells. However, no significant cytotoxicity was observed (Figure 4E). We then treated MCF-7 cells with caCD8-EVs or liposomes and identified the differential proteins using mass spectrometry. Compared with the liposomes-treated cells, 176 proteins were newly expressed and 72 proteins were highly expressed (> 4×, Figure 4F). A GO database analysis showed that many of these proteins are involved in processes such as spindle organization, regulation of ubiquitin–protein, cytokine response, intrinsic apoptosis, etc. (Figure 4G) Strikingly, the online STRING analysis revealed that some proteins related to interferon-stimulated genes (ISGs), such as STAT1, STAT2, IFIT2, GBP1, GBP2, DDX60, DDX58, PSMB9 and PSMB10, were aggregated and became key nodes in the network (Figure 4H). QPCR also confirmed the inductive expression of ISGs in EVs-treated MCF-7 cells (Figure 5E). These data suggest that interferon may play a role in the induction of cell apoptosis.

2.6 IFNγ derived from caCD8-EVs partially induce cytotoxicity against tumor cell

To investigate which interferon from caCD8-EVs induced the expression of ISG proteins, we identified the proteins in caCD8-EVs using mass spectrometry (Figure 5A). Strikingly, IFNγ expression of caCD8-EVs was approximately eight times higher than that of caCD8 cells, indicating that IFNγ was enriched in the EVs (Figure 5B). Western blot also verified the above results (Figure 5C). In addition, an IFNγ blocking antibody or IFNγR pathway inhibitor (JAK1/2 inhibitor, Ruxolitinib) significantly blocked ISG transcription in tumor cells treated with caCD8-EVs (Figure 5E). Moreover, the cytotoxic activity and apoptosis induced by caCD8-EVs was partly reversed (Figure 5D, 5F, 5G). These data suggest that IFNγ present in caCD8-EVs may induce the formation of ISGs and involved in the cytotoxic activity against tumors.

2.7 Cancer cells release and share EV-derived IFN γ

To investigate whether IFN γ exists on the surface of EVs, we used various antibodies (including antibodies against CD3, HLA, IFN γ) in an ELISA to capture EVs and detect IFN γ on the surface of EVs (Figure 6A). Unexpectedly, IFN γ was not found on the surface of EVs (Figure 6B). We wanted to next assess how intracellular IFN γ in EVs could bind to surface IFN γ R on tumor cells. Previous studies have shown that IFN γ can bind to phosphatidylserine (PS) on tumor cell membranes, become internalized, and then slowly release in an autocrine or paracrine manner to obtain a long-term cytokine effect. This process was defined as “catch and release” [38]. Therefore, we speculate that IFN γ in the EVs also undergoes a similar process to exert its effect on tumor cells.

To test this hypothesis, we set up a co-culture assay previously described [38], in which one group of cancer cells is pulsed with EVs for 24 h, washed, and co-cultured with un-pulsed cancer cells (CFSE-labeled, receptor cells). After one day of co-culture, the upregulation of p-STAT1 in receptor cells was measured by flow cytometry to reflect their IFN γ exposure. Interestingly, the level of p-STAT1 was upregulated significantly in the receptor cells following co-culture with caCD8-EVs-pulsed cells. Furthermore, p-STAT1 upregulation was reduced when cells were treated with IFN γ -blocking antibody or JAK1/2 inhibitor (Figure 6C). These data indicate that once EVs deliver IFN γ into tumor cells, IFN γ can be released by tumor cells and interact with IFN γ R on the cell surface, thereby activating the IFN γ R pathway.

To conclude, caCD8-EVs deliver IFN γ and typical killer proteins (i.e., granzyme B and perforin) to tumor cells. Subsequently, IFN γ is released by tumor cells and activates IFN γ R on the surface of surrounding cells, thereby participating in EVs-induced antitumor activities (Figure 6D).

Discussion

CaCD8 cells are characterized by a high proliferative rate *ex vivo* in adequate conditions and strong antitumor activity. These cells are major cytotoxic effector subsets of CIK cells [21, 22]. CIK cells are easily generated from PBMCs under caCD8 culture conditions and have been well established for adoptive cellular immunotherapy. An increasing number of clinical trials have demonstrated the feasibility and efficacy of CIK treatment in a wide variety of neoplasms [23, 26, 32, 39–41]. The present study found that the EVs derived from caCD8 also have marked antitumor effects both *in vitro* and *in vivo*. Phenotype analysis showed caCD8-EVs inherited some specific membrane molecules from parental cells, such as CD3, CD8, TCRs and NKG2D, but no checkpoint receptors. These components might endow caCD8-EVs the ability to recognize target cells and to prevent from the modulation of immunosuppressive molecules in the tumor microenvironment.

