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Evaluating the Influence of Different Serum-Free Culture Conditions on the Production and Function of Natural Killer Cell-Derived Extracellular Vesicles

Yunjie Wu[1] | Héloïse Chollet[2] | Amanda Sudworth[1] | Marit Inngjerdingen[1]

[1]Department of Pharmacology, Institute of Clinical Medicine, University of Oslo, Oslo, Norway | [2]Université Côte d'Azur, Nice, France

Correspondence: Marit Inngjerdingen (mariti@medisin.uio.no)

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ABSTRACT

Natural killer (NK) cells are exploited in cellular therapies for cancer. While NK cell therapies are efficient against haematological cancers, it has been difficult to target solid tumours due to low tumour infiltration and a hostile tumour microenvironment. NK-cell derived extracellular vesicles (NK-EVs) target and kill cancer cells in vitro and represent an alternative treatment strategy for solid tumours. To exploit their potential, it is necessary to standardize NK-EV production protocols. Here, we have performed a comparative analysis of EVs from the human NK-92 cell line cultured in five serum-free commercial media optimized for growth of human NK cells and one serum-free medium for growth of lymphocytes. The effect of growing the NK-92 cells in static cell cultures versus shaking flasks was compared. EVs were purified via ultracentrifugation followed by size-exclusion chromatography. We found that there were no significant differences in EV yield from NK-92 cells grown under static or dynamic conditions. However, we found clear differences between the different culture media in terms of EV purity as assessed by the enrichment of the CD63 and CD81 markers in the isolates that translated into their capacity to induce apoptosis of the colon cancer cell line HCT 116. These findings will be instructive for the design of future production protocols for therapeutic NK-cell derived EVs.

1 | Introduction

Natural killer (NK) cells are cytotoxic lymphocytes that are critical for killing infected and malignant cells. They kill through the release of granules containing cytolytic proteins such as perforin and granzymes, or via death receptors (Lanier 2024). NK cells recognize malignant cells via a balance of activating and inhibitory input, where the expression of major histocompatibility complex I normally protects cells from NK-cell mediated killing. Up-regulation of diverse stress-related proteins will engage activating receptors and initiate target cell killing if the inhibitory input is sufficiently low (Lanier 2024). Due to their natural ability to kill malignant cells while sparing healthy cells, NK cells are increasingly exploited in cancer therapies. As therapeutic products, either primary, allogeneic NK cells or the FDA-approved NK cell line NK-92 are used (Laskowski et al. 2022). NK cells have classically shown the best effect against haematological tumours, but their efficacy against solid tumours is now exploited with diverse CAR-based approaches (Kloess et al. 2019). A current limitation of their applicability for solid tumours is the hostile tumour microenvironment that suppresses the activity of cytotoxic immune cells, exemplified by the hypoxic, immunosuppressive, and acidic environment (Seliger and Koehl 2022). While these limitations in cell therapies can be overcome with improved knowledge on how to circumvent these obstacles (Ni et al. 2020), targeting of solid tumours may also be feasible

using extracellular vesicles (EVs) derived from NK cells (Farcas and Inngjerdingen 2020).

EVs have shown promise as therapeutic modalities in many diseases, although there are still several hurdles related to EV standardization, in vivo bioavailability and tissue targeting that need resolving before they can be fully exploited clinically (Lener et al. 2015; Borrelli et al. 2018; Giebel et al. 2017). Several studies have shown that NK-EVs can induce cancer cell apoptosis in vitro (Lugini et al. 2012; Jong et al. 2017; Cochran and Kornbluth 2021; Aarsund et al. 2022; Enomoto et al. 2022), and some studies have also reported tumour regression in vivo upon iv injection of NK-EVs in mouse models (Cochran and Kornbluth 2021; Wang et al. 2019). NK-EV studies have utilized either primary, peripheral blood NK cells or the human NK-92 cell line as a source for NK-EVs, and the cells are grown in serum-free medium with diverse cytokine supplements for 24–48 h. Most studies have utilized bulk EVs isolated via ultracentrifugation (UC), size-exclusion chromatography (SEC), or precipitation. We previously reported that a subset of cytolytic NK-EVs can be enriched via UC followed by density-gradient UC (Aarsund et al. 2022), indicating that refined methods for EV separation should be considered in a therapeutic context. The exact mechanism of action of NK-EVs is still not clear, but due to their content of perforin, granzymes, and miRNA, it is likely that these mediators contribute to the observed apoptotic activities (Lugini et al. 2012; Enomoto et al. 2022; Aarsund et al. 2022; Fabbri 2020; Wu et al. 2019), although it remains to be determined how this cargo is delivered to the cytosol of the target cells. NK-EVs appear to be taken up via endocytosis, and blocking interaction of NK cell receptors NKG2D or DNAM-1 with their ligands is shown to reduce the level of NK-EV mediated apoptosis (Aarsund et al. 2022; Di Pace et al. 2020).

