

**Extracellular Vesicles Derived from Natural Killer Cells as a Therapeutic
Strategy for Triple-Negative Breast Cancer**

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Abstract

Natural killer cell-derived extracellular vesicles (NK-EVs) represent a promising new dual-class of cancer immunotherapeutics, combining the intrinsic cytotoxicity of NK cells and their immunomodulatory function with the advantages of a cell-free nanovesicle system. Their small size, stability, tumour tropism, and ability to cross biological barriers position them as attractive candidates for treating hard-to-treat malignancies, such as triple-negative breast cancer (TNBC). However, their translation into clinical settings requires establishing scalable manufacturing methods, validating potency assays, and deepening the mechanistic understanding of their immunomodulatory activity. This thesis, composed of three central studies, addresses these challenges. In Study 1, a Good Manufacturing Practice-compliant biomanufacturing workflow was developed using a closed-loop, perfusion-powered hollow-fibre bioreactor system to produce NK-EVs from the clinically relevant NK92-M1 cell line continuously. Under serum-free, xeno-free, and feeder-free conditions, this process generated large quantities of viable NK cells and clinical-grade NK-EVs while retaining cytotoxic effectors and pro-inflammatory cytokines. The resulting products exhibited potent cytotoxicity against leukemic cells with minimal off-target toxicity against healthy cells, providing a scalable platform for clinical-grade NK-EV production. In Study 2, a highly sensitive resazurin phenoxazine-based viability assay was validated as a potency assay to assess the cytotoxic function of NK-EVs. The assay reliably quantified NK-EV-mediated cytotoxicity against both suspension- and adherent-cancer models, demonstrating specificity & sensitivity, linearity & range, reproducibility, and stability. Importantly, NK-EV potency correlated with effector proteins and detected loss of function in degraded samples, establishing it as a robust analytical method for biotherapeutic development. In Study 3, the immunomodulatory capacity of NK-EVs was investigated using high-dimensional flow cytometry, single-cell RNA sequencing and functional assays. NK-EVs activated and reprogrammed immune subsets, including cytotoxic CD8⁺ T cells and NK cells, enhancing coordinated anti-cancer responses in both healthy donors and breast cancer patients. Collectively, this work establishes the foundation for translating NK-EVs into a novel cell-free immunotherapy for hard-to-treat cancers.

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Preface

This thesis is a collection of the following manuscripts, with data presented as follows:

Chapter 2: NK-EV biomanufacturing and product characterization

- St-Denis-Bissonnette, F., Cummings, S. E., Qiu, S., Stalker, A., Muradia, G., Mehic, J., Mediratta, K., Kaczmarek, S., Burger, D., Lee, S.-H., Wang, L., & Lavoie, J. R. (2023). A clinically relevant large-scale biomanufacturing workflow to produce natural killer cells and natural killer cell-derived extracellular vesicles for cancer immunotherapy. *Journal of Extracellular Vesicles*, 12, e12387. <https://doi.org/10.1002/jev2.12387>.

Chapter 3: NK-EV cytotoxicity assessment

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Chapter 4: NK-EV immunomodulation assessment

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Additional work performed during the thesis is presented in the appendix and briefly discussed in Chapter 1.8.

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Abbreviations

ANOVA	Analysis of variance
AO	Acridine Orange
APCs	Antigen-Presenting Cells
BBB	Blood-Brain Barrier
CAR	Chimeric Antigen Receptor
CCK8	Cell Counting Kit 8
CM	Conditioned medium
CMC	Chemistry Manufacturing and Controls
DC	Dendritic Cells
DMEM	Dulbecco's Modified Eagle Medium
DPBS ^{-/-}	Dulbecco's phosphate-buffered saline without Ca ²⁺ /Mg ²⁺
DOX	Doxorubicin
EC ₅₀	Half maximal effective concentration
ECS	Extracellular capillary space
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
EPR	Enhanced Permeability and Retention
ER	Estrogen Receptor
EVs	Extracellular Vesicles
E: T	Effector: target
FACS	Fluorescence-activated cell sorting
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FPLC	Fast Protein Liquid Chromatography
FRET	Förster resonance energy transfer
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GMP	Good Manufacturing Practice
GNLY	Granulysin
GzmB	Granzyme B
HER2	Human Epidermal Growth Factor 2
HFB	Hollow-Fibre Bioreactor
HLA	Human leukocyte antigen
ICH	International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
ICIs	Immune Checkpoint Inhibitors
IFN- γ	Interferon-gamma
IL	Interleukin
ISO	International Organization for Standardization
LDH	Lactate Dehydrogenase
MFI	Median fluorescent intensity
miR	MicroRNA
MISEV	Minimal Information for Studies of Extracellular Vesicles
MSCs	Mesenchymal Stromal/Stem Cells
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NDDS	Nanoscale Drug Delivery System
NK cells	Natural Killer cells
NK-EVs	Natural Killer Cell-derived Extracellular Vesicles
NTA	Nanoparticle Tracking Analysis
NVEPs	Non-Vesicular Extracellular Particles
PBMCs	Peripheral Blood Mononuclear Cells
PD-1	Programmed cell death protein 1

PD-L1	Programmed death ligand-1
PDX	Patient-derived xenograft
PFN	Perforin
PI	Propidium Iodide
PTX	Paclitaxel
PR	Progesterone Receptor
PBS	Phosphate-Buffered Saline
RFP	Red Fluorescent Protein
RFU	Relative Fluorescence Units
RT-qPCR	Quantitative reverse transcriptase polymerase chain reaction
SEER	Surveillance, Epidemiology and End Results
SEM	Standard error of the mean
SF	Serum-Free
SEC	Size-Exclusion Chromatography
TIL	Tumour-Infiltrating Lymphocytes
TME	Tumour-Microenvironment; TNBC: Triple-Negative Breast Cancer
TNF α	Tumour necrosis factor alpha
UF	Ultrafiltration

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Chapter 1. Project Background

1.1. Cancer as a Leading Cause of Death, with a Focus on Triple-Negative Breast Cancer (TNBC)

Cancer is a heterogeneous collection of diseases characterized by the uncontrolled proliferation of abnormal cells arising from the accumulation of genetic, metabolic and epigenetic alterations that disrupt the regulatory networks governing cell growth, differentiation, and survival [1]. These alterations enable cells to evade normal physiological constraints, acquire proliferative autonomy, and progressively develop the capacity to invade surrounding tissues and metastasize to distant organs. As such, cancer is widely understood as a disease of dysregulated cellular growth driven by evolutionary selection acting on genetically unstable somatic cell populations.

As of early 2026, cancer is one of the leading causes of death worldwide, affecting millions of people each year, according to leading health authorities such as the World Health Organization. In fact, the number of new cancer cases globally per year is expected to rise from 20 million (2022) to 33-35 million by 2050, a 77% increase from the current level, according to the National Cancer Institute at the National Institutes of Health (United States government), the Canadian Cancer Society and other epidemiological data [2]. In Canada alone, the latest estimates indicate that there would be over a quarter of a million new cancer cases, accompanied by close to a tenth of a million cancer-related deaths in 2026. These data indicate that nearly one in five people (~20.0%) will be diagnosed with cancer over their lifetime, and approximately one in nine men (~11.1%) and one in twelve women (~8.3%) will succumb to the disease [2]. In industrialized countries, the situation is particularly concerning, with higher incidence rates due to a higher prevalence of known risk factors [3]. Examples include, but are not limited to 1) lifestyle-related factors (higher rates of tobacco and alcohol consumption, calorie-dense diets, and sedentary behaviour), 2) environmental and occupational exposure factors (air pollution, industrial chemicals, and ionizing radiation associated with medical imaging) and 3) demographic and biological factors (aging population and genetics). These high incidence and mortality rates emphasize the urgent need for continued research and

advancements [4-6]. Cancer, like many diseases, can only be treated across three axes: 1) prevention, 2) diagnosis, and 3) treatment [7]. The ten most common cancer types worldwide, based on age-standardized incidence rates (ASR; standardized surrogate for prevalence), include lung cancer, breast cancer, colorectal cancer, prostate cancer, stomach cancer, liver cancer, thyroid cancer, cervical cancer, bladder cancer, and non-Hodgkin lymphoma. Other common cancers include leukemia (ranked ~13th), kidney cancer (ranked ~14th), melanoma (ranked ~17th), and brain/central nervous system cancers (ranked ~19th). In Canada, lung and bronchus cancer (12.9%), breast cancer (12.5%), prostate cancer (11.9%) and colorectal cancer (10.4%) collectively account for nearly half of all diagnoses in 2025, according to the Canadian Cancer Society.

According to the Surveillance, Epidemiology and End Results (SEER) Program and other epidemiological studies, breast cancer is the most frequently diagnosed cancer in women (11.6 – 15.5% of all new cancer cases) and is a leading cause of cancer-related deaths (6.8 – 6.9% of all cancer deaths) [4, 8]. In Canada, the breast cancer mortality rate was 6.2% in 2025, according to the Canadian Cancer Society. Breast cancer comprises multiple subtypes, each defined by specific molecular markers and associated with unique diagnostic profiles and clinical prognoses [9-12]. Notably, the various molecular subtypes are distinguishable by the presence or absence of specific receptors, such as the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2) [13, 14]. For example, the most common subtype, ER+/HER2–, accounts for approximately 70% of all breast cancer cases.

Conversely, triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer, and it is characterized by the absence of ER, PR, or HER-2 receptors, accounting for only 11-15% of all breast cancers [15-18]. Within the TNBC classification, there are several subtypes, including basal-like, mesenchymal, and immune-modulatory subtypes, each with distinct molecular features and treatment responses [19-22]. This lack of receptor expression makes TNBC particularly difficult to treat since it does not respond to currently approved hormonal and HER-2-targeted therapies [23].

Clinically, TNBC tends to grow and spread more rapidly than other breast cancer subtypes and has a higher likelihood of recurrence and metastasis [24]. Several factors influence TNBC prognosis, including comorbidities, patient age, tumour size, lymph node involvement, and the presence of distant metastases at diagnosis [25]. TNBC is also associated with early systemic dissemination, high recurrence rates, and poor overall prognosis, particularly among younger women and those with the BRCA1 mutation (a tumour suppressor gene whose mutation is associated with a markedly increased incidence of breast and ovarian cancers) [26]. As a result, TNBC accounts for a disproportionately high number of breast cancer-related deaths. At the time of writing, the five-year survival rate for TNBC is 78.4%, while for other subtypes of breast cancer is 91.7%; SEER 21 2018-2022. However, survival rates plummet following metastasis, where the five-year survival rate for TNBC patients at the distant stage hovers around 15%. These realities underscore the urgent need for more effective and targeted therapeutic strategies to improve outcomes and survival in TNBC.

1.2. Standard of Care for TNBC

The current standard of care for TNBC involves surgical resection combined with neoadjuvant or adjuvant chemotherapy, sometimes followed by radiation therapy [27]. Depending on the tumour's size, location, and patient preference, treatment may involve either a lumpectomy (removal of the tumour with a margin of surrounding tissue; breast-conserving surgery) or a mastectomy (removal of one or both breasts). In cases of lumpectomy, radiation therapy can be used to destroy any remaining cancer cells in the breast, chest wall, or axillary lymph nodes, as it reduces the risk of local recurrence.

Chemotherapy is a cornerstone of TNBC treatment, typically employing agents such as anthracyclines (*e.g.*, doxorubicin or epirubicin), taxanes (*e.g.*, paclitaxel or docetaxel), platinum-based compounds (*e.g.*, cisplatin or carboplatin), antimetabolites (*e.g.*, capecitabine or gemcitabine), or cytoskeletal inhibitors (*e.g.*, eribulin), depending on disease stage and prior treatment history [27, 28]. It can be administered before surgery (neoadjuvant chemotherapy) to

shrink the tumour and facilitate resection, or after surgery (adjuvant chemotherapy) to eliminate residual disease and reduce the likelihood of recurrence. Moreover, most chemotherapeutic agents are unable to cross the blood-brain barrier (BBB), limiting their effectiveness against brain tumours, including TNBC metastases (~ 1/3 of TNBC patients), which currently represent a terminal stage of the disease with no curative treatment available [5, 6]. Often, these approaches are used in combination to increase the likelihood of treatment success.

Despite showing initial responsiveness to chemotherapy, most TNBC patients ultimately relapse due to the emergence of chemoresistance and the persistence of cancer stem cells [29, 30]. Considering the totality of current evidence, the absence of molecular targets, coupled with pronounced tumour heterogeneity and an immunosuppressive tumour microenvironment, continues to limit durable treatment outcomes in TNBC. Nonetheless, emerging therapeutic strategies are beginning to address these challenges.

1.3. Immunotherapy: An Emerging Cancer Therapeutic Modality

Recent advances in oncology have shifted the therapeutic landscape toward precision and immune-based approaches, collectively known as immunotherapy, which leverage the body's natural defences to target and eliminate cancer cells. Among these, key modalities include cell-based, immune checkpoint inhibitor (ICI), monoclonal antibody, oncolytic virus (OV) and recombinant cytokine therapies, each offering distinct mechanisms of action, clinical advantages, and challenges. Collectively, these innovations represent a paradigm shift away from nonspecific cytotoxic regimens toward targeted, immunologically guided cancer treatments.

First, ICIs act by blocking inhibitory pathways that suppress immune activation, restoring T-cell-mediated cytotoxicity against tumours [31]. Agents targeting PD-1, PD-L1, or CTLA-4 have demonstrated durable responses in select malignancies. In TNBC, the PD-1 inhibitor pembrolizumab, especially when combined with chemotherapy, has improved outcomes for patients with PD-L1-positive or high-tumour mutational burden disease [32]. However, these

benefits are limited to a subset of patients (~20%), and immune-related toxicities remain a concern [33].

Second, cell-based therapies, including chimeric antigen receptor (CAR)-engineered T and NK cells, represent another major innovation in immuno-oncology. These approaches involve modifying immune cells to recognize specific tumour antigens, thereby enabling potent, targeted cytotoxic responses. While CAR-T therapies have achieved some level of success in hematologic malignancies, their efficacy in solid tumours, such as TNBC, remains limited due to tumour heterogeneity, poor infiltration, and an immunosuppressive tumour microenvironment [34-41]. Moreover, their complexity, high cost, and safety risks, such as cytokine release syndrome and neurotoxicity, lymphodepletion requirement, and limited source availability (patient-derived materials, which can be compromised by disease state and prior treatments) further constrain widespread application. For instance, natural killer (NK) cell therapy has shown great promise against various hematological cancers [42, 43]. However, it has not been as effective against solid tumours due to poor trafficking, tumour infiltration and immunosuppression by the TME [44-46].

Third, OV therapy has also gained attention as a novel immunotherapeutic strategy. These engineered or naturally occurring viruses selectively infect and lyse tumour cells while stimulating systemic immune responses. The first FDA-approved OV, talimogene laherparepvec (T-VEC), has demonstrated both local and systemic antitumour activity and is under investigation for other solid tumours, including breast cancer [47]. Despite encouraging early results, no additional OV has yet been approved by regulatory agencies in the decade since T-VEC's approval [48]. Nonetheless, several OVs remain in development (*e.g.*, RP2, MVR-T3011, VG161 and Cretostimogene grenadenorepvec), employing diverse vectors and immune-modulating genes.

Collectively, these modalities underscore the transformative potential of immunotherapy in reshaping the cancer treatment landscape. However, persistent challenges related to safety, scalability, manufacturing complexity, and tumour heterogeneity highlight the need for next-generation platforms that preserve the efficacy of immune-based therapies while improving their accessibility and translational feasibility. Among these emerging approaches, extracellular vesicle

(EV)-based therapeutics represent a rapidly advancing field with the potential to overcome many of these limitations.

1.4. Extracellular Vesicle (EV)-based Therapies

A brief history: the study of EVs spans more than eight decades and has evolved from early descriptive observations to a rapidly expanding field with major translational implications [49]. The first observations of vesicle-like particles in biological fluids date back to 1946, when Chargaff and West reported the presence of small lipid particles in plasma, resembling the thromboplastic protein, following high-speed centrifugation experiments [50]. Subsequent ultrastructural studies in the 1960s and 1970s, including the description of platelet “dust” by Wolf in 1967, provided early visual evidence for membrane-derived extracellular particles [51]. In the early 1980s, vesicles released during the maturation of reticulocytes were identified and later formally termed “exosomes” by Johnstone and colleagues in 1987 [52]. Initially considered a mechanism for cellular waste disposal, EVs gained renewed attention in the 1990s when studies demonstrated their involvement in immune regulation, including antigen presentation by B-cell and dendritic cell-derived vesicles [53, 54]. The early 2000s marked a conceptual turning point with the discovery that EVs carry functional nucleic acids, including messenger RNA and microRNA, capable of modulating gene expression in recipient cells [49]. Over the past decade, advances in high-resolution imaging, omics technologies, and vesicle isolation methods have further expanded our understanding of EV biology, revealing their roles in intercellular communication, disease pathogenesis, and therapeutic delivery. The rapid growth of the field has led to the establishment of the International Society for Extracellular Vesicles (ISEV; see <https://www.isev.org/>) and the publication of the Minimal Information for Studies of Extracellular Vesicles guidelines (MISEV; now on its third edition), which aim to standardize experimental practices and improve reproducibility across EV research [55, 56].

Over the past decade, extracellular vesicles (EVs) have emerged as a promising class of cell-free immunotherapeutics with great potential for cancer treatment. EVs naturally carry lipids,

proteins, and nucleic acids reflective of their parent cells and mediate intercellular communication [28, 56, 57]. EVs encompass all nanosized lipid bilayer particles produced and released by cells, including those from plants and bacteria. They are often classified based on their origin (*i.e.*, exosomes, microvesicles/ectosomes, or apoptotic bodies) or their size (*e.g.*, small EVs are less than 200 nm) [56]. For more information about EVs, refer to the MISEV guidelines [56].

In essence, EVs play a fundamental role in intercellular (cell-to-cell) communication, immune modulation, and tissue homeostasis. Their molecular composition is shaped by the parent cell type and the extracellular context, directly influencing biological function. Isolation and purification methods also directly affect the molecular composition of vesicles, especially regarding the EV protein corona (a loosely associated layer of surface proteins that is emerging as an essential determinant of EV behaviour) [58-60]. For instance, cancer cell-derived EVs can promote tumour growth and immune evasion by transferring immunosuppressive cytokines and regulatory molecules that inhibit NK and dendritic cell activity [61]. Conversely, EVs from regenerative or immune cells can convey anti-inflammatory or antitumour effects, depending on their origin and cargo. For example, stem cell-derived EVs have demonstrated anti-inflammatory and regenerative properties, with applications extending from tissue repair to wound healing [62, 63]. In oncology, EVs are being investigated both as drug delivery systems capable of transporting therapeutic agents directly to tumour cells [64-72] and as immune modulators that enhance the activity of cytotoxic immune cells [73-75].

Unlike cell-based therapies, EVs are nonreplicating and remain functionally stable under hypoxic and immunosuppressive tumour microenvironments. These conditions often hinder the efficacy of cell-based therapies in solid tumours such as TNBC [45, 76, 77]. EVs are increasingly recognized as “off-the-shelf” biotherapeutics owing to their intrinsic biocompatibility, nanoscale size, and lipid-bilayer structure, which protect their bioactive cargo from degradation and facilitate systemic circulation [28]. Compared to synthetic nanoparticles, EVs offer enhanced biocompatibility, superior stability, reduced toxicity, and natural tropism toward recipient cells [28]. Their ability to cross biological barriers (*e.g.*, *the BBB*) enables targeted delivery and accumulation

within tumours [64-72]. The BBB is a highly selective cellular interface that protects the central nervous system by tightly regulating the passage of molecules between the bloodstream and the brain [78]. Their tumour-targeting ability largely arises from the enhanced permeability and retention (EPR) effect, driven by the abnormal vasculature of tumours [79]. They can also be filter-sterilized due to their nanoscale size, reducing the risk of contamination and making them ideal biotherapeutics. If desired, EVs can also be engineered to deliver chemotherapeutic drugs, nucleic acids, or immunomodulatory molecules, functioning as natural nanoscale drug delivery systems (NDDS). Several EV-based therapeutics have now advanced into preclinical and clinical evaluation, signaling a transition toward cell-free, vesicle-mediated immunotherapy. **Table 1** details relevant clinical trials focusing exclusively on cancer-treatment applications of EVs as of 2026.

Table 1. History of all declared clinical trials investigating EV-based therapeutics for cancer treatment. Search for exosome or extracellular vesicles (EVs) or microvesicle or microparticle or ectosome or “natural nanoparticle”, information obtained from <https://clinicaltrials.gov/>.

Trial ID	Status (last update)	Phase	Condition	EV intervention	Note
NCT01668849	Completed (2022)	I	Head & Neck Cancer	Dietary Supplement of grape exosome	Safe and reduced chemoradiation-induced oral mucositis.
NCT01294072	Recruiting (2023)	I	Colon cancer	Dietary Supplement of curcumin with plant exosomes	No update in +2 years.
NCT05375604	Terminated (2023)	I	Advanced Hepatocellular Carcinoma (HCC) and patients with liver metastases from either primary gastric cancer or colorectal cancer (CRC)	Intravenous injection of exoASO-STAT6 (CDK-004)	Trial halted; the sponsor filed for bankruptcy.
NCT06536712	Not-yet recruiting (2024)	I	Rectal Cancer	Intraperitoneal injections of Human placenta MSC-derived exosomes	Evaluating regenerative exosomes to prevent anastomotic leakage after surgery.
NCT05156229	Terminated (2022)	I/II	Cutaneous T-Cell Lymphoma	Engineered exosomes displaying IL-12 cytokine (exoIL-12, a.k.a. CDK-003)	Trial halted; the sponsor filed for bankruptcy.
NCT04592484	Completed (2022)	I/II	Advanced solid tumours (injectable lesions in e.g. TNBC, H&N cancers)	Engineered exosomes loaded with a STING agonist (exoSTING)	First-in-human intratumoral exosome therapy delivering a STING agonist to stimulate local tumour immunity. Phase I/II results showed acceptable safety, immune activation, and evidence of anti-tumour effect. No reported Participant data.

					Note: the sponsor filed for bankruptcy.
NCT03608631	Recruiting (2025)	I/II	Metastatic Pancreas Cancer with Kras ^{G12D} Mutation	Intravenous injection of Mesenchymal stromal cells-derived exosomes with KrasG12D siRNA (iExosomes)	First-in-human trial of gene-silencing exosomes targeting mutant KRAS in advanced pancreatic cancer.
NCT01159288	Completed (2018)	II	Advanced unresectable non-small cell lung cancer	Intradermal injections of tumour antigen-loaded Dendritic Cell-derived Exosomes (Dex)	The EV-based vaccine was safe and induced immune activation, although clinical benefit remains uncertain and requires larger studies.

*The information provided in this table is as accurate as available on the www.clinicaltrials.gov official website.

The most frequently reported routes of EV administration for cancer applications in pre-clinical studies are intratumoral and intravenous injection, although intranasal delivery has also been explored to enhance brain targeting, further underscoring the versatility of EVs as therapeutic agents [28]. Clinical studies to date indicate that EV administration is generally safe, with few and mild adverse effects (e.g., transient fever, vomiting, or mild hepatic dysfunction) observed in patients [80].

1.5. Natural Killer Cell-derived EVs (NK-EVs) Immunotherapeutics

Building on these advancements, natural killer cell-derived extracellular vesicles (NK-EVs) have emerged as a clinically relevant subclass of immune-derived EVs, uniquely integrating the cytotoxic potency of NK cells with the scalability and safety of cell-free biomanufacturing, positioning them as a next-generation platform for cancer immunotherapy [28, 81].

Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system that play a crucial role in tumour surveillance and viral defence [82-84]. More specifically, their activity is governed by a finely tuned balance between germline-encoded activating (*i.e.*, NKG2D, NKp30, NKp44, and NKp46; detect stress-induced ligands) and inhibitory receptors (*i.e.*, KIRs and NKG2A; survey for self MHC class I molecules on target cells). Loss or downregulation of MHC class I, a

hallmark of many tumours, removes this inhibitory tone and sensitizes targets to NK cell attack, allowing NK cells to discriminate between healthy and compromised cells in an antigen-independent manner. This is known as the missing self-hypothesis [85].

Upon this engagement, NK cells initiate cytotoxic programs through multiple coordinated mechanisms. The dominant pathway involves directed exocytosis of cytolytic granules that contain perforin and granzymes, which together induce membrane permeabilization and apoptosis. NK cells also deploy death receptor pathways, including TRAIL and FasL, to trigger caspase-mediated apoptosis in targets. Furthermore, NK cells release a broad cytokine repertoire, such as IFN γ , TNF α , GM-CSF, and chemokines (CCL3, CCL4, CCL5), which modulate the tumour microenvironment, enhance antigen presentation, and recruit additional immune subsets.

Recent work has highlighted EVs as an additional, potent arm of NK effector biology. NK-EVs generally contain the NK cell functional signature, enabling them to kill tumour cells independently of direct cell-cell contact. These vesicles retain selective surface markers, which contribute to target recognition and functional specificity. Multiple studies have shown that NK-EVs exert dose- and time-dependent cytotoxic effects across diverse cancer types, including glioblastoma, skin, colorectal, leukemia and breast cancers – see **Table 2**.

Table 2. Pre-clinical studies evaluating NK-EVs against different cancer types.

Cancer type	Example study context	Summary of NK-EV anti-tumour activity	Representative references
Glioblastoma	Glioblastoma cell lines and xenografts	Dose and time-dependent cytotoxicity, with reduced viability and increased apoptosis of glioblastoma cells.	[86, 87]
Skin cancer	Melanoma and other skin cancer models	NK-EVs induce strong cytotoxic effects and apoptosis in skin cancer cells, limiting tumour growth in preclinical models.	[88, 89]
Colorectal cancer	Colorectal carcinoma cell lines and models	NK-EVs reduce colorectal cancer cell proliferation and survival in a dose and time-dependent manner.	[87, 89-91]
Leukemia	Leukemia cell lines and primary blasts	NK-EVs show potent, selective cytotoxicity against leukemia cells while sparing non-malignant controls.	[92-95]
Breast cancer	Hormone receptor-positive and TNBC models	NK-EVs mediate dose and time-dependent killing of breast cancer cells and	[81, 86, 87, 89, 90, 94, 96-99]

enhance apoptosis in
multiple models.

Thus, NK-EV-based therapeutics hold great promise for addressing the challenges of treating cold, immunologically inert solid tumours, such as TNBC [100-104]. Recent evidence, including the work presented in this thesis, demonstrates that NK-EVs exert dual mechanisms of action: direct tumour cytotoxicity and immune reprogramming within the tumour microenvironment. By bridging innate and adaptive immunity, NK-EVs represent a new generation of off-the-shelf, cell-free immunotherapeutics with the potential to redefine treatment paradigms for TNBC and other refractory cancers.

Despite this promise, significant challenges remain before NK-EV-based therapeutics can reach full clinical maturity. Manufacturing standardization is a central obstacle: EV yield, purity, and composition vary across parent cells, culture conditions, and isolation methods, complicating reproducibility and regulatory compliance. Establishing Good Manufacturing Practice (GMP)-compliant workflows that ensure consistent product quality, potency, and safety is essential for clinical scalability. Additional challenges include optimizing cargo loading (if needed), targeting specificity, long-term stability, and potency testing, all of which directly influence therapeutic efficacy. Addressing these barriers will be critical to realizing the full potential of NK-EVs as safe, effective, and scalable cell-free immunotherapies, setting the stage for their integration into next-generation precision oncology. This thesis investigates NK-EVs as a next-generation, cell-free immunotherapy for TNBC and the overarching objective is to unite biomanufacturing innovation, functional validation, and immune reprogramming into a cohesive translational framework that advances the clinical readiness of NK-EV-based therapeutics.

1.7. Thesis Hypothesis

The overall goal of this thesis was to assess NK-EVs as a strategy for treating TNBC. The hypothesis is that NK-EVs can directly kill TNBC models and modulate the immune system, leading to a favourable anti-cancer response (**Figure 1**).

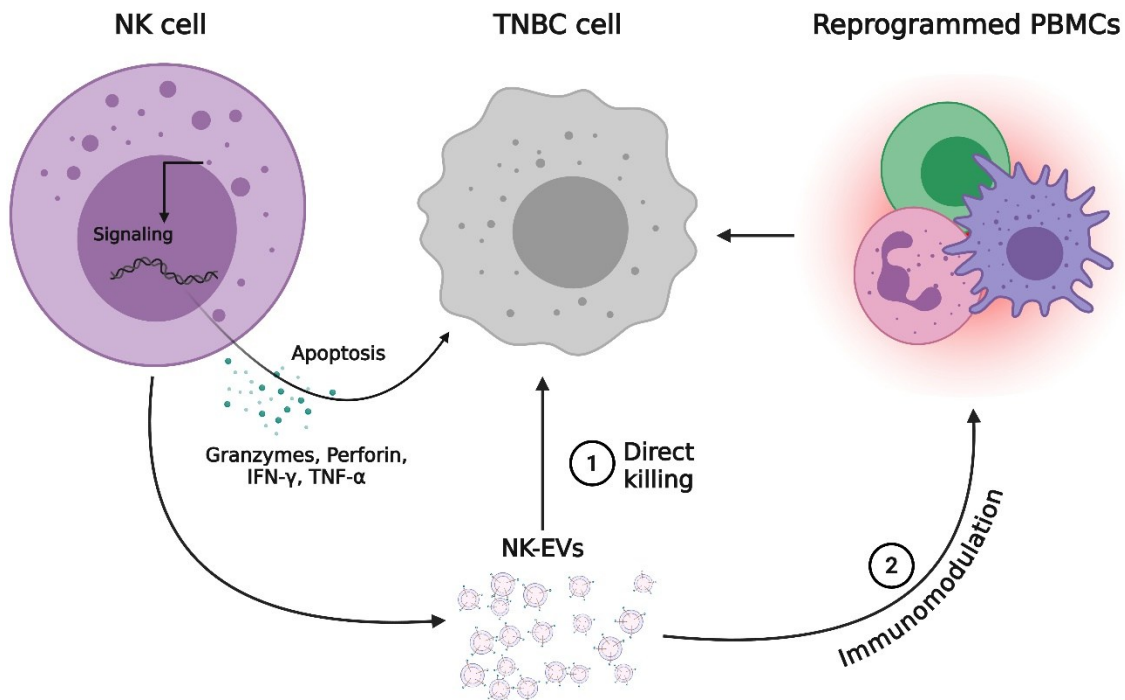


Figure 1. Graphical research hypothesis.

Natural killer (NK) cells release extracellular vesicles (NK-EVs) that carry various cytotoxic and immunoregulatory molecules. Once secreted, these NK-EVs exert two complementary effects: 1) Direct killing: NK-EVs interact with triple-negative breast cancer (TNBC) cells, delivering their cytotoxic payloads and triggering apoptosis, or 2) Immunomodulation: NK-EVs reprogram peripheral blood mononuclear cells (PBMCs), enhancing and amplifying antitumour immunity. Together, these dual functions highlight NK-EVs as multifunctional, cell-free immunotherapeutic agents that bridge innate and adaptive immune responses against cancer. This figure was created with BioRender.com. St-Denis-Bissonnette, F. (2026) <https://BioRender.com/s6kyly3>.

Chapter 2 (NK-EV biomanufacturing and product characterization) describes a clinically relevant biomanufacturing workflow to produce large quantities of clinical-grade NK-EVs and heavily characterize the NK-EVs (**thesis aim 1**). This work serves as the foundation for the other Chapters. **Chapter 3** (NK-EV cytotoxicity assessment) validates a potency assay to evaluate the cytotoxic function of NK-EVs (**thesis aim 2**). Several alternative potency assays commonly used for this purpose were also evaluated. **Chapter 4** (NK-EV immunomodulation assessment) assesses NK-EVs' reprogramming of human immunity to enhance tumour cytotoxicity using high-resolution single-cell transcriptomics and functional cytotoxicity assays (**thesis aim 3**). This study uniquely integrates single-cell transcriptomics and functional assays, using healthy-donor and cancer-patient-derived PBMCs, to reveal, for the first time, the mechanistic immunoregulatory cascade by which NK-EVs reprogram human immune cells.

Taken together, these studies address the key gaps previously outlined in the field, uniting GMP-aligned biomanufacturing, qualified potency metrics, and mechanistic insights into immune modulation, thereby establishing a coherent translational path for advancing NK-EVs toward clinical development for TNBC.

1.8. Contribution to the field

This thesis makes a direct and substantive contribution to the field by systematically addressing key scientific and technical barriers that have limited the translational progress of EV. Rather than advancing NK-EVs solely as a biologic, this work positions them as a potential therapy modality by aligning discovery science with industry-aligned translational expectations.

First, this thesis advances EV biotechnology by establishing a scalable, GMP-aligned biomanufacturing workflow for immune cell-derived EVs [105]. A closed-loop hollow fibre bioreactor system operating under serum-free (SF), xeno-free (XF), feeder-free (FF), and antibiotic-free conditions addresses a major translational gap in the field, namely the lack of platforms capable of producing large-scale, clinically relevant EV products. By demonstrating continuous, reproducible generation of high-yield, functionally active NK-EVs from a clinically relevant cell line, and characterizing the product in accordance with MISEV guidance, this work advances EV production beyond small-scale laboratory feasibility toward an industrially deployable, cost-effective process. The accompanying protocol publication further supports field-wide adoption through a standardized and transferable manufacturing and isolation framework [106].

Second, this thesis advances EV analytics through the development of a validated, stability-indicating potency assay suitable for advanced biotherapeutic development [107]. While potency testing is poorly defined across the EV field, this work rigorously validates a resazurin phenoxazine-based cytotoxicity assay with demonstrated sensitivity, reproducibility, and correlation to molecular effectors, providing a fit-for-purpose potency metric for immune EV therapeutics. Importantly, the assay enables lot-to-lot comparability, stability assessment, and degradation detection, supporting quality control and product comparability for EV-based products.

Third, this thesis advances mechanistic understanding of EV-mediated immune modulation (manuscript under peer revision at the time of writing). Using integrated single-cell transcriptomics, high-dimensional flow cytometry, and functional assays with healthy donor and cancer patient-derived immune cells, this work establishes NK-EVs as dual-function agents mediating both direct

tumour cytotoxicity and immune reprogramming. By linking single-cell transcriptional changes to functional outcomes, this thesis provides mechanistic insight relevant to early clinical translation.

Beyond the core thesis aims, this work contributes to the development of methodologies that strengthen reproducibility and industrial relevance across EV research. The demonstration that purification strategies differentially alter the EV biomolecular corona and biological potency provides actionable guidance for process development and comparability assessments (manuscript under publication with the JExBio; accepted). In parallel, the optimized TEM positive staining protocol improves structural characterization and quality assessment, supporting more reliable identity testing and product release characterization [108]. Complementary co-first-author work further demonstrates that NK-EVs are cytotoxic to both bulk tumour cells and CSC populations in TNBC models, including patient-derived xenograft (PDX) organotypic cultures (manuscript under peer review at the time of writing).

Beyond experimental research outputs, this work includes sustained contributions to the development and organization of the EV research community. The author is a founding member of the Canadian Society for Extracellular Vesicles (CanSEV) and has served on its Board of Directors since 2024, contributing to national community building, knowledge exchange, and coordination of academic and translational EV research in Canada. In addition, the author contributed to the development of the MISEV2023 guidelines [56] through detailed review and technical recommendations, with contributions recognized through co-authorship as part of the MISEV consortium, reflecting direct engagement in shaping standards for rigour, transparency, and reproducibility in EV research. Private sector endeavours are excluded from this thesis.

Collectively, this thesis advances the EV field from exploratory biology toward biotechnology translation by integrating GMP-aligned manufacturing, validated potency analytics, and mechanistic immune profiling, contributing to the maturation of EVs as a credible and industrially viable class of biotherapeutics.

Chapter 2. NK-EV biomanufacturing and product characterization

Preface: The following chapter consists of a research article for which I am the primary author. This article, published in the Journal of Extracellular Vesicles (JEV) in 2023, lays the foundation for this thesis by demonstrating how NK-EVs are produced using a scalable biomanufacturing workflow.

Citation: St-Denis-Bissonnette, F., Cummings, S. E., Qiu, S., Stalker, A., Muradia, G., Mehic, J., Mediratta, K., Kaczmarek, S., Burger, D., Lee, S.-H., Wang, L., & Lavoie, J. R. (2023). A clinically relevant large-scale biomanufacturing workflow to produce natural killer cells and natural killer cell-derived extracellular vesicles for cancer immunotherapy. *Journal of Extracellular Vesicles*, 12, e12387. <https://doi.org/10.1002/jev2.12387>.

Contributions: JRL, LW and FSTDB conceived the idea. FSTDB and GM performed the Hollow-Fibre Bioreactor EV production and cell expansion. FSTDB and SQ contributed to cell co-culture assays. FSTDB and AS acquired flow cytometry data, and JRL, LW and FSTDB contributed to the flow analyses. FSTDB and SK performed NK92 cell radiation. FSTDB performed the NTA experiments, and JRL, FSTDB and SEC contributed to the NTA analyses. FSTDB and SC performed the surface and cytokine MACSplex experiments, and JRL, FSTDB, and SEC contributed to the MACSplex analyses. SEC and JM performed the western blot experiment, and JRL, SEC and FSTDB contributed to the data interpretation. FSTDB and GM performed the additional EV characterization assessment (protein, nucleic acid, and endotoxin). KM performed RT-qPCR for common viral pathogens, and JRL, LW, KM and FSTDB contributed to the data interpretation. FSTDB performed the EV cytokilling assay, and JRL, LW, FSTDB, and SEC contributed to the cytotoxicity analyses. JRL, LW and FSTDB performed the statistical analyses. All authors contributed general intellectual input to the experimental design, participated in the data analysis, and discussed the results. JRL, LW and FSTDB wrote the manuscript and assembled the figures. All authors reviewed and edited the manuscript and approved its final version.

Abstract

Natural killer cell-derived extracellular vesicles (NK-EVs) have shown promising potential as biotherapeutics for cancer due to their unique attributes as cytotoxic nanovesicles against cancer cells and immune-modulatory activity towards immune cells. However, a biomanufacturing workflow is needed to produce clinical-grade NK-EVs for preclinical and clinical applications. This study established a novel biomanufacturing workflow using a closed-loop hollow-fibre bioreactor to continuously produce NK-EVs from the clinically relevant NK92-M1 cell line under serum-free, xeno-free, and feeder-free conditions following GMP-compliant conditions. The NK92 cells grown in the bioreactor for three continuous production lots resulted in large quantities of both NK cell and NK-EV biotherapeutics at the end of each production lot (over 10^9 viable cells and 10^{13} EVs), while retaining their cytotoxic payload (granzyme B and perforin), pro-inflammatory cytokine (interferon-gamma) content and cytotoxicity against the human leukemic cell line K562 with limited off-target toxicity against healthy human fibroblast cells. This scalable biomanufacturing workflow has the potential to facilitate the clinical translation of adoptive NK cell-based and NK-EV-based immunotherapies for cancer with GMP considerations.