To allow for cell-to-cell communication, EVs are required to fuse either with the plasma membrane or with the endosomal membrane after endocytic uptake [3, 34]. EV uptake can be visualized directly using fluorescent dyes [34]. Unlike some common methods such as flow cytometry and confocal microscopy, live cell imaging allows for monitoring of EVs uptake successively. Surprisingly, when the cells began to divide and become rounded, they captured more EVs compared with non-dividing cells. The more active

proliferation of tumor cells allows for more EVs binding compared with normal cells, which partially explains the specific killing of tumor cells by caCD8-EVs.

Cell division has traditionally been subdivided into a series of steps according to the alignment of chromosomes and the cytoskeleton: prophase, prometaphase, metaphase, anaphase and telophase. During mitosis, cells alter their volume and surface topology rapidly [42]. De novo membrane synthesis would be impractical to accommodate these changes, as it requires considerable time and resources [43]. Instead, cells undergo membrane reshaping, growth and delivery. For example, new membrane can be added from internal stores via trafficking events such as exocytosis or endocytic recycling or membrane blebbing [44]. The current study showed another possible mechanism of membrane compensation in dividing cells through EVs fusion. But how exogenous EVs bind to rounding cells remains unknown. Protein-protein interactions or changes in physical features, such as surface charge or osmotic pressure, might contribute to this process. The precise mechanism needs to be further explored.

Cytotoxic proteins (e.g., granzyme B and perforin) contained in lytic granules are known to mediate cytolytic activity of CTL or NK cells [14]. Upon target cell recognition and conjugation, these active effectors are released into the synapse. Perforin, a pore forming protein, activates clathrin- and dynamin-dependent endocytosis, which results in the internalization and delivery of granzyme B into the target cell cytosol [45]. Granzyme B is a serine protease that triggers activation of the caspases – 9, -3, -7 cascade, which leads to target cell apoptosis [46]. EVs secreted by CTL or NK cells also carry these killer effectors, which have been implicated in the killing of target cells [15–18]. Similarly, the present study found granzyme B and perforin are abundant in the caCD8-EVs, which might be involved in EVs-triggered apoptosis against cancer cells, at least to some extent.

Through protein mass spectrometry analysis, we unexpectedly detected IFN γ enriched in the EVs. Blocking the IFN γ pathway with an antibody or JAK1/2 inhibitor partially attenuated caCD8-EVs-induced apoptosis. IFN γ is a critical cytokine that promotes innate and adaptive immune responses. It also exerts direct anti-proliferative and pro-apoptotic effects on a wide variety of tumor cells [47, 48]. Several studies have shown IFN γ increases the susceptibility of cancer cells to extrinsic and intrinsic pathways of apoptosis by regulating the expression of apoptosis-related proteins, such as Fas, caspase proteins, survivin and Bim [49–52]. In addition, some ISGs have been reported to involve in apoptosis under the various stimulation, such as IFIT2, DDX58, GBP2, UBE2L6 [53–56]. Therefore, our data propose a possibility that EVs-derived IFN γ participate in EVs-induced apoptosis to cancer cells.

It is interesting that the functional consequences of IFN γ signaling from EVs are dependent on internal IFN γ as opposed to surface IFN γ . Notably, Oyler-Yaniv et al. reported a mode of action of IFN γ , named “catch and release”. IFN γ can bind to PS on the surface of cancer cells and be internalized. IFN γ is then slowly released in an autocrine or paracrine manner and activates the IFNGR signal pathway to drive long-term cytokine-response [38]. Similarly, the present study found that after caCD8-EVs delivered IFN γ to tumor cells, the IFN γ was secreted by tumor cells and acted on surrounding IFNGR, thereby activating the transcription of ISGs. The time-dependent cytotoxic assay might reflect the slow release of IFN γ from

tumor cells to a certain extent. Following treatment with caCD8-EVs for 48 h, cytotoxicity gradually took effect and peaked at 96 h. Moreover, in the EVs IFN γ might be bound to PS because the inner leaflet of EVs contains PS [57]. This binding might facilitate IFN γ absorption by tumor cells. However, further research is needed to understand the precise mechanisms of IFN γ releasing from tumor cells, as well as IFN γ packaging into EVs.