In order to progress towards therapeutic exploitation of NK-EVs, it is necessary to develop robust and reproducible production protocols that yield high numbers of functional EVs. Several strategies to enhance the production yield of NK-EVs are already reported, including bioreactor based approaches such as the seesaw-motion bioreactor system by leveraging mechanical stimuli (Wu et al. 2022) and the hollow-fibre bioreactor (St-Denis-Bissonnette et al. 2023). Another hurdle is the lack of knowledge regarding optimal culture media conditions that affect EV yield and quality. Several studies have indicated that the media in which cells are cultured can significantly influence their metabolic activity (Patel et al. 2017; Palviainen et al. 2019) and, consequently, the quantity and the molecular composition of the EVs they produce (Li et al. 2015). Currently, the field lacks a comprehensive analysis of how different media conditions specifically affect NK-EV production. The culturing protocols used for producing NK-EVs are highly variable. Donor cells (primary NK cells, NK-92 cells, or NK3.3 cells) have been cultured in either RPMI-1640 with 10% exosome-depleted FBS (Lugini et al. 2012; Jong et al. 2017; Cochran and Kornbluth 2021; Wu et al. 2022; Neviani et al. 2019), Advanced RPMI-1640 (Enomoto et al. 2022), serum-free AIM V (Aarsund et al. 2022; Aarsund et al. 2022), or serum-free/xeno-free ImmunoCult-XF T cell Expansion Medium (St-Denis-Bissonnette et al. 2023) in the presence of cytokines that enhance NK cell activation and proliferation. Several different serum-free and xeno-free culture media tailored specifically for human NK cells have been developed in recent years to support the growing interest in NK cell therapies and the need for GMP-grade, efficient expansion of highly functional NK cells.

In this paper we have systematically tested and compared five different commercial serum-free and xeno-free cell culture media to determine an optimal medium for the production of NK-EVs and compared it against a more broadly tailored serum-free media. We chose the NK-92 cell line as donor cells to reduce the variability that would be seen using primary NK cells of different donor origins. We further compared the effect of culturing NK-92 cells under standard static conditions with shaking conditions in order to test whether dynamic culturing would induce a higher rate of EV release. NK-EVs were purified via UC followed by SEC, and EV yield, physiochemical characteristics, and anti-apoptotic activity were compared. Our study provides critical insights for the development of scalable EV-based strategies to accelerate the application of NK cell-derived EVs as cancer therapy.

2 | Materials and Methods

2.1 | Cell Cultures

NK-92 cells (ATCC CRL-2407) were maintained in Advanced RPMI 1640 (ThermoFisher) supplemented with 20% heat inactivated FBS (Merck), 100 U/mL penicillin-streptomycin (ThermoFisher), 1× GlutaMAX supplement (ThermoFisher), 50 nM 2-mercaptoethanol (ThermoFisher), and human recombinant IL-2 (R&D Systems) at 50 ng/mL. The cells were maintained at a density of 0.5 million cells/mL. For EV production, NK-92 cells were spun at 350 × *g* for 8 min, and washed once in PBS prior to culturing in serum-free media. NK-92 cells (5×10^7 cells) were resuspended in 65 mL of serum-free media with 10 ng/mL of recombinant human IL-15 (R&D Systems) and cultured for 48 h. Six different serum-free media optimized for culturing of NK cells were tested: CellGenix GMP SCGM (xeno-free, Sartorius [abbreviated GMP SCGM]), CTS NK-Xpander Medium (xeno-free, ThermoFisher [abbreviated CTS]), Immunocult NK Cell Base Medium (xeno-free, StemCell Technologies, abbreviated Immunocult), ExCellerate Human NK Cell Expansion Media, Xeno-Free (R&D Systems, abbreviated ExCellerate XF), and ExCellerate Human NK Cell Expansion Media, Animal Component Free (R&D Systems, abbreviated ExCellerate ACF). Additionally, AIM V (ThermoFisher) which is formulated for serum-free culture of lymphocytes and has been previously used in our research, was used. All cultures were in parallel set up as static cultures or as a shaking culture. For the shaking cultures, a CO_2 resistant shaker platform (ThermoFisher) was set to 125 rpm, and cells were cultured in conical Corning cell-culture Erlenmeyer flasks with ventilated caps (125 mL flasks, Merck). Four separate biological experiments were performed. For assessment of viability, samples of NK-92 cells were taken at 48 h, and the cells were pelleted in an Eppendorf centrifuge, pellets resuspended in 200 µL PBS with 2% FBS, and propidium iodide. The samples were acquired by flow cytometry (LSRFortessa, BD Biosciences) and analysed by the FlowJo v10.8.1 software (TreeStar).

2.2 | NK Cell Phenotyping

Aliquots of NK-92 cells were harvested after 48 h in serum-free cultures under either shaking or static conditions, and stained (0.5×10^6 cells/sample) in 100 µL PBS with 2% FBS with anti-CD56 (1:200; B159-AF647, BD Biosciences) for 20 min at 4 C in the dark, washed three times in PBS with 2% FBS, and analysed using flow cytometry (LSRFortessa, BD Biosciences) and FlowJo v10.8.1 software (TreeStar).

2.3 | EV Isolation by UC and SEC

Cell cultures were harvested and spun at 2000 × *g* for 30 min. The supernatants were then ultracentrifuged using the SW41Ti rotor at 38,500 rpm (180,000 g avg) in a Beckman Coulter Optima LE-80K for 2 h and 15 min (k-factor 124, complete sedimentation 'cut-off' size 50 nm). The crude EV pellets were resuspended in 500 µL of Dulbecco's Phosphate Buffered Saline (DPBS, Sigma-Aldrich), protein was measured via Nanodrop, and the EVs were further purified through SEC using qEV columns (35 nm, Izon) using 500 µL of EV isolate as input. Thirty fractions of 400 µL were separated and collected using the qEV automatic fraction collector (Izon) based on gravity, following the manufacturer's instructions. The absorbance of each fraction at 280 nm was measured using a Nanodrop Spectrophotometer (ThermoFisher) to determine protein concentration and EV distribution. Fractions containing EVs (F7-F11) were combined and concentrated using Amicon Ultra 0.5 mL centrifugal filters (10 kDa MWCO, Merck). The EV concentrates were brought to 100 µL, and the protein concentration was measured by both Nanodrop and BCA (bicinchoninic acid) assay (ThermoFisher), and the samples were frozen at −80°C for downstream experiments.