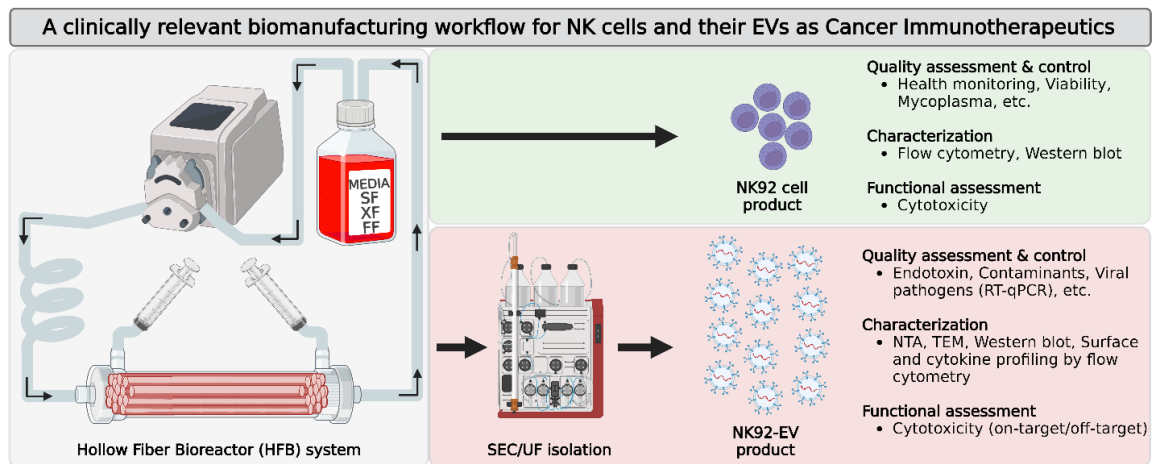


Figure 2. Graphical abstract of Biomanufacturing of NK92 cells and derived extracellular vesicles in a closed-loop hollow fiber bioreactor (HFB).

Schematic representation of the biomanufacturing workflow to generate large quantities of clinical-grade NK92 cells and their derived extracellular vesicles (NK92-EVs). NK92 cells are seeded into a closed-loop HFB and cultured in a serum-free (SF), xeno-free (XF) and feeder-free (FF) medium. NK92 cells are expanded in the hollow fiber cartridge and harvested at the end of the production cycle. The EV-rich conditioned medium is collected and processed daily for later EV purification by Size Exclusion Chromatography (SEC) coupled with Ultrafiltration (UF) sequentially before filter sterilization of EVs. NK92 cells and NK92-EVs are characterized and assessed through multiple

assays, and their cytotoxicity against K562 leukemia target cells is evaluated in a co-culture assay. This figure was created with BioRender.com.

Introduction

Natural Killer (NK) cells are immune cells that play a crucial role in the body's defence against virus-infected, malignant, and stressed cells [82-84]. NK cells possess the ability to kill these abnormal cells through a combination of activating and inhibitory receptors, and by releasing cytotoxic granules, which can contain granzymes, such as granzyme B (GzmB), perforin (PFN) and pro-inflammatory cytokines, including interferon-gamma (IFN γ) [109-112]. For example, when cytotoxic granules enter cancer cells, they trigger apoptosis, a process of programmed cell death [111, 112]. NK cells have inherent therapeutic value for cancer immunotherapy due to their intrinsic cytolytic properties, where they can produce extracellular vesicles (EVs) with cytolytic payloads [83, 113-115]. EVs are small vesicles released by cells that carry various types of biomolecules, including proteins, RNA, and DNA [28, 55, 57]. When produced from certain immune cells, EVs are emerging as new biotherapeutics for cancer treatment due to their unique carrier and physiochemical properties and their ability to be extrinsically loaded with drugs or nucleic acids [55, 116-124]. Native, engineered, or drug/nucleic acid-loaded EVs are being investigated as cancer immunotherapies due to their potential to accumulate within solid tumours, to carry a wide range of biomolecules, and to cross biological barriers which maximize their biodistribution; all characteristics that may help to overcome some of the challenges of treating cold (low-immunogenic) solid tumours that are difficult to infiltrate with T and NK cells [100, 101, 103, 104, 125, 126].

Among the various EV types, those derived from NK cells (NK-EVs) have demonstrated promising cytolytic and immunomodulatory properties [63, 65, 66, 127-136]. These properties are due, in part, to the presence of a combination of intravesicular proteins (PFN, GzmA, GzmB and GNLY), miRNA payload, and NK-related receptors (*i.e.*, NKG2D, FasL/CD178, DNAM-1/CD226, and TRAIL/CD253) [65, 66, 103, 128-130, 133, 135, 137-139]. Given the potential of NK-EVs in cancer immunotherapy, it is vital to establish a scalable, good manufacturing practice (GMP)-

compliant biomanufacturing process that can produce large quantities of EVs in a consistent manner [140-142]. While some progress has been made in the large-scale production of NK cells, NK-EVs currently lag, requiring additional research and development [143-151]. This process should involve GMP-compliant cell culture conditions, a bioreactor system, and a scalable EV isolation process to consistently yield functional, high-purity NK-EVs [140, 152-156]. In addition, to maintain the batch-to-batch consistency of a qualified product, it is necessary to address specific challenges that may arise during the production of clinical-grade NK-EVs. Moreover, current methods for NK-EV production have limitations, such as the reliance on non-scalable feeder systems or the use of culture media containing animal components, which may hinder the clinical translatability of EVs [127].

In order to address these current gaps in NK-EV production, this study establishes a novel and cost-effective clinically relevant biomanufacturing workflow using a closed-loop hollow-fiber bioreactor (HFB) system to culture and expand NK92 cells under GMP-compliant conditions using a chemically-defined, serum-free (SF) and xeno-free (XF) medium in a feeder-free environment (**Figure 2**). The NK92 cell line, derived from malignant non-Hodgkin's lymphoma, has been extensively tested and approved for use in clinical settings to treat various diseases, including cancer, without causing cytokine release syndrome, immune effector cell-associated neurotoxicity syndrome, or graft-versus-host disease [157-160]. Additionally, NK cells have the potential to be used in an allogenic manner, making them a readily available "off-the-shelf" option to treat multiple patients [37, 161, 162]. As a proof-of-principle of the capacity and feasibility of this new biomanufacturing process, we generated three consecutive production lots using the same HFB cartridge, each lasting 5-7 days. As a result, we generated over 10^9 NK92 cells and large quantities of NK92-EVs ($>10^{13}$) suitable for clinical application. Importantly, our approach significantly improved the NK92 cell viability and cytotoxicity compared to traditional flask-based culture methods. The purified NK92-EV product generated from the bioreactor-cultured NK92 cells and purified using scalable size exclusion chromatography and ultrafiltration techniques demonstrated a high-purity profile and retention of the NK92 essential characteristics such as expression of surface markers, cytokine payload, and strong cytotoxicity against human K562 leukemia cells.

In summary, our cell and EV biomanufacturing approach provides a reliable and cost-effective means of generating clinical-grade NK92 cells and NK-EVs for cancer immunotherapies, paving the way for their clinical translation.

Materials and Methods

Cell Culture Condition Parameters

NK92-MI cells (ATCC, CRL-240) purchased at passage 2 were cultured in RPMI-1640 (Gibco, cat#11875-93) with 0.1% 2-Betamercaptoethanol (Gibco, cat#2289319), 20mM HEPES (Gibco, cat#15630-080) and 10% Heat Inactivated FBS (Gibco, cat#12484-028) for five days before switching to ImmunoCult-XF T Cell Expansion Medium (StemCell Technologies, cat#10981) for 14 days of acclimatization. NK92-MI cells are interleukin (IL)-2 self-sufficient, simplifying their clinical use while overcoming toxicity issues related to exogenous IL-2 [163]. From this point onward, the NK92-MI cell terminology will be simplified to NK92 cells. ImmunoCult-acclimatized NK92 cells (passage 9) were then expanded for 18 days (passage 15) for seeding into the hollow-fibre cartridge (FiberCell Systems, cat#P3202). In flasks, NK92 cell density was maintained between $3\text{--}6 \times 10^5$ cells/mL. Human leukemic K562 cells (ATCC, CRL-3343) purchased at passage 2 were cultured in RPMI-1640 with 10% heat-inactivated FBS, where the density was maintained between $1\text{--}4 \times 10^5$ cells/mL. FreeStyle 293-F (or 293F) cells (Thermo Fisher Scientific, R790-07) were cultured in FreeStyle 293 Expression Medium (Gibco, cat#12338018) on a resistant shaker (Thermo Fisher Scientific, cat#88881127) at 135 RPM, where the density was maintained between $1\text{--}3 \times 10^6$ cells/mL. All cell lines were cultured at 37°C in a 5% CO₂ incubator, except the 293F cell line, which was cultured at 37°C in an 8% CO₂ incubator. Cell counts were performed on the Cellometer Auto 2000 Viability Counter (Nexcelom BioScience LLC) using the ViaStain Acridine Orange and Propidium Iodide (AO/PI) Staining Solution (ESBE Scientific, cat#CS2-0106) with the immune cell AO/PI program (channel 1: 470/535 nm for 0.5 seconds of exposure and channel 2: 540/605 nm for 3 seconds of exposure). During the NK92 cell expansion phase, Population Doubling Time (PDT) was calculated as follows: $(t_2 - t_1)/3.32 \times (\log n_2 - \log n_1)$ where “t” is time

and “n” number of cells. HFB-produced NK92 cell normalized fold expansion was calculated by dividing the number of harvested cells by the number of seeded cells and normalized by dividing by the length of the culture in days.

Mycoplasma testing

The Mycoplasma PCR detection kit (Abcam, cat#ab289834) was used to measure the presence or absence of mycoplasma in NK92 conditioned media (from flask expansion and HFB production lots), following the manufacturer’s protocol. PCR products were run on a 1% agarose gel made using agarose (Thermo Fisher Scientific, cat#R0491), 0.5X UltraPure TAE Buffer (Invitrogen, cat#15558042) and SYBR Safe DNA Gel Stain (Invitrogen, cat#S33102). The Quick Load 1Kb Plus DNA ladder (NEB, cat#N0469S) and samples were loaded with Blue Juice Gel Loading Buffer (Invitrogen, cat#10816015) before running the electrophoresis program for 30 minutes. Visualization was performed on the Dual LED Blue/White Light Transilluminator (Invitrogen, cat#LB0100).

NK92 cell radiation

NK92 cells were treated with a dose of 10 Gy (clinical gold standard dose) delivered by the X-RAD 320 (Marietta X-Ray & Precision X-Ray) at the University of Ottawa Preclinical Imaging Core to assess the cytotoxicity of the cells following the clinical protocol [164]. In addition, cell viability was measured by AO/PI daily for five days. Microscopic images were taken using the EVOS FL Auto 2 Imaging System (Invitrogen, cat#AMAFD2000) by the EVOS 4X/0.13 Fluorite Objective (Invitrogen, cat#AMEP4680).

Hollow-Fiber Bioreactor (HFB) Cell Culture Condition Parameters and EV production

The HFB cartridge was primed according to the manufacturer’s procedure using sterile Dulbecco’s phosphate-buffered saline without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (DPBS^{-/-}, Gibco, cat#14190250) and ImmunoCult-XF T Cell Expansion Medium. The medium was replaced before seeding cells into the cartridge, and cell health metric baselines were acquired where 0.5 mL of medium was drawn from the medium reservoir to verify glucose content using a glucose meter (AccuCheck Guide Glucose meter, Model 930), L-Lactate using the L-Lactate Assay Kit (Abcam, cat#ab65331) and pH with pH

Strips-Mquant 5-10 (Millipore Sigma, cat#1.09533). Then, a cell suspension of 20 mL containing 1.0×10^8 flask-expanded NK92 cells (passage 15) in ImmunoCult-XF T Cell Expansion Medium was injected into a medium hollow-fibre cartridge (20kDa Molecular Weight Cut-Off, 4000 cm², polysulfone fibre cartridge with oxygenator of 4 m; FiberCell Systems, cat#C2011) according to the manufacturer's procedure. The HFB cartridge was put on the duet 93202 Flow Control Pump (FiberCell Systems) at 37°C in a 5% CO₂ incubator, where the medium volume in the extracellular capillary space (ECS), 29 mL, was set at a flow rate of 22, the equivalent of 88 mL/min for the first day of culture. Afterwards, the flow rate for the remaining culture was set to 25, the equivalent of 100 mL/min. On average, the medium in the reservoir (250-500 mL) was replaced every 1-2 days to maintain the glucose content above 50% of the initial levels found in the medium. Cell health surrogates were monitored daily, where 0.5 mL of the medium was drawn from the reservoir to monitor glucose, L-lactate, and pH. Daily, 20 mL of pre-warmed ImmunoCult-XF T Cell Expansion Medium was injected from the left ECS port to push an equivalent volume of EV-rich conditioned medium (CM) through the right ECS port for EV collection. Harvested EV-rich CM was first centrifuged at 300xg for 5 min to remove cells and then centrifuged at 2000xg for 10 min to remove cell debris. Cleared EV-rich CM was equally aliquoted (~9.5 mL) into two Corning 15 mL tubes (Corning, cat#430791) and stored at -80°C until processing. After 8 days of culture in the HFB, 126 mL of EV-rich CM was harvested, thereby generating two aliquots of 63 mL of EV-rich CM, representing production lot 1. To continue producing EV-rich CM using this biomanufacturing workflow, the NK92 cells were harvested from the ECS of the HFB by performing the "HFB-cell harvest protocol": 60 mL of pre-warmed ImmunoCult medium was injected through the left ECS port and pushed back-forth at least ten times to loosen up cells before completely being pushed and collected by a syringe through the right ECS port. This was repeated a total of two times for the first production lot (120 mL of ImmunoCult medium used for HFB-cell harvest) and a total of three times for subsequent production lots (180 mL of ImmunoCult medium was used for HFB-cell harvest) to account for the larger number of NK92 cells present in the cartridge. Harvested cells were counted using AO/PI as detailed in the "Cell Culture Condition Parameters" section and centrifuged at 300xg for 5 min. At this point, a cell suspension of 20 mL containing 1×10^8 HFB-

produced NK92 cells was seeded into the same HFB cartridge for continuous EV production to create subsequent lots using the same process. The remaining HFB-produced NK92 cells were cryopreserved in CryoStor CS10 freezing medium (Sigma, cat#C2874) using a Mr. Frosty Freezing Container. Subsequent production lots 2 and 3 lasted 6 days of culture, each generating two aliquots of 45 mL of EV-rich CM (90 mL per production lot).

Immunophenotypic analysis of NK92 cells

Immunophenotypic analysis of NK92 cells was performed on flask-cultured and HFB-produced NK92 cells. Cells were counted and suspended in ImmunoCult-XF T Cell Expansion Medium, where 5×10^5 cells were seeded per well in a U-bottom 96-well plate (Costar, cat#163320) without stimulation (basal expression level) or with stimulation using 12.5 ng of phorbol-12-myristate-13-acetate (PMA; StressMarq, cat#SIH-508-1MG) and 250 ng of Ionomycin (IO; Stemcell Technologies, cat#73722) for 4h, in 250 μ L final volume. PE-Cy7-CD107 α (H4A3) (BioLegend, cat#328618) was added at the beginning of the assay to assess the degranulation potential (activation status) of the NK92 cells. After 4h of incubation, cells were transferred into a V-bottom plate (Costar, cat#249662) and centrifuged at 400xg for 5 min at 4°C. Cells were then suspended into a solution of 0.6 μ g Fc blocking IgG (R&D systems, cat#MAB002) and LIVE/DEAD Fixable Blue Dead Stain (Life Technologies, cat#L23105) and incubated for 10 min at 4°C, protected from light. Afterwards, 100 μ L of titrated antibodies solution containing BV711-CD16 (3G8) (BioLegend, cat#302044), BV605-CD56 (5.1H11) (BioLegend, cat#362538) and BV785-CD69 (FN50) (BioLegend, cat#310932) was added to corresponding wells, using 10% BV stain buffer plus (BD Bioscience, cat#566385) in flow buffer, for 30 min at 4°C, protected from light. The samples were washed twice with flow buffer before fixation and permeabilization using the Fixation/Permeabilization Solution Kit (BD Biosciences, cat#554714), per the manufacturer's instructions. Afterwards, 100 μ L of titrated intracellular antibodies solution made of BV421-IFN γ (4S.B3) (BioLegend, cat#502532), BV650-Tumour necrosis factor alpha (TNF α) (Mab11) (BioLegend, cat#502938) and AF647-GzmB (QA16A02) (BioLegend, cat#372220) was added to corresponding wells, using the same staining buffer composition. To maximize the detection of

intracellular cytokines, the corresponding cells were treated with Protein Transport Inhibitor GolgiPlug-brefeldin A (BD Bioscience, cat#555029) and GolgiStop-monensin (BD Bioscience, cat#554724) for 4h before the end of the stimulation period. After 30 min of staining at 4°C, protected from light, the cells were washed twice with the permeabilization buffer. Following labelling, samples were transferred to 5 mL FACS tubes and analyzed by flow cytometry using the BD FACS Aria™ III Fusion flow cytometer (BD Biosciences) equipped with a 70 µm nozzle assay (BD Biosciences; P/N647339) and the BD FACSDiva Software (v9.0.2). A minimum of 100 000 events per sample were collected at the single-cell CD56-positive gate. The data was analyzed using FlowJo V10.8.1 (FlowJo LLC, USA) after cleaning the data with the FlowAI plugin (v2.3.1).

Cytotoxicity analysis of NK92 cells

Cytotoxicity analysis of NK92 cells was performed on flask-cultured and HFB-produced NK92 cells. All cell counts were performed using AO/PI, and all cells were suspended in ImmunoCult-XF T Cell Expansion Medium. In a U-bottom 96-well plate (Costar, cat#163320), 1×10^5 of K562 cells labelled with 2 µM of CellTrace™ CFSE Cell Proliferation kit (Invitrogen, cat#C34554) were seeded per well. Effector NK92 cells (non-irradiated or irradiated) or 293F (negative control) were used at various effector: target (E:T) ratios and co-cultured for 4h. Afterwards, the cells were transferred into a V-bottom plate (Costar, cat#249662) and centrifuged at 400xg for 5 min at 4°C. The cells were washed once with flow buffer before being suspended in a final staining solution containing 1 µM of Sytox Blue Nucleic Acid Stain (Invitrogen, cat#S11348). Samples were transferred to 5 mL FACS tubes and analyzed by flow cytometry using the BD FACS Aria™ III Fusion flow cytometer (BD Biosciences) equipped with a 70 µm nozzle assay (BD Biosciences; P/N647339) and the BD FACSDiva Software (v9.0.2). A minimum of 50 000 events per sample were collected at the single-cell CFSE-positive gate. The data was analyzed using FlowJo V10.8.1 (FlowJo LLC, USA) after cleaning the data with the FlowAI plugin (v2.3.1). Cytotoxicity percentage was calculated as follows where dead control represents K562 heat shocked for 15 min at 70°C (dead cell % in heat shock control), and the K562 only control represents spontaneous death (dead cell % K562 only control).

$$\% \text{ Cytotoxicity} = \left(\frac{\text{CFSE}^+ \text{ dead cell \% in sample}}{\text{CFSE}^+ \text{ dead cell \% in heat shock control}} \right) - \left(\frac{\text{CFSE}^+ \text{ dead cell \% in k562 only control}}{\text{CFSE}^+ \text{ dead cell \% in heat shock control}} \right)$$

NK92-EV purification from the EV-rich CM by Fast Protein Liquid Chromatography using size-exclusion chromatography (SEC)

As described in the “Hollow-Fiber Bioreactor (HFB) Cell Culture Condition Parameters and EV production” section, the EV-rich CM was harvested daily. Half of the total NK92-EV-rich CM harvested from each lot was processed for EV purification (63/126 mL was processed for lots 1 and 45/90 mL for lots 2 and 3). 293F EV-rich CM (30 mL) was processed for EV isolation to generate control EVs. The EV-rich CM was centrifuged at 10 000xg for 30 min after thawing. Then, the EV-rich CM was incubated for 4 hours at 37°C with 50 U/mL Benzonase Nuclease (Millipore Sigma, cat#E1014-25KU) and 1.5 mM of MgCl₂ (Millipore Sigma, cat#M1028), while moderately shaking for nucleic acid digestion. Then, the samples were loaded into a 60 mL syringe (BD Biosciences, cat#309653). For SEC-based EV purification, two inline pre-packed HiScreen Capto Core 700 (Cytiva, cat#17548115) for a total bed height of 20 cm were prepared on the AKTA Fast Protein Liquid Chromatography (GE Lifesciences, cat#29022094) according to the manufacturer for the clean-in-place and column priming procedures, using filtered DPBS^{-/-}. Filtered-DPBS^{-/-} and filtered water were generated using the 0.1 µm vacuum filtration unit Filtropur V50 (Sarstedt, cat#83.3941.002). The ultraviolet absorbance was zeroed after the reading stabilized while filtered-DPBS^{-/-} was running at the instrument. EV-rich CM was loaded on the column attached to the instrument at a flow velocity of 150 cm/h or flow rate of 1.2 mL/min. Fractionation of the sample, 5 mL collected in Eppendorf tubes (Eppendorf, cat#30119487), was initiated once the ultraviolet absorbance level passed the 230 mAU mark and stopped when the ultraviolet absorbance level dropped below the 1600 mAU mark. All fractions falling between this ultraviolet absorbance range were combined and concentrated using sterilized (rinsed with 70% ethanol) and filtered-DPBS^{-/-} pre-equilibrated Centricon Plus-70 Centrifugal Filter (Millipore Sigma, cat#UFC701008) at 4000xg for 25 minutes. The final retentate was collected by inverting the filter into the collection cup and spinning the assembly at 1000xg for 2 minutes. To ensure sterility, the recovered solution was filtered at 0.22 µm with a pre-wet (filtered-DPBS^{-/-}) Acrodisc Syringe Filter (Pall Corporation,

cat#PN4602). The final concentrated solution of NK92-EV was kept at 4°C and used for various analyses within a week.

Monitoring EV purification efficiency through nucleic acid and protein content analysis

To monitor the purification efficiency of EVs, various aliquots were collected throughout the isolation process (pre/post-benzonase, individual fractions, combined fractions and final concentrated solution) and nucleic acid (DNA and RNA) content was assessed by the Qubit dsDNA HS Assay Kits or Qubit dsDNA BR Assay Kits (Invitrogen, cat#Q33231 and cat#Q33262, respectively) and the Qubit RNA High Sensitivity (Invitrogen, cat#Q32855) on the Qubit Flex Fluorometer (Invitrogen, cat#Q33327; v1.5.0) using the Qubit Flex Assay Tube Strips (Invitrogen, cat# Q33252). EV protein content was assessed by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, cat#23227).

Nanoparticle tracking analysis (NTA) of NK92-EVs

Purified EV samples were analyzed by NTA using the NanoSight NS300 (Malvern, UK), equipped with a 488 nm laser and a sCMOS camera. For capture settings, a camera level of 14 was used for all samples, and a detection threshold of 15 was used for analysis resulting in approximately 30-70 particles per frame. After stabilizing at room temperature, each sample was thoroughly vortexed before preparing an appropriate EV dilution (using filtered-DPBS^{-/-}) to obtain a final volume of 1 mL in a 1 mL syringe (Thermo Fisher Scientific, cat#MB9204560TF-LAB) to be loaded on the instrument. Roughly half of the syringe content was pushed manually through the instrument until the camera observed stable particles per frame. The syringe was then loaded on the syringe pump (Harvard Apparatus, cat#98-4730), which was used for acquisition capture in flow mode. Each sample was run using the following script with NTA v3.0 software: 23°C, one capture of 1 min at a flow speed of 10. After the script was completed, the flow rate was increased to 1000 for 10-15 seconds before being returned to 10. This sequence was repeated until five captures were taken per sample. This acquisition method allows sampling and analysis of the sample through the syringe, assuring an accurate representation of the entire sample. The five captures were averaged and analyzed using the NTA v3.0 software (Malvern Instruments).

Transmission Electron Microscopy (TEM) Analysis of a single NK92-EV

Purified EV samples (2×10^9) were added to a Vivaspin 300 kDa filter (Sartorius, cat#VS0651), pre-equilibrated with filtered D-PBS^{-/-} and centrifuged at 2000xg for 3 minutes. Next, the EVs were washed with 100 uL of 4% paraformaldehyde (PFA; Electron Microscopy Sciences, cat#15710) three times to ensure complete collection. The resulting wash volumes were combined into a low-bind Eppendorf tube to yield 300 uL of fixed-EV solution. Later, a 50 uL drop of fixed-EV solution was added to parafilm, where a glow-discharged (45 sec) formvar-carbon coated EM grid (Electron Microscopy Sciences; cat#FCF300-CU) was added on top of the fixed-EV drop and rested for 10 min. Next, each grid was rinsed in one drop of 50 uL filtered D-PBS^{-/-} for three minutes before processing for double contrast staining. Here, each grid was placed in one drop of UranylLess EM Stain (Electron Microscopy Sciences; cat#22409) combined with one drop of Lead citrate (Electron Microscopy Sciences; cat#22410) for 30 seconds before rinsing five times into one drop of 50 uL D-PBS^{-/-} for three minutes. Then, each grid was allowed to air dry for at least 30 min before imaging on the EI Tecnai G2 Spirit Twin TEM with a Lab6 emitter, operating at 120kV, at Carleton Nano Imaging Facility, Canada. The images were acquired with an Eagle camera with a 16K resolution. All steps were performed at room temperature.

Endotoxin assessment of NK92-EVs

Purified EV samples were analyzed and used for endotoxin assessment using the Pierce Chromogenic Endotoxin Quant Kit (Life Technologies, cat#PIA39552; highly sensitive end-point chromogenic endotoxin detection assay based on the amebocyte lysate method for research use only [not GMP-compliant]) as per the manufacturer's instructions for a dose of 1×10^9 EVs.

Bacterial contamination assessment of NK92 cells and NK92-EVs

NK92-MI cell and EV media samples were cultured at 37°C overnight and over 5 days in LB broth (BioBasic, cat#SD7002) where bacterial contamination was evaluated using positive controls (*E. coli* HST08 strain) and negative controls (water). The optical density at 600 nm (OD₆₀₀)

was measured using the Microplate Reader (BioTek Synergy H1 Multimode Reader; cat#SH1M2G-SN) using the Gen5 software (3.14).

Viral panel assessment of NK92 cells and NK92-EVs by quantitative reverse transcription PCR (RT-qPCR)

NK92-MI cells and NK92-EVs from each lot were assessed for the presence of several viruses, including Cytomegalovirus (CMV), Epstein-Barr Virus (EBV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Human Immunodeficiency Virus 1 and 2 (HIV-1/2), Human Papillomavirus type 16 (HPV-16), and Human T-lymphotropic Virus (HTLV). NK92-MI cells total RNA was extracted from using the RNeasy kit (Qiagen, cat#74104), as per manufacturer instructions. NK92-EVs total RNA was extracted using the Trizol (Thermo Fisher, cat#15596026) and chloroform (Thermo Fisher, cat#C295-4) method (2.5:1) after which they were thoroughly vortexed and allowed to incubate at room temperature for 10 minutes. Phase separation was performed by centrifuging samples at 12,000xg and 4°C for 15 minutes. The upper aqueous phase was collected and mixed with 10% sodium acetate (3 M, pH 5.5) from Thermo Fisher (cat#AM9740), 20 µg/mL glycogen (Thermo Fisher, cat#FERR0551), and an equivalent volume of 100% ethanol. Samples were thoroughly vortexed and allowed to incubate overnight at -80°C. Following incubation, RNA pellets were obtained through centrifugation at 16,000xg and 4°C for 30 minutes, washed twice with 70% ethanol and centrifugated at 16,000xg and 4°C for 10 minutes, air-dried for 10 minutes, and then resuspended in nuclease-free water (A_{260}/A_{280} and $A_{260}/A_{230} \geq 2.00$). cDNA was synthesized from mRNA using the iScript cDNA Synthesis Kit (Bio-Rad, cat#1708890), as per manufacturer instructions. Gene expression levels were determined using the Bio-Rad MyiQ real-time PCR system (Bio-Rad, cat#170-9770) where the reaction mixture consisted of 50% SYBR Green (Bio-Rad, cat#1708880), 37.5% nuclease-free water, 5% of forward/reverse primers, and 2.5% cDNA. Specific primers were used for each virus (Forward and reverse gene primers: CMV (ACC GGA GAA GAG CCC ATG TC / CCA GTG CCC GCA GTT TTT ATT), EBV (CTT GGA GAC AGG CTT AAC CAG ACT CA / CCA TGG CTG CAC CGA TGA AAG TTA T), GAPDH (AAT GGG CAG CCG TTA GGA AA / GCG CCC AAT ACG ACC AAA TC), HBV (AAG CTG TGC CTT GGG TGG CTT / CGA GAT TGA GAT CTT CTG CGA C), HCV (CTG GGC AAT TGG TTC GGT TG /

CAG AGC AAG GTG TTG TTG CC), HIV-1 (GAC GGT ACA GGC CAG ACA AT / CTT GAT GCC CCA GAC TGT GA), HIV-2 (TGG GAG ATG GGC GCG AG / TTC ATT CGC TGC CCA CAC AA), HPV-16 (TCA AAA GCC ACT GTG TCC TGA / CGT GTT CTT GAT GAT CTG CAA) and HTLV (CCA TAC AAC CCC ACC AGC TCA G / GGT GGA TTT GCC ATC GGG TT). Reaction conditions consisted of one cycle at 95°C for 20 seconds (denaturation-step), one cycle at 95°C for 3 seconds (synthesis-step), and one cycle at 60°C for 30 seconds (annealing-step), repeated for a total of 45 cycles. A cycle threshold (CT) difference of at least 5 cycles from the 35-cycle cut-off value and above the Y-axis threshold of 100 RFU was considered a positive result.

Western blot detection of NK92-EV protein markers

Immediately after purification of NK92-EV samples, 200 µL aliquots were lysed in 5X RIPA buffer (Alfa Aesar, cat#J62524-AE) with the addition of 1X HALT protease and phosphatase inhibitors (Thermo Fisher Scientific, cat#78441) and transferred to a 1.5 mL Protein LoBind tube (Eppendorf, cat#0030.108.116). Each sample was then put on an end-over-end shaker (LabQuake Shaker) and incubated for 30 min at 4°C. The sample was spun briefly to collect the EV lysate before sonicating on ice (3 x 10-second pulses at 20% amplitude). Next, the EV protein lysate was centrifuged at 14000×g for 5 min at 4°C. The recovered supernatant was stored at -80°C until processing, and protein content was assessed by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, cat#23227). For western blot analysis, 10 µg of cell protein lysates and 30 µg of NK92-EV protein lysates were combined with 4X LI-COR Protein Loading Buffer (LI-COR, cat#928-40004) and Bolt 10X Sample Reducing Agent (Invitrogen, cat#B0009) to a final concentration of 1X. Samples were boiled for 5 min and then loaded onto precast Bolt Bis-Tris Plus 4–12% SDS-PAGE gels (Invitrogen, cat#NW04120BOX). Gels were run using a MOPS buffer system (Invitrogen, cat#B0001) for 30 min at 200 V. Gels were transferred to a Millipore Immobilon FL PVDF membrane (Millipore, cat#IPFL00005) using the Bolt Mini Module wet transfer system for one hour at 20V. Following the transfer, membranes were dried. Membranes were then reactivated in methanol, rinsed in filtered-DPBS^{-/-} for 5 min at room temperature and washed once in distilled water for 5 min on an orbital shaker at speed 4 (~300 RPM). For target detection, membranes were incubated in the iBind blocking buffer (Invitrogen, cat#SLF2019) for 1 hour at room temperature on

an orbital shaker at approximately 300 RPM. Using the iBind Western Blot System (Invitrogen, cat#SLF1000), membranes were probed with the following primary antibodies diluted in iBind solution (Invitrogen, cat#SLF2019): anti-Calnexin (Cell Signalling Tech, cat#2433S, 1:5000), anti-GM130 (BD Transduction Laboratories, cat#610822, 1:500), anti-Flotillin-1 (BD Transduction Laboratories, cat#610820, 1:500), anti-GAPDH (GeneTex, cat#GTX627408; 1:1000), anti-GzmB (Cell Signalling Tech, cat#17215S, 1:1000). The following secondary antibodies were diluted 1:4000 in iBind solution: IRDye 800CW Goat anti-Mouse (LI-COR, cat#925-32210) and IRDye 800CW Goat anti-Rabbit (LI-COR, cat#925-32211). Following antibody incubation, membranes were rinsed in distilled water twice for 2 minutes and imaged. All image captures were performed at 800 nm using the LI-COR Odyssey CLx NIR imager.

Immunophenotyping of surface antigens of NK92-EVs using multiplex bead-based flow cytometry analysis

Purified EV samples were assessed for surface antigen expression using the MACSPlex Exosome kit human (Miltenyi Biotec, cat#130-108-813), as previously described, with minor modifications [2]. Briefly, 1×10^{10} EV samples were processed, per the manufacturer's recommendations, using the "overnight protocol for the assay using 1.5 mL tubes" on a temperature control orbital shaker set at 4°C and 450 RPM. EVs in each sample were captured using 40 µL of MACSPlex Exosome Capture Beads where the final volume was brought up to 340 µL of filtered-DPBS^{-/-}. EVs were labelled using the anti-CD63 and anti-CD81 cocktail MACSPlex Exosome Detection Reagent. Following labelling, samples were processed by flow cytometry using the BD FACSymphony A1 (BD Biosciences) and the BD FACSDiva Software (v9.0.2). A minimum of 10 000 events per sample were collected. The data was analyzed using FlowJo V10.8.1 (FlowJoLLC, USA). For data processing, buffer-only control (filtered-DPBS^{-/-}) was used for background subtraction for each bead population; negative values were zeroed. In addition, the IgG isotype control or REA control signal was subtracted after background subtraction, and negative values were zeroed.

Cytokine profiling of NK92-EVs using multiplex bead-based flow cytometry analysis

Immediately after purification of NK92-EV samples, 200 μ L aliquots were lysed in 0.2 mL of lysis buffer composed of PBS^{-/-}, 0.2% Triton X-100 (Sigma, cat#T9284) and 1X HALT protease and phosphatase inhibitors (Thermo Fisher Scientific, cat#78441) and transferred to a 1.5 mL Protein LoBind tube (Eppendorf, cat#0030.108.116). Each sample was then put on an end-over-end shaker (LabQuake Shaker) and incubated for 30 min at 4°C. The sample was then spun briefly to collect the EV lysate before sonicating on ice (3 x 10-second pulses at 20% amplitude). Next, the EV protein lysate was centrifuged at 14,000 $\times$ g for 5 min at 4°C. The recovered supernatant was stored at -80°C until processing, and protein content was assessed by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, cat#23227). For cytokine assessment of IL-2, IL-4, IL-6, IL-10, IL-17A, IL-21, MCP-1 (CCL2), GM-CSF, IFN γ , TNF α , GzmB and PFN, the human MACSPlex Cytotoxic T/NK Cell Kit (Miltenyi Biotec, cat#130-125-800) was used as per the manufacturer's instructions for the "Filter Plate protocol." The standards, samples and filter plate were prepared exactly per the manufacturer's recommendations. Samples and standards were incubated with capture beads for 2 hours with mixing (orbital shaker, 450 RPM), protected from light. Following washes, all samples and standards were incubated with the first detection reagent (biotin-conjugated anti-cytokine antibodies) for 1 hour with mixing (orbital shaker, 450 RPM), protected from light. Then, following washes, samples and standards were then incubated with the second detection reagent (anti-biotin-APC) for 30 minutes with mixing (orbital shaker, 450 RPM), protected from light. Following final washes, samples were suspended in 200 μ L of MACSPLEX buffer to be processed by flow cytometry using the BD FACSymphony A1 (BD Biosciences) and the BD FACSDiva Software (v9.0.2). A minimum of 5000 events per sample were collected. Each group was conducted with four technical replicates. The data was analyzed using FlowJo V10.8.1 (FlowJoLLC, USA). The PBS buffer was added as a control for median fluorescent intensity (MFI) background subtraction for data processing, and negative values were zeroed. For data processing, the concentration (x-values) was logarithmically transformed for interpolating a five-parameter logistic curve fit (5PL, X-values are log) where the sample's MFI was extrapolated to obtain the log-value of the concentration. The natural log was applied to obtain the cytokine concentration in pg/mL. The cytokine concentration was normalized by the respective protein

concentration (mg/mL) determined by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, cat#23227).

Cytotoxicity Assessment of NK92-EVs by PrestoBlue Assay

The cytotoxicity of NK92-EVs was assessed by PrestoBlue assay after 5h co-incubation with the K562 target cell line. K562 cells were counted using ViaStain AO/PI Staining Solution (ESBE Scientific, cat#CS2-0106) and suspended in RPMI media (Gibco, cat#A18967-01) containing 10% Exosome-Depleted FBS (Gibco, cat#A2720803). Four thousand viable cells per well were seeded in 96-well Flat Clear Bottom Black Polystyrene TC-treated Microplates (Costar, cat#3603). Maintenance (non-treated cell) and lysed cells are used as controls for cytotoxicity. Effector NK92-EVs and negative control 293F-EVs were added at different concentrations (such as 1×10^5 , 1×10^7 , 1×10^9 , 1×10^{10} , and 1×10^{11} particles/mL) and incubated with the K562 target cells (150 μ L/well) for 5 hours. Cell viability was measured as per the PrestoBlue Assay (Life Technologies, cat#A13261), as per manufacturer's instructions. Fluorescence intensities were measured, after 90 minutes of incubation, by the Microplate Reader (BioTek Synergy MX) using the Gen5 software (v2.09) with excitation of 560 ± 9 nm and emission of 590 ± 9 nm. Each group was conducted with four technical replicates and processed for dose-response analysis using a non-linear regression for inhibition effect with a variable slope (four parameters) on normalized data using the K562-only control RFU.

Off-target toxicity assessment of NK92-EVs against fibroblast cells by PrestoBlue assay

Human BJ fibroblast cells (ATCC CRL-2522), provided by Dr. Lisheng Wang at passage 5), were used as model to assess the off-target toxicity of NK-EVs. BJ cells were cultured in MEM with Earle's & NEAA (StemCell tech, cat#36550) with 10% Fetal Bovine Serum (FBS, Gibco, cat#12483020) at 37°C in a 5% CO₂ incubator where the density was maintained between $3-4 \times 10^4$ cells/cm²; passaged every 2-3 days. Trypsinization was performed after washing the cells with sterile Dulbecco's phosphate-buffered saline without Ca²⁺/Mg²⁺ (DPBS⁻, Gibco, cat#14190250) and TrypLe select (Gibco, cat#12563-029). Cell counts were performed on the Cellometer Auto 2000 Viability Counter (Nexcelom BioScience LLC) using the ViaStain Acridine Orange and

Propidium Iodide (AO/PI) Staining Solution (ESBE Scientific, cat#CS2-0106) with the immune cell AO/PI program (channel 1: 470/535 nm for 0.5 seconds of exposure and channel 2: 540/605 nm for 3 seconds of exposure). The off-target toxicity of NK92-EVs was assessed by PrestoBlue assay after 5h co-incubation with the target cell line. K562 leukemic cells were used as a control for on-target cytotoxicity. Using a 96-well Flat Clear Bottom Black Polystyrene TC-treated Microplates (Costar, cat#3603), 5000 K562 leukemic target cells (suspension cells), or 1000 BJ target cells (adherent cells) were seeded per well in their respective medium. Maintenance (non-treated cell) and lysed cells are used as controls for cytotoxicity. Each cell line was maintained in its respective culture medium with 10% Exosome-Depleted FBS (Gibco, cat#A2720803) and allowed to settle into the plate for two hours before adding effector NK92-EVs at various concentrations (ratio of 3 000, 30 000, 300 000, 3 000 000 and 15 000 000 effector EVs to target cells) and incubated with the target cells (150 μ L/well) for 5 hours. Cell viability was measured with the PrestoBlue Assay as detailed in the “Cytotoxicity Assessment of NK92-EVs by PrestoBlue Assay” section.