Conclusions

In conclusion, the present study reveals a phenotype and antitumor effects of EVs derived from caCD8 cells. These nanovesicles deliver not only the typical killer proteins (i.e., granzyme B and perforin), but also IFN γ to tumor cells, at least in part accounting for the mechanism of cytotoxicity induced by caCD8-EVs. In regard to their nanometer size and the lack of checkpoint molecules, caCD8-EVs can potentially be applied as an auxiliary approach to overcome some of the limitations or defects of CIK cell immunotherapy, thereby enhancing overall therapeutic effect.

Abbreviations

CAR-T: Chimeric antigen receptor T cells, CaCD8: Cytokine-activated CD8 $^{+}$ T cells, CIK: Cytokine-induced killer cells, CTLs: Cytotoxic T lymphocytes, DCs: Dendritic cells, EVs: Extracellular vesicles, ISGs: Interferon-stimulated genes, LFQ: Label-free quantification, NKG2D: Natural-killer group 2, member D, NK: natural killer cells, NTA: Nanoparticle Tracking Analysis, PS: Phosphatidylserine, PBMCs: Peripheral blood mononuclear cells,

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Cancer Hospital of Tianjin Medical University (Approved No., E2016055).

All experiments involving mice were approved by the Animal Ethical and Welfare Committee of Tianjin Medical University Cancer Institute and Hospital (Approved No., AE2018121).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

We declare here none of our authors has financial or other conflicts of interest that might be construed as influencing the results or interpretation of our study.

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Authors' contributions

Lin Zhang contributed to the experimental studies and manuscript preparation. Yang An, Xuena Yang, Limei Wang, Zhenyu Ji and Wenwen Yu contributed to the experimental studies. Quanyong Yang and Baihui Li contributed to statistical analysis. Shuai Meng contributed to liposomes construction. Feng Wei and Lili Yang contributed to the data acquisition and literature research. Xiubao Ren conceived and designed the entire study and revised manuscript critically. All authors read and approved the manuscript.

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References

1. P. Wu, B. Zhang, D.K.W. Ocansey, W. Xu, H. Qian, Extracellular vesicles: A bright star of nanomedicine, *Biomaterials*, 269 (2021) 120467.
2. A. Möller, R.J. Lobb, The evolving translational potential of small extracellular vesicles in cancer, *Nature reviews. Cancer*, 20 (2020) 697-709.
3. M. Mathieu, L. Martin-Jaular, Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication, *Nature cell biology*, 21 (2019) 9-17.
4. F. Teng, M. Fussenegger, Shedding Light on Extracellular Vesicle Biogenesis and Bioengineering, *Advanced science (Weinheim, Baden-Wurttemberg, Germany)*, 8 (2020) 2003505.
5. J. Kowal, M. Tkach, Dendritic cell extracellular vesicles, *International review of cell and molecular biology*, 349 (2019) 213-249.
6. M. Farcas, M. Inngjerdigen, Natural killer cell-derived extracellular vesicles in cancer therapy, *Scandinavian journal of immunology*, 92 (2020) e12938.
7. N. Seo, Y. Shirakura, Y. Tahara, F. Momose, N. Harada, H. Ikeda, K. Akiyoshi, H. Shiku, Activated CD8(+) T cell extracellular vesicles prevent tumour progression by targeting of lesional mesenchymal cells, *Nature communications*, 9 (2018) 435.
8. T. Huyan, H. Li, H. Peng, J. Chen, R. Yang, W. Zhang, Q. Li, Extracellular Vesicles - Advanced Nanocarriers in Cancer Therapy: Progress and Achievements, *International journal of nanomedicine*, 15 (2020) 6485-6502.