2.4 | Transmission Electron Microscopy

Purified NK-EVs (10 µg) were added on top of a formvar carbon-coated copper grid for 1 h. Excess fluid was removed by blotting with filter paper. The grids were rinsed by dipping in PBS three times and dried using filter paper. The grid with EVs was further fixed by adding a drop of 2.5% glutaraldehyde before washing the grids five times with distilled water and contrasted by adding a drop of 2% uranyl acetate. Finally, the grids were rinsed quickly with ice-cold 1.8% methylcellulose and 0.4% uranyl acetate (MC/UA). The grids were air-dried for 20 min and examined using an FEI Tecnai 120 kV transmission electron microscope G2 Spirit TEM (FEI, The Netherlands) equipped with a Morada digital camera and RADIUS imaging software.

2.5 | Western Blotting

EVs (40 µg) were mixed with 10× RIPA buffer and incubated for 5 min on ice, supplemented with 4× SDS sample buffer and boiled at 90°C for 5 min. The samples were run on 12% SDS-PAGE Criterion gels (Bio-Rad) under non-reducing conditions. After transfer onto PVDF membranes (ThermoFisher Scientific), membranes were blocked with 5% dry milk and incubated overnight at 4°C with primary antibodies against CD63 (Ts63, 1:500) and CD81 (M38, 1:500) from ThermoFisher Scientific, and against granzyme B (#2103A, 1:500) from R&D Systems. Blots were probed with goat anti-rabbit IgG-HRP or goat anti-mouse IgG-HRP (Bio-Rad) and developed by Pierce ECL Western blotting Substrate (ThermoFisher).

2.6 | Nanoparticle Tracking Analysis

EV media from different conditions were diluted in PBS to yield a target concentration within 10^8 particles and were analysed using a Nanosight NS500 nanoparticle tracking analyser with a 488 nm-laser (Malvern Panalytical). Samples were analysed under constant flow conditions (flow rate = 20) at 25°C, and 3 × 60-s videos were captured. Data were analysed using NTA software with a detection threshold of 5 and bin size of 2, and the different dilutions were taken into account. Three biological replicates were measured.

2.7 | Phenotypic Bead Assay

Ten micrograms of the EV isolates were incubated with 1 µL of aldehyde/sulphate/latex beads (1/200 in PBS, ThermoFisher Scientific) in 250 µL of DPBS overnight at room temperature with gentle rotation. As a negative control, beads were incubated in DPBS alone. Next, 25 µL of 1 M glycine were added to a final concentration of 100 mM, the samples incubated another 30 min at room temperature, and centrifuged at 2400 × *g* for 5 min. The beads were washed three times with PBS and 0.5% FBS. Samples were stained for 20 min at 4°C in 50 µL PBS and 0.5% FBS with either PE-conjugated mouse IG1 isotype (BD Biosciences), 5 µL anti-CD63-PE (H5C6, ThermoFisher) and 5 µL anti-CD81-PE (TAPA-1, BioLegend). Samples were washed twice with PBS and 0.5% FBS, and analysed by flow cytometry (LSRFortessa, BD Biosciences) and FlowJo v10.8.1 software (TreeStar).

2.8 | Apoptosis Assay

The colorectal carcinoma cell line HCT 116 (ATCC CCL-247) was used as a target cell for measuring NK-EV-induced apoptosis. The cells were maintained in complete RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin-streptomycin (ThermoFisher), and 50 nM 2-mercaptoethanol (ThermoFisher). Cells were seeded at 20,000 cells/well in 96-well flat bottom plates 24 h prior to the assay, and treated with 40 µg of EVs from the different conditions for 36 h in a humidified cell incubator at 37°C. PBS alone was used as a negative control. The samples were incubated together with 2.5 µM of Caspase-3/7 Green Detection Reagent (ThermoFisher). At the end of the assay, the cells were isolated and analysed for caspase 3/7 activity via flow cytometry (LSRFortessa, BD Biosciences).

2.9 | Statistical Analysis

All data are expressed as mean ± SEM of at least three independent experiments using GraphPad Prism. Statistical analyses were performed using the Friedman one-way ANOVA test.