EV-TRACK

We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV230058) [165].

Statistical analysis

Data were expressed as the means $\pm$ the standard deviation (S.D.) or standard error of the mean (S.E.M.), without outlier exclusion. When a logarithmic transformation was performed, details were added in the respective section. The data were normalized to the control group for relative comparison, as indicated in each figure legend. The number of experimental and technical replicates used is indicated in the figure legends. Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software Inc., LaJolla, CA, USA). One-way ANOVA and Two-way ANOVA followed by post hoc tests (Tukey's multiple comparisons) and unpaired Student's t-test were used and identified in the figure legend. Results were considered significant when $p < 0.05$.

Results

ImmunoCult-acclimatized NK92 cells displayed their NK92 properties

To generate clinical-grade NK92-EV products, the NK92 cells were cultured under feeder-free conditions in ImmunoCult-XF, a chemically defined SF/XF medium. After 3-5 days of recovery in RPMI-1640 with 10% FBS, the cells were flask-expanded in ImmunoCult medium for 18 days. A total of 1×10^8 viable NK92 cells were expanded for use in the HFB system (**Figure 3A**). Acridine Orange and Propidium Iodide (AO/PI) viability measurements indicated an average NK92 cell viability of $68.43 \pm 3.72\%$ during the expansion phase (**Figure 3B**). The average population doubling time (PDT) was 3.10 ± 1.32 days (**Figure 3C**). As expected, the ImmunoCult-cultured NK92 cells expressed CD56 and lacked CD16 (**Figure 3D**). Upon stimulation with PMA/IO, the cells also expressed the activation marker CD69 and the degranulation marker CD107 α (**Figure 3D**). CD107 α -expressing NK92 cells demonstrated a response to 4 hours of PMA/IO stimulation, as indicated by a decrease in the MFI of GzmB (basal MFI of 19972 relative fluorescence units [RFU] vs stimulated MFI of 4286 RFU) and increased in the MFIs of IFN- γ (basal MFI of 1607 RFU vs stimulated MFI of 6917 RFU) and TNF α (basal MFI of 1539 RFU vs stimulated MFI of 2292 RFU) (**Figure 3E**). To test the cytotoxic functionality of the ImmunoCult-acclimatized NK92 cells, we performed a 4-hour *in vitro* cytokilling assay against the human leukemic target cell line K562 (a gold standard cell line for testing cytotoxicity of NK92 cells). We observed cytotoxicity in a dose-dependent manner by the NK92 cells against K562 cells, with a 1:1 E:T ratio yielding $31.93\% \pm 9.99\%$ lysis and a 10:1 E:T ratio yielding $68.54\% \pm 13.68\%$ lysis (**Figure 3F**).

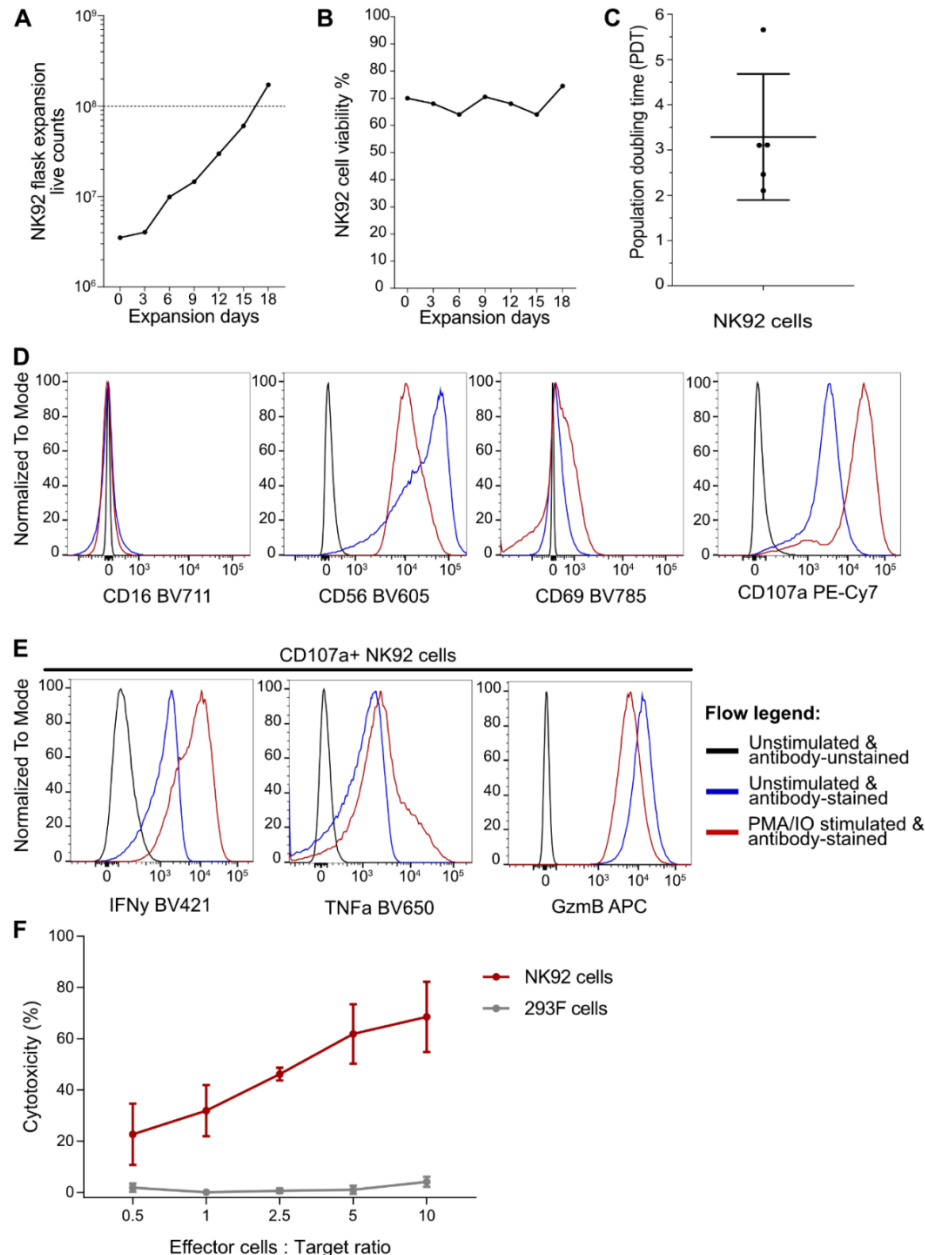


Figure 3. ImmunoCult-acclimatized NK92 cells retained NK properties.

(A) The growth curve of flask-expanded NK92 cells in ImmunoCult medium (xeno-free and serum-free) shows live cell counts (dotted line representing the minimal number of cells required for seeding a medium cartridge for the hollow-fiber bioreactor system (n=1 experimental replicate). (B) Flask-expanded NK92 cell viability during cell expansion (n=1 independent experimental replicate with technical duplicates). (C) Flask-expanded NK92 cell population doubling time (PDT) in days during cell expansion (mean $\pm$ S.D., n=5 independent experimental replicates). (D) Representative antigenic profile of flask-expanded NK92 cells cultured in ImmunoCult medium and, analyzed by flow cytometry to detect CD16, CD56, CD69 and CD107 α . (E) Representative cytokine profile of flask-expanded CD107 α + NK92 cells by flow cytometric analysis to detect IFN γ , TNF α and GzmB. (F) Flask-expanded NK92 cells in ImmunoCult medium show cytotoxicity against leukemia K562 cells at various effector: target (E:T) ratios compared to negative effector control 293F cells. Cytotoxicity was determined by a 4-hour standard *in vitro* cytotoxicity assay using flow cytometry (mean $\pm$ S.D., n=3 independent experimental replicates).

HFB-produced NK92 cells in the ImmunoCult environment displayed their NK properties

To continuously generate EV-rich CM from ImmunoCult-acclimatized NK92 cells, we used the HFB cartridge from FiberCell Systems. First, 1×10^8 flask-expanded live NK92 cells were seeded into the cartridge and maintained in ImmunoCult medium (**Figure 4A**).

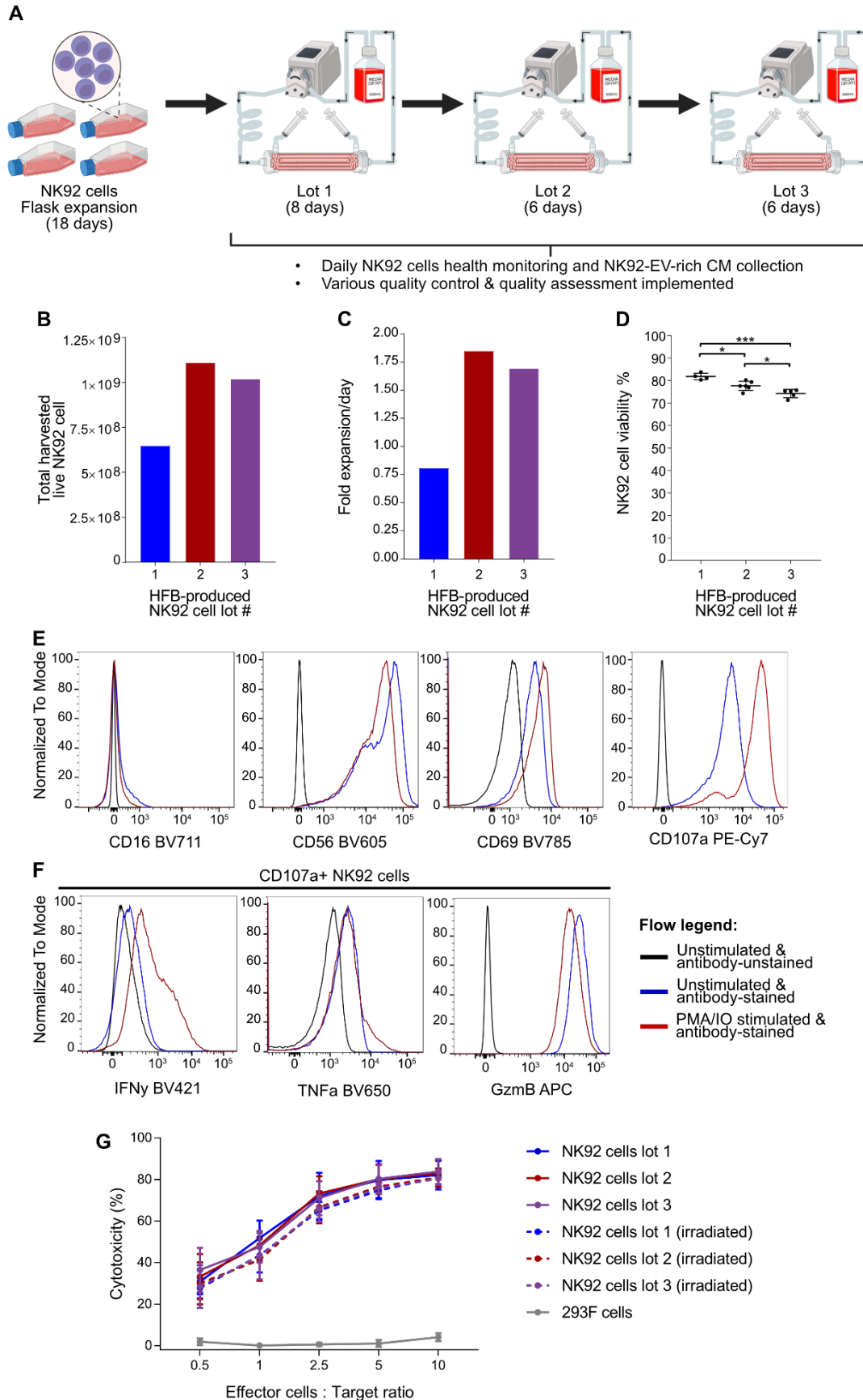


Figure 4. HFB-produced NK92 cells retained their NK properties.

(A) Biomanufacturing workflow design where NK92 cells were flask-expanded before HFB inoculation in ImmunoCult medium. Continued HFB NK92 production was performed for three separate lots over 20 days (lots 1 to 3) continuously, where harvested cells were re-seeded into the same cartridge after each production lot. EV-rich CM was collected daily and stored at -80°C until further purification. (B) Total harvested live HFB-produced NK92 cells from each production lot. (C) Fold expansion of HFB-produced NK92 cells per day of culture (fold expansion/day). (D) Viability of HFB-produced NK92 cells (mean $\pm$ S.D., n=4-6 from independent experimental replicates with technical duplicates). One-way ANOVA with Tukey's multiple comparisons test was applied where * $p \leq 0.05$ and *** $p \leq 0.001$. (E) Representative antigenic profile of HFB-produced NK92 cells analyzed by flow cytometry to detect CD16, CD56, CD69 and CD107 α . (F) Representative cytokine profile of HFB-produced CD107 α + NK92 cells analyzed by flow cytometry to detect IFN- γ , TNF α and GzmB. (G) Both non-irradiated and irradiated HFB-produced NK92 cells from lots 1 to 3 exhibit cytotoxicity against leukemia K562 target cells at various effector: target (E:T) ratios. Cytotoxicity was determined by a 4-hour standard *in vitro* cytotoxicity assay using flow cytometry (mean $\pm$ S.D., n=3 independent experimental replicates). Two-way ANOVA with Tukey's multiple comparisons test was applied. Panel A was created with BioRender.com.

The cells were cultured for three continuous production lots (lots 1, 2, and 3) over 20 days using the same HFB cartridge. Production lots 2 and 3 were reseeded with 1×10^8 cells harvested from lots 1 and 2, respectively. Production lot 1 consisted of 8 days of culture, while production lots 2 and 3 consisted of 6 days of culture before cell retrieval from the cartridge. To monitor the health of the NK92 cells in the bioreactor, we assessed the glucose, lactate, and pH levels in the medium reservoir daily (**Figure 5**). Glucose levels were maintained above 2.5 g/L (corresponding to 50% consumption) by replacing the medium as needed (**Figure 5**). Lactate levels remained below 8 g/L (except for the fifth day of culture during production lot 1) and stabilized with fewer fluctuations (**Figure 5**). The pH of the reservoir medium from all three lots remained constant over the monitoring period between 7.0-8.0 (**Figure 5**).

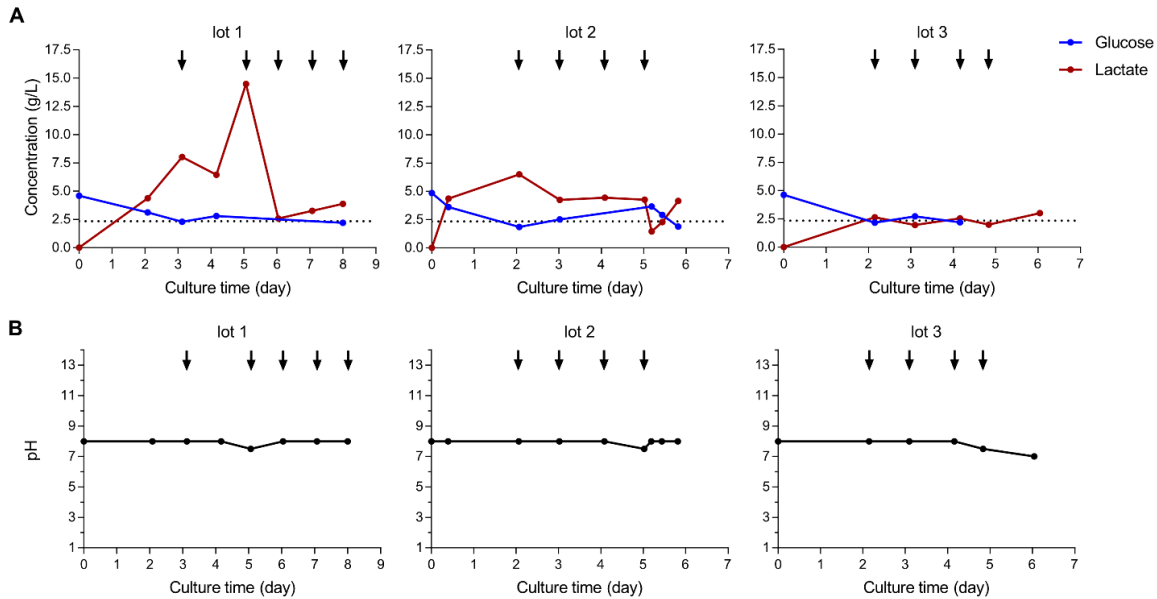


Figure 5. NK92 cell health metrics monitoring in the Hollow Fiber Bioreactor (HFB) system under ImmunoCult medium condition.

(A) Cell health metric monitoring for glucose concentration (the hachured line represents 50% consumption) and L-lactate content of reservoir medium of HFB production lots 1 to 3. (B) pH monitoring of reservoir medium of HFB production lots 1 to 3. Arrows indicate medium change.

HFB-produced NK92 cells were harvested at the end of each production lot, where 6.48×10^8 , 1.11×10^9 , and 1.02×10^9 viable cells were collected from production lots 1 to 3, respectively, which is within the clinical dose range (10^6 - 10^8 cells per kilogram [166, 167]) for each production lot (**Figure 4B**). The normalized fold expansion per day of culture was 0.81, 1.85, and 1.70 for production lots 1 to 3, respectively (**Figure 4C**). The viability of the NK92 cells was $81.82 \pm 1.46\%$, $77.65 \pm 2.05\%$, and $74.12 \pm 1.84\%$ for production lots 1 to 3, respectively (**Figure 4D**). The antigenic profile of NK92 cells produced by the HFB method was similar to that of NK92 cells grown in flasks. Both types of cultured NK92 displayed their CD16 and CD56 expression characteristics of NK92 cells and responsiveness to PMA/IO stimulation (MFI increase of CD69 and CD107 α) (**Figure 4E**). Similarly, CD107 α -presenting NK92 HFB-produced cells also responded to PMA/IO stimulation (MFI increase of IFN- γ and TNF α , and MFI decrease of GzmB) (**Figure 4F**). They also displayed cytotoxicity against the human leukemic target cell line K562 (**Figure 4G**). All HFB-produced NK92 cell lots showed a dose-dependent K562 cytotoxicity effect where a 1:1 E:T ratio (NK92:K562) yielded $49.19 \pm 2.81\%$ K562 lysis and a 10:1 E:T ratio yielded $82.99 \pm 0.85\%$ lysis. The NK cell

cytotoxicity was not affected by radiation, where the 1:1 and 10:1 E:T ratios (NK92:K562) yielded similar results ($42.59 \pm 1.16\%$ and $80.82 \pm 0.39\%$ K562 lysis, respectively).

All production lots tested negative for mycoplasma by PCR (**Figure 6**) and negative for bacterial growth (**Figure 7**).

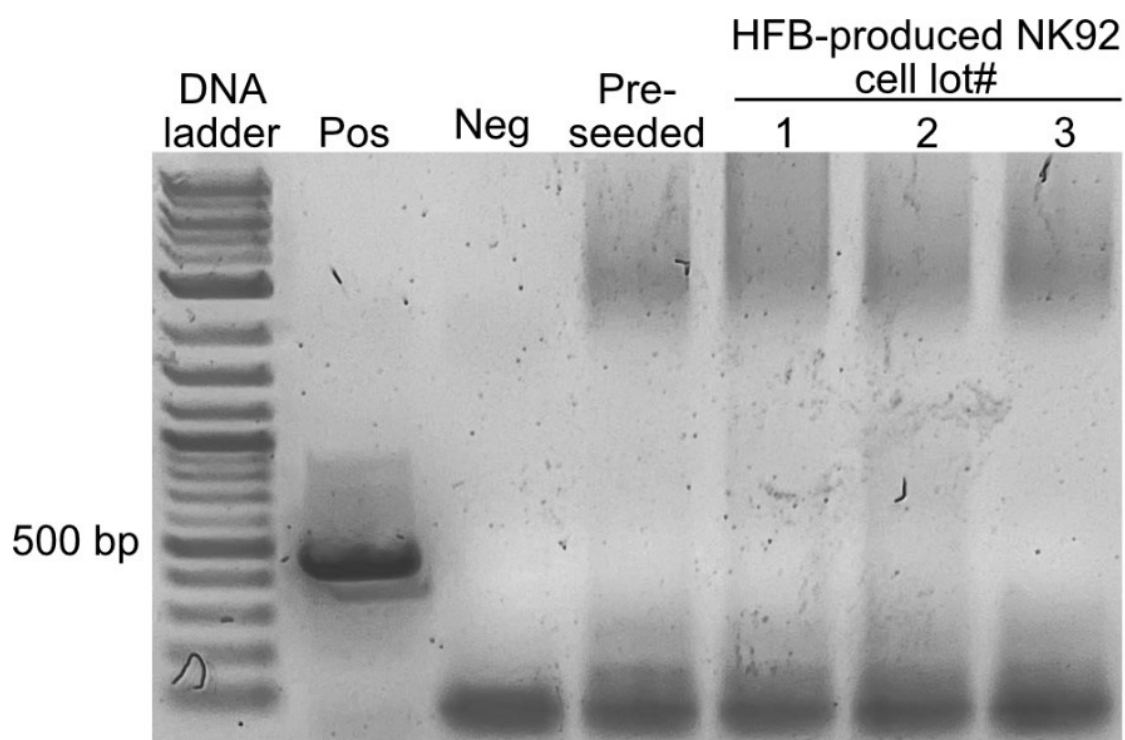


Figure 6. Mycoplasma testing of conditioned medium from NK92 cells.

Mycoplasma testing by PCR, using the Mycoplasma PCR detection kit (Abcam, cat#ab289834), on 1% agarose gel showed the absence of mycoplasma for pre-HFB seeding of NK92 cells and HFB-produced NK92 cells from production lots 1-3. Positive control represents a PCR product band at 500bp (control provided with the kit), whereas the negative control (water provided with the kit) does not yield any PCR product.

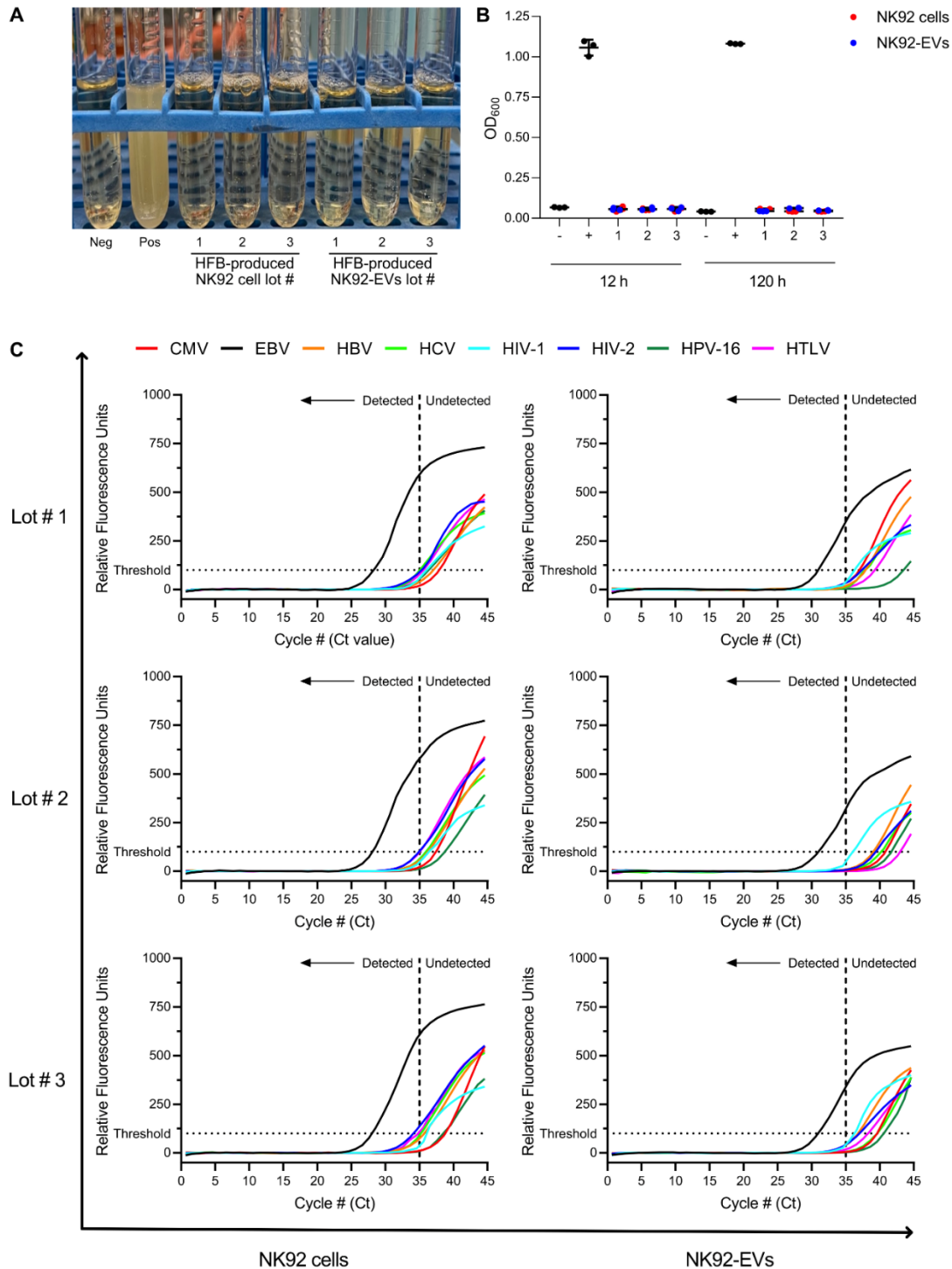


Figure 7. Bacterial and viral assessment of NK92 cells and NK92-EVs by quantitative reverse transcription PCR (RT-qPCR).

(A) HFB-produced NK92 cells and NK92-EVs from production lots 1-3 were cultured for 120 hours in LB broth, showing absence of bacterial growth/contamination. (B) OD₆₀₀ readings of bacterial culture assessment where NK92 cells are shown in red and NK92-EVs are shown in blue. (C) The presence/absence of CMV, EBV, HBV, HCV, HIV-1/2, HPV-16 and HTLV in HFB-produced NK92 cells and NK92-EVs were assessed by RT-qPCR (mean, n=1 experimental replicate with technical duplicates). Y-axis threshold set at 100 RFU and X-axis threshold set at 35 cycles.

Comparable HFB-produced NK92 cell yield and viability were obtained when repeating the biomanufacturing workflow, demonstrating reproducibility (**Figure 8**).

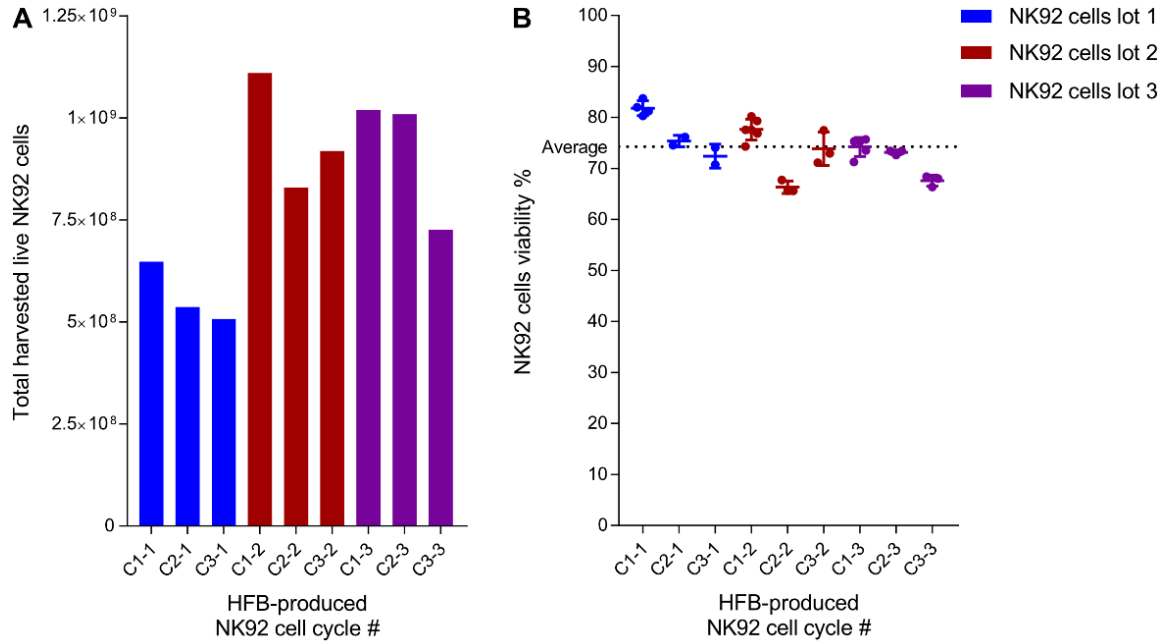


Figure 8. Reproducibility assessment of NK92 cell biomanufacturing workflow in three independent biomanufacturing cycles (C1, C2, C3).

(A) Total harvested live HFB-produced NK92 cells. (B) Viability of HFB-produced NK92 cells (mean $\pm$ S.D., $n=2-3$ from independent experimental replicates with technical duplicates). Legend: C stands for “cycle”.

Since radiation is a safety requirement for the clinical use of NK92 cells, we analyzed cell viability and morphology after radiation. (**Figure 9**). We found a significant reduction in viability (p -value < 0.0001) in all HFB-produced NK92 cell production lots after the first two days post-radiation (**Figure 9A**). On day four, the viability of irradiated cells stabilized at $28.47 \pm 1.29\%$, $24.83 \pm 1.61\%$, and $28 \pm 2.72\%$ for production lots 1 to 3, respectively (**Figure 9A**). In contrast, the viability of non-irradiated NK92 cells remained at $71.5 \pm 5.64\%$, $70.77 \pm 1.12\%$, and $70.03 \pm 5.88\%$ on day four for production lots 1 to 3, respectively. Additionally, we observed the effect of radiation on cell morphology at day four and found that cell growth was fully inhibited, as indicated by the absence of cluster formation, a typical characteristic of NK92 cells (**Figure 9B**).

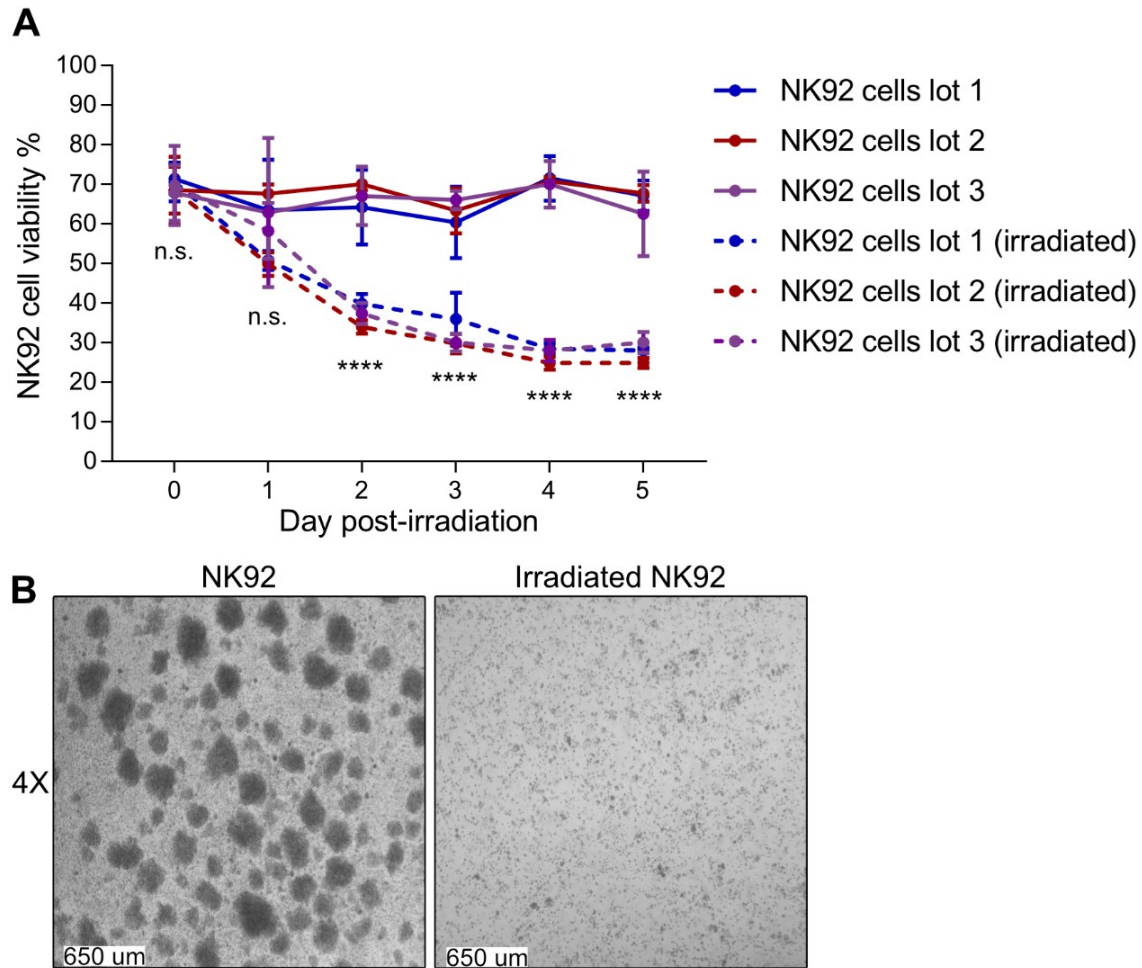


Figure 9. Viability analysis of HFB-produced NK92 cells after radiation.

(A) Following radiation of HFB-produced NK92 cells, viability was measured over five days (mean $\pm$ S.D., $n=3$ from independent experimental replicates with technical duplicates). Two-way ANOVA with Tukey's multiple comparisons test was applied where **** $p \leq 0.0001$. (B) Representative microscopic images (phase contrast, 4X objective) of non-irradiated NK92 cells (left) and irradiated NK92 cells (right) were taken on day 4 after radiation.

Interestingly, HFB-produced NK92 cells had significantly higher viability than flask-cultured NK92 cells under the same ImmunoCult medium conditions (**Figure 10A**); the difference between the two was $9.19 \pm 1.63\%$. Additionally, HFB-produced NK92 cells demonstrated greater cytotoxicity against K562 cells compared to flask-cultured NK92 cells (**Figure 10B**). The greatest difference (p -value < 0.0001) was observed at an E:T ratio of 2.5:1, with a 25.89% increase in K562 lysis for HFB-produced cells compared to flask-expanded cells. The effective concentration (EC_{50}) of HFB-produced NK92 cells that resulted in 50% of the maximum cytotoxic response against K562

cells was 1.16 E:T ratio comparatively to an EC_{50} of 2.39 E:T ratio for flask-cultured NK92 cells (**Figure 10C**), further supporting the greater cytotoxicity of NK cells produced from the HFB biomanufacturing system versus flask-based.

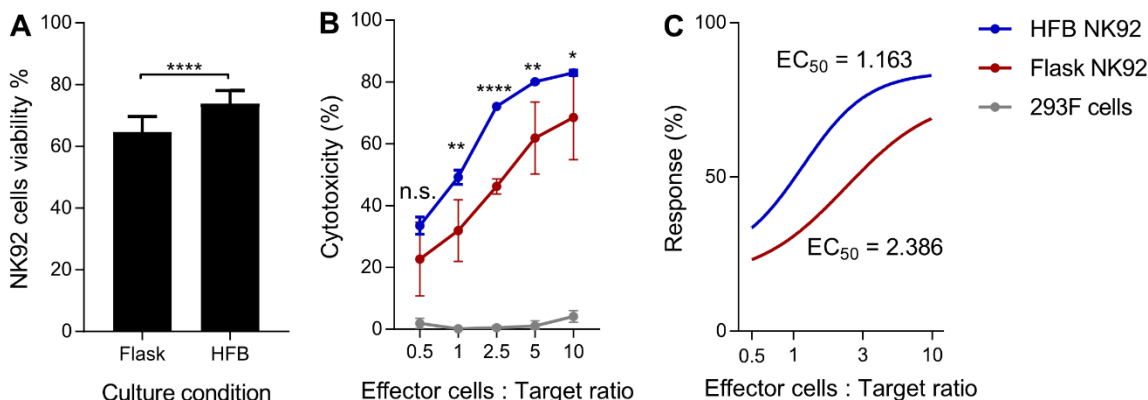


Figure 10. Comparative analysis of cell viability and cytotoxicity of flask-expanded and HFB-produced NK92 cells.

The effect of cultivation conditions (flask-expansion vs HFB-production) on the NK92 cell viability and cytotoxicity were assessed under the ImmunoCult medium condition. The data of all flask-expanded NK92 cells (originally presented in Figure 1) and the three HFB production lots (originally presented in Figure 2) were averaged and compared. (A) Cell viability analysis comparing flask-expanded NK92 cells (mean $\pm$ S.D., $n=7$ independent experimental replicates) versus HFB-produced NK92 cells (mean $\pm$ S.D., $n=15$ independent experimental replicates). An unpaired t-test (two-tailed) was applied where **** $p \leq 0.0001$. (B) Cytotoxicity analysis against leukemia K562 target cells at various effector to target (E:T) ratios comparing flask-expanded NK92 cells (mean $\pm$ S.D., $n=3$ experimental replicates) versus HFB-produced NK92 cells (mean $\pm$ S.D., representing the average of HFB production 1-3 lots where each lot were assessed for $n=3$ experimental replicates). 293F cells were used as a negative effector control (mean $\pm$ S.D., $n=3$ experimental replicates). Two-way ANOVA with Tukey's multiple comparisons test was applied where * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$. (C) Dose-response of K562 viability loss caused by HFB-produced or flask-expanded NK92 cell treatment.

At the end of each production lot, HFB-produced NK92 cells were harvested and cryopreserved in a GMP-compliant CryoStor CS10 preservation medium. After three months of storage in liquid nitrogen, the cells were recovered, and their viability was measured. The recovery of total live HFB-produced NK92 cells was $80.56 \pm 1.74\%$, $91.80 \pm 9.66\%$, and $90.20 \pm 9.96\%$ for production lots 1 to 3, respectively (**Figure 11A**). Viability measurements using AO/PI staining were $78.67 \pm 0.76\%$, $72.67 \pm 0.76\%$, and $75.00 \pm 4.36\%$ for production lots 1 to 3, respectively (**Figure**

11B), demonstrating that all production lots retained their viability after three months of storage in liquid nitrogen.