9. L. Barile, G. Vassalli, Exosomes: Therapy delivery tools and biomarkers of diseases, *Pharmacology & therapeutics*, 174 (2017) 63-78.
10. J.M. Pitt, F. André, S. Amigorena, J.C. Soria, A. Eggermont, G. Kroemer, L. Zitvogel, Dendritic cell-derived exosomes for cancer therapy, *The Journal of clinical investigation*, 126 (2016) 1224-1232.
11. C. Théry, M. Boussac, P. Véron, P. Ricciardi-Castagnoli, G. Raposo, J. Garin, S. Amigorena, Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles, *Journal of immunology* (Baltimore, Md. : 1950), 166 (2001) 7309-7318.
12. C. Théry, A. Regnault, J. Garin, J. Wolfers, L. Zitvogel, P. Ricciardi-Castagnoli, G. Raposo, S. Amigorena, Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73, *The Journal of cell biology*, 147 (1999) 599-610.
13. S. Viaud, M. Terme, C. Flament, J. Taieb, F. André, S. Novault, B. Escudier, C. Robert, S. Caillat-Zucman, T. Tursz, L. Zitvogel, N. Chaput, Dendritic cell-derived exosomes promote natural killer cell activation and proliferation: a role for NKG2D ligands and IL-15Ralpha, *PloS one*, 4 (2009) e4942.
14. L. Lugini, S. Cecchetti, V. Huber, F. Luciani, G. Macchia, F. Spadaro, L. Paris, L. Abalsamo, M. Colone, A. Molinari, F. Podo, L. Rivoltini, C. Ramoni, S. Fais, Immune surveillance properties of human NK cell-derived exosomes, *Journal of immunology* (Baltimore, Md. : 1950), 189 (2012) 2833-2842.
15. A.Y. Jong, C.H. Wu, J. Li, J. Sun, M. Fabbri, A.S. Wayne, R.C. Seeger, Large-scale isolation and cytotoxicity of extracellular vesicles derived from activated human natural killer cells, *Journal of extracellular vesicles*, 6 (2017) 1294368.
16. P.J. Peters, H.J. Geuze, H.A. Van der Donk, J.W. Slot, J.M. Griffith, N.J. Stam, H.C. Clevers, J. Borst, Molecules relevant for T cell-target cell interaction are present in cytolytic granules of human T lymphocytes, *European journal of immunology*, 19 (1989) 1469-1475.
17. P.J. Peters, J. Borst, V. Oorschot, M. Fukuda, O. Krähenbühl, J. Tschopp, J.W. Slot, H.J. Geuze, Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes, *The Journal of experimental medicine*, 173 (1991) 1099-1109.
18. W. Fu, C. Lei, S. Liu, Y. Cui, C. Wang, K. Qian, T. Li, Y. Shen, X. Fan, F. Lin, M. Ding, M. Pan, X. Ye, Y. Yang, S. Hu, CAR exosomes derived from effector CAR-T cells have potent antitumour effects and low toxicity, *Nature communications*, 10 (2019) 4355.
19. N. Blanchard, D. Lankar, F. Faure, A. Regnault, C. Dumont, G. Raposo, C. Hivroz, TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex, *Journal of immunology* (Baltimore, Md. : 1950), 168 (2002) 3235-3241.
20. J. Wahlgren, L. Karlson Tde, P. Glader, E. Telemo, H. Valadi, Activated human T cells secrete exosomes that participate in IL-2 mediated immune response signaling, *PloS one*, 7 (2012) e49723.
21. M. Franceschetti, A. Pievani, G. Borleri, L. Vago, K. Fleischhauer, J. Golay, M. Introna, Cytokine-induced killer cells are terminally differentiated activated CD8 cytotoxic T-EMRA lymphocytes, *Experimental hematology*, 37 (2009) 616-628.e612.
22. P.H. Lu, R.S. Negrin, A novel population of expanded human CD3+CD56+ cells derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency, *Journal of*