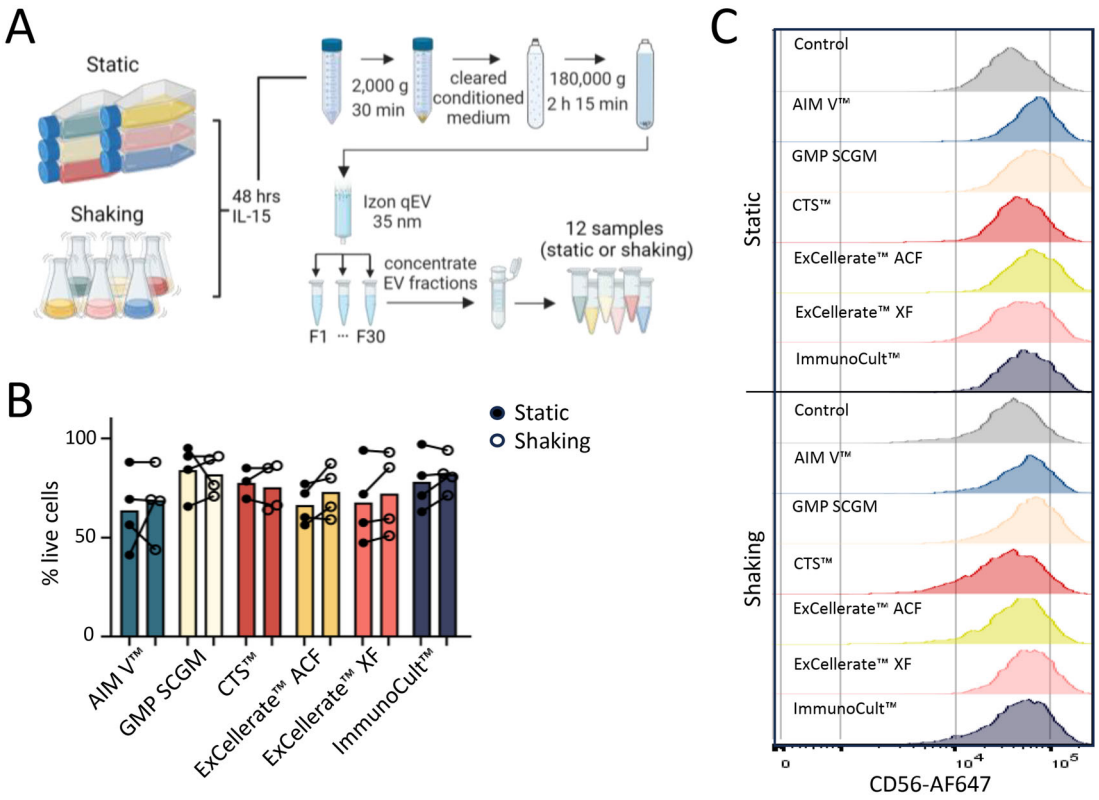

FIGURE 1 | NK cell characteristics after different serum-free culture conditions. (A) Workflow for static and shaking NK-92 cultures, and purification of EVs. (B) Viability of NK-92 cells after 48 h of culture in serum-free media under static or shaking conditions supplemented with IL-15 was assessed by propidium iodide by flow cytometry. Control samples were NK-92 cells grown in Advanced RPMI medium with 20% FBS. Data are presented as the mean ± SD of four separate experiments. Statistical analysis performed using the Friedman one-way ANOVA test indicated no statistical difference between the different culture conditions. (C) Expression levels of CD56 on NK-92 cells after 48 h of culture in serum-free media under static or shaking conditions. One representative experiment of three.

2.10 | EV-TRACK

We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV240140) (Van Deun et al. 2017).

3 | Results

3.1 | Comparing NK-92 Cell Viability and Characteristics After 48 h of Culture in Different Serum-Free Culture Conditions

Five different culture media optimized for serum-free and xeno-free growth of NK cells were chosen from four different manufacturers. AIM V, a specialized serum-free medium for lymphocytes, has been used in our previous research, and was also included. Cells were grown either as standard static cultures or in specialized Erlenmeyer flasks for cell culturing, and EVs were isolated as indicated in the experimental workflow outlined in Figure 1A. Initial viability optimization experiments determined that the NK-92 cells tolerated shaking at 125 rpm (data not shown). Upon 48 h of culture in the different media supplemented with IL-15, we did not observe any difference in viability under static or shaking conditions (Figure 1B). The viability was comparable across the five media specialized for NK cell cultures and the AIM V medium. We next evaluated by flow cytometry whether the different media and culture conditions affected the phenotype of the NK-92 cells, and showed that the expression levels of the NK-cell marker CD56 were comparable across all samples (Figure 1C). This indicates that the phenotypic integrity of NK-92 cells was maintained regardless of the culture medium used.

3.2 | Serum-Free Media Tailored for NK Cells Results Differ in Their EV Yields and Soluble Protein Contamination

We next examined the effect of the different media conditions on NK-EV production. NTA analysis was performed on pre-cleared culture supernatants, and showed that the EVs predominantly fell within the 150–200 nm median size range, and there were no large differences between media or culture conditions (Figure 2A). The simultaneously measured particle concentrations, however, varied across the different culture conditions. There were no statistical differences between static and shaking conditions, with the lowest particle yield observed with AIM V media for cells grown in static conditions (Figure 2B), and with a tendency for higher particle counts from NK cells grown in the NK-cell formulated media under shaking conditions compared to static conditions.