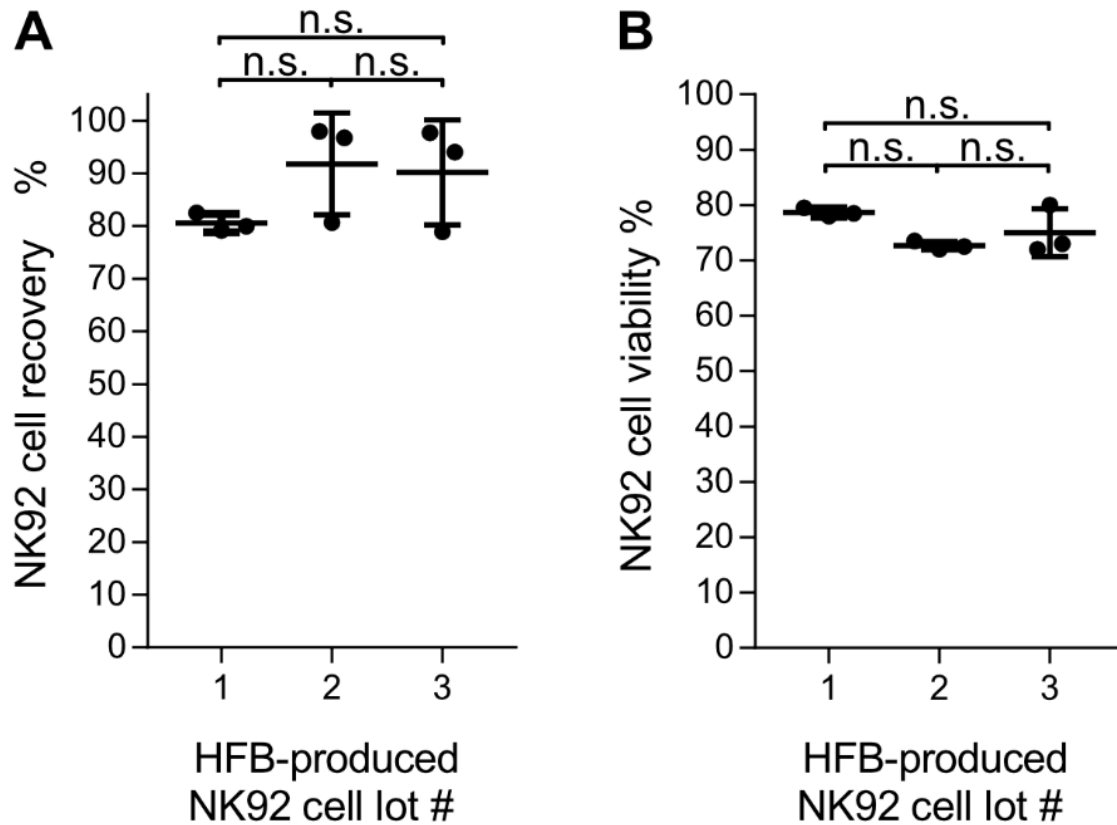


Figure 11. Recovery and viability of cryopreserved HFB-produced NK92 cells from each production lot.

After three months of storage in liquid nitrogen, HFB-produced NK92 cells from each production lot were thawed to assess cell recovery and viability. (A) HFB-produced NK92 cell recovery of each production lot (mean $\pm$ S.D., $n=3$ from independent experimental replicates). One-way ANOVA with Tukey's multiple comparisons test was applied; n.s. not significant. (B) HFB-produced NK92 cell viability of each production lot (mean $\pm$ S.D., $n=3$ from independent experimental replicates with technical duplicates). One-way ANOVA with Tukey's multiple comparisons test was applied; n.s. not significant.

HFB-produced NK92 cells yielded large quantities of small NK92-EVs

The EV-rich CM was collected daily from the bioreactor and purified through differential centrifugation, SEC coupled with ultrafiltration and filtration (**Figure 12**).

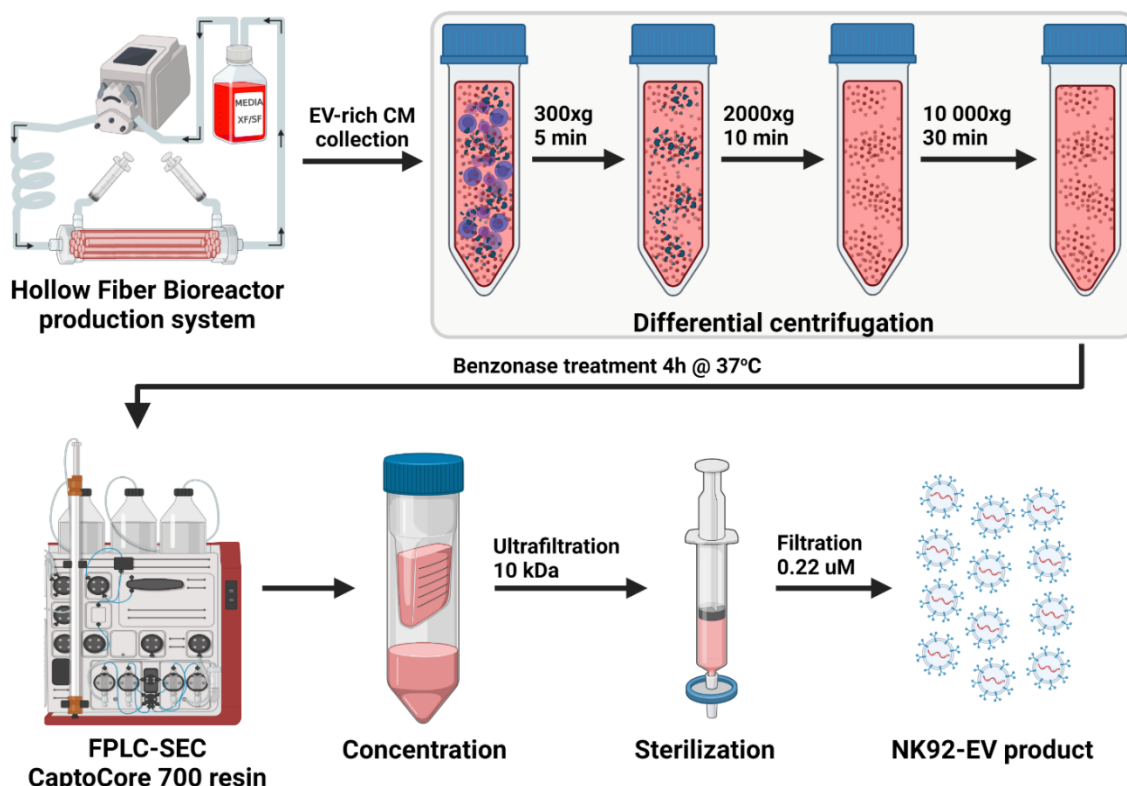


Figure 12. Schematic representation of the scalable NK92-EV isolation process using SEC coupled with ultrafiltration.

After the daily collection of EV-rich conditioned medium (CM), the solution was centrifuged at 300xg for 5 min to remove cells and then centrifuged at 2000xg for 10 min to remove cell debris at 4°C. Cleared EV-rich CM was equally aliquoted and stored at -80°C until further processing. Once ready for isolation of NK92-EVs, a frozen aliquot was thawed at room temperature and centrifuged one more time at 10 000xg for 30 min to ensure the removal of cellular debris. Then, the EV-rich CM was incubated for 4 hours at 37°C with Benzonase (Nuclease) to digest nucleic acids present in the solution. Next, the samples were processed by FPLC-SEC for EV purification using HiScreen Capto Core 700 resin. Fractions were combined and concentrated using ultrafiltration before filtering the EV-rich isolate to ensure the sterility of the final NK92-EV product. This figure was created with BioRender.com.

During purification, BCA and Qubit assays were used to monitor the presence and removal of non-EV-related proteins and extracellular nucleic acids. Benzonase treatment before SEC resulted in a significant reduction of double-stranded (ds) DNA in the EV-rich CM (p-value < 0.001) but had no measurable effect on protein or RNA levels (**Figure 13**).

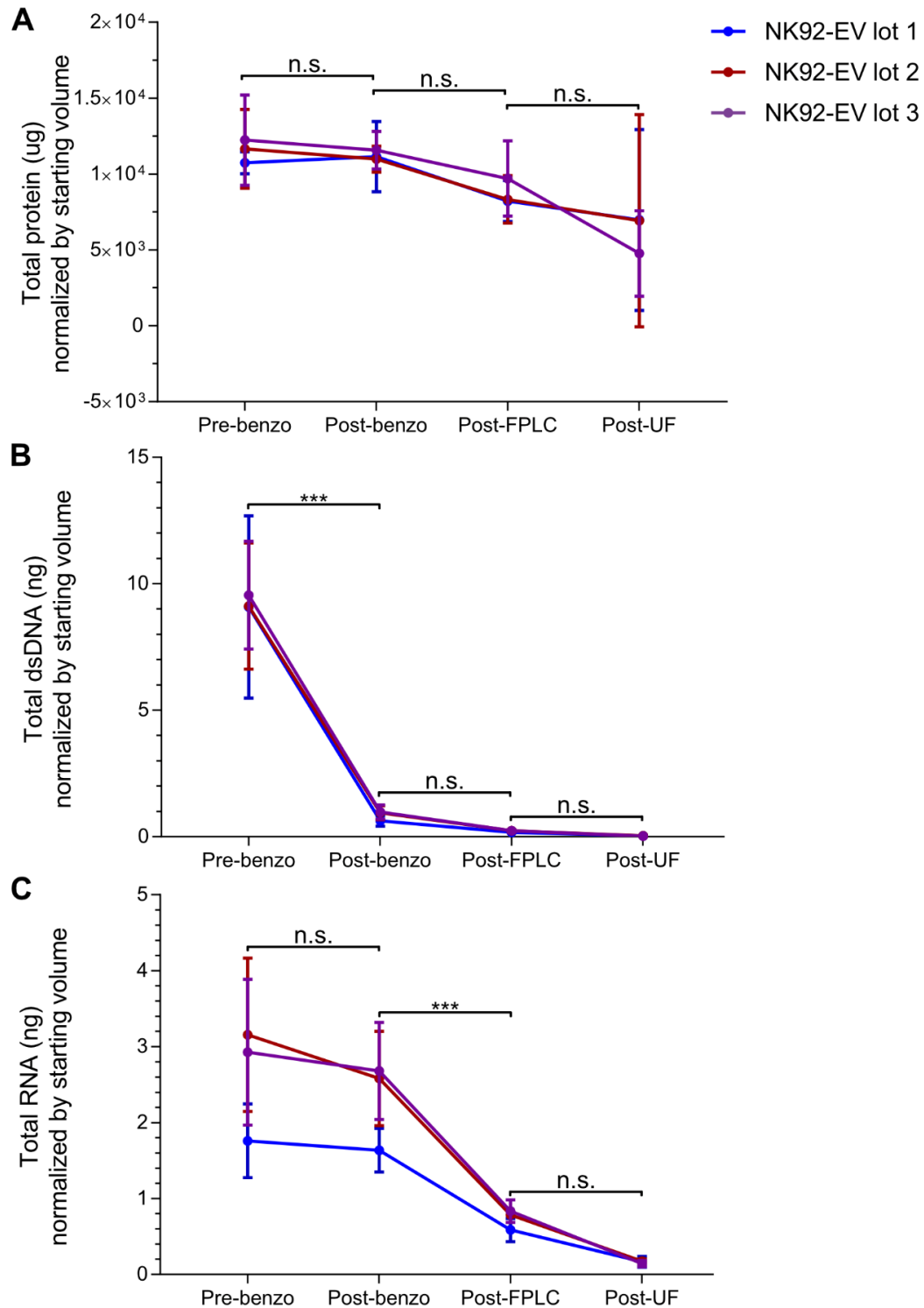


Figure 13. Analysis of protein and nucleic contaminants during the NK92-EV isolation process.

Quantification of proteins in intact EV samples by BCA assay, normalized to starting volume of EV-rich CM (A), extravesicular dsDNA by Qubit assay (B) and extravesicular RNA by Qubit assay (C) (mean $\pm$ S.D., $n=3$ from independent isolations). Two-way ANOVA with Tukey's multiple comparisons test was applied where *** $p \leq 0.001$.

NTA showed that the NK92-EVs generated in all three production lots had a size range of 71.48-161.34 nm (**Figure 14A** and **Table 3**), fitting the definition of small EVs [55, 168]. The 0.2 μ m sterilization filtration step did not significantly affect the size distribution or the yield (with only 10.77% loss of particles) of the NK92-EVs, as the size range remained relatively unchanged at 75.40-170.27 nm when unfiltered (**Figure 15** and **Table 4**).

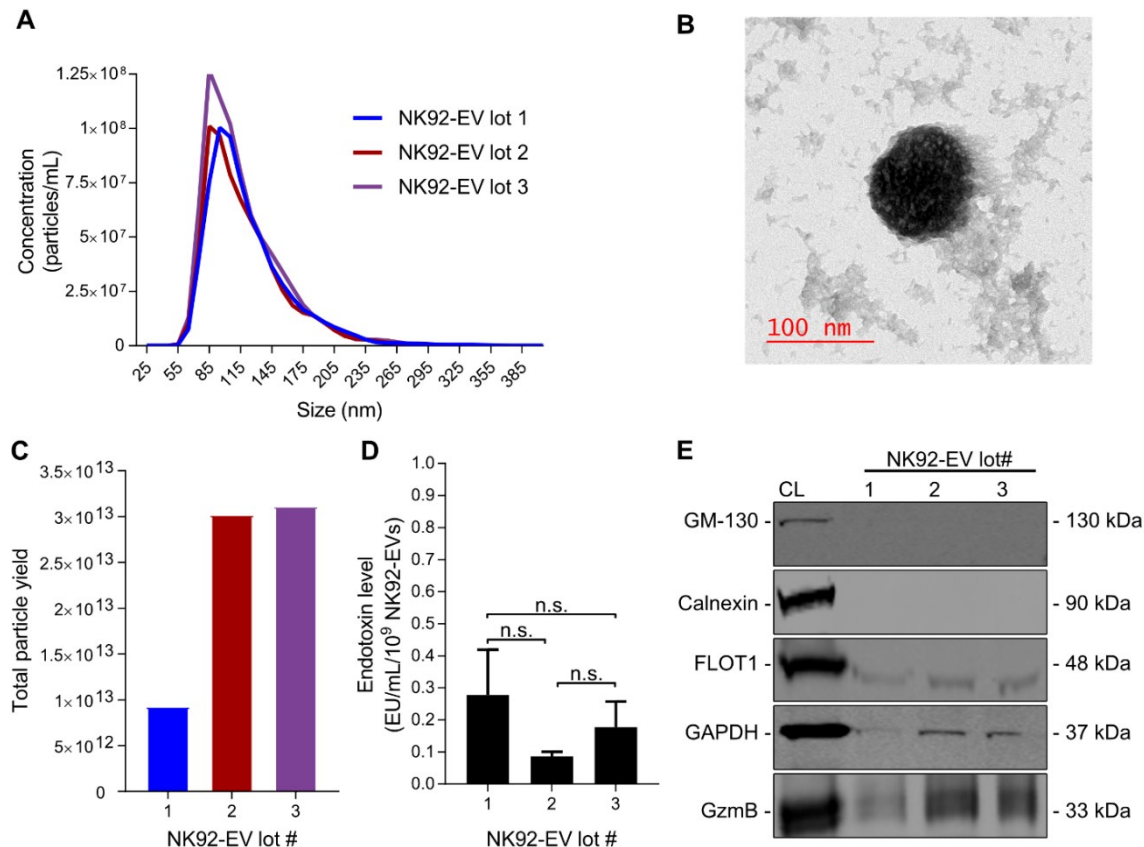


Figure 14. HFB-produced NK92 cells yielded large quantities of small NK92-EVs.

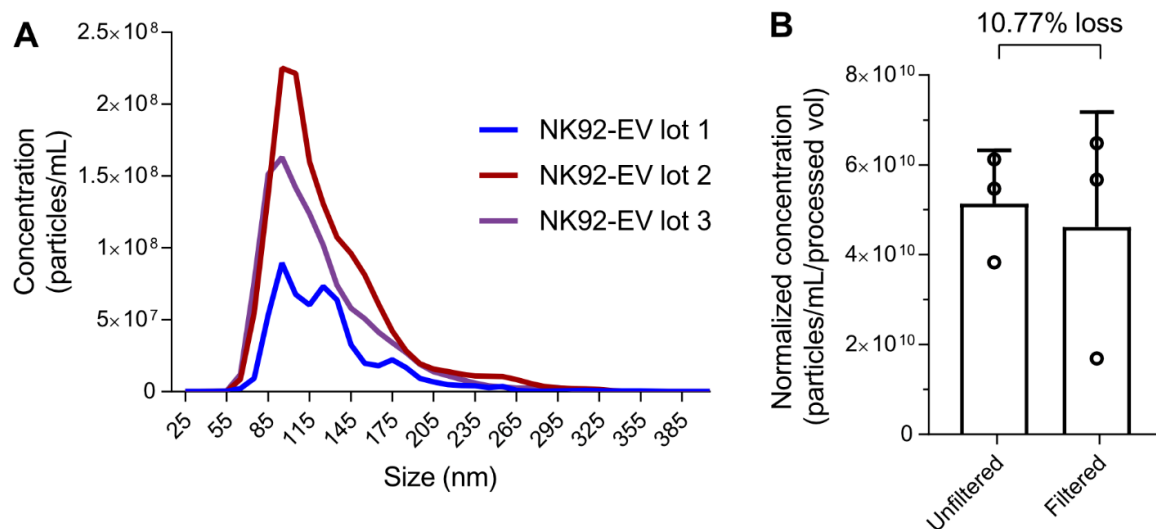
(A) NTA histogram plots of nanoparticle size and concentration (mean, $n=3$ independent experimental replicates with five technical replicates) of filtered NK92-EVs. (B) Contrast-stained TEM image of a single NK92-EV. (C) Total particle yield of NK92-EV preparations. (D) Endotoxin analysis of NK92-EVs for a dose of 1×10^9 EVs (mean $\pm$ S.D., $n=3$ independent experimental replicates with technical duplicates). One-way ANOVA with Tukey's multiple comparisons test was applied where n.s. = not significant. (E) Western blot analyses of NK92 protein lysate and derived-EV protein lysate for cellular contaminants (GM-130 and calnexin), EV (flotillin 1 (FLOT1) and GAPDH) and NK (granzyme B; GzmB) markers.

Table 3. Average size distribution metrics (nm) of filtered NK-EVs across 3 lots and 3 isolations for n=9 individual experiments.

NTA metric	Average mean (nm)	Average S.D. (nm)
D10	71.48	1.24
D50	99.77	1.51
D90	161.34	3.68
Mean	115.11	2.57
Mode	94.23	2.85
SD	43.43	8.45

Table 4. Average size distribution metrics (nm) of unfiltered NK-EVs across 3 lots and 1 isolation for n=3 individual experiments.

NTA metric	Average mean (nm)	Average S.D. (nm)
D10	75.40	3.50
D50	106.00	4.26
D90	170.27	1.79
Mean	121.23	3.25
Mode	96.43	3.06
SD	42.80	2.00

**Figure 15. NTA characterization of non-filtered NK92-EVs.**

(A) NTA histogram plots of nanoparticle size and concentration (mean, n=3 independent experimental replicates with five technical replicates) of unfiltered NK92-EVs; (B) Comparative particle yield of unfiltered vs filtered NK-EVs (mean $\pm$ S.D., n=3 independent experimental replicates). Yield difference was calculated by Bland-Altman comparison (percentage difference).

Single vesicle visualization below the 200 nm size was confirmed by TEM (**Figure 14B**). Additional TEM images representing various nanostructures and demonstrating the size range of the NK92-EVs in the preparation are shown in **Figure 16**. When purifying NK92-EVs from half of the total EV-rich CM generated from each lot (*i.e.*, 51 mL on average/isolation), we purified a total NK92-EV yield of 9.31×10^{12} (solution volume ~ 8.00 mL), 3.02×10^{13} (solution volume ~ 3.86 mL), and 3.12×10^{13} (solution volume ~ 1.61 mL) NK92-EVs for production lots 1-3, respectively (**Figure 14C**). Cumulatively, this corresponds to an average yield of 2.36×10^{13} NK92-EVs, more than sufficient for preclinical dosing compared to other large-scale EV production studies [168-170]. This corresponds to an average NK92-EV concentration of 1.18×10^{12} NK92-EVs/mL (**Figure 17A**).

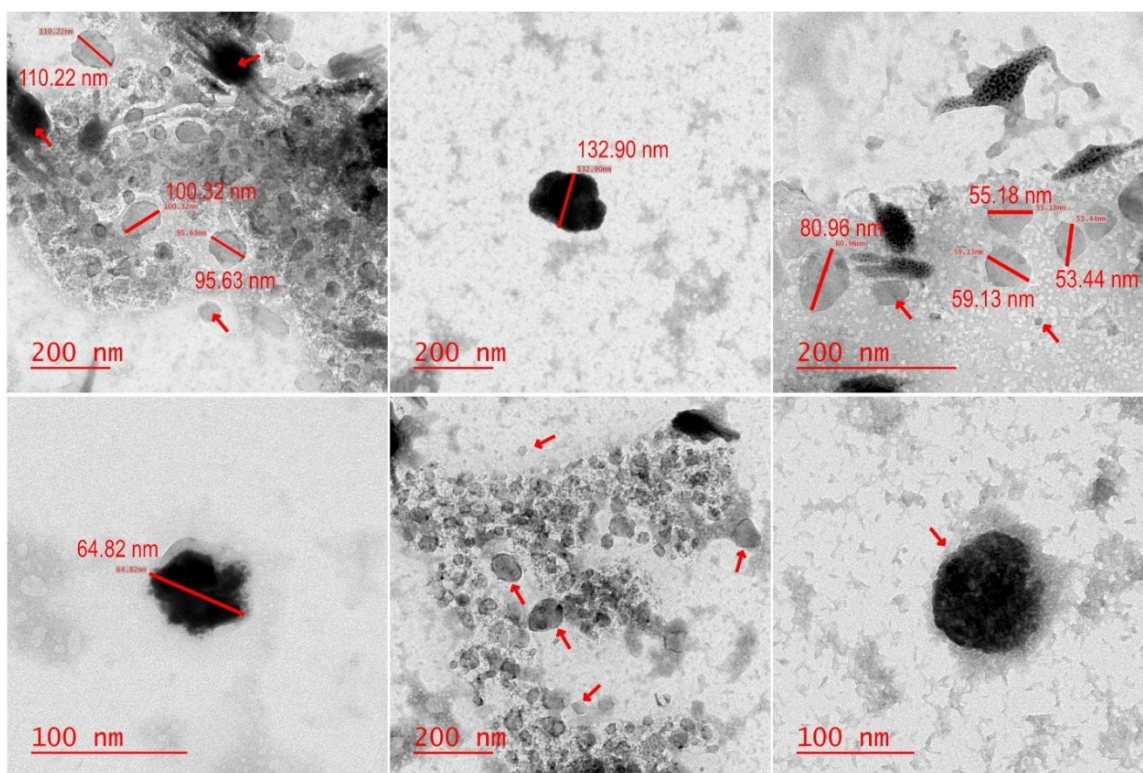


Figure 16. Additional transmission electron microscopy (TEM) images of NK92-EV showing various nanostructures present in the NK-EV preparations.

Arrows and size bars for some visible particles are provided, demonstrating the size range of the particles found in the NK-EV preparations.

Importantly, the starting materials (DF-PBS, unconditioned medium (UCM; ImmunoCult-XF T Cell Expansion Medium) and FPLC-processed UCM) used in the EV biomanufacturing process do not appear to contribute to the NK92-EV yield (**Figure 17A**) as background particles from these materials accounted only for a very small percentage. For instance, using the same isolation process and starting volume of media, the FPLC-processed UCM had a concentration of 1.11×10^9 particles/mL as measured by NTA. In comparison, NK-EVs had 1.18×10^{12} particles. Notably, this represented merely 0.094% of non-vesicular extracellular particles (NVEPs) in NK-EV products.

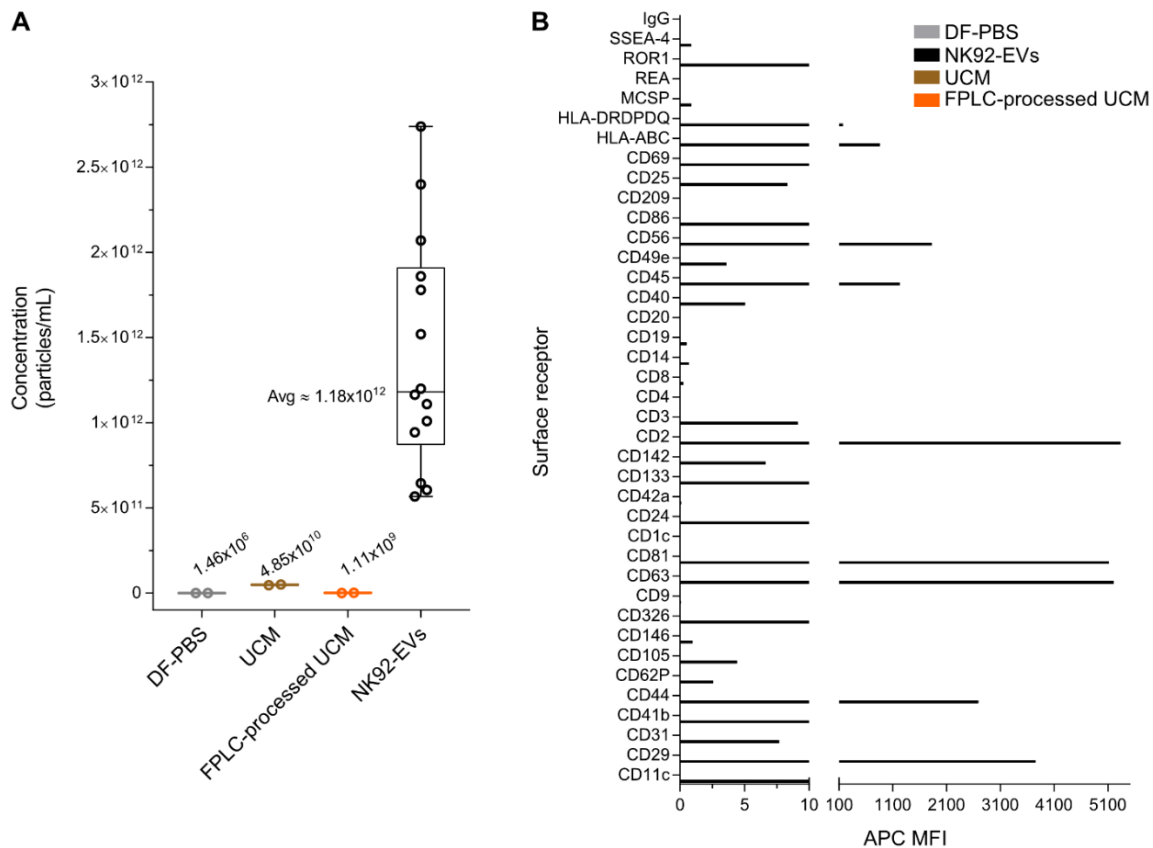


Figure 17. Starting materials contain very limited endogenous EVs or particles as per NTA and EV antigenic signature assessment.

(A) Box-whisker plot of particle concentration of starting material: double filtered (DF)-PBS, unconditioned medium (UCM), FPLC-processed UCM (mean, $n=2$ independent experimental replicates) *versus* particle concentration of HFB-produced NK92-EVs (mean, $n=14$ independent experimental replicates). Note: the concentration factor is not accounted for in this analysis for NK92-EVs. (B) UCM and FPLC-processed UCM do not display an EV antigenic signature compared to the averaged HFB-produced NK92-EV antigenic signature. Relative median fluorescent intensity (MFI) levels of the surface antigenic profile of intact UCM and FPLC-processed

UCM particles and DF-PBS (mean, n=3 experimental replicates, with value all at zero, undetectable) *versus* HFB-produced NK92-EVs particles (mean, representing the average of HFB-produced NK92-EVs from production 1-3 where each lot was assessed for n=4 experimental replicates with technical duplicates) normalized to a dose of 1×10^{10} particles.

Endotoxin analyses revealed low levels of endotoxins in all three production lots (0.28 ± 0.24 , 0.07 ± 0.03 and 0.18 ± 0.14 EU/mL/ 10^9 NK92-EV for production lots 1-3, respectively) (**Figure 14D**). Bacterial contaminations were absent following LB culture (**Figure 7A/B**). Furthermore, common viral entities commonly assessed for clinical cell- and gene-based biotherapeutics (*i.e.*, CMV, HBV, HCV, HIV-1/2, HPV-16 and HTLV [171, 172]) were negative for both HFB-produced NK92 cells and NK92-EVs, which share similar physiochemical characteristics with EVs and could potentially be co-isolated in the final product if present (**Figure 7C** and **Table 5**). As expected, we confirmed the presence of EBV in both HFB-produced NK92 cells and NK92-EVs, as this cell line is known to contain EBV viral DNA sequences. Western blot analysis confirmed the presence of EV protein markers flotillin-1 (FLOT1) and GAPDH in all NK92-EV production lots and the presence of GzmB in all three production lots (**Figure 14E**). No cellular protein contaminants, such as GM130 and calnexin, were detected in the NK92-EV protein lysates from any production lots.

Table 5. Viral entities quantitative reverse transcription PCR (RT-qPCR) CT difference results.

Target Virus/Gene	NK92-MI cells			NK92-EVs		
	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3
Cytomegalovirus (CMV)	37.64	37.21	37.94	39.42	39.01	38.84
Epstein-Barr Virus (EBV)	27.73	27.37	27.29	30.23	30.49	30.12
Hepatitis B Virus (HBV)	34.4	34.86	34.73	37.1	37.14	37.5
Hepatitis C Virus (HCV)	33.65	33.79	33.24	35.59	36.68	34.87
Human Immunodeficiency Virus 1 (HIV-1)	32.81	32.31	32.53	35.1	36.65	34.9
Human Immunodeficiency Virus 2 (HIV-2)	34.68	34	33.14	36.69	36.1	35.94
Human Papillomavirus type 16 (HPV-16)	35.2	36.07	35.4	39.77	39.15	39.27
Human T-lymphotropic Virus (HTLV)	34.76	34.44	34.68	39.51	39.73	39.22

HFB-produced NK92-EVs display antigenic signature and cytokine profile of NK cells

To determine the antigenic profile of NK92-EVs produced in the HFB, we performed flow cytometry analysis on the EVs using a multiplex assay for 37 surface antigenic markers (**Figure 18**).

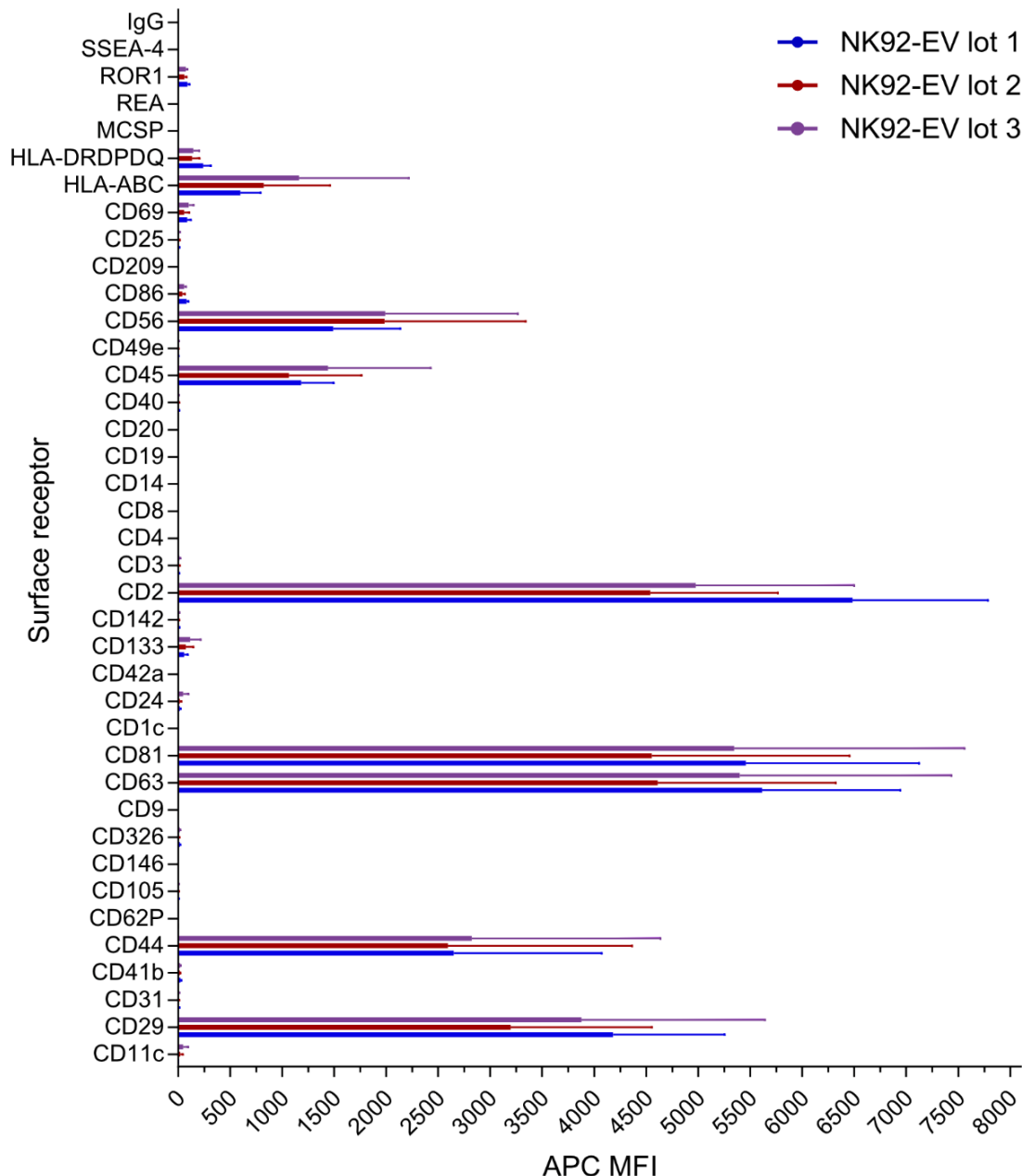


Figure 18. HFB-produced NK92-EVs display an NK antigenic signature while presenting classical EV markers.

Relative median fluorescent intensity (MFI) levels of the surface antigenic profile of intact NK92-EVs for production lots 1 to 3 (mean $\pm$ S.D., $n=4$ independent experimental replicates with technical duplicates) normalized to a dose of 1×10^{10} NK92-EVs.

We detected CD2, CD29, CD44, CD45, and CD56 antigens expression in all three production lots and confirmed the absence of CD3, CD4, CD8, CD16, and CD19, which is consistent with the NK92 cell profile. We also measured the presence of glycosylphosphatidylinositol (GPI)-anchored tetraspanins CD63 and CD81, but not CD9, and human leukocyte antigen (HLA)-ABC consistently across all three production lots. In addition, we observed minimal levels of CD69, CD86, CD133, and HLA-DR/DP/DQ.

To determine the cytokine profile of NK92-EVs produced in the HFB, we performed a multiplex flow cytometry assay for 12 cytokines associated with NK cell cytotoxicity using EV lysates (**Figure 19**).

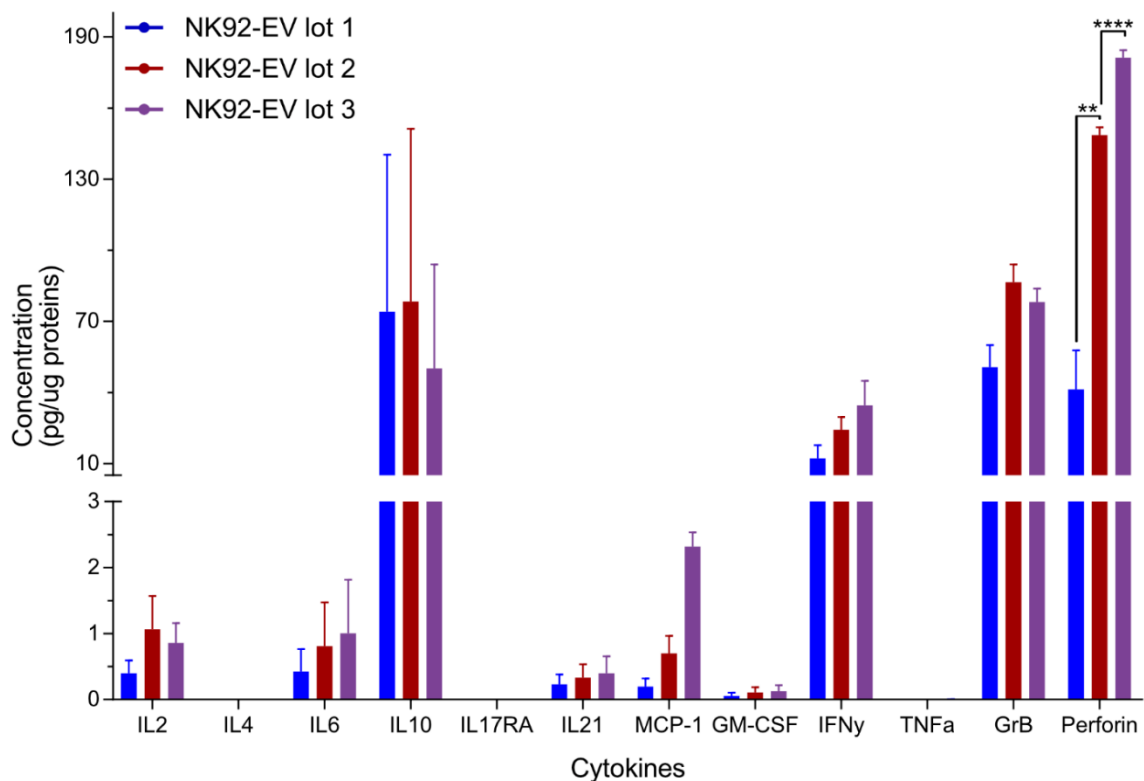


Figure 19. Cytokine profiling of HFB-produced NK92-EVs.

Quantitative assessment of cytokine profile of NK92-EVs from production lots 1-3 (mean $\pm$ S.E.M., $n=3$ independent experimental replicates with technical duplicates) normalized to protein content obtained by BCA Protein assay. Two-way ANOVA with Tukey's multiple comparisons test was applied where $**p \leq 0.0015$ and $****p \leq 0.0001$.