- immunology (Baltimore, Md. : 1950), 153 (1994) 1687-1696.
23. J.J. Mata-Molanes, M. Sureda González, B. Valenzuela Jiménez, E.M. Martínez Navarro, A. Brugarolas Masllorens, Cancer Immunotherapy with Cytokine-Induced Killer Cells, Targeted oncology, 12 (2017) 289-299.
 24. N.S. Yaacob, N.F. Ismail, Comparison of cytotoxicity and genotoxicity of 4-hydroxytamoxifen in combination with Tualang honey in MCF-7 and MCF-10A cells, BMC complementary and alternative medicine, 14 (2014) 106.
 25. F. Zhao, W.T. Klimecki, Culture conditions profoundly impact phenotype in BEAS-2B, a human pulmonary epithelial model, Journal of applied toxicology : JAT, 35 (2015) 945-951.
 26. L. Liu, W. Zhang, X. Qi, H. Li, J. Yu, S. Wei, X. Hao, X. Ren, Randomized study of autologous cytokine-induced killer cell immunotherapy in metastatic renal carcinoma, Clinical cancer research : an official journal of the American Association for Cancer Research, 18 (2012) 1751-1759.
 27. L. Zhang, J. Wang, F. Wei, K. Wang, Q. Sun, F. Yang, H. Jin, Y. Zheng, H. Zhao, L. Wang, W. Yu, X. Zhang, Y. An, L. Yang, X. Zhang, X. Ren, Profiling the dynamic expression of checkpoint molecules on cytokine-induced killer cells from non-small-cell lung cancer patients, Oncotarget, 7 (2016) 43604-43615.
 28. M. Shen, C. Dong, X. Ruan, W. Yan, M. Cao, D. Pizzo, X. Wu, L. Yang, L. Liu, X. Ren, S.E. Wang, Chemotherapy-Induced Extracellular Vesicle miRNAs Promote Breast Cancer Stemness by Targeting ONECUT2, Cancer research, 79 (2019) 3608-3621.
 29. J.L. Hood, R.S. San, S.A. Wickline, Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis, Cancer research, 71 (2011) 3792-3801.
 30. A. Bigdeli, S. Palchetti, D. Pozzi, M.R. Hormozi-Nezhad, F. Baldelli Bombelli, G. Caracciolo, M. Mahmoudi, Exploring Cellular Interactions of Liposomes Using Protein Corona Fingerprints and Physicochemical Properties, ACS nano, 10 (2016) 3723-3737.
 31. L.M. Doyle, M.Z. Wang, Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis, Cells, 8 (2019).
 32. D. Sangiolo, G. Mesiano, L. Gammaitoni, V. Leuci, M. Todorovic, L. Giraudo, C. Cammarata, C. Dell'Aglio, L. D'Ambrosio, A. Pisacane, I. Sarotto, S. Miano, I. Ferrero, F. Carnevale-Schianca, Y. Pignochino, F. Sassi, A. Bertotti, W. Piacibello, F. Fagioli, M. Aglietta, G. Grignani, Cytokine-induced killer cells eradicate bone and soft-tissue sarcomas, Cancer research, 74 (2014) 119-129.
 33. Z. Li, S. Tan, S. Li, Q. Shen, K. Wang, Cancer drug delivery in the nano era: An overview and perspectives (Review), Oncology reports, 38 (2017) 611-624.
 34. L.A. Mulcahy, R.C. Pink, D.R. Carter, Routes and mechanisms of extracellular vesicle uptake, Journal of extracellular vesicles, 3 (2014).
 35. J.Y. Ong, J.Z. Torres, Dissecting the mechanisms of cell division, The Journal of biological chemistry, 294 (2019) 11382-11390.
 36. B.J. Thompson, Developmental control of cell growth and division in Drosophila, Current opinion in cell biology, 22 (2010) 788-794.