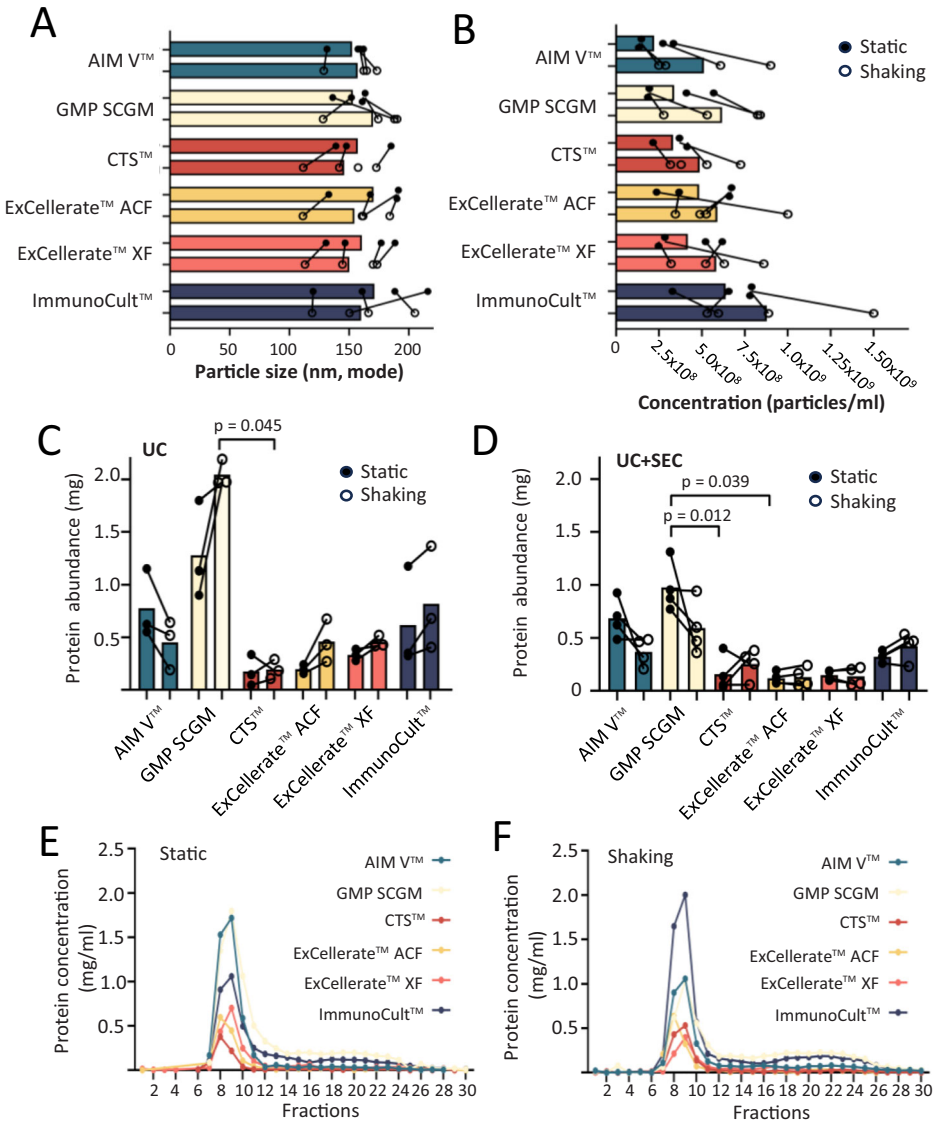

FIGURE 2 | EV isolate concentrations obtained after NK-92 cell cultures in different serum-free media conditions. (A) Particle size or (B) particle concentrations measured by NTA analysis of pre-cleared culture media. Data are presented as the mean ± SD of four separate experiments. Statistical analysis performed using the Friedman one-way ANOVA test indicated no statistical difference between the different culture conditions. Protein concentration of EV isolates obtained after ultracentrifugation (C) or after additional purification by SEC (D) from indicated NK-92 culture conditions measured via NanoDrop spectrophotometer or BCA assay, respectively. Data are presented as the mean ± SD of four separate experiments. Statistical analysis was performed using the Friedman one-way ANOVA test. (E)–(F) Protein concentration measurements of 30 SEC fractions obtained when purifying ultracentrifuged EV isolates from indicated cultures. One representative experiment of four. SEC, size-exclusion chromatography.

To further estimate the yield of EVs, crude EVs were isolated by UC, and protein concentration was measured. As shown in Figure 2C, the crude EV isolates derived from AIM V, GMP SCGM, and ImmunoCult media contained higher levels of proteins compared to CTS, ExCellerate XF, and ExCellerate ACF media. Except for AIM V and CTS, there were tendencies in each biological experiment for higher protein abundance in samples from shaking conditions. After a second SEC-purification step to remove soluble protein contaminants (Figure 1A), the protein abundance was measured via BCA, and the total protein measured was more similar between static and shaking conditions, and for the GMP SCGM medium, protein abundance was dramatically reduced for the shaking condition (Figure 2D). The protein concentration distribution of each SEC fraction as measured via Nanodrop is shown in Figures 2E and 2F. Two distinct peaks were observed: the first peak corresponds to EVs, and the second peak indicates soluble protein contaminants. The second peak was more pronounced for fractions obtained with the GMP SCGM and ImmunoCult media, consistent with higher initial protein concentrations.