We found elevated levels of IFN- γ , GzmB, and PFN in all three NK92-EV production lots relative to the other cytokines tested, which suggests a potential cytotoxic signature for the HFB-produced NK92-EVs. Moreover, we detected elevated levels of IL-10 and some IL-2, IL-6, IL-21, MCP-1, and GM-CSF in all NK92-EV lysates relative to the other cytokines assessed, indicating a potential immunomodulatory signature. Although not statistically significant, we measured lower levels of each cytokine, except for PFN, in NK92-EV lysate from lot 1, compared to NK92-EV lysate from lots 2 and 3. IL-4, IL-17RA, and TNF α were not detectable in NK92-EV production lots. Additionally, we confirmed undetectable surface antigenic EVs markers in the UCM and FPLC-processed UCM (**Figure 17B**), confirming the low abundance of endogenous EV particles in the starting materials.

HFB-produced NK92-EVs demonstrate dose-dependent cytotoxicity against human leukemic cell line K562

To evaluate the cytotoxicity of NK92-EVs produced in the HFB, we tested the effect of different doses of NK92-EVs on the human leukemic cell line K562 for a 5-hour treatment. All NK92-EV lots resulted in a robust and consistent dose-dependent decrease in K562 cell viability (**Figure 20A**). The effective concentration (EC_{50}) of NK92-EVs that produced 50% of the maximum response against K562 cancer cells was calculated to be 7.35×10^9 , 1.05×10^{10} , and 8.22×10^9 particles/mL for lots 1 to 3, respectively, with an average EC_{50} of $8.68 \times 10^9 \pm 1.60 \times 10^9$ particles/mL (**Figure 20B**). The cytotoxicity amongst the different NK92-EV lots did not show a significant difference (p-value >0.95), indicating that the biomanufacturing process can produce large quantities of NK92-EVs with consistent cytotoxicity across production lots.

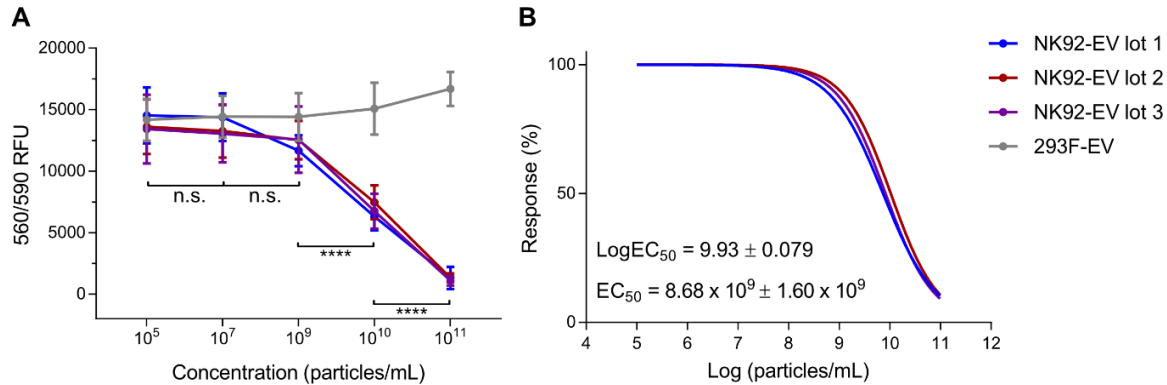


Figure 20. HFB-produced NK92-EVs demonstrate a dose-dependent cytotoxicity against human leukemic cell line K562.

Leukemia K562 cells were treated at various concentrations of HFB-produced NK92-EVs and 293F-EVs (negative control) for 5 hours before processing with endpoint PrestoBlue viability assay. (A) PrestoBlue assay where Relative Fluorescence Unit (RFUs) represent cell viability (mean ± S.E.M., n=3 independent experimental replicates with technical quadruplets). Two-way ANOVA with Tukey's multiple comparisons test was applied where ****p ≤ 0.0001 and n.s. = not significant. (B) Dose-response of K562 viability loss caused by HFB-produced NK92-EVs treatment.

Limited off-target toxicity was confirmed using comparable doses of NK92-EVs against healthy human fibroblast cells (**Figure 21**).

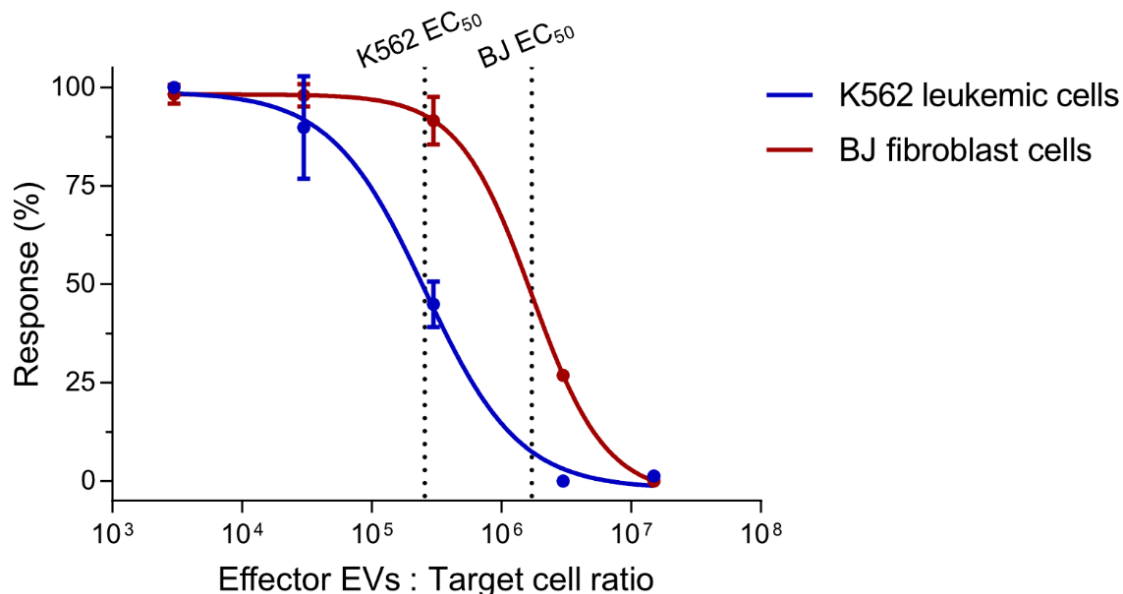


Figure 21. HFB-produced NK92-EVs showed limited off-target toxicity against human BJ fibroblast cells in comparison to K562 leukemic cells.

Dose-response (viability loss) of BJ fibroblast cells and K562 leukemic cells treated at comparable effector HFB-produced NK92-EVs to target cell ratio for 5 hours before processing with endpoint PrestoBlue viability assay (mean ± S.D., n=2 independent experimental replicates with technical duplicates).

Discussion

Cancer is a leading cause of death worldwide, and the development of effective treatments is an unmet medical need. Given the potential of NK-EVs in cancer immunotherapy, a GMP-compliant, scalable, and reproducible biomanufacturing workflow to consistently produce large quantities of high-quality, functional NK cells and EVs is critical for clinical applications [141, 142, 168, 169, 171, 173-178]. Therefore, we established a new biomanufacturing process using a GMP-compliant, closed-loop HFB system to culture and expand NK92 cells in a SF/XF medium under feeder-free conditions, producing large quantities of clinical-grade NK92 cells and NK92-EVs that could be used for clinical translation of NK and NK-EV therapies.

One crucial step in generating biotherapeutics for clinical application is selecting a cell source as well as GMP-compliant and clinically relevant cell culture conditions [140-142]. While most reported methods of generating NK cells or NK-EVs have relied on primary NK cells [143, 145-151], the finite lifespan of primary NK cells and donor-to-donor differences introduce significant variability in production [179-181]. Additionally, current methods for primary NK cell expansion rely on immortalized feeder-cells, which results in the co-isolation of feeder-cell EVs with the desired NK-EV product [28, 182]. By using the clinically relevant NK92-MI cell line, it is possible to avoid the use of a feeder system for producing clinical-grade NK-EVs. The NK92-MI cell line is easily expanded and retains most NK cell characteristics, including CD45 and CD56 receptor expression and robust cytotoxicity against various cancer types. Furthermore, it can be expanded for a prolonged period, compared to primary NK cells, without adding exogenous IL-2 [162, 163, 183]. This approach eliminates the contaminations of feeder-cell EVs and reduces variability between different production lots. Moreover, a chemically-defined medium free of animal-derived components (SF/XF) eliminates the risk of contamination of animal products, related variability and decreased immunogenic potential [155, 173, 184]. Importantly, SF/XF media has been associated with increased EV yield as it supports high cell densities and maintains the expanded cells' phenotype and function [185]. To this end, we used the chemically defined ImmunoCult-XF T Cell Expansion Medium to culture the NK92-MI cells (herein shortened to "NK92 cells") in the HFB system and produced NK92 cells and NK92-EVs under SF/XF and feeder-free conditions. This is

the first study to show that this culture system can be used to grow and culture NK92 cells while maintaining the NK92 signature and characteristics, such as phenotype, morphology, and cytotoxicity.

Another crucial step in generating biotherapeutics for clinical use is selecting an appropriate bioreactor platform [179, 186]. Among the available options, hollow-fiber bioreactors (HFBs) stand out as closed-loop systems that offer several benefits; these include improved control over critical process parameters, reduced maintenance, and reduced hands-on time, ultimately leading to lower costs and better product quality [143, 153, 171, 186, 187]. In addition, HFBs allow for high-density cell cultivation, making it possible to generate large quantities of biotherapeutics continuously and rapidly [140]. Compared to most commercially available bioreactors, this biomanufacturing workflow is an easy to implement and cost-effective approach for cancer immunotherapies. Together, this new strategy serves as a proof-of-concept where we produced large quantities (the equivalent of clinical dose) of high-quality NK92 cells (approximately 1×10^8 viable cells per adult or the equivalent of $10^6 - 10^8$ cells per kg generated for treatment [166, 167]) in less than a week and in a cost-effective manner. The cell products underwent rigorous culture-condition control and quality verification through a variety of assays, including assessments of viability, monitoring of key cell culture parameters (such as temperature, gas levels, pH levels, glucose consumption, and lactate production), and testing for mycoplasma, bacterial and viral contaminations. Quality control of the cells in culture is vital for ensuring lot-to-lot consistency of the EVs produced, therefore assessment of the overall health of the cells in culture through these surrogate markers is an integral quality verification step in the EV production workflow [171, 180, 181]. Harvested HFB-produced NK92 cells remained viable under the SF/XF and feeder-free conditions in the HFB system and tested negative for mycoplasma contamination and common viral entities.

HFB-produced NK92 cells displayed their NK92 cell characteristics (*e.g.*, high expression of CD56 and absence of CD16) [162, 163, 183]. In addition, the cells were characterized as negative for CD3/CD4/CD8 and positive for CD45 (data not shown). Interestingly, HFB-produced

NK92 cells exhibited significantly higher viability than those grown in flasks. Furthermore, the HFB-produced NK92 cells demonstrated improved cytotoxicity against leukemia K562 cells compared to flask-cultured NK92 cells. Despite being irradiated for clinical safety to prevent their uncontrolled growth, the ability of those irradiated NK92 cells to kill cancer cells remains unchanged [188]. These results were consistent across production lots, indicating the robustness and reproducibility of the HFB biomanufacturing workflow. The enhanced viability and cytotoxicity of HFB-produced NK92 cells could be attributed to the 3D culture environment increasing cell-to-cell contact and providing improved conditions for cell growth and survival [189-191]. This finding is particularly promising for clinical translation, as high cell viability and functionality are crucial for cell-based therapies' success [188, 192, 193]. When the NK92 cell line was originally described by Klingemann in 1994, the group reported 84% cytotoxicity against K562 cells at an E:T ratio of 5:1 after 4 hours of treatment, assessed through the ⁵¹-chromium release assay [183]. Recent studies have reproduced these findings [194, 195]. In our study, HFB-produced NK92 cells exhibited a similar cytotoxic effect when subjected to identical experimental conditions where an E:T ratio of 5:1 yielded 80% of K562 cell death after 4 hours of treatment.

We observed some minor differences in the yield of NK92 cells between the first production lot and subsequent lots (lots 2 and 3). Given that each production lot, except for the first one, was done using HFB-produced NK92 cells harvested from the HFB system, it is possible that a significant number of NK92 cells remained inside the cartridge after cell harvest, which could explain the yield disparity between the first and the subsequent lots. In addition, the NK92 cells that were transitioned from the flask system to the HFB system (during lot 1) needed some time to acclimate to the HFB environment, which could have initially hampered their growth.

HFBs are also promising for producing EVs at scale, yielding approximately 40-fold more EVs/mL of CM than traditional flask-based methods [156, 168]. Here, we employed a scalable SEC-based process utilizing Capto Core 700, combined with ultrafiltration and filtration steps, to isolate EVs from NK cell CM [168] and produced large quantities of clinical-grade cytotoxic NK92-EVs. Future studies will assess the feasibility of our approach and explore alternatives, such as

tangential flow filtration (TFF), for cell removal as opposed to differential cell centrifugation to be more amenable for processing larger volumes, if needed.

The resulting EVs had a size below 200 nm, ranging from 72.80-164.00 nm. This size makes filtration-based sterilization possible, enabling a scalable production and isolation process to meet preclinical and clinical demands [55, 168]. From approximately 51 mL of CM (representing half of the CM of each production lot), we isolated on average, 2.36×10^{13} NK92-EVs, corresponding to a yield of approximately 4.62×10^{11} NK92-EVs/mL of CM. When measuring background particle yield by NTA in our different starting materials to understand its possible contribution to the final NK-EV product during biomanufacturing process, we found a low particle count in all of them (*i.e.*, < 0.1% of non-vesicular extracellular particles (NVEPs)). This emphasizes the importance of using a chemically defined culture medium without serum for producing therapeutic EVs. Lastly, this production yield is more than sufficient for preclinical work based on the average dose of 3.37×10^8 EVs/kg of body weight calculated by Gupta et al., 2021 [196]. Future studies should consider the disease and patient-specific context that may affect NK-EV clinical dosage and route of administration.

The safety and quality profiles of NK92-EVs were demonstrated by the absence of mycoplasma contamination, low endotoxin levels (≤ 0.5 EU/ mL, representing the clinical upper limit [171, 175, 197]), no bacterial growth, and the absence of commonly tested viruses (*i.e.*, CMV, HBV, HCV, HIV-1/2, HPV-16 and HTLV with the exception of EBV, as the NK92 cell line is known to contain EBV viral DNA sequences). Additionally, common cellular protein contaminants were also absent [198]. Moreover, the NK92-EV products from different lots exhibited an NK92-like signature with CD2, CD45, and CD56 and an EV signature with CD63 and CD81 but not CD9, consistent with previous studies with primary NK-EVs [65, 66, 199]. Furthermore, cytokine profiling revealed GzmB, PFN, and IFN γ as predominant cytotoxic cytokines, which have been previously identified as important effectors in the cytotoxicity of NK-EVs [65, 66, 127, 130, 133, 137].

While this biomanufacturing workflow is not indication-specific, we validated the cytotoxicity of the HFB-produced NK92-EVs using K562 leukemia cells as proof-of-concept cell line. In

accordance with previous reports, the different NK92-EV lots we generated demonstrated consistent dose-dependent cytotoxicity against K562 cells [63, 65, 133, 199]. For example, Lugini et al. [63] showed a dose-dependent cytotoxicity with approximately 10% of K562 cell lysis after 24 hours of treatment with activated NK-EVs. Di Pace's group [199] also derived EVs from NK cells isolated from peripheral blood mononuclear cells and showed a range of 20-80% cytotoxicity against K562 cells in a dose-dependent manner after 24 hours of treatment. Similarly, Enomoto et al. [133] showed a dose-dependent effect where approximately 40% of K562 cell lysis was achieved after 24 hours of treatment with NK92-EVs, using the WST-1 viability assay (akin to the PrestoBlue viability assay used in our study). Cochran and Kornbluth showed approximately 25-30% of K562 cell lysis after 48 hours of NK3.3-EV (NK3.3 is a cell line from a healthy donor) treatment and a dose-dependent effect. Subsequent flow cytometric assessment from this group revealed approximately 50% of K562 cell death after 24 hours of treatment. Of note, all of these studies showed dose-dependent cytotoxicity effects of NK-EVs against the K562 cells, although using a longer time of EV treatment (24-48 hours) for most reports [65, 133, 199] than what we reported here (5 hours). Indeed, we obtained 50% of K562 cell lysis within 5 hours of NK92-EV treatment with approximately 1×10^9 NK92-EVs. Comparatively, Lugini et al. showed approximately 20% of K562 cell lysis at the 5 hour time point of EV treatment. Directly comparing these different reports proved challenging due to differences in EV source and biomanufacturing process, isolation technique and experimental conditions used in the K562 cell lysis assay. Having standardized potency assays for evaluating NK-EVs' cytolytic function would facilitate comparisons across studies in the future.

In summary, we present a novel biomanufacturing workflow using a closed-loop HFB system under SF/XF and feeder-free conditions to produce clinical-grade NK cells with improved viability and cytotoxicity while yielding large quantities of clinical-grade NK92-EVs containing anti-cancer cytokines and displaying cytotoxic function against leukemia cells. This study addresses preclinical and clinical demands by providing a cost-effective and reliable approach for further *in vitro* and *in vivo* investigation of cancer immunotherapies using NK cells and NK-EVs.

Chapter 3. NK-EV cytotoxicity assessment

Preface: The following chapter consists of a research article for which I am the primary author. This article was published in the *Journal of Extracellular Biology* (JExBio) in 2024 and represents a direct follow-up to the manuscript detailed in **Chapter 2**.

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Contributions: JRL, LW and FSTDB conceived the idea. FSTDB cultured and maintained all the cell lines. AS generated the RPF-MDA-MB-231 cell line. FSTDB and GM performed the biomanufacturing of NK-EVs. FSTDB performed EV isolation and characterization (NTA assessment, protein and nucleic acid quantification). GM performed the ELISA assessment, and FSTDB performed the analyses. FSTDB performed all EV cytotoxicity assays (flow- and plate-based), and SQ contributed to processing flow cytometry samples. AS acquired flow cytometry data, and GM performed AO/PI counting. JRL, LW and FSTDB contributed to the cytotoxicity analyses. FSTDB and MK performed the bioactivity assessment (EV isolation, EV characterization and EV cytotoxicity assays) with the help of JM for NTA assessment. FSTDB performed additional validation of the cell viability assay. JRL, LW and FSTDB performed the statistical analyses. All authors contributed general intellectual inputs on the experimental design, contributed to the data analysis, and discussed the results. JRL, LW and FSTDB wrote the manuscript and assembled the figures. All authors reviewed and edited the manuscript and approved its final version.

Abstract

Natural killer cell-derived extracellular vesicles (NK-EVs) are candidate biotherapeutics against various cancers. However, standardized potency assays are necessary for a reliable assessment of NK-EVs' cytotoxicity. This study aims to thoroughly evaluate a highly sensitive resazurin phenoxazine-based cell viability potency assay (measurement of the cellular redox metabolism) for quantifying the cytotoxicity of NK-EVs against leukaemia K562 cells (suspension model) and breast cancer MDA-MB-231 cells (adherent model) *in vitro*. The assay was evaluated based on common analytical parameters set forth by regulatory guidelines, including specificity, selectivity, accuracy, precision, linearity, range, and stability. Our results revealed that this resazurin-based cell viability potency assay reliably and reproducibly measured a dose-response of NK-EVs' cytotoxic activity against both cancer models. The assay showed precision with 5% and 20% variation for intra-run and inter-run variability. The assay signal showed specificity and selectivity of NK-EVs against cancer target cells, as evidenced by the diminished viability of cancer cells following a 5-hour treatment with NK-EVs, without any detectable interference or background. The linearity analysis of target cancer cells revealed strong linearity for densities of 5000 K562 and 1000 MDA-MB-231 cells per test with a consistent range. Importantly, NK-EVs' dose-response for cytotoxicity showed a strong correlation ($|r| \sim 0.8$) with the levels of known cytotoxic factors associated with the NK-EVs' corona (FasL, GNLY, GzmB, PFN, and IFN- γ), thereby validating the accuracy of the assay. The assay also distinguished cytotoxicity changes in degraded NK-EVs, indicating the ability of the assay to detect the potential loss of sample integrity. Compared to other commonly reported bioassays (*i.e.*, flow cytometry, cell counting, lactate dehydrogenase release assay, DNA-binding reporter assay and confluence assay), our results support this highly sensitive resazurin-based viability potency assay as a high-throughput and quantitative method for assessing NK-EVs' cytotoxicity against both suspension and adherent cancer models for evaluating NK-EVs' biotherapeutics.

Introduction

Research in extracellular vesicle (EV)-based biotherapeutics in cancer treatment is soaring worldwide [178, 200-203]. However, a considerable barrier to the advancement of natural killer cell-derived EVs (NK-EVs) as a therapy for cancer has been the lack of validated standardized potency assays to measure NK-EVs' cytotoxic activity [28, 57, 63, 103, 105, 128, 129, 132, 204-208]. Such standardized quantitative potency assays must meet key attributes aligned with regulatory guidelines, such as the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [175, 178, 202, 209]. Other challenges include: *i)* comparing novel EV-based therapeutics across studies, EV lots/preparations, and individual experiments [105]; *ii)* the need for more essential quality controls and consistent dosing methodologies [105, 175, 178, 202].

Historically, flow cytometry and various plate-based assays have been used to evaluate the potency of various biotherapeutics, including both cell and EV products [62, 63, 65, 66, 130, 131, 133, 135, 136, 199, 210-214]. While many of these assays have also been used to assess the cytotoxic effect of NK-EVs in various cancer cell models, to the best of our knowledge, no published studies have yet systematically investigated specificity, selectivity, accuracy, precision, linearity, range, and stability of these potency assays.

To this end, we evaluated a high sensitivity (HS) cell viability assay, PrestoBlue HS cell viability assay, which relies on reducing resazurin to resorufin (detectable by absorbance or fluorescence), which measures the metabolic activity of living cells. Here, we perform a rigorous analysis of the performance of this highly sensitive resazurin-based cell viability assay against other commonly reported assays in the field using the NK92-M1 cell line-derived-EVs, which is a clinically relevant model [65, 66, 105, 130, 131, 133-136]. This resazurin-based reagent, marketed by Thermo Fisher Scientific, is promoted as an exceptional pure resazurin solution with better signal-to-background ratio compared to the standard version.

The assay performance and suitability were based on the evaluation of common analytical parameters such as accuracy (comparing measured values to a known reference), precision

(measuring consistency across repeated measurements, formally known as Intermediate Precision, and reported as an intra-run and inter-run variation), linearity (confirming accurate and proportional results across analyte concentrations), range (the interval between the lowest and the highest reportable results), specificity and selectivity (assessing the accurate identification of the intended analyte without interference), and stability (evaluating the integrity and performance of the product over time-based on bioactivity levels). Additionally, we performed Spearman dose-response correlations using levels of known NK-EVs' cytotoxic factors (*i.e.*, Fas Ligand - FasL, Granulysin - GNLY, Granzyme B - GzmB, Perforin - PFN, and Interferon-gamma - IFN- γ) to validate the accuracy of the potency assay.

This is the first study to comprehensively provide common analytical parameters supporting the utility of a highly sensitive resazurin phenoxazine-based cell viability assay as a reliable high-throughput and quantitative potency assay to measure the cytotoxicity of NK-EVs against cancer cell models in comparison to other commonly used potency assays (*e.g.*, flow cytometry, cell counting, lactate dehydrogenase (LDH) release assay, DNA-binding reporter assay and confluence assay). This cell viability assay's high sensitivity, fast and user-friendly features render it an accessible and reliable method for assessing the potency of NK-EVs' cytotoxic products. This work contributes to the standardization and development of potency assays for EV-based cancer therapeutics, fostering advancement in the clinical translation of EV-based products.

Materials and Methods

Cell culture parameters

The human leukaemia K562 cell line (ATCC, CCL-243) was cultured in RPMI-1640 with 10% Fetal Bovine Serum (FBS, Gibco, cat#12483020). Cells were maintained between $1 - 6 \times 10^5$ cells/mL and passaged every 2 - 3 days. The human breast cancer cell line MDA-MB-231 tagged with Red Fluorescent Protein (RFP) was cultured in Hams F-12 Medium (Corning cat#10-080-CV) and high glucose DMEM with GlutaMAX Supplement (Gibco, cat#10566016) at 1:1 ratio with 10% FBS. During passaging, cell detachment was performed using TrypLe select (Gibco, cat#12563-

029) after washing the cells with sterile Dulbecco's phosphate-buffered saline without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (DPBS⁻, Gibco, cat#14190250). Cells were maintained between $3 - 4 \times 10^4$ cells/cm² and passaged every 2 - 3 days. The FreeStyle 293F cell line (Thermo Fisher Scientific, R790-07) was cultured in FreeStyle 293 Expression Medium (Gibco, cat#12338018) to produce non-therapeutic control EVs (*i.e.*, 293F-EVs), as previously done [105]. Cells were maintained between $1 - 3 \times 10^6$ cells/mL on a heat-resistant shaker (Thermo Fisher Scientific, cat#88881127) at 135 RPM. Cell lines were cultured at 37°C in a 5% CO₂ incubator, except the 293F cell line, which was cultured at 37°C in an 8% CO₂ incubator. Cell counting was performed on the Cellometer Auto 2000 Viability Counter (Nexcelom BioScience LLC) using the ViaStain Acridine Orange and Propidium Iodide (Acridine Orange/Propidium Iodide) Staining Solution (ESBE Scientific, cat#CS2-0106) with the immune cell AO/PI program (channel 1: 470/535 nm for 0.5 sec of exposure and channel 2: 540/605 nm for 3 sec of exposure).

Biomanufacturing of NK-EVs from NK92-MI cells

As previously done, NK-EVs were manufactured using a large-scale biomanufacturing workflow that adheres to Good Manufacturing Practices and uses NK92-MI cells [105]. Briefly, the biomanufacturing of NK92-MI cells and NK-EVs was performed using a closed-loop hollow fibre bioreactor to generate large quantities of clinical-grade NK-EVs. NK92-MI cells were seeded into the hollow fibre cartridge and cultured in serum-free, xeno-free, and feeder-free medium - ImmunoCult-XF T Cell Expansion Medium (StemCell Technologies, cat#10981). NK92-MI cells were expanded in the cartridge and harvested at the end of the production cycle. The EV-rich conditioned medium (CM) was collected and stored at -80°C daily until further processing.

Purification of NK-EVs by fast protein liquid chromatography (FPLC) using size-exclusion chromatography (SEC) and ultrafiltration (UF)

Purification of NK-EVs was performed as previously described with slight modifications [105]. Thawed NK-EVs-rich CM was centrifuged at $10,000 \times g$ for 30 min before being loaded into a 60 mL syringe (BD Biosciences, cat#309653) for SEC-based EV purification. SEC was performed on the AKTA Fast Protein Liquid Chromatograph (GE Lifesciences, cat#29022094) using the

HiScale chromatography column 10/40 (Cytiva, cat#29360550) packed with CaptoCore 700 resin (Cytiva, cat#17548102) for a total bed height of 20 cm. The column/resin was stored in 20% ethanol (Commercial Alcohols, cat#P006EAAN). Clean-in-place and priming procedures were performed using the following filtered solutions; filtration performed using the 0.1 µm vacuum filtration unit Filtropur V50 (Sarstedt, cat#83.3941.002): cleaning solution (0.5M NaOH (Supelco, cat#SX0607N-6) and 30% Isopropyl Alcohol (EMD, cat#PX1834-1) in water), water and DPBS^{-/-} (Fisher, cat#BP399-1) according to manufacturer protocol. The ultraviolet absorbance was zeroed after the reading stabilized while running filtered-DPBS^{-/-} through the instrument. The NK-EVs-rich CM was loaded on the column at a flow velocity of 150 cm/h (flow rate of 2.0 mL/min). Sample fractionation, collected into 15 mL tubes (Corning, cat#430791), was initiated once the ultraviolet absorbance level passed the 230 mAU mark and stopped when the ultraviolet absorbance level dropped below the 1600 mAU mark. To ensure sterility, the fractionated sample was combined before filtration at 0.22 µm with a pre-wet (filtered-DPBS^{-/-}) Acrodisc Syringe Filter (Pall Corporation, cat#PN4612). Then, the FPLC-retentate solution was concentrated using a pre-equilibrated (sterilized with 70% ethanol and rinsed twice with filtered-DPBS^{-/-}) 10 kDa Centricon Plus-70 Centrifugal Filter - regenerated cellulose membrane (Millipore Sigma, cat#UFC701008) at 4,000 x g for 15 - 25 min to attain a concentration factor of 40 - 45 X pre-concentration volume. The FPLC-retentate was mixed using a serological pipette after 15 min of spinning using the Centricon apparatus. Then, the FPLC-retentate was further spun for an extra 10 min. The final retentate was collected by inverting the filter into the collection cup and spinning the assembly at 2,000 x g for 2 min. NK-EVs were stored at 4°C, and their use was limited to 7 days post isolation for the entire study, except for the experiment section entitled "Characteristics and bioactivity assessment of NK-EVs following 25°C treatment over time". Each experimental replicate was made from a different NK-EVs' isolation.

Nanoparticle tracking analysis (NTA) of NK-EVs

NTA analysis was performed as previously described with slight modifications [105]. Purified NK-EVs samples were thoroughly vortexed before preparing two EV dilutions within the working range of the system using filtered-DPBS^{-/-} and analyzed using the NanoSight NS300 (V3.4

software; Malvern, UK), equipped with a 488 nm laser and a sCMOS camera. For capture settings, the camera level was set to 14 and the detection threshold to 15 for analysis, resulting in approximately 30 - 80 particles per frame. Two dilutions were run, one ranging from 30 - 50 particles per frame and the other from 50 - 80 particles per frame (dilutions of 1500X and 1000X, respectively). After temperature-stabilizing the instrument at 23°C, approximately 1 mL of NK-EVs sample was loaded in a 1 mL syringe (Thermo Fisher Scientific, cat#MB9204560TF-LAB), where roughly half of the syringe content was manually injected into the chamber of the instrument until the camera observed stable particles per frame. The syringe was then mounted on the syringe pump (Harvard Apparatus, cat#984730) for acquisition capture in flow mode (flow speed 30) for one capture of 1 min. After the acquisition, the flow rate was increased to 1000 for ~10 sec before returning to 30. This sequence was repeated for a total of five captures. This acquisition method allows sampling and analysis of the sample through the syringe, assuring an accurate representation of the entire sample. To ensure maximal reproducibility and accuracy, the EV concentration was calculated for each NK-EVs preparation by averaging the dilution-corrected concentration of both dilutions ran on NTA, where each dilution captures were averaged.

ELISA assessment and protein quantification of NK-EVs' corona

ELISA assessment of purified intact NK-EVs was performed per the manufacturer's protocol to assess the level of the following cytotoxic factors that are likely associated with the NK-EVs' corona: FasL/TNFSF6 (R&D Systems, cat#DFL00B), GNLY (Abcam, cat#ab256402), GzmB (R&D Systems, cat#DGZB00), PFN (Abcam, cat#ab46068), and IFN- γ (R&D Systems, cat#DIF50C). Readings were made using the Microplate Reader (BioTek Synergy H1 Multimode Reader; cat#SH1M2G-SN; Gen5 software V3.14). PBS buffer was added as a control for background subtraction in data processing, and negative values were zeroed. Protein quantification was performed using the Qubit Protein BR Assay Kit (Invitrogen, cat#A50669) on the Qubit 4 Fluorometer according to the manufacturer's protocol, using triplicates.

Resazurin-based cell viability assay: Potency evaluation of NK-EVs treated cancer cells

Using 96-well Flat Clear Bottom Black Polystyrene TC-treated Microplates (Costar, cat#3603), 5000 K562 target cells (suspension cells) or 1000 MDA-MB-231 target cells (adherent cells) were seeded per well; 4 technical replicates per condition. Cells were maintained in Fluorobrite DMEM (Gibco, cat#A18967-01) with 10% Exosome-Depleted FBS (Gibco, cat#A2720803), where the final well volume was normalized to 250 μ L final. Untreated cells, 1X of Triton-X 100 (Sigma, cat#T-9284) detergent-treated cells and 293F-EVs were used as controls. Cells were allowed to settle into the plate for 1 - 2 hours before adding effector EVs (NK-EVs and negative control 293F-EVs) at various effector EVs to target cancer cell ratios: 500; 5,000; 50,000; 500,000; and 5,000,000. After 5 hours of treatment at 37°C with 5% CO₂, PrestoBlue HS Cell Viability Reagent Assay (Invitrogen, cat#P50200) was added to each well as per manufacturer protocol. Plate incubation was performed at 37°C in a 5% CO₂ incubator and protected from light for 90 min (maximizing the assay sensitivity). Fluorescence was measured (Ex/Em 560/590 $\pm$ 9 nm) with a Microplate Reader (BioTek Synergy H1 Multimode Reader; cat#SH1M2G-SN; Gen5 software V3.14), temperature-stabilized at 37°C to reduce temperature-variation on fluorescence measurements. Before acquisition, the plate reader mixed the plate for 60 sec at 350 RPM to homogenize the content in each well. Technical replicates were averaged for analysis and then corrected for background before performing a dose-response analysis using a non-linear regression for inhibition effect showing the log(inhibitor) vs. normalized response - variable slope without constraint; the Hillslope and EC₅₀ were recorded.

Resazurin-based cell viability assay: Evaluation of linearity and range

To assess this resazurin-based cell viability assay linearity, K562 cells or MDA-MB-231 cells were seeded, ranging from 0 cells to 50,000 cells per well (0, 10, 50, 100, 500, 750, 1000, 2500, 5000, 10,000, 25,000 and 50,000), in 96-well Flat Clear Bottom Black Polystyrene TC-treated Microplates. To assess this resazurin-based cell viability assay range, 5000 K562 cells/well were seeded at varying levels of viability (0, 5, 10, 20, 25, 37.5, 50, 62.5, 75, 80, 90% and "max; as per AO/PI counting,"), which was achieved by mixing the appropriate ratio of untreated cells to heat-shocked (30 min at 70°C) treated K562 cells) Alternatively, 1000 MDA-MB-231 cells/well were

seeded with varying levels of viability (cells treated with varying concentrations of sodium dodecyl sulfate solution (SDS; Sigma, cat#71736-500 mL) for 30 - 60 min). MDA-MB-231 cells were allowed to settle and adhere to the bottom of the plate for at least 12 - 24 hours before linearity and range assessment. The culture media was changed prior to evaluation to remove un-attached MDA-MB-231 cells. Well volume was normalized to 100 μ L final using Fluorobrite DMEM with 10% FBS. Cell viability assessment was performed as detailed in the section “Resazurin-based cell viability assay: Potency evaluation of NK-EVs treated cancer cells.”

PrestoBlue HS cell viability assay: Evaluation of reagent incubation time

Using 96-well Flat Clear Bottom Black Polystyrene TC-treated Microplates (Costar, cat#3603), 5000 K562 target cells were seeded per well; 4 technical replicates per condition. Cells were maintained in Fluorobrite DMEM (Gibco, cat#A18967-01) with 10% Exosome-Depleted FBS (Gibco, cat#A2720803), where the final well volume was normalized to 100 μ L. Untreated cells and 1X of Triton-X (Sigma, cat#T-9284) detergent-treated cells were used as samples. After 30 min of treatment at 37°C with 5% CO₂, the PrestoBlue HS Cell Viability Reagent Assay (Invitrogen, cat#P50200) was added to each well, where plate incubation was performed at 37°C in a 5% CO₂ incubator and protected from light. Fluorescence was measured (Ex/Em 560/590 $\pm$ 9 nm) with a Microplate Reader (BioTek Synergy H1 Multimode Reader; cat#SH1M2G-SN; Gen5 software V3.14), temperature stabilized at 37°C to reduce temperature-variation on fluorescence measurements at the following time points: 5, 15, 30, 45, 60, 90, 120, 150 and 180 min post-PrestoBlue HS Cell Viability Reagent addition. Before acquisition, the plate reader mixed the plate for 60 sec at 350 RPM to homogenize the content in each well. For analysis, technical replicates were averaged and then corrected for background.

Characteristics and bioactivity assessment of NK-EVs following 25°C treatment over time

After NK-EVs' purification, the final product was thoroughly vortexed before being equally aliquoted in Eppendorf Protein LoBind 0.5 mL tubes (Eppendorf, cat#022431064). NK-EVs aliquots were treated at 25°C for three weeks to promote product degradation. Weekly, NTA was used to assess the NK-EVs' characteristics for size and concentration quantification (as detailed above)

and selected plate-based assays (PrestoBlue HS Cell Viability assay and Cell Counting Kit 8 Assay (Sigma-Aldrich, cat#96992) - CCK8) to assess NK-EVs' bioactivity. Cytotoxicity assessment of NK-EVs was performed using 96-well Flat Clear Bottom Black Polystyrene TC-treated Microplates, in which 5000 K562 target cells (suspension cells) were seeded per well in duplicates. Untreated cells and media only were used as controls. K562 cancer cells were maintained in Fluorobrite DMEM with 10% Exosome-Depleted FBS. Well volume was normalized to 100 µL final using the cell culture medium, where the plate was maintained at 37°C with 5% CO₂. We used 1X CCK8 (450 nm absorption) for continuous assessment (every hour for the first 8 hours and then every 24 hours until 72 hours). Cell Viability assessment was performed as detailed in the section "Resazurin-based cell viability assay potency evaluation of NK-EVs treated cancer cells." Cell viability was calculated as follows, where RFU is relative fluorescence units:

$$\% \text{ Cell viability} = \left(\frac{\text{EV treated cells RFU}}{\text{Untreated cells RFU}} \right) \times 100\%$$

Various plate-based assays for cell death evaluation following NK-EV treatment

Using 96-well Flat Clear Bottom Black Polystyrene TC-treated Microplates, 5000 K562 target cells (suspension cells), labelled with 1 µM of CellTrace™ CFSE Cell Proliferation kit (Invitrogen, cat#C34554), or 1000 MDA-MB-231-RFP target cells (adherent cells) were seeded per well; 4 technical replicates per condition. Cells were maintained in Fluorobrite DMEM with 10% Exosome-Depleted FBS, where the final well volume was normalized to 250 µL. Untreated cells, 1X of Triton-X 100 (Sigma, cat#T-9284) detergent-treated cells and 293F-EVs are used as controls. Cells were allowed to settle into the plate for 1 - 2 hours before adding effector EVs (NK-EVs and negative control 293F-EVs) at various effector EVs to target cancer cells: ratio of 500; 5000; 50,000; 500,000; and 5,000,000. For continuous monitoring using an image-based cytometer (qIBC), measurements were made for cell confluence (label-free), cell cytotoxicity using 0.008X CellTox Green Cytotoxicity Assay (Promega, cat#G8742) and cell reporter-loss: CFSE signal (K562) or RFP signal (MDA-MB-231-RFP) using the CELLCYTE X instrument (CYTENA; C-2111171, HeartOS V6.1.1) at 37°C with 5% CO₂. Each well was assessed by capturing four overlaid images using a 10X objective (Resolution: 0.345 µm/pixel and Field of View: 0.8 x 0.7 mm), a red laser

(Ex/Em 580-598 / 612-680), a green laser (Ex/Em 473-491 / 502-561 nm) and a blue laser (Ex/Em 370-410 / 429-462 nm). The qIBC data was analyzed using the CELLCYTE Studio software (V2.6.0). We also continuously monitored an environmentally-controlled Microplate Reader using 1X Cell Counting Kit 8 (450 nm absorption) Assay (CCK8; Sigma-Aldrich, cat#96992). After 5 hours of treatment at 37°C with 5% CO₂, 50 µl aliquot of each well was transferred into a new 96-well Flat Clear Bottom Black Polystyrene Microplate and processed as per the CyQuant LDH Cytotoxicity (Ex/Em 560/590 ± 9 nm) Assay (Life Technologies, cat#C20302), where plate incubation was performed at 37°C in a 5% CO₂ incubator and protected from light. Fluorescence intensities were measured by the Microplate Reader (BioTek Synergy H1 Multimode Reader; cat#SH1M2G-SN; Gen5 software V3.14). For analysis, technical replicates were averaged and then corrected for background. LDH release cytotoxicity assay results were calculated as follows, where RFU is relative fluorescence units:

$$\% \text{ Cytotoxicity by LDH assay} = \left(\frac{\text{Compound treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \right) \times 100\%$$

AO/PI cell counting and flow cytometry for cell death evaluation following NK-EV treatment

Using 24-well Clear TC-treated Plates (Costar, cat#3524), 50,000 K562 target cells (suspension cells) or 50,000 MDA-MB-231-RFP target cells (adherent cells) were seeded per well; 4 technical replicates per condition. Cells were maintained in Fluorobrite DMEM (Gibco, cat#A18967-01) with 10% Exosome-Depleted FBS (Gibco, cat#A2720803), where the final well volume was normalized to 350 µL. Untreated cells, 1X of Triton-X 100 (Sigma, cat#T-9284) detergent-treated cells and 293F-EVs are used as controls. Cells were allowed to settle into the plate for 1 - 2 hours before adding effector EVs (NK-EVs and negative control 293F-EVs) at various effector EVs to target cancer cells: ratio of 500; 5000; 50,000; 500,000; and 5,000,000. After 5 hours of treatment at 37°C with 5% CO₂, technical replicates were combined into an Eppendorf Protein LoBind 2 mL tube (Eppendorf, cat#022431102) and centrifuged at 300 x g for 3 min at 4°C. The MDA-MB-231-RFP cells were processed with additional steps: after collecting the supernatant and rinsing the wells with Dulbecco's phosphate-buffered saline without Ca²⁺/Mg²⁺ (DPBS⁻, Gibco, cat#14190250), the cells were detached using TrypLe select (Gibco, cat#12563-029) for 5 min.