37. J.M. Rojo, R. Bello, P. Portolés, T-cell receptor, *Advances in experimental medicine and biology*, 640 (2008) 1-11.
38. J. Oyler-Yaniv, A. Oyler-Yaniv, M. Shakiba, N.K. Min, Y.H. Chen, S.Y. Cheng, O. Krichevsky, N. Altan-Bonnet, G. Altan-Bonnet, Catch and Release of Cytokines Mediated by Tumor Phosphatidylserine Converts Transient Exposure into Long-Lived Inflammation, *Molecular cell*, 66 (2017) 635-647.e637.
39. Y. Meng, Z. Yu, Y. Wu, T. Du, S. Chen, F. Meng, N. Su, Y. Ma, X. Li, S. Sun, G. Zhang, Cell-based immunotherapy with cytokine-induced killer (CIK) cells: From preparation and testing to clinical application, *Human vaccines & immunotherapeutics*, 13 (2017) 1-9.
40. K. Pan, X.X. Guan, Y.Q. Li, J.J. Zhao, J.J. Li, H.J. Qiu, D.S. Weng, Q.J. Wang, Q. Liu, L.X. Huang, J. He, S.P. Chen, M.L. Ke, Y.X. Zeng, J.C. Xia, Clinical activity of adjuvant cytokine-induced killer cell immunotherapy in patients with post-mastectomy triple-negative breast cancer, *Clinical cancer research : an official journal of the American Association for Cancer Research*, 20 (2014) 3003-3011.
41. Y. Zhu, H. Zhang, Y. Li, J. Bai, L. Liu, Y. Liu, Y. Qu, X. Qu, Efficacy of postoperative adjuvant transfusion of cytokine-induced killer cells combined with chemotherapy in patients with colorectal cancer, *Cancer immunology, immunotherapy : CII*, 62 (2013) 1629-1635.
42. N. Ramkumar, B. Baum, Coupling changes in cell shape to chromosome segregation, *Nature reviews. Molecular cell biology*, 17 (2016) 511-521.
43. J.G. Carlton, H. Jones, U.S. Eggert, Membrane and organelle dynamics during cell division, 21 (2020) 151-166.
44. T. Kiyomitsu, I.M. Cheeseman, Cortical dynein and asymmetric membrane elongation coordinately position the spindle in anaphase, *Cell*, 154 (2013) 391-402.
45. J. Thiery, D. Keefe, S. Saffarian, D. Martinvalet, M. Walch, E. Boucrot, T. Kirchhausen, J. Lieberman, Perforin activates clathrin- and dynamin-dependent endocytosis, which is required for plasma membrane repair and delivery of granzyme B for granzyme-mediated apoptosis, *Blood*, 115 (2010) 1582-1593.
46. D. Martinvalet, Mitochondrial Entry of Cytotoxic Proteases: A New Insight into the Granzyme B Cell Death Pathway, *Oxidative medicine and cellular longevity*, 2019 (2019) 9165214.
47. M.A. Kursunel, G. Esendagli, The untold story of IFN- γ in cancer biology, *Cytokine & growth factor reviews*, 31 (2016) 73-81.
48. H. Ikeda, L.J. Old, R.D. Schreiber, The roles of IFN gamma in protection against tumor development and cancer immunoediting, *Cytokine & growth factor reviews*, 13 (2002) 95-109.
49. J.K. Lee, T.J. Sayers, A.D. Brooks, T.C. Back, H.A. Young, K.L. Komschlies, J.M. Wigginton, R.H. Wiltout, IFN-gamma-dependent delay of in vivo tumor progression by Fas overexpression on murine renal cancer cells, *Journal of immunology (Baltimore, Md. : 1950)*, 164 (2000) 231-239.
50. C. Ruiz-Ruiz, C. Ruiz de Almodóvar, A. Rodríguez, G. Ortiz-Ferrón, J.M. Redondo, A. López-Rivas, The up-regulation of human caspase-8 by interferon-gamma in breast tumor cells requires the induction and action of the transcription factor interferon regulatory factor-1, *The Journal of biological chemistry*, 279 (2004) 19712-19720.

51. E. Pizzoferrato, Y. Liu, A. Gambotto, M.J. Armstrong, M.T. Stang, W.E. Gooding, S.M. Alber, S.H. Shand, S.C. Watkins, W.J. Storkus, J.H. Yim, Ectopic expression of interferon regulatory factor-1 promotes human breast cancer cell death and results in reduced expression of survivin, *Cancer research*, 64 (2004) 8381-8388.
52. J. Barthson, C.M. Germano, F. Moore, A. Maida, D.J. Drucker, P. Marchetti, C. Gysemans, C. Mathieu, G. Nuñez, A. Jurisicova, D.L. Eizirik, E.N. Gurzov, Cytokines tumor necrosis factor- α and interferon- γ induce pancreatic β -cell apoptosis through STAT1-mediated Bim protein activation, *The Journal of biological chemistry*, 286 (2011) 39632-39643.
53. M. Stawowczyk, S. Van Scoy, K.P. Kumar, N.C. Reich, The interferon stimulated gene 54 promotes apoptosis, *The Journal of biological chemistry*, 286 (2011) 7257-7266.
54. R. Besch, H. Poeck, T. Hohenauer, D. Senft, G. Häcker, C. Berking, V. Hornung, S. Endres, T. Ruzicka, S. Rothenfusser, G. Hartmann, Proapoptotic signaling induced by RIG-I and MDA-5 results in type I interferon-independent apoptosis in human melanoma cells, *The Journal of clinical investigation*, 119 (2009) 2399-2411.
55. J. Wang, H. Min, B. Hu, X. Xue, Y. Liu, Guanylate-binding protein-2 inhibits colorectal cancer cell growth and increases the sensitivity to paclitaxel of paclitaxel-resistant colorectal cancer cells by interfering Wnt signaling, *Journal of cellular biochemistry*, 121 (2020) 1250-1259.
56. H. Li, L. Xing, N. Zhao, J. Wang, N. Zheng, Furosin Induced Apoptosis by the Regulation of STAT1/STAT2 and UBA7/UBE2L6 Genes in HepG2 Cells, *International journal of molecular sciences*, 19 (2018).
57. T. Skotland, N.P. Hessvik, K. Sandvig, A. Llorente, Exosomal lipid composition and the role of ether lipids and phosphoinositides in exosome biology, *Journal of lipid research*, 60 (2019) 9-18.