3.3 | Similar EV Morphology in EV Isolates From the Different Serum-Free Culture Conditions

Morphological analysis of the EVs using TEM revealed no large differences in EV structure across the different media conditions with vesicles in the range 50–200 nm in size. Electron-dense

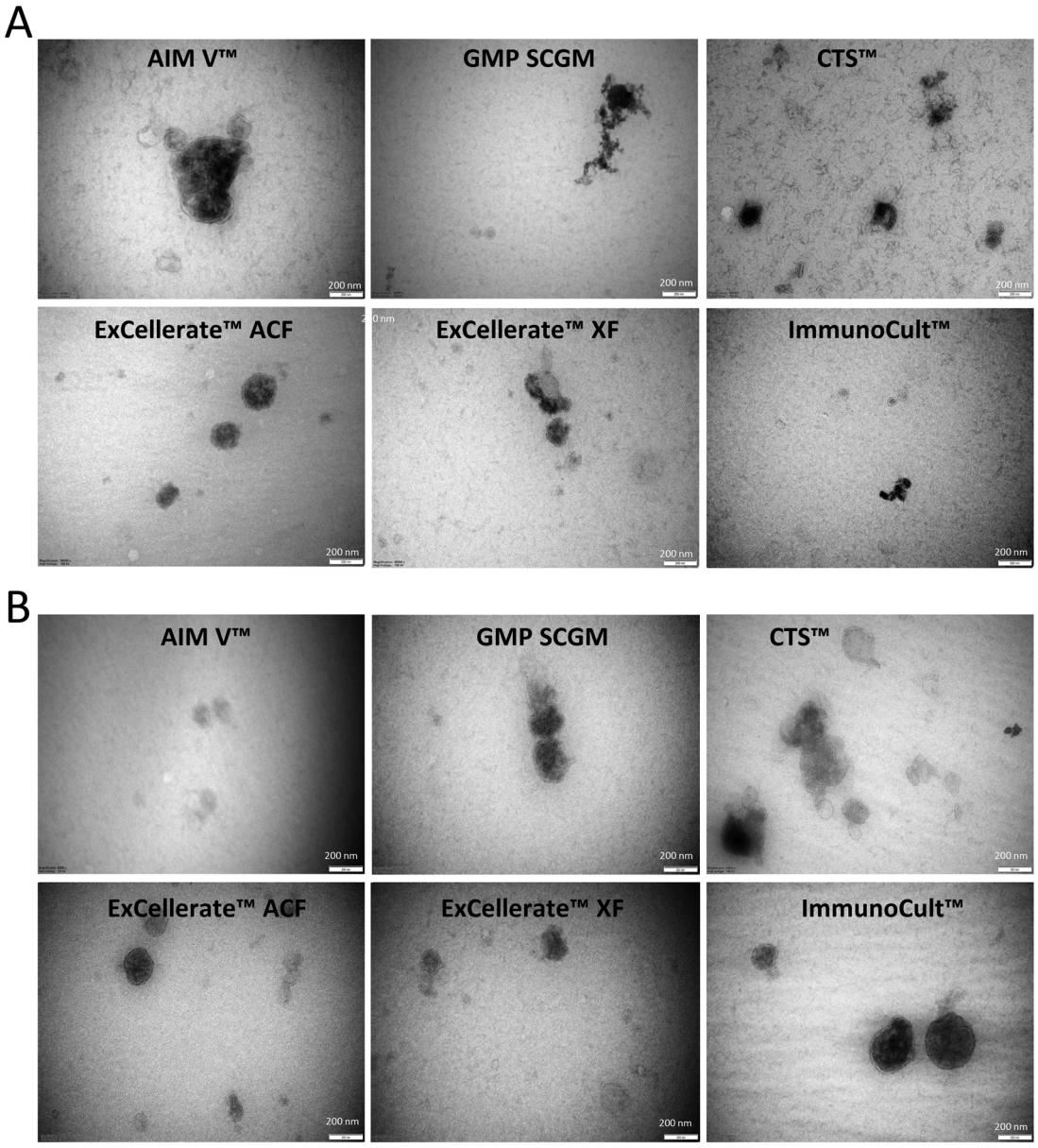

FIGURE 3 | TEM images of purified EVs derived from different serum-free NK cell culture media. (A) TEM images of EVs obtained from static culture conditions. (B) TEM images of EVs obtained from shaking culture conditions. Scale bar 200 nm. Images are representative of 8–10 images from each sample.

EVs in the size-range 150–200 nm were found in all isolates, as previously reported for NK-EVs (Figures 3A and 3B) (Aarsund et al. 2022; Federici et al. 2020). Also, smaller 50–100 nm, translucent cup-shaped vesicles were present in all isolates. No significant impact on EV morphology was therefore evident with either culture condition.

3.4 | Enrichment of Functional CD63+ Granzyme B+ EVs From NK-92 Cultures in ExCellerate and CTS

We next assessed EV phenotype via analysing the presence of tetraspanins CD63 and CD81. Equal amounts of protein from the different EV isolates obtained after SEC were coupled to beads and assessed for expression levels of the tetraspanins via flow cytometry. Surprisingly, we found high levels of CD63 and CD81 on EV isolates obtained from cell cultures in ExCellerate ACF, followed by Excellerate XF and CTS, with low levels detected in the other isolates (Figure 4A). This was further confirmed by Western blotting (Figure 4B), which showed strong bands for CD63 in EVs obtained from both ExCellerate and CTS conditions compared to the other three conditions, indicating that these isolates may contain a higher enrichment of EVs. CD81 levels appeared comparable across isolates. Granzyme B is a central cytotoxic mediator found in NK-EVs, and we further show that this marker appears enriched in EV isolates from the CTS and ExCellerate XF media (Figure 4B).