Then, each solution was transferred to its corresponding supernatant into the Eppendorf Protein LoBind tube. This maximizes the collection of all the cells present in a well for analysis. Cells were washed once with PBS, after a spin at 300 x g for 3 min, to remove effector EVs before suspending the cell solution at approximately 1×10^6 cells/mL using PBS (approx. 220 μ L). In duplicates, an aliquot of 20 μ L was acquired for cell counting using ViaStain AO/PI Staining Solution (ESBE Scientific, cat#CS2-0106). The remaining solution was transferred into 5 mL FACS tubes containing a final resuspension buffer of 1 μ M Sytox Blue Nucleic Acid Stain (Invitrogen, cat#S11348). Samples were allowed to incubate for at least 15 min on ice before flow cytometry acquisition. All samples were acquired on the BD FACSymphony A1 (BD Biosciences) and analyzed on the BD FACSDiva Software (V9.0.2). A minimum of 150,000 events per sample were collected at the single-cell gate. Raw data was analyzed using FlowJo V10.9.0 (FlowJoLLC, USA).

EV-TRACK

We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV230983) [165].

Statistical Analysis

Data were expressed as the means $\pm$ the standard deviation (S.D.) or the standard error of the mean (S.E.M.) as indicated in the figure legend. Data were normalized to the control group for relative comparison, as indicated in each figure legend. The number of experimental and technical replicates used is also indicated in figure legends. Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software Inc., LaJolla, CA, USA). Two-way ANOVA followed by post hoc tests (Tukey's or Sidak's multiple comparisons) were used and identified in the figure legend. Results were considered significant when $p < 0.05$. To assess the accuracy of potency assays, we performed Spearman correlations between the outcome of the assay (response induced by NK-EVs) and the level of cytotoxic factors, where the response was transformed to its logarithmic value (log of RFU), and the cytotoxic factor dose was transformed to its root square value (sqr of cytotoxic factor ng/mg of proteins) for better data representation. Correlation coefficient values (ρ) are defined as perfectly negative (-1), strongly negative (-0.8),

moderately negative (-0.5), weakly negative (-0.2), no association (0), weakly positive (0.2), moderately positive (0.5), strongly positive (0.8), perfectly positive (1).

Results

This study aims to thoroughly evaluate potency assays for quantifying NK-EVs' cytotoxicity, starting with a highly sensitive resazurin-based cell viability assay (measurement based on redox metabolism), based on common analytical parameters, including specificity, selectivity, accuracy, precision, linearity, range, and stability.

Resazurin-based cell viability assay reaffirms a dose-dependent cell viability loss in leukaemia cells after treatment with NK-EVs

First, cell viability loss following NK-EVs treatment was reaffirmed using a highly sensitive resazurin-based cell viability assay, as previously shown [105]. Cytotoxic NK-EVs or negative control 293F-EVs were incubated at various concentrations with the leukaemia cell line K562 (gold-standard cell model for NK cytotoxicity [215]) for over 5 hours. As shown in **Figure 22A-i**, the cell viability assay revealed a dose-response of K562 cell viability loss after treatment with NK-EVs. In contrast, control 293F-EVs did not exhibit a cytotoxic dose-response, indicating a specific effect attributed to cytotoxic effectors of NK-EVs. The EC₅₀ of NK-EVs' cytotoxic effect was measured at an E: T ratio (Effector: Target) of 392,351 particles per K562 cell, as shown in **Figure 22A-ii**.

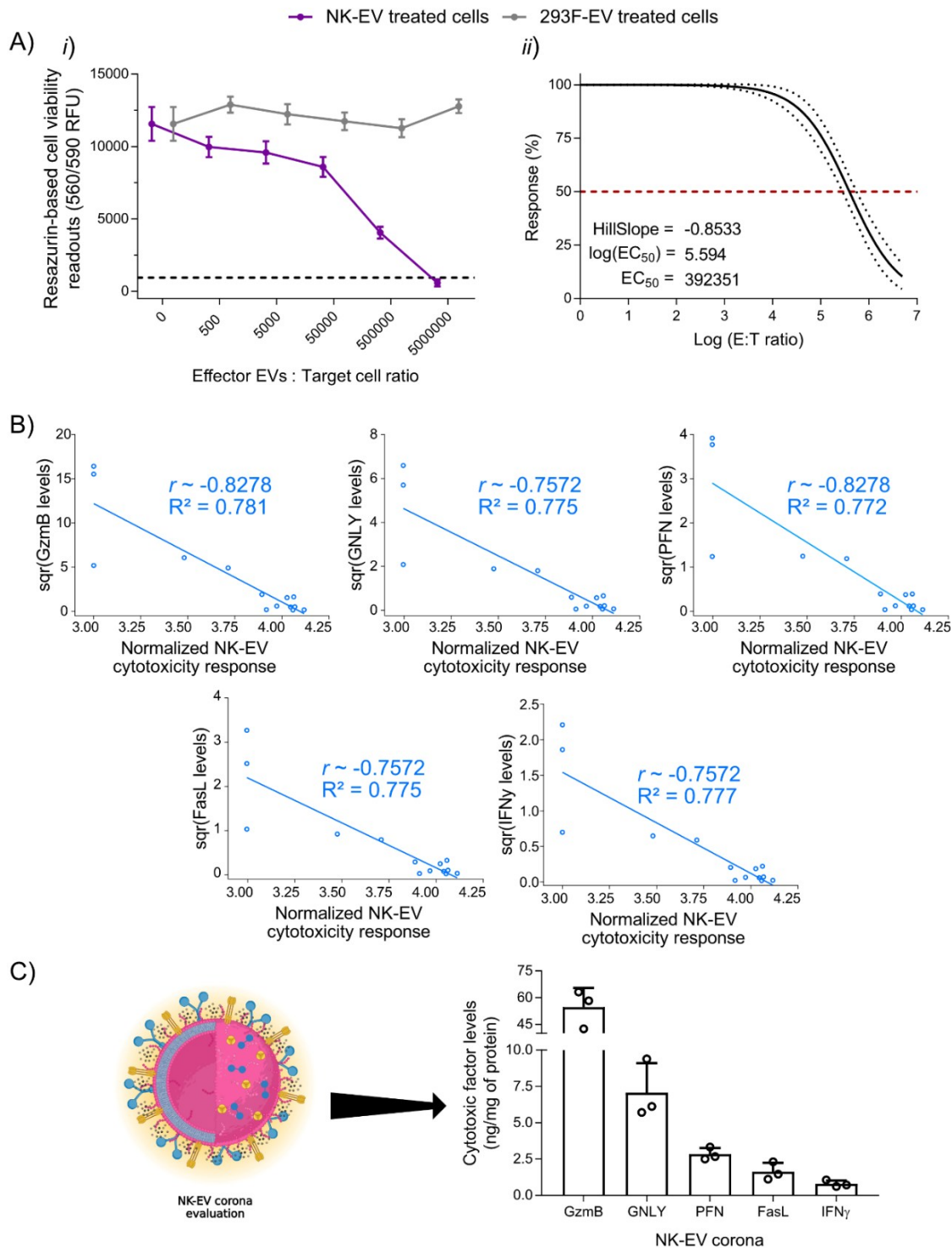


Figure 22. Resazurin-based cell viability assay reaffirms a dose-dependent cell viability loss in leukaemia cells after NK-EV correlating with levels of cytotoxic factors.

(A) Leukaemia K562 cells were treated for 5 hours with various E: T ratios of NK-EVs or negative control 293F-EVs, followed by endpoint resazurin-based cell viability assay. The results are presented in (i) RFU (Relative Fluorescence Units) readouts (black dashed line represents lysed K562 cell control; detergent-treated) and (ii) EC_{50} curve analysis with variable slope for NK-EV treatment with 95% confidence interval/prediction bands (red dashed line represents 50% response). Data are shown as mean $\pm$ S.E.M. from 7 independent experiments, each with technical

quadruplets. (B) Spearman correlations of selected NK-EV's corona cytotoxic factors (GzmB, GNLY, PFN, FasL and IFN- γ) detected by ELISA in relation to cell viability assay using leukaemia K562 cells (n=14 correlations pairs where one pair (a dot in the figure) represents the correlation between the level of a cytotoxic factor and the cell viability assay readout for a given NK-EV dose). (C) NK-EVs corona cytotoxic factors (schematically shown) were assessed by ELISA and presented as the protein-normalized levels (ng/mg of protein) of a given cytotoxic factor (GzmB, GNLY, PFN, FasL and IFN- γ) using total protein levels (mg) detected by Qubit Protein Assay. Data are shown as mean $\pm$ S.D. from 3 independent experiments, each with technical duplicates.

Resazurin-based cell viability assay shows excellent dose-dependent correlations to known cytotoxic factors of NK-EVs' corona in leukaemia K562 cells

To validate the accuracy of this cell viability assay as a reliable potency assay for NK-EVs' cytotoxicity against target cancer cells, the levels of cytotoxic factors (*i.e.*, FasL, GNLY, GzmB, PFN, and IFN- γ) that are likely associated with the NK-EVs' corona (*i.e.*, a layer of molecules embedded to the EV external surface, thereby excluding the intravesicular content) were assessed by ELISA and used for dose-response correlations. The levels of each cytotoxic factor (square root transformed) were correlated to the corresponding cell viability assay RFU values (logarithmically transformed). Data transformation was done to optimize data visualization. As shown in **Figure 22B**, dose-response of the cell viability assay strongly correlated with all cytotoxic factors that mediate cell death in target K562 cells ($|\rho| \sim 0.757 - 0.828$ and $R^2 \sim 0.772 - 0.828$). Notably, all cytotoxic factors correlated similarly, as shown in **Table 6**. Comparatively, 293F-EV control did not yield a dose-response correlation, as shown in **Figure 23** and **Table 7**. The abundance of cytotoxic factors in the NK-EVs' corona across different batches of product preparations is depicted in **Figure 22C**. NK-EVs' corona is composed of GzmB (54.63 ng $\pm$ 6.27 ng), GNLY (7.08 ng $\pm$ 1.16 ng), PFN (2.83 ng $\pm$ 0.25 ng), FasL (1.63 ng $\pm$ 0.36 ng) and IFN- γ (0.80 ng $\pm$ 0.14 ng), normalized to the protein levels (mg) in solution.

Table 6. Spearman correlational analyses of selected NK-EV's corona cytotoxic factors detected by ELISA in relation to PrestoBlue viability assay responses in leukaemia K562 cells.

Coefficient value (ρ) are defined as perfectly negative (-1), strongly negative (-0.8), moderately negative (-0.5), weakly negative (-0.2), no association (0), weakly positive (0.2), moderately positive (0.5), strongly positive (0.8), perfectly positive (1). Each correlation was made using n=14 data points acquired from n=3 experimental replicates with appropriate technical replicates.

Potency Assay		PrestoBlue HS	
Assay quality / robustness		Z' factor	0.9340
Spearman correlation by cytotoxic factor	GzmB	Rho (ρ)	-0.7403
		P value	0.0071
		R ²	0.7640
	PFN	Rho (ρ)	-0.7403
		P value	0.0071
		R ²	0.7596
	FasL	Rho (ρ)	-0.6940
		P value	0.0104
		R ²	0.8010
	IFN- γ	Rho (ρ)	-0.6940
		P value	0.0104
		R ²	0.7876
	GNLY	Rho (ρ)	-0.6940
		P value	0.0104
		R ²	0.7823
	Average	Rho (ρ)	0.7125
		P value	0.0091
		R ²	0.7789

Table 7. Spearman correlational analyses of selected 293F-EV's corona cytotoxic factors detected by ELISA in relation to PrestoBlue viability assay responses in leukaemia K562 cells.

Coefficient value (ρ) are defined as perfectly negative (-1), strongly negative (-0.8), moderately negative (-0.5), weakly negative (-0.2), no association (0), weakly positive (0.2), moderately positive (0.5), strongly positive (0.8), perfectly positive (1). Each correlation was made using n=14 data points acquired from n=3 experimental replicates with appropriate technical replicates.

Potency Assay		PrestoBlue HS	
Spearman correlation by cytotoxic factor	GzmB	Rho (ρ)	0
		P value	0.999
		R ²	0
	PFN	Rho (ρ)	0
		P value	0.999
		R ²	0
	FasL	Rho (ρ)	0
		P value	0.999
		R ²	0
	IFN- γ	Rho (ρ)	0
		P value	0.999
		R ²	0
	GNLY	Rho (ρ)	0
		P value	0.999
		R ²	0
	Average	Rho (ρ)	0.0000
		P value	0.9990
		R ²	0.0000

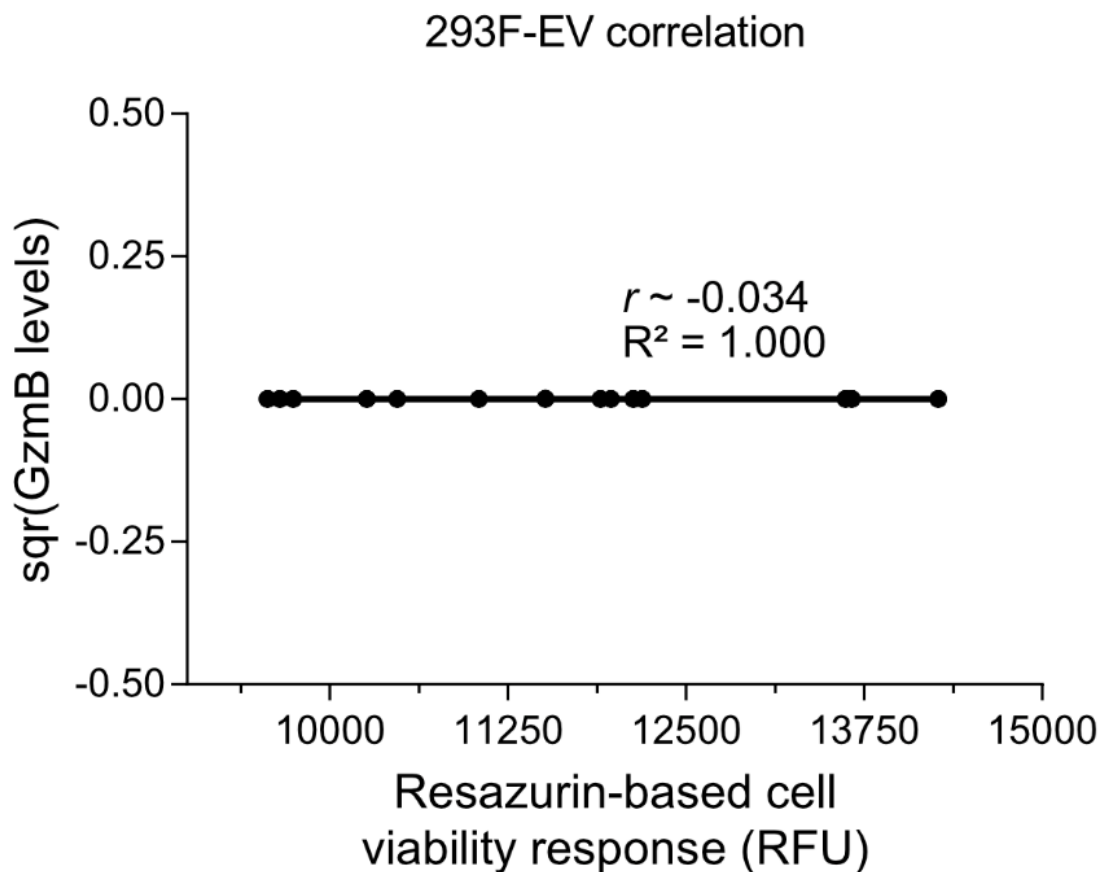


Figure 23. Corroborative evidence from Spearman correlational analysis.

Spearman correlations of 293F-EV's corona for GzmB detection by ELISA, in relation to the resazurin-based cell viability assay responses in leukaemia K562 cells (n=14 correlations pairs where one pair (a dot in the figure) represents the correlation between the level of a cytotoxic factor and the cell viability assay readout for a given NK-EV dose).

Resazurin-based cell viability assay shows precision with K562 cells

To assess this resazurin-based cell viability assay precision, the coefficient of variation (% CV, defined as the ratio of the standard deviation to the mean) from the results in **Figure 22A** was plotted. As shown in **Figure 24A**, intra-run variability (between technical quadruplets within each assay) ranges from 2.94% CV - 9.63% CV with a mean of 6.15% CV. Similarly, inter-run variability (between independent assays) ranges from 13.48% CV - 24.46% CV with a mean of 20.05% CV, as shown in **Figure 24B** and **Table 8**.

Table 8. PrestoBlue viability assay variability (precision; intra-run and inter-run coefficient of variation) for K562 cells.

Samples	Mean	S.D.	CV%
<i>Intra-run</i>			
1	15444.50	2215.63	14.35
2	9986.50	1728.18	17.31
3	23623.00	1995.21	8.45
4	25618.25	919.10	3.59
5	15847.67	420.69	2.65
6	12882.33	828.54	6.43
7	11930.00	659.91	5.53
8	5587.00	257.71	4.61
9	5435.00	21.21	0.39
10	9754.50	340.13	3.49
11	10577.00	247.26	2.34
12	8507.33	124.04	1.46
13	5287.33	179.92	3.40
14	5221.00	231.93	4.44
15	11930.00	659.91	5.53
16	5307.75	62.90	1.19
17	4149.75	75.45	1.82
18	8939.50	128.96	1.44
19	9806.75	70.10	0.71
20	6319.00	356.38	5.64
21	3864.00	442.65	11.46
Average CV%	-	-	5.06
<i>Inter-run</i>			
1	13218.20	2453.91	18.56
2	6007.53	1404.54	23.38
3	4697.80	1085.96	23.12
4	14255.35	2274.54	15.96
5	13154.55	1773.56	13.48
6	13073.60	3197.18	24.46
7	10439.60	2375.74	22.76
8	6240.95	1355.51	21.72
Average CV%	-	-	20.43

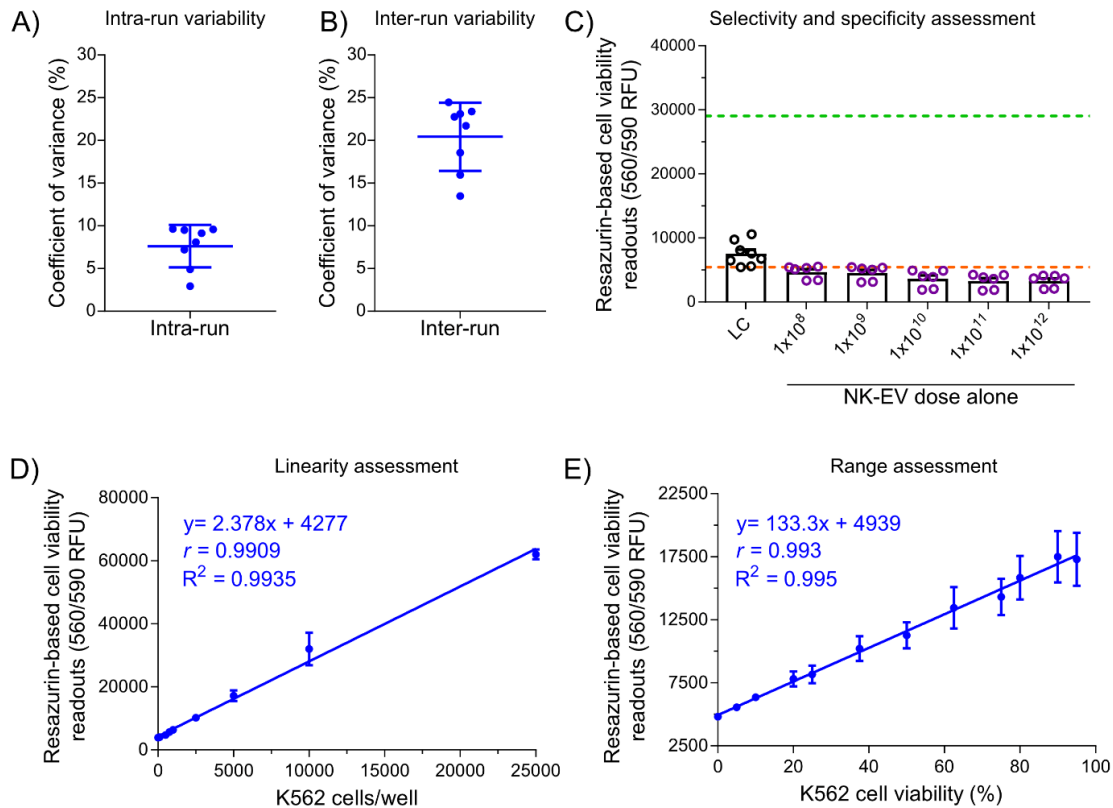


Figure 24. Analytics of highly sensitive resazurin-based cell viability assay conducted with K562 leukaemia cells.

(A) The mean coefficient of variance $\pm$ S.D., representing the intra-run variability in the resazurin-based cell viability assay, was calculated from 7 independent experiments, each with technical quadruplets. Each independent experiment had 8 samples, including six NK-EV doses, untreated K562 cells and detergent-treated K562 cells. (B) The mean coefficient of variance $\pm$ S.D., representing the inter-run variability in the resazurin-based cell viability assay, was calculated from 7 independent experiments, each with technical quadruplets. Each independent experiment had 8 samples, including six NK-EV doses, untreated K562 cells and detergent-treated K562 cells. (C) Fluorescence readings (RFU) of varying NK-EV doses for resazurin-based cell viability assay assessment are presented as mean $\pm$ S.E.M. from 8 independent experiments, each with technical duplicates. The green and orange dashed line represents the upper (5000 K562 cells alone without any NK-EV treatment) and lower (media only without any cells or NK-EVs) assay readouts, respectively, based on controls. Legends: LC – lysed cells. (D) Linearity assessment of resazurin-based cell viability assay (0 - 50,000 K562 cells/well). The data are presented as mean $\pm$ S.E.M. from 4 independent experiments, each with technical duplicates. (E) Range assessment of resazurin-based cell viability assay (5000 K562 cells/well with varying levels of viability). The data are presented as mean $\pm$ S.E.M. from 4 independent experiments, each with technical duplicates.

Resazurin-based cell viability assay exhibits both selectivity and specificity in detecting the loss of cell viability and shows excellent linearity and range with K562 cells

To evaluate the selectivity and specificity of this resazurin-based cell viability assay, varying concentrations of purified NK-EVs were introduced into a plate containing only the cell viability reagent without any cancer cells present. Consequently, the contribution of NK-EVs alone on the assay readings, specifically assessing their background and interference, was evaluated. As depicted in **Figure 24C**, the assay readouts remained unaffected by adding NK-EVs alone, regardless of the dose. This affirms that the assay readouts specifically reflect a decline in target cell viability and are not impacted by NK-EVs alone, thus showing no measurable interference.

The incubation time for this highly sensitive resazurin-based cell viability reagent was comprehensively evaluated to assess the assay's rapidity and sensitivity using the 96-well plate format, as shown in **Figure 25**. A minimum of 30 minutes of incubation is required to achieve statistical differences ($p < 0.0005$), yielding a fold-difference close to 13 for untreated K562 cells (5842.93 ± 833.14 RFU) over detergent-treated K562 cells (469.38 ± 81.24 RFU). Shorter incubation times did not yield statistically significant results ($\alpha = 0.05$). Comparatively, 90 minutes ($p < 0.0001$) of incubation allowed for a fold-difference close to 18 when comparing untreated K562 cells ($12,845.80 \pm 1758.80$ RFU) to detergent-treated K562 cells (734.56 ± 92.87 RFU). The assay signal for untreated K562 cells increased proportionally over time, whereas the signal for detergent-treated cells barely increased.

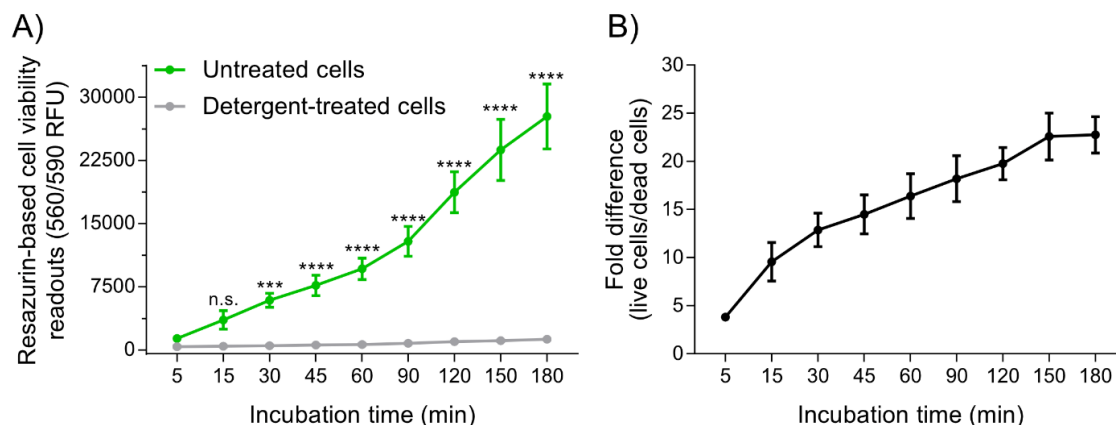


Figure 25. Resazurin-based cell viability assay shows a rapid turnaround time for endpoint assessment.

The viability of K562 target cells was assessed using the resazurin-based cell viability reagent immediately after seeding K562 cells in a 96-well plate, with fluorescence intensities measured at various time points. (A) Resazurin-based cell viability assay mean RFU readouts $\pm$ S.E.M. of untreated K562 cells compared to detergent-treated cells. Two-way ANOVA was performed, followed by post hoc tests (Sidak's multiple comparisons) where *** $p \leq 0.0005$ and **** $p \leq 0.0001$. (B) Fold-difference of untreated K562 cells compared to detergent-treated cells. The data are presented as mean $\pm$ S.E.M. from 4 independent experiments, each with technical quadruplets.

Moreover, the linearity of this resazurin-based cell viability assay was assessed by seeding K562 cells in a 96-well plate, ranging from 0 to 50,000 cells, and performed a linear regression analysis. The assay exhibited a linear response capable of detecting a minimum of 100 cells per well and a maximum of 25,000 cells per well, highlighting its high sensitivity. Using the 96-well plate format without allowing cell replication, robust linearity for 100 - 10,000 K562 cells/well ($R^2 \sim 0.994$ and $|\rho| \sim 0.991$) was demonstrated, as depicted in **Figure 24D**. Similarly, the assay range was assessed by seeding 5000 K562 cells/well with varying levels of viability, artificially generated by mixing the appropriate ratio of untreated cells to heat-shocked treated K562 cells. Using the 96-well plate format and without allowing cell replication, the assay could distinguish between low and high levels of cell viability ($R^2 \sim 0.995$ and $|\rho| \sim 0.993$), as shown in **Figure 24E**.

Resazurin-based cell viability assay effectively discriminates changes in NK-EVs' bioactivity following a 25°C treatment over time

To evaluate the ability of this resazurin-based cell viability assay to distinguish changes in bioactivity, NK-EVs were subjected to a 25°C treatment over three weeks to expedite NK-EVs' degradation. Importantly, the 25°C treatment temperature was intentionally selected to accelerate product degradation over a short period, unlike the more commonly used storage temperature for EVs (4°C, -20°C or -80°C), which favours product stability. Functional potency is a stability-indicating quality attribute for investigational biotherapeutics, including NK-EVs. To evaluate the stability of 25°C-treated NK-EVs cell viability was assessed using this resazurin-based cell viability assay, and particle size range distribution, and concentration by Nanoparticle Tracking Analysis (NTA) every week.

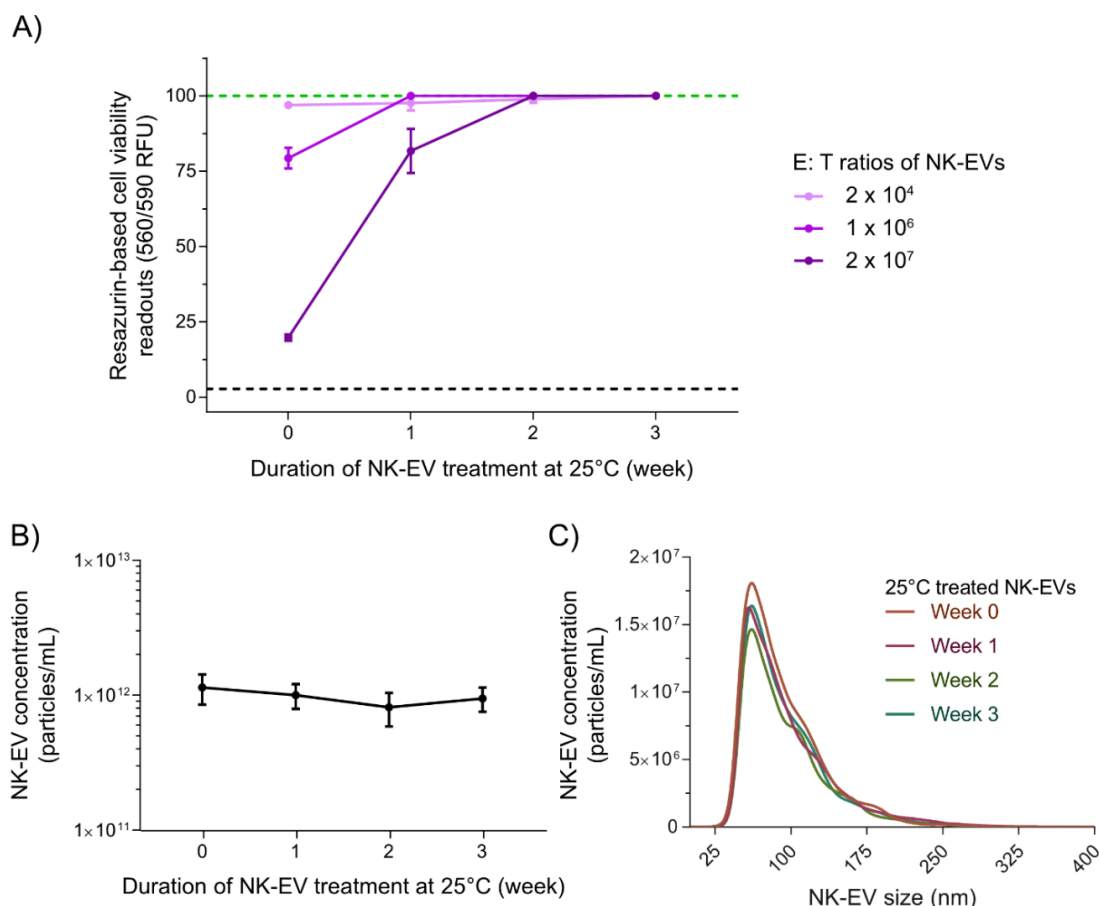


Figure 26. Resazurin-based cell viability assay distinguishes changes in bioactivity after exposure of NK-EVs to 25°C treatment over time using K562 leukaemia cells.

(A) K562 cells were treated for 5 hours with various E: T ratios of NK-EVs (after incubation at 25°C for 0, 1, 2, and 3 weeks), where their viability was assessed using the resazurin-based cell viability assay. The black dashed line represents lysed K562 cell control (detergent-treated) and the green dashed line represents untreated K562 cells. NK-EV dose information per K562 cell: low dose (2×10^4), medium dose (1×10^6) and high dose (2×10^7). Data are presented as mean $\pm$ S.E.M. from 4 independent experiments, each with technical duplicates. (B) NK-EV particle product concentration (particles/mL) measured over time by NTA, presented as mean $\pm$ S.D. from 4 independent experiments, each with technical duplicates. (C) NK-EV product size distribution after exposure to 25°C for 0, 1, 2, and 3 weeks, measured by NTA, shown as mean from 4 independent experiments, each with 10 technical replicates.

In this experiment, three NK-EVs doses were selected to depict the dose-response effect against gold-standard leukaemia cells, as shown in **Figure 26A**. After EV isolation, NK-EVs at time 0 yielded cytotoxicity against K562 cells, except for the low dose (2×10^4 NK-EVs per K562 cell). For instance, the medium dose (1×10^6 NK-EVs per K562 cell) and the high dose (2×10^7 NK-EVs per K562 cell) generated $20.69\% \pm 3.43\%$ and $80.26\% \pm 1.04\%$ cytotoxicity, respectively. However, after one week of treatment at 25°C, the functionality of the NK-EVs was significantly impaired. Notably, the medium dose (1×10^6 NK-EVs per K562 cell) could no longer yield cytotoxicity. In contrast, the high dose (2×10^7 NK-EVs per K562 cell) only produced an $18.30 \pm 7.30\%$ cytotoxic effect. This represents approximately a 62% potency difference for the same dose equivalency between untreated and temperature-treated NK-EVs for the high treatment dose. Moreover, NK-EVs' cytotoxicity was entirely reduced by the second week of treatment, highlighting the ability of this resazurin-based cell viability assay to detect changes in the NK-EVs product bioactivity. Interestingly, despite this loss of function, the product size distribution and concentration remained highly consistent over time, as depicted in **Figure 26B-C** and **Table 9**, suggesting that EV size and concentration are not stability-indicating attributes.

Table 9. Properties of the size distribution in the NK-EV product following 25°C treatment do not change over time.

NK-EV product NTA size metrics (D10 - size below which 10% of all particles are located, D50 - size below which 50% of all particles are located, D90 - size below which 90% of all particles are located, the mean, the mode and the S.D.) of NTA measurements, presented as mean $\pm$ S.D. from 5 independent experimental replicates with technical duplicates.

Week 0											Average
D10	76.1	81.3	76.6	75.3	74.6	75	79.6	80.2	75.2	77.9	77.18
D50	108.4	115.1	102	103.1	103.4	103	109	108	105.8	107.7	106.55
D90	166.8	176.6	169.4	169.1	170	164.6	175.8	172.9	174.7	181.5	172.14
Mean	116.1	123.7	114.4	115.3	114.3	113.9	121.1	118.6	116.2	122.2	117.58
Mode	88.6	95.9	84	88	82.1	82.9	84.9	86.9	89.4	87.3	87
S.D.	40	41.9	40.8	41.2	40.1	39.8	42.4	39.4	40.5	51.8	41.79
Week 1											Average
D10	78.4	76	75.1	76.3	79.2	74	79.3	78.7	74.4	77.7	76.91
D50	107.2	106.9	103.9	105.6	109	104.5	110.2	109.4	103.1	103.7	106.35
D90	169.8	176.8	172.5	180.1	186.8	184.8	180.7	189.4	168.8	169.6	177.93
Mean	118.1	120.2	116.3	119.1	121.5	118.2	123.3	123	114.4	115	118.91
Mode	83	98.7	78.9	78.3	85.4	79.6	83.1	81.7	92.7	86.1	84.75
S.D.	42	47.1	43.7	45.4	46.6	47.2	48.2	46.4	41.6	40.4	44.86
Week 2											Average
D10	78.4	78	80.2	77.9	78.2	77.2	78.3	77.7	77.3	77.2	78.04
D50	108.3	106.8	109.2	108.2	106.1	105	106.3	107.5	109.3	107.6	107.43
D90	176	166	180.4	168.3	174.8	179.6	178.3	173.2	177.1	171.4	174.51
Mean	118.5	115.8	120.4	118.5	117	117.9	118.6	118.9	119.3	118.1	118.3
Mode	84.3	98.7	88.5	84.8	83.1	84.1	86.6	81.7	85.8	86.2	86.38
S.D.	40.5	36.5	41.1	41.6	40.7	43.9	43.4	42.6	43.2	42.9	41.64
Week 3											Average
D10	78	79.2	77.7	81.6	76.4	75.4	78.6	75.3	77.4	75.9	77.55
D50	108.3	108.7	105	107.3	106.2	103.9	107.3	105.6	113.1	112.3	107.77
D90	173.8	172.2	170.3	180.1	162.3	173.3	192.5	167.7	188.2	178.8	175.92
Mean	119.3	118.5	117.8	119.7	115.5	116.3	120.5	115.3	124.3	117.9	118.51
Mode	84.1	92.3	85.9	92.2	84.8	93.6	83.3	80.7	83.5	78.4	85.88
S.D.	43.7	40.5	43	40.6	41	42.2	47.1	40.9	46.8	48	43.38

Direct comparison of various commercially available resazurin-based cell viability assays

During the internal review process, one recurring commentary was the comparison of the PrestoBlue assay to other commercially available resazurin-based potency assays. We proceeded without this data set for the submission, but we were curious to know the answer. **Figure 27** shows that the PrestoBlue assay is superior to other commercially available resazurin-based potency

assays (CellTiterBlue and AlamarBlue), mainly because of its higher signal-to-noise ratio, resulting in less background and higher sensitivity.