Figures

Figure 1

Figure 1

Characterization of caCD8 EVs A) Schematic representation of caCD8 cultures and the collection of caCD8-EVs. CD8+ T cells were sorted from PBMC and stimulated with anti-CD3 mAb and a combination of cytokines. After 12–15 days, the supernatants were collected for EVs preparation. B) The morphology of caCD8- EVs was detected using transmission electron microscopy (left) and scale bars were shown. The size of EVs was measured using NTA, where the peak of particles was observed at an 88 nm

diameter (right). C) Western blot analysis of EVs related markers (HRS, Alix, TSG101 and CD63) and a cells marker (GM130). β -actin was used as a loading control. D-E) Immunophenotypic and checkpoint molecule analysis of caCD8 cells or caCD8-EVs linked to latex beads (4 μ m diameter). The black area of the histograms corresponds to the isotype controls of the respective antibodies, and the red line indicates positive fluorescence.

Figure 2

Figure 2

Cytotoxicity of caCD8 EVs in vitro and in vivo A) Anti-proliferative activity of caCD8-EVs against breast and lung cancer cells. Cell lines were incubated with vehicle (DPBS) or caCD8-EVs (200 µg/ml) for 96 h. Cell viability was measured using a CellTiter-Glo Luminescent assay. Relative cell viability in the presence of EVs was normalized to the vehicle-treated controls after background subtraction. The horizontal dotted red line shows 50% growth. Two non-cancerous cell lines, MCF10A and BEAS-2B, were used as controls. B) CaCD8-EVs exert cytotoxic effects in a time-dependent manner. Cell lines were incubated with vehicle or caCD8-EVs (200 µg/ml). Cell viability was measured at 24 h, 48 h, 72 h and 96 h. Cell growth curves were drawn at the indicated days. C) Cancer cells were treated with purified caCD8-EVs (200 µg/ml) from six donors. Other EVs derived from different cancer cells, MCF-7, H520, HH (cutaneous T cell lymphoma) and liposomes were chosen as controls. Cell viability was measured after 96 h. D) Liposomes were analyzed by transmission electron microscope and NTA. E) A pre-treatment mouse model was used to evaluate the antitumor effects of caCD8-EVs. Tumor cells (2×10^5) were pre-treated with liposomes or caCD8-EVs (200 µg/ml) in vitro for 48 h and then inoculated subcutaneously into bilateral flanks of mice, respectively. Tumor volumes were measured at the indicated days and tumor growth curves were drawn. F) A therapeutic mouse model was used to evaluate the antitumor effect of caCD8-EVs. Tumor cells (5×10^5) were inoculated subcutaneously in one flank. Once tumor masses grew to about 30 mm³ (approximately one week), mice were intravenously injected with vehicle (DPBS, 100 µl), liposomes or caCD8-EVs (200 µg, 100 µl) every two days for two weeks. Tumor volumes were measured at the indicated days and tumor growth curves were drawn. *p < 0.05, **p < 0.01. ***p < 0.001.

Figure 3

Figure 3

CaCD8 EVs show enhanced binding to dividing cells A) Representative dynamic images (Live Cell Imaging System) of H520 cell uptake of CFSE-labeled caCD8-EVs (100 $\mu\text{g/ml}$). Scale bar, 50 μm . B) The intensity of fluorescence was calculated using Image J software. C) Tumor cells (MCF-7, H520) and non-cancerous cells (MCF-10A, BEAS-2B) were cultured in vitro for 48 h with caCD8-EVs (100 $\mu\text{g/ml}$) or vehicle. Flow cytometry detection of CD3 positive cells. Top, representative images from flow cytometry,

bottom, statistical analysis. D) Cell proliferation was evaluated using cell number count. **p < 0.01.***p < 0.001.