Finally, we assessed the functional activity of the isolated EVs by testing their apoptotic effects on HCT 116 cancer cells. Forty micrograms of each EV isolate was applied to the cancer cells, and caspase 3/7 activity were measured after 36 h of culture. EVs

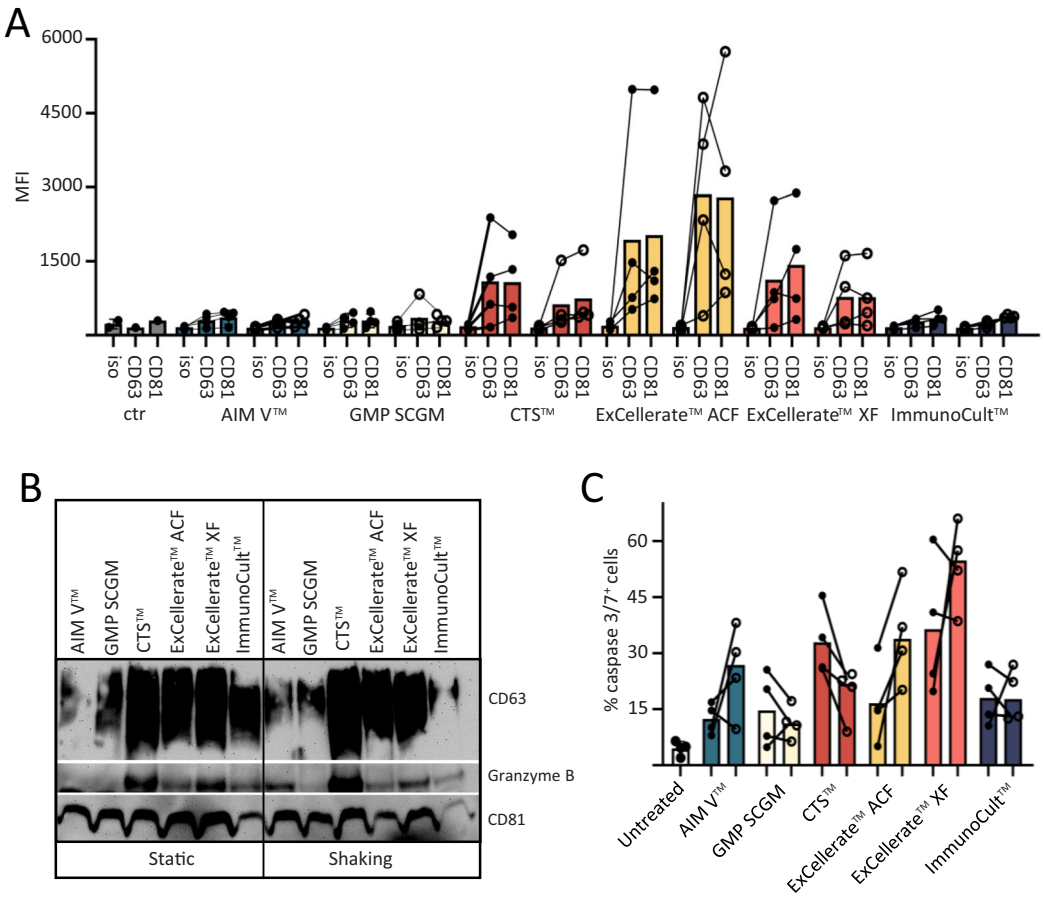

FIGURE 4 | EV phenotyping and functional analysis. (A) Equal amounts of EVs were captured on aldehyde/sulphate latex beads and stained with either isotype control antibody, anti-CD63 or anti-CD81 antibodies, and expression levels measured by flow cytometry. Empty beads were included as a negative control (ctr). Data are presented as the mean ± SD of four separate experiments. Statistical analysis performed using the Friedman one-way ANOVA test indicated no statistical difference between the different culture conditions. (B) Western blot analysis using 40 μg of indicated EV isolates. Representative of three independent experiments. (C) Measurement of apoptosis in HCT 116 cancer cells after 36 h as detected with Caspase 3/7 green detection reagent, using 40 μg purified EVs from the indicated NK-92 culture conditions. Data are presented as the mean ± SD of four separate experiments. Statistical analysis performed using the Friedman one-way ANOVA test indicated no statistical difference between the different culture conditions.

derived from both ExCellerate media formulations and from the CTS media exhibited the highest apoptotic activity (Figure 4C). There were no significant differences in the activity of EVs obtained from either static or shaking conditions, although for some samples there was a small trend for more active EVs obtained from shaking conditions in the case of the ExCellerate and AIM V media, and the opposite for the CTS medium.

4 | Discussion

There is a high need for novel immunotherapeutic strategies to treat cancer. NK-EVs may be one such treatment modality, given their innate capacity to target cancer cells and their potential unperturbed function in a hostile tumour microenvironment. In order to develop NK-EVs as a therapeutic product, it is critical to define optimal culture conditions for NK cells that result in high yields of functional EVs. Here, we have tested five different commercial serum-free and xeno-free culture media that are specifically manufactured for GMP-grade culture of human NK cells. The five media were compared against the traditional serum-free medium AIM V, which is tailormade for serum-free cultures of human lymphocytes, and that we have previously used for detailed characterizations of NK-EVs (Aarsund et al. 2022; Aarsund et al. 2022). We report that there are considerable differences in the yield and purity of EVs isolated from the different NK-92 cell cultures.

As expected, the viability of the NK-92 cells was comparable between the five NK-cell specialized serum-free media after 48 h of culture, and lower in the AIM V medium. This probably reflects the efforts to formulate media specially tailored towards NK cells. CD56 expression levels were similar, indicating that the different serum-free media did not impact the NK-92 phenotype.