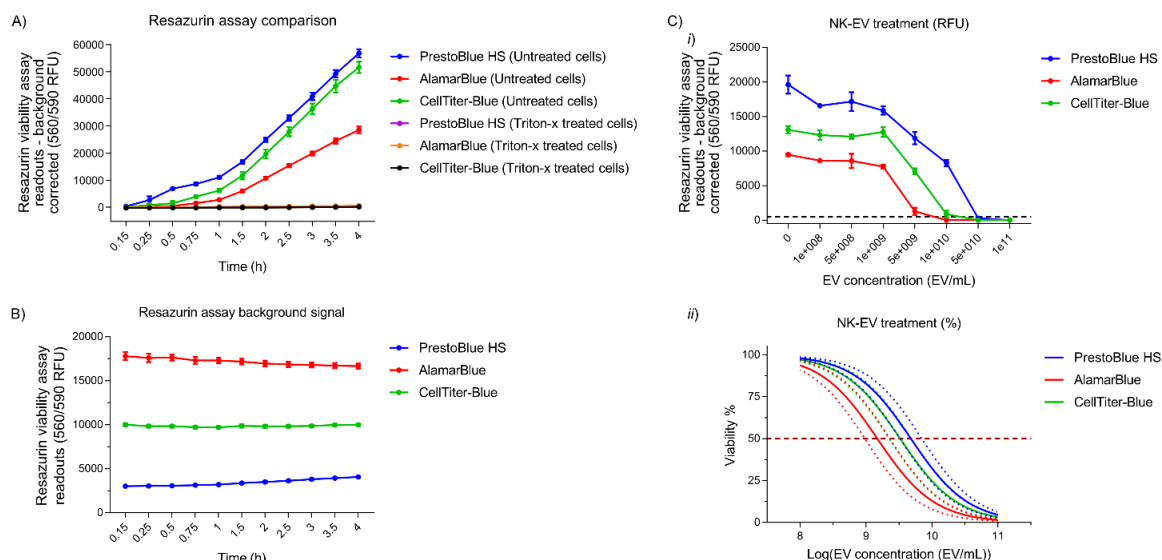


Figure 27. Direct comparison of commercially available resazurin cell viability assays.

(A) K562 cell viability was assessed using various commercially available resazurin cell viability reagents immediately after seeding 5000 K562 cells in a 96-well plate with or without Triton-x treatment, with background-corrected relative fluorescence intensities (RFU) measured at various time points; 0.15 – 4 hours. (B) Background signal of various commercially available resazurin cell viability reagents (assay media only / no cells) in a 96-well plate, with RFU measured at various time points; 0.15 – 4 hours. (C) Leukaemia K562 cells were treated for 3 hours with various E: T ratios of NK-EVs, followed by various commercially available resazurin cell viability assays. The results are presented in (i) background-corrected RFU readouts (black dashed line represents lysed K562 cell control; detergent-treated) and (ii) EC50 curve analysis for NK-EV treatment with 95% confidence interval/prediction bands (red dashed line represents 50% response). The data are presented as mean $\pm$ S.E.M. from 3 independent experiments, each with technical duplicates.

Assessment of the resazurin-based cell viability assay using adherent breast cancer cell model

Since this resazurin-based cell viability assay showed promising results in evaluating the cytotoxic effect of NK-EVs using the gold-standard leukaemia cell line K562 (suspension model), we asked whether it also performed well using adherent cancer cells as targets. Following a similar methodology, cytotoxic NK-EVs or negative control 293F-EVs were incubated at various concentrations to treat breast cancer MDA-MB-231 cells over 5 hours. The potency of NK-EVs was assessed using this resazurin-based cell viability assay. As shown in **Figure 28A-i**, this resazurin-

based cell viability assay revealed a dose-response of MDA-MB-231 cell viability loss after NK-EVs' treatment. The EC_{50} of NK-EVs' cytotoxic effect was measured at an E: T ratio of 655,055 NK-EVs' particles per MDA-MB-231 cell, as shown in **Figure 28A-ii**. There was a similar correlational trend for all cytotoxic factors that mediate cell death in target MDA-MB-231 cells ($|r| \sim 0.631 - 0.653$ and $R^2 \sim 0.747 - 0.827$), as shown in **Figure 28B**.

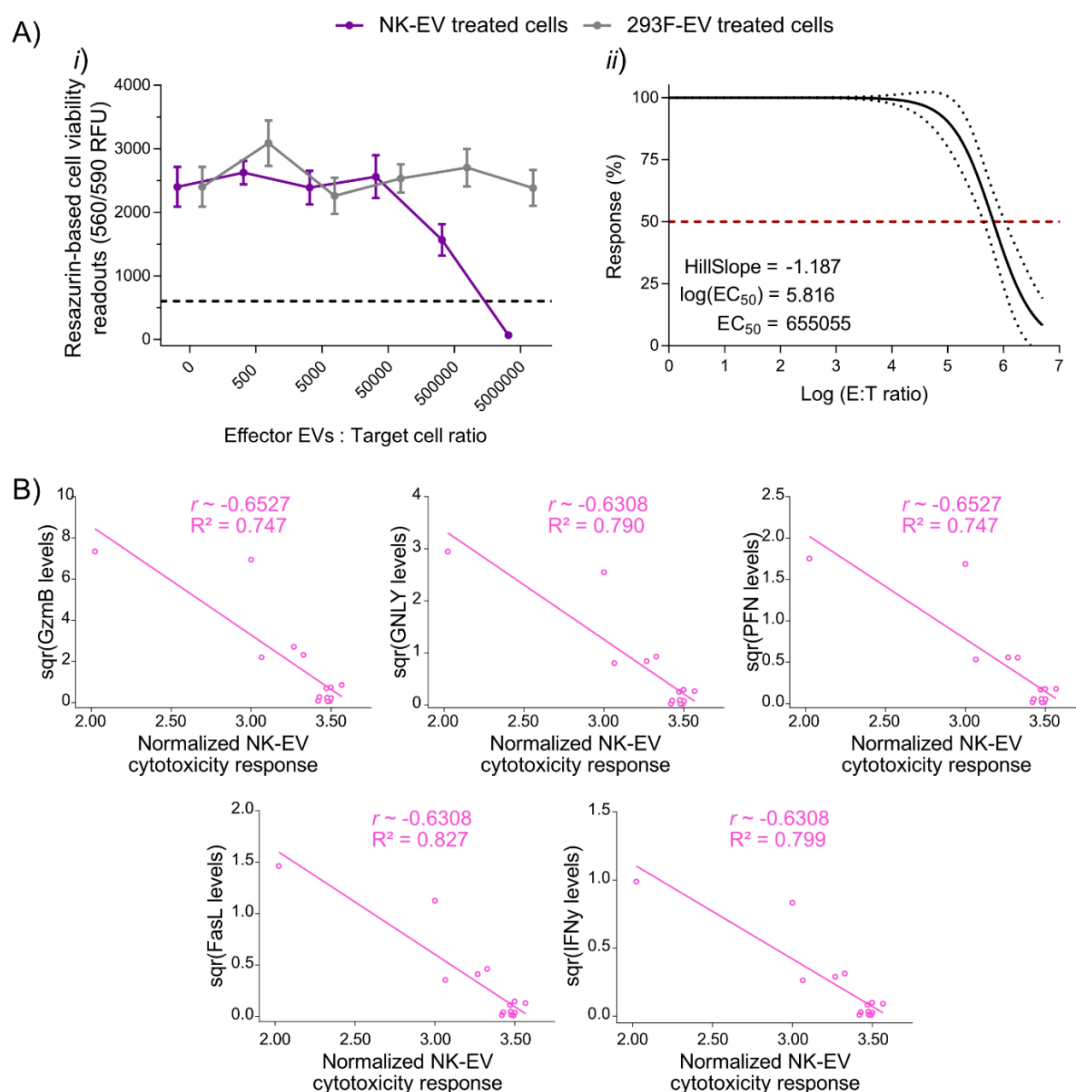


Figure 28. Resazurin-based cell viability assay reveals a dose-dependent cell viability loss in adherent breast cancer MDA-MB-231 cells upon NK-EV treatment correlating with levels of cytotoxic factors.

(A) Breast cancer MDA-MB-231 cells were treated for 5 hours with various E: T ratios of NK-EVs or negative control 293F-EVs, followed by endpoint resazurin-based cell viability assay. The results are presented in (i) RFU (black dashed line represents lysed cell control) and (ii) EC_{50} curve analysis with variable slope for NK-EV treatment with 95% confidence interval/prediction bands

(red dashed line represents 50% response). Data are shown as mean $\pm$ S.E.M. from 6 independent experiments, each with technical quadruplets. (B) Spearman correlations of the selected NK-EVs corona cytotoxic factors (GzmB, GNLY, PFN, FasL and IFN- γ) in relation to cell viability assay responses in MDA-MB231 cancer cells (n=14 correlations pairs where one pair (a dot in the figure) represents the correlation between the level of a cytotoxic factor and the cell viability assay readout for a given NK-EV dose).

This resazurin-based cell viability assay exhibited precision for intra-run variability ranges from 3.12% CV - 6.79% CV with a mean of 5.53% CV, as shown in **Figure 29A**. Similarly, the inter-run variability ranges from 17.69% CV - 25.74% CV with a mean of 22.5% CV, as shown in **Figure 29B** and **Table 10**. Additionally, this resazurin-based cell viability assay signal showed excellent linearity and range in breast cancer MDA-MB-231 cells, using the 96-well plate format, allowing the cells to adhere to the well and washing away unadhered cells. **Figure 29C** shows robust linearity for 100 - 10,000 MDA-MB-231 cells/well ($R^2 \sim 0.998$ and $|\rho| \sim 1.000$). Similarly, the assay range was assessed by seeding 1000 MDA-MB-231 cells/well with varying levels of viability artificially generated after treatment with different concentrations of SDS (anionic detergent). As shown in **Figure 29D**, the assay could distinguish between low and high viability ($R^2 \sim 0.816$ and $|\rho| \sim 0.833$).

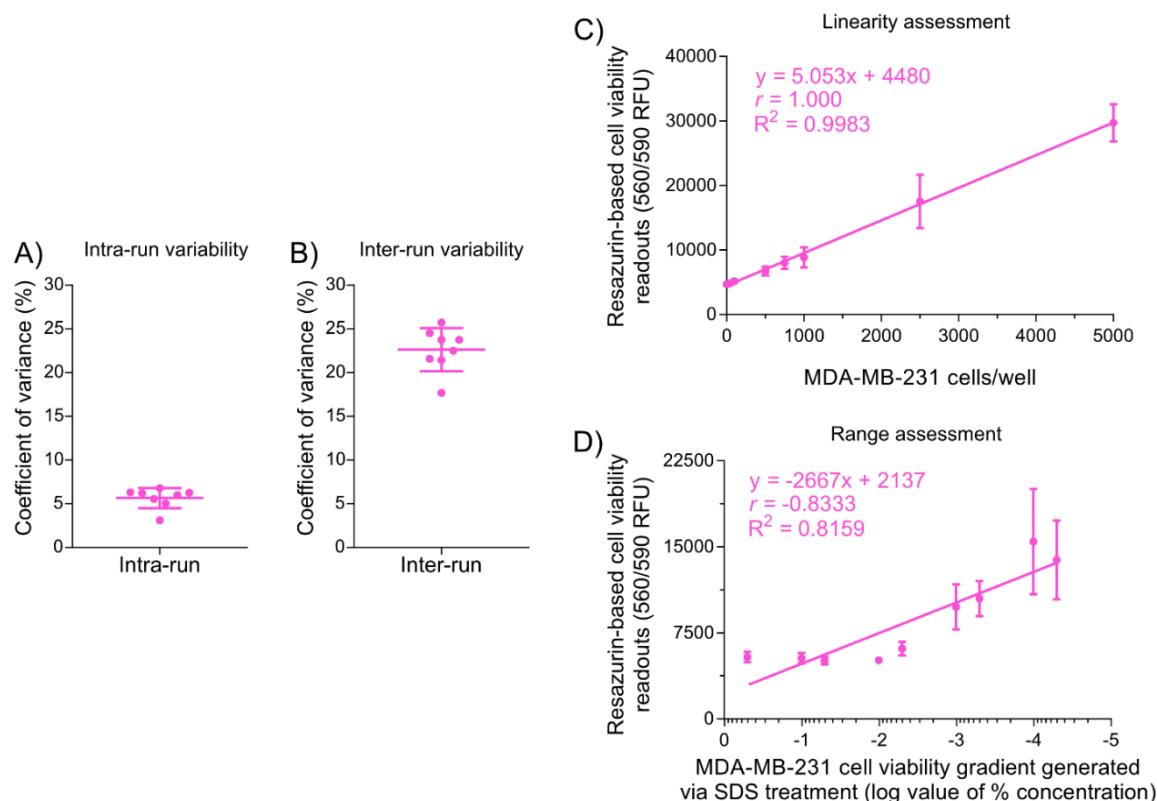


Figure 29. Analytics of highly sensitive resazurin-based cell viability assay conducted with adherent MDA-MB-231 breast cancer cells.

(A) The mean coefficient of variance $\pm$ S.D., reflecting the intra-run variability in the resazurin-based cell viability assay, was calculated from 6 independent experiments, each with technical quadruplets. Each independent experiment had 8 samples, including six NK-EV doses, untreated MDA-MB-231 cells, and detergent-treated MDA-MB-231 cells. (B) The mean coefficient of variance $\pm$ S.D., reflecting the inter-run variability in the resazurin-based cell viability assay, was calculated from 6 independent experiments, each with technical quadruplets. Each independent experiment had 8 samples, including six NK-EV doses, untreated MDA-MB-231 cells, and detergent-treated MDA-MB-231 cells. (C) Linearity assessment of resazurin-based cell viability assay (0 - 50,000 breast cancer MDA-MB-231 cells/well). The data are presented as mean $\pm$ S.E.M. from 4 independent experiments, each with technical duplicates. (D) Range assessment resazurin-based cell viability assay (1000 breast cancer MDA-MB-231 cells/well with varying levels of viability). The data are presented as mean $\pm$ S.E.M. from 4 independent experiments, each with technical duplicates.

Table 10. PrestoBlue viability assay variability (precision; intra-run and inter-run coefficient of variation) for MDA-MB-231 cells.

Samples	Mean	S.D.	CV%
<i>Intra-run</i>			
1	6381.50	422.24	6.62
2	5573.00	163.00	2.92
3	5406.00	66.79	1.24
4	4892.75	290.18	5.93
5	4813.75	37.88	0.79
6	4168.00	79.33	1.90
7	11502.50	610.59	5.31

8	8372.50	660.89	7.89
9	7955.00	170.20	2.14
10	10321.33	45.94	0.45
11	8335.50	271.29	3.25
12	7641.75	339.62	4.44
13	8520.33	726.20	8.52
14	8171.00	179.57	2.20
15	6657.50	355.67	5.34
16	9472.50	659.61	6.96
17	8768.00	216.01	2.46
18	6657.50	355.67	5.34
19	6332.00	355.27	5.61
20	5202.00	149.84	2.88
21	4900.00	432.75	8.83
Average CV%	-	-	4.34
Inter-run			
1	8381.93	2157.27	25.74
2	7075.81	1681.79	23.77
3	6240.50	1347.46	21.59
4	8555.50	1834.60	21.44
5	8398.00	1994.66	23.75
6	8875.92	2176.79	24.52
7	7190.21	1618.45	22.51
8	5914.07	1046.38	17.69
Average CV%	-	-	22.63

Alternative potency assays for NK-EVs' functional assessment show limitations

Alternative potency assays were evaluated under the same conditions as this resazurin-based cell viability assay, including assay length, seeding density (except for non-plate-based assays) and various NK-EVs doses. Initially, the CCK8 viability assay, another metabolism-based assay, was investigated, allowing for continuous monitoring of cell viability (72 hours of treatment). Both cell models (K562 and MDA-MB-231 cells) exhibited an increasing difference in CCK8 dye readings over time between high treatment doses (*i.e.*, 2×10^6 NK-EVs per K562 cell) compared to untreated cells and the cells treated with lower doses of NK-EVs, as shown in **Figure 30**. For the highest dose of NK-EVs (2×10^6 NK-EVs per K562 cell), it took approximately 3 and 48 hours to observe significant signal divergence from non-treated K562 cells and non-treated MDA-MB-231

cells, respectively. However, the seeding density of 5000 cells/well (suspension K562 cells) resulted in the loss of the assay range after approximately 48 hours of culture (**Figure 30A-i**). This was not the case for the seeding density of 1000 cells/well for adherent MDA-MB-231 cells. The CCK8 viability assay also strongly correlated with GzmB levels ($|r| \sim 0.8$ with statistical significance and $R^2 \sim 0.637$), as shown in **Figure 30B**. Importantly, CCK8 assay readings, like those of the resazurin-based cell viability assay, are specific to a loss of cell viability and are not influenced by NK-EVs alone, as shown in **Figure 30C**. To simplify the assay precision analysis, the intra/inter-run variability was calculated using untreated cancer cells over time, and the results are comparable to the other samples. As shown in **Figure 30D**, the intra-run variability ranges from 1.12% CV - 8.97% CV with a mean of 1.84% CV. As shown in **Figure 30E**, inter-run variability ranges from 0.42% CV - 7.98% CV with a mean of 1.32% CV.

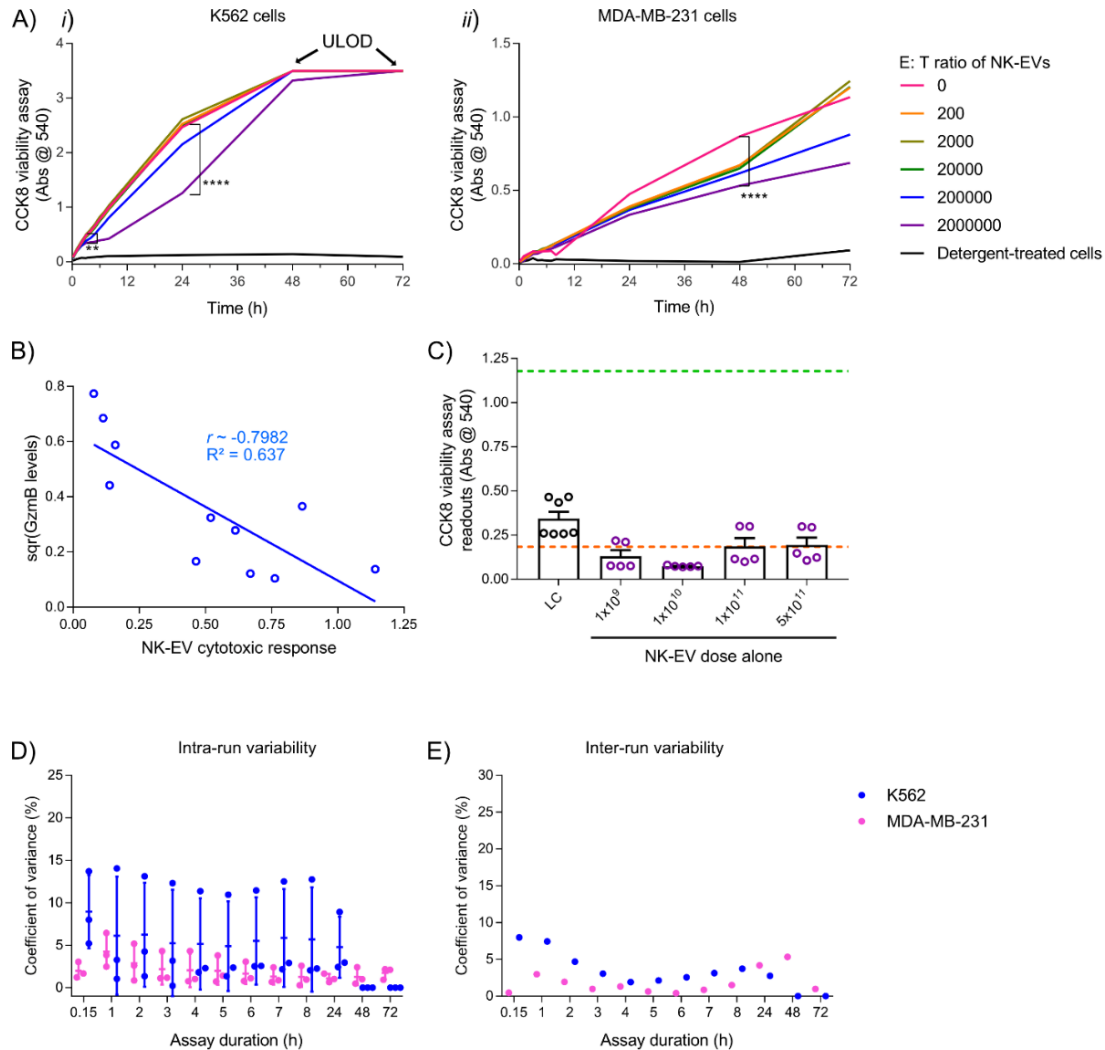


Figure 30. CCK8 viability assay in evaluating NK-EVs' cytotoxicity against K562 leukemia and MDA-MB-231 breast cancer cells.

(A) K562 cells (i) and MDA-MB-231 cells (ii) were treated for 72 hours with various E: T ratios of NK-EVs or negative control 293F-EVs, followed by continuous CCK8 viability assay assessment. The results are presented as absorbance readouts. Data are shown as mean from 3 independent experiments, each with technical triplicates. Legend: ULOD – upper limit of detection. Two-way ANOVA was performed, followed by post hoc tests (Tukey's multiple comparisons) where $**p \leq 0.01$ and $****p \leq 0.0001$. (B) Spearman correlations of GzmB NK-EV's corona, detected by ELISA, in relation to CCK8 viability assay responses in leukaemia K562 cells ($n=14$ correlations pairs where one pair (a dot in the figure) represents the correlation between the level of a cytotoxic factor and the CCK8 viability assay readout for a given NK-EV dose). (C) Selectivity and specificity of CCK8 viability assay of NK-EVs alone (no cancer cells except for controls). The green dashed line represents the upper (5000 K562 cells alone without any NK-EV treatment) and the orange dashed line represents the lower (media only without cells or NK-EVs) assay readouts. Data are shown as mean $\pm$ S.E.M. from 7 independent experiments, each with technical duplicates. Legends: LC – lysed cells. (D) The mean coefficient of variance $\pm$ S.D. over time, reflecting the intra-experiment variability in CCK8 viability absorbance readouts for untreated K562 (blue) or untreated MDA-MB-231 (pink) cells; results were calculated using data from 3 independent experiments, each with technical triplicates. (E) The mean coefficient of variance over time, reflecting the inter-run variability in CCK8 viability absorbance readouts for untreated K562 or untreated MDA-MB-231 cells; the results were calculated using data from 3 experiments, each with technical triplicates.

Further, the endpoint lactate dehydrogenase (LDH) release assay, often used as a surrogate for cytotoxicity via LDH release indicative of cell membrane damage during cell death processes, was also investigated. The measured signal was not selective to NK-EVs' cytotoxicity but rather to endogenous LDH present in NK-EVs, as evidenced by quantitative assessment in **Figure 31A**, where even NK-EVs alone (in the absence of cells) could yield a signal indicative of LDH presence. Furthermore, a relatively low dose of 1×10^8 NK-EVs generated an LDH signal comparable to the one from the detergent-treated cell control (theoretical maximum).

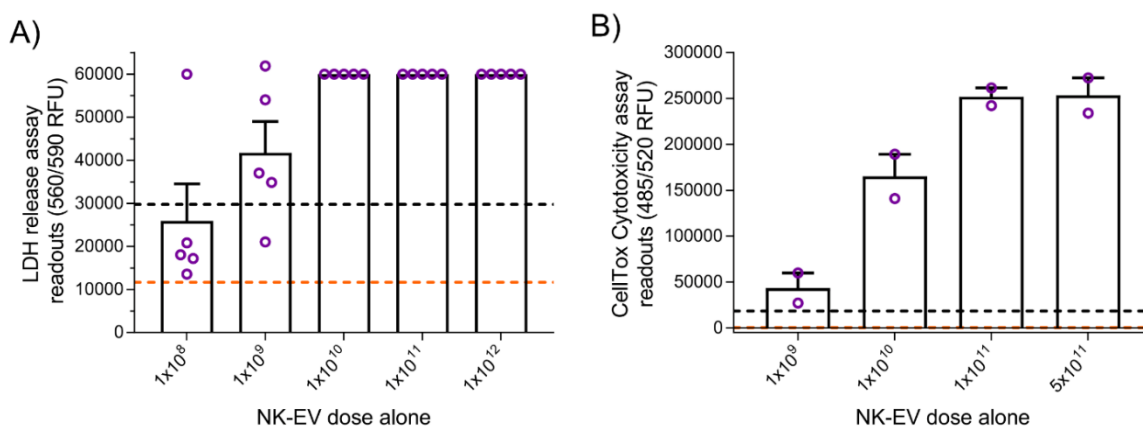


Figure 31. LDH release assay and CellTox Green Cytotoxicity assay show limited selectivity for NK-EVs-induced cell death.

Selectivity assessment of (A) LDH release assay and (B) CellTox Green Cytotoxicity assay using NK-EVs alone (no cancer cells except for controls). Data are shown as mean $\pm$ S.E.M. of 5 independent experiments for LDH release assay and 2 independent experiments for CellTox Green Cytotoxicity assay, each with technical duplicates. The black dashed line represents the upper (5000 detergent-treated K562 cells) and the orange dashed line represents the lower (media only without cells or NK-EVs) assay readouts.

Next, the endpoint CellTox DNA-binding dye cytotoxicity assay was also investigated. The assay signal was determined to be non-selective, where quantitative assessment demonstrated an artificially generated assay signal when measuring DNA presence via the CellTox dye in NK-EVs alone (without the presence of cells), as shown in **Figure 31B**. This indicates the presence of endogenously loaded DNA in NK-EVs.

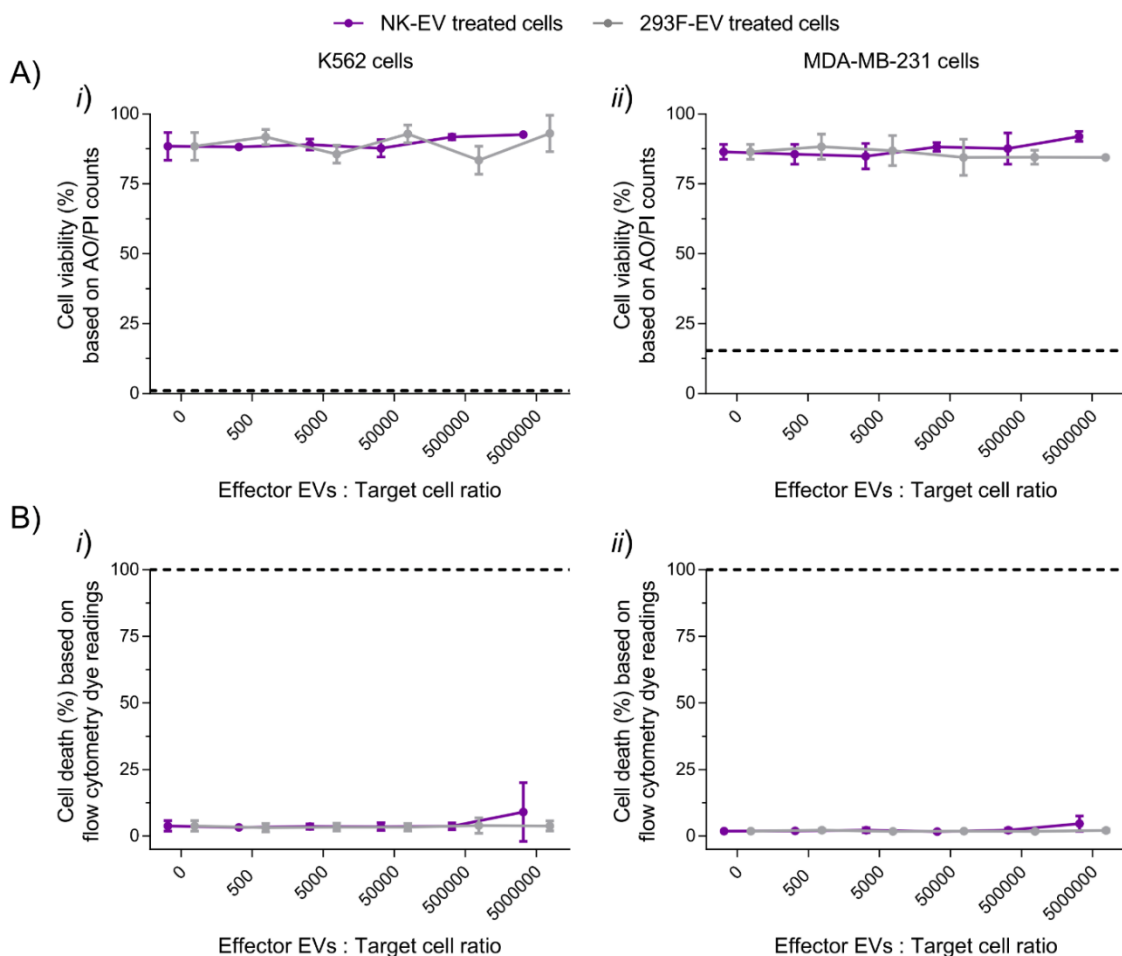


Figure 32. Endpoint cell death evaluation using AO/PI cell counting and flow cytometry in cancer cells shows poor cytotoxic dose-response following NK-EV treatments.

K562 leukaemia cells (i) or MDA-MB-231 breast cancer cells (ii) were treated for 5 hours with various E: T ratios of NK-EVs or negative control 293F-EVs, followed by endpoint cell counting and flow cytometric assay assessment. (A) Cell viability based on AO/PI counting. (B) Cell death based on Sytox Blue dye (flow cytometry). Data are shown as mean $\pm$ S.E.M. from 3 independent experiments, each with technical quadruplets. The dashed lines represent the heat shock-treated cell control.

Lastly, AO/PI counting and flow cytometry methods for evaluating the cytotoxic effect of NK-EVs were investigated. As shown in **Figure 32**, AO/PI cell counting, and flow cytometry methods could not yield the expected NK-EVs' cytotoxic dose-response in both K562 and MDA-MB-231 cell models despite these assays working correctly according to controls. **Figure 33** depicts the flow cytometry gating strategy.

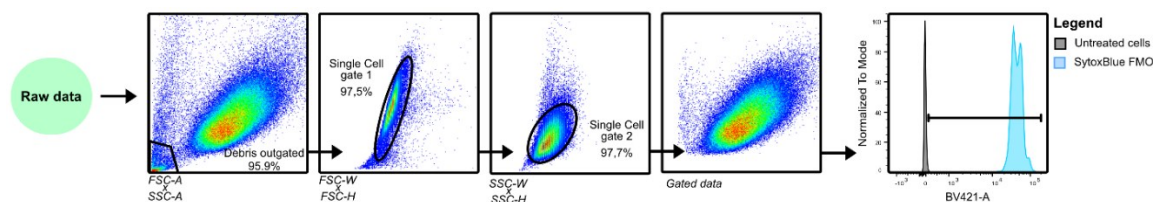


Figure 33. Flow cytometric gating strategy with control.

Cell debris and doublets were gated out using both forward and side scatter gates and applied before analyzing flow cytometry files for cell death using the SYTOX Blue Dead Cell stain. Legend: gray – unstained cells and cyan – SYTOX Blue stained cells.

Discussion

Potency assays are critical requirement for the development of commercially available cell and gene therapy products. Accordingly, potency assays should align with the published guidelines set forth by the ICH (ICH Q6B, ICH Q2(R2), ICH Q8), which are adopted and implemented internationally by multiple regulatory agencies. Potency assays, whether applied to biological or non-biological, are defined as methods capable of predicting a product's specific ability to achieve the desired clinical outcome using appropriate laboratory tests, providing representative measurements of the therapeutic effect [178, 202, 209]. Adopting a standardized potency assay helps set acceptance limits for both the release and validation of drug substance, ensuring results meet specified criteria. Such test should possess key attributes, including the ability to (1) assess potential therapeutic function, (2) provide quantitative measurements, (3) utilize reference materials if possible, and (4) contribute to confirmation of product identity. Additional requirements are needed for clinical manufacturing and product release [178, 202, 208, 209].

As the interest in EV biotherapeutics in cancer treatment continues to grow, there is a critical need to establish, refine and standardize potency assays for cytotoxic EV products [202]. These assays are crucial building blocks for developing and advancing therapeutic applications using EVs. For *in vitro* assays, various techniques, including flow cytometry [63, 65, 66, 133, 135, 199, 212, 213, 216], cell counting [66, 127, 130], a range of plate-based assays [64-66, 105, 130, 131, 136, 210-214], and reporter loss assays [127, 135, 216] have been employed for this purpose. Nonetheless, challenges persist in the reliable evaluation of EVs' therapeutic potential against cancer, hindering progress [105, 202]. These challenges encompass various aspects of the

preclinical assessment, including a lack of standardized or even proven assays, inconsistent dosing methodology (*i.e.*, NTA vs protein dosing), essential quality controls, and inadequate time points or cell models [175, 178]. Potency assays should also allow for discrimination between an active and inactive or degraded form of the product (bioactivity levels) [178, 202, 209]. Adhering to regulatory guidelines is crucial, and potency assays play a vital role in their development [175]. Pursuing potential novel EV-based therapeutics and comparing results between studies, EV lots/preparation, and individual experiments is complicated and sometimes only possible with a standardized evaluation process. Therefore, establishing standardized assays to robustly assess cytotoxic NK-EVs is urgently needed.

To address this demand, we assessed a highly sensitive resazurin phenoxazine-based cell viability potency assay (PrestoBlue HS Cell Viability Reagent) for evaluating the cytotoxic effect of NK-EVs against cancer cells, building on promising results in our previous report [105]. This product is a ready-to-use resazurin-based solution serving as a cell health indicator, using the reducing power of living cells to measure viability quantitatively. Other metabolism-based cell viability assays, such as alamarBlue (resazurin-based reagent), CellTiter-Blue cell viability assay (resazurin-based reagent) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide cell viability assay (MTT, a non-resazurin-based reagent) are also available. A 2014 study by Xu et al. showed that resazurin-based assays (*i.e.*, PrestoBlue cell viability assay (standard version) and alamarBlue cell viability assay) are more sensitive than the MTT cell viability assay [217]. The authors proposed two rationales to substantiate this assertion: *i)* MTT primarily measures mitochondrial dehydrogenase activity, reduced mainly by oxidoreductase enzymes utilizing NADH, whereas those resazurin-based assays involve various mitochondrial enzymes and accept electrons from NADPH, FADH, FMNH, NADH, and cytochromes, and *ii)* the reagent solubility difference where MTT formazan is insoluble comparatively to soluble resazurin. Additionally, resazurin reagents are non-toxic compared to the MTT reagent and can be detected by both absorbance and fluorescence methods, offering more flexibility. The primary difference between commercially available resazurin reagents is the purity level, resulting in a more sensitive assay with reduced background noise. For example, the PrestoBlue cell viability assay (HS and standard

version) is marketed as a faster assay with higher sensitivity (the manufacturer advertises a minimum 10-minute incubation) over the alamarBlue cell viability assay (manufacturer advertises a minimum 60-minute incubation). Although the alamarBlue cell viability assay has not been evaluated in this study, we have illustrated that the PrestoBlue HS cell viability assay enables rapid determination of cell viability within 30 minutes of incubation to attain statistically significant results using the 96-well plate format. This result is consistent with previous study [217]. It is conceivable that the advertised 10-minute incubation time may yield statistical significance when using the 384-well plate format. Prolonged incubation increases the assay resolution by amplifying the signal difference (fold-difference) between positive and negative sample readouts. In this study, 90-minute incubation was done to maximize the assay resolution. Nonetheless, the PrestoBlue HS cell viability assay incubation time is relatively short.

To evaluate the reliability and utility of resazurin-based cell viability assay for NK-EVs' cytotoxicity measurements against cancer cells, our potency assay validation plan adhered to the regulatory guideline criteria mentioned earlier, including precision, accuracy, linearity, range, specificity, selectivity, and stability (changes in bioactivity). The assay robustness was confirmed and validated internally but not further investigated; see the general chapter *Biological Assay Validation* [218]. This assay reliably evaluates the potency of NK-EVs' cytotoxicity by generating reproducible dose-response curves in both suspension and adherent cancer cell models. Additionally, the cytotoxic dose-response strongly correlates with recognized NK cytotoxic factor levels (*i.e.*, FasL, GNLY, GzmB, PFN, and IFN- γ), with readouts specifically indicating a loss of target cancer cell viability and distinguishing varying levels of NK-EVs' bioactivity. This is crucial according to the ICH Q2(R2) guidelines [219]. We demonstrated that resazurin-based cell viability assay is a validated method for identifying changes in key quality attributes of NK-EV product from NK-EVs during storage. This stability-indicating test reliably detects alterations in the products' characteristics over time. In summary, we have methodologically demonstrated that this highly sensitive resazurin-based cell viability assay is well-suited to evaluate the cytotoxic function of NK-EVs biotherapeutics.

Generally, most plate-based assays require minimal manipulations and enable extensive data collection and analysis, thereby improving statistical reproducibility [220, 221]. For a comprehensive evaluation, it is advisable to incorporate a minimum of five doses to investigate the dose-response, as recommended by the MISEV2023 and ICH guidelines [56]. Furthermore, reporting results as functional units can facilitate clinical translation and cross-study comparison [105, 202]. In this context, the functional unit can be derived by generating a non-linear regression to obtain the EC₅₀ and Hillslope values. The EC₅₀ represents the concentration required to elicit a response halfway between the baseline and maximum responses. This would translate to a 50% cytotoxic response from NK-EVs against the target cancer cells. Therefore, regardless of the specific doses utilized in a study, reporting the EC₅₀ (ideally for both particle-based or protein-based dosing; EV/mL or E: T ratio and protein/mL or protein quantity) and the Hillslope value could alleviate the challenge of comparing results across studies, EV preparations, and individual experiments, thereby standardizing the assessment of EV therapeutics.

We also investigated alternative potency assays commonly used to assess NK-EVs' cytotoxicity, revealing significant limitations. Notably, flow cytometry is a widely used method for characterizing and evaluating cell-based products. However, it has shown poor utility in evaluating NK-EVs' cytotoxicity in our study. The same trend was also observed for AO/PI cell counting (dual stain working via Förster resonance energy transfer - FRET), despite its recognized utility for cell counting in cell passaging by the International Organization for Standardization (ISO) [222-224]. Theoretically, comparable results would be anticipated with Trypan Blue staining, a more commonly used method than AO/PI for cell viability assessment, but it is known to be less accurate than AO/PI counting [222-224]. Importantly, these assays were tested using cancer cells treated with the same effector NK-EVs to target cell ratios as used in the resazurin-based cell viability assay. While the highest dose tested yielded complete cancer cell killing according to this resazurin-based cell viability assay, neither flow cytometry nor AO/PI cell counting revealed cell killing. We postulate bias is introduced during the necessary cell-washing steps, impacting downstream measurements where dying cancer cells are washed away and lost. For example, cells with compromised structural integrity (*e.g.*, lysed and dying cells) are likely lost in this process, resulting in unreliable

analysis. This would be akin to cells treated with a detergent (*i.e.*, Triton-X), where significantly fewer cells would be detectable by flow cytometry or cell counting. Despite their widespread use to assess the functionality of NK-EVs our study was unable to accurately and reliably assess NK-EVs potency using these methods.