Figure 4

Figure 4

Apoptosis of cancer cell induced by CaCD8-EVs and its protein bioinformatics analysis A) Tumor cells were treated with liposomes or caCD8-EVs (200 µg/ml) for 96 h. Flow cytometry was then performed for apoptosis detection. Left, representative images. Right, statistical analysis. B) Western blot for apoptosis

assessment. Western blot was conducted to detect cleaved caspase-3/caspase-9 and PARP following cell treatment with liposomes or caCD8-EVs. C) Western blot for perforin and granzyme B expression in caCD8 cells and caCD8-EVs. D) The expression of perforin and granzyme B in caCD8 cells and EVs were quantified by LFQ analysis of mass spectrometry. E) Effect of RNA derived from caCD8-EVs on cancer cells. The tumor cells were seeded into 96-well plates and then transfected with the RNA extracted from caCD8-EVs (200 µg/ml). Following 96 hours of culture in vitro, cell viability was measured using a CellTiter-Glo Luminescent assay. Vehicle (RNAiMAX, ThermoFisher) was used as controls. RNA extraction was performed by TRIZOL LS (ThermoFisher). F) MCF-7 cells were treated with liposomes or caCD8-EVs (200 µg/ml) for 48 h. Mass spectrometry identified differential gene expression. Compared with liposomes-treated cells, a total of 176 proteins were newly expressed and 72 proteins were upregulated (> 4×) in caCD8-EVs-treated cells (positive proteins). A total of 540 proteins were not expressed and 125 proteins were downregulated in caCD8-EVs-treated cells. There were 2283 co-expressed proteins in both liposomes- and EVs-treated cells. G) GO classification was performed for BP analysis of the positive proteins. The graph showed the top 10 biological processes. H) STRING analysis for protein-protein interactions of the positive proteins. **p < 0.01. ***p < 0.001.

Figure 5

Figure 5

IFN γ derived from caCD8-EVs partially induce tumor cell cytotoxicity A) Heatmap for differential proteins identified by mass spectrometry between caCD8-EVs and caCD8 cells. B) The graph showed the representative differential proteins. C) Western blot analysis was performed to measure the presence of IFN γ in caCD8-EVs. D) Cytotoxic activity induced by caCD8-EVs was partially reversed by blocking the IFN γ pathway. IFN γ Ab, IFN γ blocking antibody, Rux, JAK1/2 inhibitor, Ruxolitinib. MCF-7 and H520 cells

were treated with caCD8-EVs (200 µg/ml) in the presence of IFN γ Ab (10 µg/ml) or Rux (0.6 µM) for 96 h. Cell viability was then detected and calculated. E) Several ISG expressions were detected by qPCR following treatment of MCF-7 cell with caCD8-EVs (200 µg/ml) for 48 h. IFN γ Ab (10 µg/ml) or Rux (0.6 µM) were added to block the IFN γ pathway. Vehicle and liposomes were used as controls. F) Flow cytometry detection of apoptosis induced by IFN γ derived from caCD8-EVs. Cells were treated as Figure 5D. Vehicle was used as controls. Left, representative images of MCF-7 cell, Right, statistical analysis. G) Western blot analysis of apoptosis induced by IFN γ derived from caCD8-EVs. Cells were treated as Figure 5D. Vehicle was used as controls. **p < 0.01.***p < 0.001.

Figure 6

Figure 6

Cancer cells release and share EV-derived IFN γ A) Schematic of ELISA to measure the IFN γ concentration on the surface of caCD8 EVs. ELISA plates were coated with by different capture antibodies (IFN γ , HLA, CD3 Ab), respectively. When the EVs were captured, the molecules on the surface were detected by test antibodies. B) ELISA of IFN γ on caCD8 EVs. Testing HLA and CD3 were for positive controls. C) For IFN γ release and sharing assays, one cohort of cancer cells was treated with caCD8-EVs (200 μ g/ml), washed,

and co-cultured with untreated receptor cells (CFSE-labeled). P-STAT1 in receptor cells was measured using flow cytometry. IFN γ Ab (10 μ g/ml) or Rux (0.6 μ M) were added to block the IFN γ pathway. D) Illustration of the mechanism of caCD8-EVs induction of cancer cells cytotoxicity. GZMB, granzyme B, PFN, perforin. **p < 0.01. ***p < 0.001.

Supplementary Files

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