The NK-92 cells were subjected to dynamic culture conditions in the shaking cultures, with the rationale that the shaking process could result in higher EV production due to the mechanical stimulus, as previous reports have indicated that dynamic cultures can increase EV yield (Cha et al. 2018). Although a report using a seesaw bioreactor reported high yields (Wu et al. 2022), this was not directly compared to a standard static NK-92 cell culture.

The physiological context of NK-EV release is still unclear, and their release may well be relevant in more static conditions within tissues. We did not observe any statistical differences in NK cell viability, measured particle concentration, or protein abundance in static versus shaking cultures, but observed a trend for more particles and protein after UC in the shaking conditions in each biological replicate. This trend was levelled out after SEC-purification, indicating higher levels of protein and other smaller particles in the cell supernatants upon shaking. In this respect, culturing the NK cells under static conditions or at least less dynamic mixing could be the most advantageous. Morphologically, we did not observe any differences in EVs isolated from the different culture conditions. Phenotypically, all EV samples were positive for both CD63 and CD81, and negative for CD9 (data not shown), and the static or shaking cultures did not impact this distribution. This tetraspanin profile of NK-EVs has also previously been reported for NK-92 and primary NK-cell derived EVs (Cochran and Kornbluth 2021; Aarsund et al. 2022; St-Denis-Bissonnette et al. 2023).

The EV yield as measured by NTA was assessed directly from pre-cleared culture supernatants prior to UC. The rationale was to quantify particles in their naïve form, avoiding the problems of EV aggregation after UC and EV loss due to the purification protocol. The particle concentration was largely comparable across all samples, with a tendency for lower concentrations with the AIM V medium and higher with the ImmunoCult medium. The measured particle concentrations were not reflected by the protein concentration profiles obtained after UC and SEC. It was evident that there were fractionally more proteins present in EV isolations from the AIM V, SGM SCGM, and ImmunoCult media. We have no detailed information on the composition of the different media, as this is proprietary information, but it would not be surprising that some media formulations may lead to more soluble protein carry-over during the EV purification. We implemented a two-step purification scheme of UC followed by SEC, as we in pilot experiments observed large differences in measured protein concentrations after UC. In our study, EV enrichment was assessed with protein concentration measurements, and we observed an overall reduction in protein concentrations after the two-step procedure in all samples. Regardless, there were still higher protein levels detected in EV isolates derived from NK-92 cultures in AIM V, SGM SCGM, and ImmunoCult media after the two-step procedure, indicating that there was likely still soluble protein remaining with the isolated EVs.

Supporting this notion was the observation that there was lower EV activity and lower CD63 and CD81 content per microgram of EV isolates with AIM V, SGM SCGM, and ImmunoCult media. EVs isolated from ExCellerate and CTS media appeared to contain purer EV isolates, as demonstrated via the phenotypic bead-EV capture assay and Western blot where CD63 was highly enriched. Functionally, the most active EV samples were from the ExCellerate and CTS media cultures, reflective of the apparent higher enrichment of EVs in these isolates. This implies that while there may be similar amounts of EVs released into culture with the different media types, the media formulations impact the purity of the final EV product. Moreover, there may be qualitative differences in the generated EVs, as demonstrated with the apparent different levels of granzyme B detected between the different EV isolates, and there was a clear correlation between the level of detected granzyme B and the caspase activity induced by the EVs. We and others have previously indicated that granzyme B may be necessary for the observed apoptotic effects (Lugini et al. 2012; Cochran and Kornbluth 2021; Aarsund et al. 2022; Aarsund et al. 2022).

It is evident from the data that there is considerable heterogeneity of the measured parameters between each biological replicate, despite careful effort to keep the culture conditions the same for each of the four reported experiments. However, we did observe similar trends between each experiment, and some of the heterogeneity may also result naturally from natural variations in readouts for the different methods applied. This study also highlights the challenge of developing standardized protocols for developing batches of EVs with comparable profiles, as would be needed for further preclinical development. For upscaled production, it will be necessary to culture the donor cells under carefully controlled bioreactor conditions to monitor and control metabolic parameters, as changes in pH, metabolites, and nutrients will impact the final EV product (Palviainen et al. 2019). There are also studies indicating that EV purity may not necessarily translate into the most optimal function, as sometimes co-purified protein may be of importance (Takov et al. 2019). Several critical studies have been performed in the field of mesenchymal stem-cell derived EVs to define optimal EV preparations for therapeutic applications, addressing the need to control several parameters, including, but not limited to, cell confluency, harvesting time, physical culture conditions, and medium composition (Lener et al. 2015; Maumus et al. 2020; Tertel et al. 2023; Madel et al. 2023). These studies highlight continued efforts towards standardization, which will have to be tailored towards the particular donor cell type and possibly also the intended usage of the EVs.

In conclusion, this study provides a detailed comparison of six different serum-free and xeno-free media for NK-92 cell culture, highlighting their effects on cell viability, EV yield, composition, and functional activity. These findings are crucial for optimizing future production protocols for therapeutic EVs derived from NK-92 cells.

Author Contributions

Yunjie Wu: Conceptualization; formal analysis; methodology; supervision; validation; visualization; writing - original draft; writing - review and editing. **Héloïse Chollet**: Conceptualization; methodology; data curation; formal analysis; writing - original draft. **Amanda Robin Sudworth**: Conceptualization; data curation; formal analysis; methodology; validation; visualization; writing - original draft; writing - review and editing. **Marit Inngjerdingen**: Conceptualization; data curation; formal analysis; funding acquisition; methodology; project administration; supervision; visualization; writing - original draft; writing - review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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