Similarly, we conducted several additional plate-based assays and observed that confluence and reporter loss assays are unsuited for the brief assessment period (< 5 hours) due to their limited resolution and sensitivity when measured by an image-based cytometer (data not shown). Alternatively, an extended assessment period could be employed to discern differences in the NK-EVs' effect, as shown by other groups [136, 213]. On average, the treatment duration of 24 to 48 hours was necessary before significant changes became evident, with longer treatment durations amplifying the cytotoxic effect of NK-EVs. For example, our study found that the CCK8 assay, which exhibited potential for continuous evaluation of NK-EVs' cytotoxicity (showing great precision, signal readout specificity and selectivity), requires a longer duration to discern differences between the treatments and the controls. Of note, the CCK8 assay, using WST-8 reagent as the tetrazolium salt, resembles the previously mentioned MTT assay, which uses MTT reagent as the tetrazolium salt. According to the manufacturer, both tetrazolium salts are reduced to formazan in metabolically active cells. However, only the formazan produced by CCK8 is soluble and non-toxic. Therefore, the CCK8 assay, along with other assays such as confluence, reporter loss and bioelectronic/impedance-based assays, could be helpful for live monitoring assessment of NK-EVs' cytotoxic effect against cancer cells as an "orthogonal procedure comparison" potency assay per the ICH guidelines. Live monitoring assays offer cost-effective and efficient approaches to overcoming limitations associated with traditional endpoint assays (*i.e.*, insufficient information from a single data point and indirect and potentially destructive biochemical detection measures). However, some will require specialized and often expensive equipment (*i.e.*, bioelectronic/impedance-based assays and quantitative image-based cytometer instruments). While these assays could prove valuable for evaluating outcomes over extended durations, their effectiveness as potency assays for NK-EVs should be carefully considered, particularly in light of the swift and rapid cytotoxic effect of NK-EVs.

Lastly, it is important to note that endogenous components present in NK-EVs can significantly affect potency assessment, especially when these are directly involved in the assay principle, as observed in LDH release assay and CellTox Green Cytotoxicity. For instance, despite dose-response results for these assays (data not shown), we observed an artificial increase in NK-EVs' readouts (*i.e.* background/interference) based on NK-EVs alone, leading to false-positive cytotoxicity readouts and lack of sensitivity. This outcome is logical, given that LDH is a well-known protein commonly found in EVs (see ExoCarta Top 100 database) and will interfere with an LDH release-based potency assay [225]. Similarly, the known presence of nucleic acid molecules in EVs prevents the application of a potency assay relying on DNA-binding dyes. Consequently, despite their frequent use in the literature, these two assays cannot accurately and reliably measure NK-EVs' cytotoxicity, as their readouts lack selectivity to cell death.

In conclusion, our study reveals that this highly sensitive resazurin phenoxazine-based cell viability assay is an analytical method suitable for potency evaluation of cytotoxic NK-EVs. This assay also demonstrates that other types of EVs, such as the 293F-EVs shown in this study, lack cytotoxicity potential. However, we have not conducted cytotoxic experiments on other types of EVs. This work highlights the importance of reproducibility, reliability, and high-throughput capabilities, aligning with and meeting regulatory guidelines for potency assays. Notably, our results suggest that the highly sensitive resazurin-based cell viability assay has the potential to predict whether a particular NK-EVs' preparation can achieve its intended therapeutic effects and may enable the comparison of EV preparations produced across batches, manufacturing platforms or from different producer cells. Furthermore, this assay stands out for its cost-effectiveness (< CAD 0.09/test), safety (non-toxic), disposability, rapid turnaround (1 - 5 hours), ease of use and access, and potential for automation. Researchers and regulatory agencies' efforts towards standardization and recognition of the necessity for reliable and reproducible potency assays signify encouraging progress. Continued research endeavours will help to overcome challenges and advance the field of EV-based therapeutics, facilitating clinical translation.

Chapter 4. NK-EV immunomodulation assessment

Preface: The following chapter is a manuscript (under revision at Molecular Therapy Oncology) for which I am the primary author. This article represents a direct follow-up to the work detailed in **Chapter 2**.

Citation: St-Denis-Bissonnette, F., Mediratta, M., Ho, N., Kirkby, M., Stalker, A., Muradia, G., Shrestha, A., Ardolino, M., Lee, S.-H., Burger, D., Mer, A., Wang, L., Lavoie, J. R. Natural Killer Cell-Derived Extracellular Vesicles Reprogram Human Immunity to Enhance Tumour Cytotoxicity. Under revision by Molecular Therapy Oncology. Please find the final version of this manuscript once it is published.

Contributions: F.S.D.B., L.W., and J.R.L. conceptualized the project. F.S.D.B. and K.M. designed the experiments. F.S.D.B. and G.M. generated the NK-EV material. F.S.D.B. and K.M. performed the experiments. F.S.D.B. and A.S. performed flow cytometry data acquisition and analysis. F.S.D.B. and K.M. performed all Incucyte (live cell imaging assay) experiments, data analysis and interpretation. A.S. created the breast RFP cell lines. N.H. performed scRNA-sequencing data analysis and interpretation. F.S.D.B., K.M. and N.H. performed data visualization. F.S.D.B., L.W., and J.R.L. managed the project. F.S.D.B. wrote the initial manuscript. All authors read and approved the final manuscript.

Abstract

Natural killer (NK) cells are part of the body's first line of defense, rapidly destroying stressed, infected or cancerous cells. These immune cells release nanosized biological packages called extracellular vesicles (EVs), which transfer molecular signals between cells and influence immune function. Although natural killer cell-derived extracellular vesicles (NK-EVs) have shown the ability to directly kill cancer cells, how they shape the broader human immune response has remained unclear. Here, NK-EVs were shown to reprogram cellular human immunity to enhance tumour cytotoxicity using single-cell transcriptomics and functional cytotoxicity assays. When human peripheral blood mononuclear cells (PBMCs) from healthy and cancer-affected patients (*i.e.*, immunosuppressed system) were exposed to NK-EVs, widespread shifts in gene activity occurred across key immune populations, notably CD8⁺ T cells and NK cells. These changes enhanced the cells' ability to recognize and eliminate tumour targets and were consistent across all donors, independent of disease status. These findings demonstrate that NK-EVs act as potent messengers that activate and coordinate both the innate and adaptive arms of immunity. This work advances understanding of immune communication and highlights NK-EVs as promising, cell-free candidates for the next generation of cancer immunotherapies that unite potency with clinical scalability.

Introduction

Natural killer (NK) cells are essential effectors of innate immunity, capable of eliminating virus-infected and malignant cells without prior sensitization [82, 84]. NK cells play critical immunoregulatory roles not only through direct cell-cell interactions and secretion of cytotoxic granules and cytokines, but also via extracellular vesicles (EVs; more commonly referred to as exosomes) [105, 226]. These NK cell-derived EVs (NK-EVs) are membrane-bound nanoscale vesicles with potent anti-tumour activity [56, 57, 103]. They are capable of inducing apoptosis in cancer cells while sparing healthy tissues [128, 131, 227]. Compared with cell-based therapies, NK-EVs are less susceptible to tumour resistance mechanisms, can be manufactured at scales,

and are amenable to off-the-shelf use, highlighting their potential as promising candidates for solid tumour immunotherapy [28, 228].

In addition to their cytotoxic cargo (e.g., perforin, granzymes, etc.), NK-EVs carry immunomodulatory payload capable of shaping immune responses [63, 107]. For instance, prior studies have shown that NK-EVs can activate T cells and reprogram myeloid cells toward pro-inflammatory phenotypes in a monocyte-dependent manner [62, 132]. These findings suggest the potential of NK-EVs to bridge innate and adaptive immunity. However, most of these studies rely on EVs derived from primary NK cells, which limits yield, reproducibility, and clinical scalability. Furthermore, to the best of our knowledge, no studies have yet utilized human peripheral blood mononuclear cells (PBMCs) derived from cancer patients to assess the potential of NK-EV to rescue their function. Broader immunomodulatory effects and their underlying molecular events remain underexplored.

To this end, NK-EVs were produced through a clinically relevant, scalable biomanufacturing workflow using the NK92-MI cell line [105]. Their immunomodulatory effects were evaluated on PBMCs from both healthy donors and patients with triple-negative breast cancer (TNBC). Effector immune cell populations driving cytotoxic reprogramming were delineated using flow cytometry, live-cell killing assays, and single-cell RNA sequencing (scRNA-seq) transcriptomic. By integrating these approaches, this study uncovers novel insights into coordinated lymphoid-myeloid engagement and consistent cytotoxic reprogramming, providing a strong rationale for NK-EVs as a next-generation cancer immunotherapeutic.

Materials and Methods

Cell culture parameters

NK92-MI cells (purchased from ATCC; CRL-2408) and PBMCs were cultured in serum-free, xeno-free, and feeder-free medium, ImmunoCult-XF T Cell Expansion Medium (StemCell Technologies, cat#10981), and maintained at $3-8 \times 10^5$ cells/mL. PBMCs were also cultured with 5% Heat-Inactivated Fetal Bovine Serum (HI-FBS, Gibco, cat# 12484-028). Healthy PBMCs (3

donors) and triple-negative breast cancer (TNBC)-derived PBMCs (3 donors) were purchased from StemCell Technologies. Donor characteristics are summarized in **Table 11**. Human TNBC cell line MDA-MB-231 (purchased from ATCC; HTB-26) was cultured in DMEM (Wisent, cat#319-015-CL) supplemented with 10% FBS (Wisent, cat#080450). Human breast cancer cell line MCF7 (purchased from ATCC; HTB-22) was cultured in EMEM (Wisent, cat#320-005-CL) supplemented with 10% FBS, 1% sodium pyruvate (Gibco, cat#11360070), 1% MEM non-essential amino acids (Gibco, cat#11140050), 10 µg/mL insulin (Sigma-Aldrich, cat#91077C), and 1.5 mg/mL sodium bicarbonate (Sigma-Aldrich, S5761). Ethics approval for experiments involving human-derived PBMC samples was obtained from the Health Canada-Public Health Agency of Canada Research Ethics Board under protocol number REB#2022-028H. Cell counts were performed on the Cellometer Auto 2000 Viability Counter (Nexcelom BioScience LLC) using the ViaStain Acridine Orange and Propidium Iodide (AO/PI) Staining Solution (ESBE Scientific, cat#CS2-0106) with the immune cell AO/PI program (channel 1: 470/535 nm for 0.5 sec of exposure and channel 2: 540/605 nm for 3 sec of exposure).

Table 11. PBMC donor information.

	Donor-H1	Donor-H2	Donor-H3	Donor-D1	Donor-D2	Donor-D3
Supplier	StemCell technologies					
Cat #	70025.1	70025.2	70025.1		200-0435	
Lot #	2301805101	191180608C	2302801112	240780701C	240882701C	240882702C
Donor #	888608699	1100242634	110046452	122887923	122933693	122873250
Anti-coagulant	Acid-citrate-dextrose solution A	Acid-citrate-dextrose solution A	Acid-citrate-dextrose solution A	Sodium Heparin	Sodium Heparin	Sodium Heparin
Health status	Healthy	Healthy	Healthy	TNBC	TNBC	TNBC
Diagnosis details	N/A	N/A	N/A	Invasive Ductal Carcinoma	Invasive Ductal Carcinoma	Invasive Ductal Carcinoma
Breast localization	N/A	N/A	N/A	N/A	Right	Left
ER levels	N/A	N/A	N/A	N/A	Weak (4.1%)	Negative (0%)
PR levels	N/A	N/A	N/A	N/A	Negative (0%)	Weak (1%)
HER-2 levels	N/A	N/A	N/A	N/A	Borderline (2+)	Weak (1+)
Age	40	34	55	70	53	49
Gender	Female	Female	Female	Female	Female	Female
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	African American
Smoking status	No	No	No	Yes	No	No
HIV-1/2, HepB, HepC	Free	Free	Free	Free	Free	Free
Previous treatments	N/A	N/A	N/A	N/A	RT to rt breast	N/A
Current treatments	N/A	N/A	N/A	AC/Keytruda	Keytruda/Abraxane	Carbo/Taxol/Keytruda
Current Medications	N/A	N/A	N/A	benadryl/lidocaine, buspirone, celexa, crestor, onco wash-maalox/oxycodone-acetaminophen, rizatriptan, trazadone, vit D3, vit B-12	Vit B12, albuterol sulfate HFA, hydrocodone-acetaminophen, lidocaine-prilocaine, melatonin	Ativan, claritin, diphenhydramine, folic acid, lidocaine-prilocaine methylprednisolone, norethindrone, promethazine, topical cream, vit B-12

PBMC subtype depletion

Using the EasySep Human Positive Selection Kit from StemCell Technologies, healthy PBMCs were depleted of CD4 (cat#17852) or CD8 (cat#17853) or CD33 (cat#17876) or CD56 (cat#17855) expressing cells using the EasySep magnet (StemCell Technologies, cat#18000) and separation buffer (Dulbecco's phosphate-buffered saline (Life Technologies, cat#14190-250), 2% FBS (Gibco, cat#A56705-01) and 1mM EDTA (Thermo Fisher, cat#R1021). Combo groups were processed concurrently and not sequentially. Depletions were verified using the flow cytometry uptake panel (details below).

NK-EV biomanufacturing from NK92-MI cells

NK-EVs were manufactured using a clinically relevant and scalable biomanufacturing workflow that adheres to Good Manufacturing Practices and utilizes NK92-MI cells (IL-2 self-sufficient). Our previous publication provides comprehensive details about the biomanufacturing process, the NK-EV product-related characteristics [105]. Briefly, the biomanufacturing of NK92-MI cells and NK-EVs was performed using a closed-loop hollow fibre bioreactor to generate large quantities of clinical-grade NK-EVs. NK92-MI cells are expanded in the cartridge and harvested at the end of the production cycle. Modification in this study: NK92-MI cells were cultured in GMP-certified ImmunoCult-XF medium (StemCell Technologies, cat# 100-0956) with EV-rich conditioned medium (CM) collection performed twice daily (stored at -80°C, daily until further processing).

NK-EV purification by fast protein liquid chromatography (FPLC) using size-exclusion chromatography (SEC) and ultrafiltration (UF)

NK-EV purification was performed as previously described with slight modifications [105]. Thawed NK-EV-rich CM was centrifuged at 10,000 x g for 30 min before being loaded into a 60 mL syringe (BD Biosciences, cat#309653) for SEC-based EV purification. SEC was performed on the AKTA Fast Protein Liquid Chromatograph (GE Lifesciences, cat#29022094) using the HiScale chromatography column 10/40 (Cytiva, cat#29360550) packed with CaptoCore 700 resin (Cytiva, cat#17548102) for a total bed height of 20 cm. The column/resin is stored in 20% ethanol

(Commercial Alcohols, cat#P006EAAN). Clean-in-place and priming procedures were performed using the following filtered solutions; filtration performed using the 0.1 μm vacuum filtration unit Filtropur V50 (Sarstedt, cat#83.3941.002): cleaning solution (0.5M NaOH (Supelco, cat#SX0607N-6) and 30% Isopropyl Alcohol (EMD, cat#PX1834-1) in water), water and DPBS^{-/-} (Fisher, cat#BP399-1) according to manufacturer protocol. The ultraviolet absorbance was zeroed after the reading stabilized while running filtered-DPBS^{-/-} through the instrument. The NK-EV-rich CM was loaded onto the column at a flow velocity of 150 cm/h (flow rate of 2.0 mL/min). Sample fractionation was initiated once the ultraviolet absorbance level reached 230 mAU and stopped when the absorbance level dropped below 1,600 mAU. To ensure sterility, the fractionated sample was combined before filtration at 0.22 μm with a pre-wet (filtered-DPBS^{-/-}) Acrodisc Syringe Filter (Pall Corporation, cat#PN4612). Then, the solution was concentrated using a pre-equilibrated (sterilized with 90% ethanol and rinsed twice with filtered-DPBS^{-/-}) 10 kDa Centricon Plus-70 Centrifugal Filter - regenerated cellulose membrane (Millipore Sigma, cat#UFC701008) at 4,000 x g for 25 min; concentration factor of 40 - 45 X pre-concentration volume. The final retentate was collected by inverting the filter into the collection cup and spinning the assembly at 2,000 x g for 2 min. NK-EV products were stored at 4°C, and their use was limited to 7 days post-isolation for the duration of the study.

Nanoparticle tracking analysis (NTA)

NTA analysis was performed as previously described, with slight modifications [105]. Purified EV samples were analyzed by NTA using the NanoSight NS300 (Malvern, UK), equipped with a 488 nm laser and an sCMOS camera. For capture settings, the camera level is 14, and the detection threshold is 15 for analysis, resulting in approximately 30 - 70 particles per frame. Two dilutions were run on the NTA, one ranging from 30 to 50 particles per frame and the other from 50 to 70 particles per frame. After stabilizing at room temperature, the sample was thoroughly vortexed before preparing an appropriate EV dilution (using filtered DPBS^{-/-}) to obtain a final volume of 1 mL in a 1 mL syringe (Thermo Fisher Scientific, cat#MB9204560TF-LAB) for loading onto the instrument. Roughly half of the syringe content was pushed manually through the instrument until

the camera observed stable particles per frame. The syringe was then mounted on the syringe pump (Harvard Apparatus, cat#984730), used for acquisition capture in flow mode using the following NTA script (V3.4 software): 23°C, one capture of 1 m at a flow speed of 30. After the script was completed, the flow rate was increased to 1,000 for ~10 sec before returning to 30. This sequence was repeated until five captures were taken per sample. This acquisition method allows for the sampling and analysis of the sample through the syringe, ensuring an accurate representation of the entire sample. The five captures of each dilution were averaged separately by the NTA V3.4 software (Malvern Instruments). To ensure maximal reproducibility between experimental replicates, the EV concentration was calculated for each preparation by averaging the dilution-corrected concentration of both dilutions run on NTA.

Flow cytometric assessment of NK-EV association with PBMCs (flow cytometry uptake panel)

CFSE staining of NK-EVs, as previously done [229]: NK-EVs were incubated with approximately 300 μ M of carboxyfluorescein diacetate succinimidyl ester (CFSE; Life Technologies, cat# C34554) in 1.5 mL Protein LoBind tubes (Eppendorf, cat# 0030.108.116) for 60 min with gentle mixing (450 RPM on an orbital shaker) at room temperature. The same volume of D-PBS combined with an equal amount of CFSE was incubated as a negative control for the assay. Zeba spin desalting columns (7 kDa MWCO, 2 mL; Thermo Fisher Scientific, cat#89890) were used to separate stained EVs from unbound CFSE. Briefly, columns were prepared according to the manufacturer's instructions, with three D-PBS washes. Then, CFSE-stained NK-EVs and PBS control were loaded into the columns and spun at 1500 x g for 2 min. Recovered products were processed for NTA to determine particle concentrations. Likewise, fluorescence (Ex/Em 494/521 $\pm$ 10 nm) was measured using a Microplate Reader (BioTek Synergy H1 Multimode Reader; cat#SH1M2G-SN; Gen5 software V3.14) with a 96-well Flat Clear Bottom Black Polystyrene TC-treated Microplate (Costar, cat#3603) in duplicate.

PBMC treatment with CFSE-stained NK-EVs: A few days before the assay, healthy peripheral blood mononuclear cell (PBMC) donor samples were cultured according to the "Cell

Culture Parameters” section. Using a U-bottom 96-well plate (Costar, cat#163320), 2.5×10^5 cells were seeded per well using a pipette repeater. Cells were maintained in ImmunoCult-XF T Cell Expansion Medium, with the final well volume normalized to 200 μ L and incubated at 37°C with 5% CO₂. Samples include untreated cells, 1×10^{11} EVs/mL CFSE-stained NK-EVs and an equal volume of CFSE-stained PBS. Notably, the dosing volume was limited to 25% of the total assay volume.

Flow cytometry processing and acquisition: After 4, 24, 48, and 72 hours of incubation, PBMCs were collected and processed for flow cytometry. Similarly, PBMC subtype depletions were also processed for flow cytometry. Cells were transferred into a V-bottom plate (Costar, cat#249662) and centrifuged at 400 x g for 5 min at 4°C. After washing the cells with flow buffer (PBS + 2% FBS), cells were stained in 100 μ L of titrated antibodies solution containing CD3-AF700 (BD Biosciences, cat#557917), CD4-APC (BD Biosciences, cat#555349), CD8-BV421 (BD Biosciences, cat#562428), CD14-BV510 (BD Biosciences, cat#740163), CD16-BV711 (BioLegend, cat#302044), CD19-BUV737 (BD Biosciences, cat#612756), CD45-APC-R700 (BD Biosciences, cat#566041), CD56-BV605 (BioLegend, cat#362538) and HLA-DR-PE (BD Biosciences, cat#555812) with added 10% BV stain buffer plus (BD Biosciences, cat#566385) on a temperature control orbital shaker set at 4°C and 350 RPM for 30 min, protected from light. Cells were washed twice with flow buffer (PBS + 2% fetal bovine serum, FBS) and resuspended in acquisition buffer (1.5 μ M propidium iodide; Life Technologies, cat# P3566). Samples were transferred into 5 mL FACS tubes and analyzed by flow cytometry using the BD FACSAria III Fusion flow cytometer (BD Biosciences) equipped with a 70 μ m nozzle (BD Biosciences; P/N 647339) and the BD FACSDiva Software (v9.0.2). A minimum of 100,000 events per sample were collected at the single-cell live gate.

Flow cytometry data analysis: Using FlowJo (v11.0.1; FlowJo LLC, USA) and R Project software (v4.4.3), raw data were cleaned with the FlowAI plugin (v2.3.2). "GoodEvents" were processed to remove obvious debris, doublets, and dead cells, and the clean files containing all the events with embedded compensation parameters were exported. FlowAI enhances data quality

by detecting and removing outliers and anomalous events based on flow rate, signal stability, and dynamic range. For normalization purposes, the total number of events was set to 100,000 single viable cells per file using the DownSample plugin (v3.3.1). All concatenated treatment group files within a single donor were combined into a single meta-file, representing all possible events across all groups. This meta-file was used to perform fitSNE and FlowSOM analyses; plugins from the FlowJo-PluginPlay-Bundle-v1.1.0_Windows10_R-v4.2.2. Each treatment group was subsequently identified in the meta-file using the “Sample ID” function. Algorithm information: Fast interpolation-based t-SNE (fitSNE) is an unsupervised nonlinear dimensionality reduction algorithm for flow cytometry data that improves convergence properties and handles challenging datasets better than t-distributed Stochastic Neighbour Embedding (tSNE) analysis. Here, fitSNE analysis was performed with 5000 iterations and 200 perplexity on compensated fluorescent parameters to provide the most accurate visual representation of the PBMC subsets across samples. Comparatively, FlowSOM is a state-of-the-art SOM for analyzing flow cytometry data, providing a clear overview of marker behaviour across cells and detecting otherwise missed subsets. FlowSOM analysis was performed against 12 meta-clusters using default settings on compensated fluorescent parameters. Subset identification was manually performed based on antigen expression and cross-referenced against the FlowSOM output. FlowSOM clusters comprising <0.1% of total events were excluded to reduce noise. Populations <1% were interpreted with caution due to potential misclassification from low event counts and statistical strength. Limited marker resolution constrained detailed characterization of myeloid and T cell subsets. Each file was individually evaluated at each step through the analysis to ensure the absence of artifacts that could interfere with downstream analysis. This processing scheme enables unbiased cross-sample comparison within a single donor. Cross-donor comparison is not possible due to inherent biological variation across donors in marker-level expression, rendering the fitSNE analysis problematic.

Single-cell RNA-sequencing (scRNA-sequencing) assessment of NK-EV-stimulated PBMCs

PBMC treatment NK-EVs: the healthy and diseased PBMC donor samples were cultured according to the “Cell Culture Parameters” section. Treatment groups: Group 1) EV-treated healthy

PBMCs (3 donors); Group 2) untreated healthy PBMCs (3 donors); Group 3) EV-treated diseased PBMCs (3 donors); Group 5) untreated diseased PBMCs (3 donors). Using a U-bottom 96-well plate, 2.5×10^5 cells were seeded per well using a pipette repeater. Cells were maintained in ImmunoCult-XF T Cell Expansion Medium, with the final well volume normalized to 200 μ L and incubated at 37°C with 5% CO₂. Samples include untreated cells, 5×10^{11} EVs/mL NK-EVs. Notably, the dosing volume was limited to 55% of the total assay volume.

Single-cell RNA-sequencing (Sample collection, fixation and permeabilization): Healthy and diseased PBMCs were stimulated for 72 hours and then processed for scRNA-sequencing using the 16-plex Chromium Next GEM Single Cell Fixed RNA Sample Prep Kit (10X Genomics, cat#1000414) according to the manufacturer's protocol. Briefly, selected treatment groups of PBMCs from the previous section were collected into a 1.5 mL Eppendorf tube and centrifuged at 400 x g for 5 min at 4°C. After washing the cells with fresh media, samples were resuspended in 1 mL for cell counting. After washing the cells with ice-cold PBS, samples were resuspended in 1 mL of fixation buffer. After 22 hours of incubation at 4°C, the cells were spun and resuspended in ice-cold quenching buffers. Cells were counted once more. To maximize sample integrity, enhancer buffer and 10% glycerol were added. Fixed and permeabilized samples were stored at -80 °C. Once all samples were acquired and ready for library construction and probe hybridization, they were processed using the V2 barcoding chemistry of 10X Genomics by the StemCore Laboratories at the Ottawa Hospital Research Institute (OHRI, Canada). scRNA-sequencing was performed on 4 pooled libraries (4x4) using the NovaSeq X platform. Libraries were pooled into four sequencing runs, generating between 0.65 and 1.07 billion reads per pool. The total number of cells recovered across all samples was 143,476, with a median recovery of 9,909 cells per sample (targeting 10,000 cells per sample). Reads per cell ranged from 4,238 to 42,454, with a mean of approximately 19,129 reads per cell. scRNA-sequencing yielded a median of 2,469 genes per cell and 4,198 unique molecular identifiers (UMIs) per cell, reflecting robust transcriptome capture and library complexity across all samples. Confidently mapped reads were consistently high ($\geq 95\%$). No significant quality concerns were observed, and all libraries were deemed suitable for downstream analysis. scRNA-sequencing analysis: Cells were filtered to retain those with 500-4,500 detected

genes, 500-14,000 total UMI counts, and <10% mitochondrial gene content. Doublets were identified and removed using scDblFinder. After quality control, 88,213 high-quality cells were retained for downstream analysis. Gene expression data were normalized using SCTransform v2 with mitochondrial gene percentage regression. Principal component analysis was performed, with optimal dimensionality determined using an elbow method. Batch effects were corrected using Harmony integration by donor ID. Hierarchical clustering was performed using TooManyCells, a spectral clustering algorithm that constructs a tree-based visualization of cell relationships through recursive bipartitioning on SCTransform-normalized expression data. UMAP dimensionality reduction was applied for two-dimensional visualization. Cell type identification was performed through a multi-level hierarchical approach using output from hierarchical clustering. Manual curation was performed using established canonical marker genes to validate and refine automated annotations. Four major immune cell populations were identified and characterized by their distinctive expression signatures: B cells (*MS4A1*, *CD19*, *PAX5*, *IGHD*), CD4+ T cells (*CD4*, *CCR4*, *FOXP3*), CD8+ T cells (*CD8A*, *CCR5*, *GZMH*), and NK cells (*NCAM1*, *KLRD1*, *PRF1*). Differential gene expression analysis was conducted using a stratified paired design with DESeq2 on pseudobulk aggregated data. For each cell type, separate analyses were performed for TNBC and healthy donor populations, followed by meta-analysis to identify genes with: (1) TNBC-specific responses, (2) healthy donor-specific responses, or (3) consistent responses across both populations. Genes were considered significantly differentially expressed at FDR < 0.05. Genes were ranked by log fold change and p-value for functional enrichment analysis. Pathway analysis was performed using MSigDB C2 collections (Size of gene sets within 20 to 400 genes). P-values were adjusted for multiple testing using the Benjamini-Hochberg method. Pathways were considered significantly enriched at FDR < 0.05.

Live-cell imaging cytotoxicity assay

MDA-MB-231 and MCF7 cell lines were tagged with RFP by lentiviral transduction of the Incucyte NuLight Red (Sartorius, cat#4625), followed by puromycin (Tocris Bioscience, cat#4089) selection. RFP-tagged cells were seeded in a 96-well plate (1×10^5 cells/well) and treated with 5

µg/mL mitomycin c (Cayman Chemical, cat#11435) for 1 hour at 70% confluence. Of note, treatment with mitomycin c was not performed in cell-depleted assays. Cells were subsequently washed twice with pre-warmed media and incubated in phenol red-free DMEM (Gibco, cat#A1896701) containing unstimulated (MOCK) or NK-EV-stimulated (NK-EV) healthy or diseased PBMCs. Healthy donor PBMC subtype depletions were also tested for the live-cell monitoring assay. NK-EV-stimulated PBMCs were stimulated for 72 hours before the live-cell imaging cytotoxicity assay. Green Annexin V dye for apoptosis (Sartorius, cat#4642) was added to all cells, and then the cells were placed in the Sartorius Incucyte S3, maintained at 37°C in a 5% CO₂ incubator for live-cell imaging. Phase-contrast, green-, and red-channel images were captured every 4 hours for 96 hours. Target breast cancer cell confluence was quantified as total red fluorescence intensity (RCU × µm²/image) and normalized to cell density at 0 h. Annexin V⁺ early apoptotic cell count was normalized by RFP⁺ breast cancer cell count. All analysis was completed on the Sartorius Incucyte 2023A Rev2 software.

Data availability (EV-TRACK, Gene Expression Omnibus (GEO) from NCBI)

All relevant data were submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV250010) [165]. All scRNA-sequencing raw data generated in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) repository under accession code GSE309543. The analysis code is publicly available on GitHub at the following link: <https://github.com/merlab/iMod>.

Statistical analysis

Data were expressed as the mean ± standard deviation (S.D.) or standard error of the mean (S.E.M.). The data were normalized to the control group for relative comparison, as indicated in each figure legend. The number of experimental and technical replicates used is indicated in the figure legends. Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software Inc., LaJolla, CA, USA), where a p-value of < 0.05 was considered statistically significant and significance differences are marked with a single (p< 0.05), double (p< 0.01), triple (p< 0.001), or quadruple (p< 0.0001) asterisk. Two-way ANOVA followed by post hoc tests (Tukey's or Sidak's multiple comparisons) was used and identified in the figure legend.

Results

NK-EVs show broad PBMC association

NK-EVs were produced using a scalable biomanufacturing workflow process utilizing the NK92-MI cells and were comprehensively characterized for product identity and safety, in accordance to MISEV guidelines [105].

To evaluate the association of NK-EVs with healthy human PBMCs (n=3), NK-EVs were fluorescently labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) dye and incubated with PBMCs under various conditions (**Figure 34A**). Quantitative fluorescence measurements confirmed a significant increase in CFSE signal in NK-EV-treated samples relative to all controls ($p < 0.0001$), validating effective and specific dye incorporation (**Figure 34B**). Residual unbound dye was efficiently removed using the cleaning column, as reflected by the minimal CFSE signal observed in the CFSE-PBS control. Nanoparticle Tracking Analysis (NTA) confirmed that labelling did not alter NK-EV size distribution or concentration (**Figure 34C**).

Following co-incubation of PBMCs with CFSE-labelled NK-EVs over 72h, high-dimensional flow cytometry (11 colours), combined with fitSNE and FlowSOM clustering enabled robust classification of uptake or association across immune cell subsets (**Figure 34D** and **Figure 35**), including B cells ($5.18 \pm 0.58 \%$), $CD4^+$ T cells ($55.86 \pm 2.38 \%$), $CD8^+$ T cells ($28.83 \pm 0.58 \%$), double-negative (DN) T cells ($2.89 \pm 0.22 \%$), NKT cells ($0.05 \pm 0.01 \%$), dendritic cells ($0.21 \pm 0.3 \%$), cytotoxic NK cells ($6.05 \pm 0.97 \%$), immunoregulatory NK cells ($0.48 \pm 0.05 \%$), and monocyte-T cell conjugates ($CD14^+$ monocyte associated with $CD4^+$ or $CD8^+$ T cells; $2.38 \pm 0.03 \%$), collectively representing $\geq 99\%$ of PBMCs (no treatment with NK-EVs). Fewer than 1% of events could not be assigned to known populations. These distributions, including a $CD4^+/CD8^+$ T cell ratio of ~ 1.9 , are consistent with expected values for healthy human PBMCs [230]. A negligible CFSE signal was observed in PBS and CFSE-PBS controls, whereas strong CFSE positivity was detected across multiple immune subsets in the NK-EV-treated conditions (**Figure 34D**). Representative results from one donor are shown; donor-specific patterns are provided in **Figures 36-38**.

Quantitative assessment of PBMCs treated with CFSE-labelled NK-EVs revealed no significant differences in subset distribution (**Figure 34E**). However, the CFSE mean fluorescence

intensity (normalized to CFSE-stained PBS control) showed pronounced enrichment of NK-EVs in monocyte-CD4⁺ T cell conjugates (CD14⁺ monocyte associated with CD4⁺ T cells; ~73K RFU) and dendritic cells (~20K RFU), corresponding to ~20-fold and ~6-fold higher levels, respectively, compared with the average PBMC subsets exposed to CFSE-labelled NK-EVs (~4K RFU) (**Figure 34F**). By contrast, B cells, NK cells, DN T cells, CD4⁺ T cells and CD8⁺ T cells exhibited minimal NK-EV uptake (mean RFU of 3.0K). Despite these differences in density, the proportion of CFSE-positivity cells was elevated across all subsets (**Figure 34G**). Collectively, these results suggest that NK-EVs interact broadly with diverse immune populations, but preferentially associate with myeloid lineages, particularly monocytes and dendritic cells.

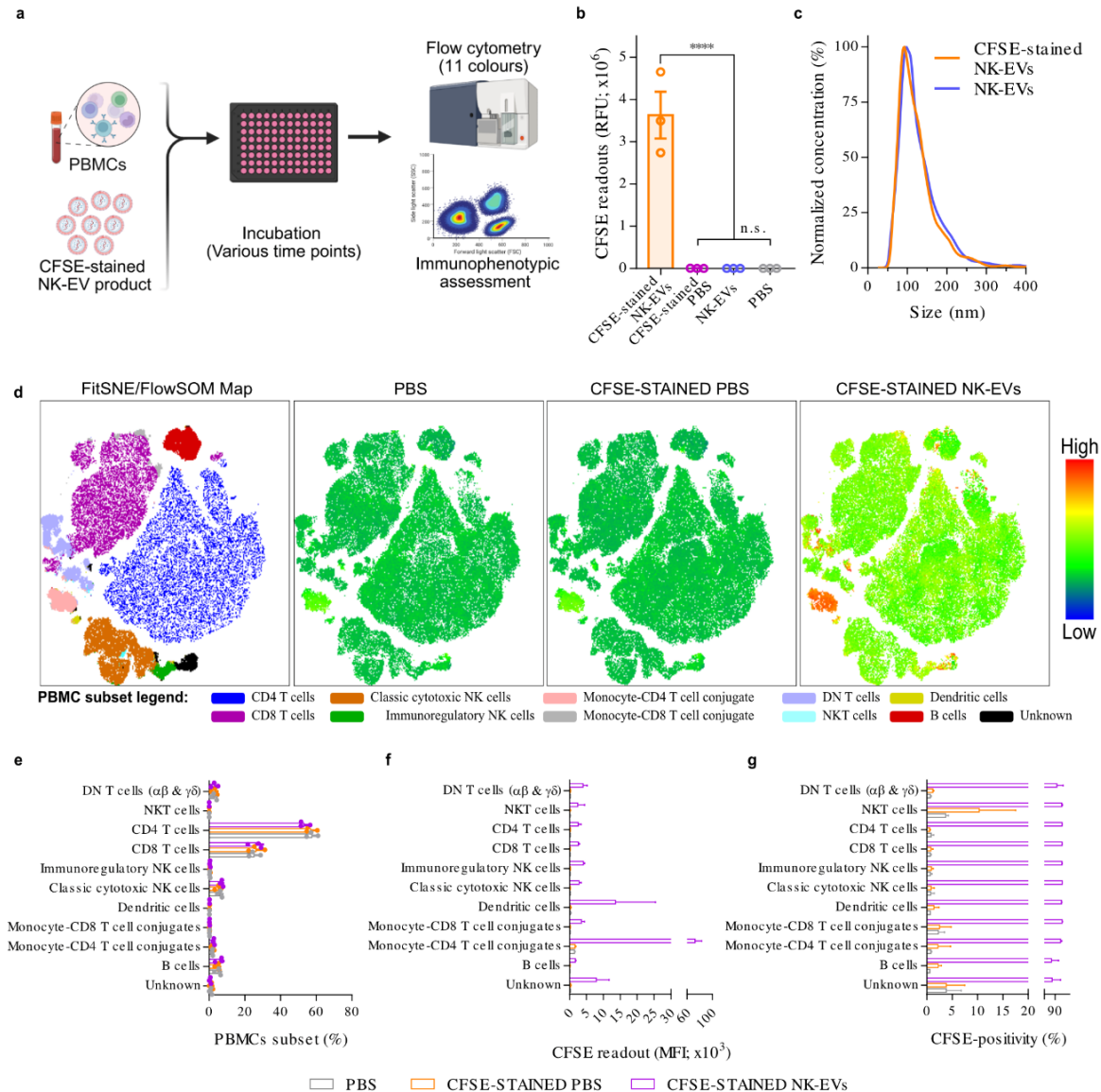


Figure 34. NK-EVs demonstrate broad PBMC uptake with selective myeloid cell enrichment by flow cytometry.

A) Experimental design of PBMCs obtained from a healthy donor incubated with CFSE-stained NK-EVs or controls, CFSE-stained PBS or PBS. B) CFSE level quantified following staining using a plate reader (mean $\pm$ S.E.M.; N=3 experimental replicates with technical duplicates). One-way ANOVA followed by post hoc Tukey's multiple comparison was performed, where $p < 0.0001$ (****), n.s., non significant). C) NK-EV concentration measured by Nanoparticle Tracking Analysis on the NS300 (mean $\pm$ S.D. from 3 experimental replicates with five technical replicates). D) FitSNE & FlowSOM map showing the identification of PBMC cell subtypes with visual confirmation of PBMCs treated with CFSE-staining of NK-EVs compared to CFSE-stained PBS and PBS controls. E) Flow cytometry analysis of PBMC subsets following exposure to CFSE-stained EVs or controls after 72 hours of incubation (mean $\pm$ S.D.; N=3 biological replicates), where i) subset distribution, ii) CFSE-MFI, and iii) CFSE positivity are shown.

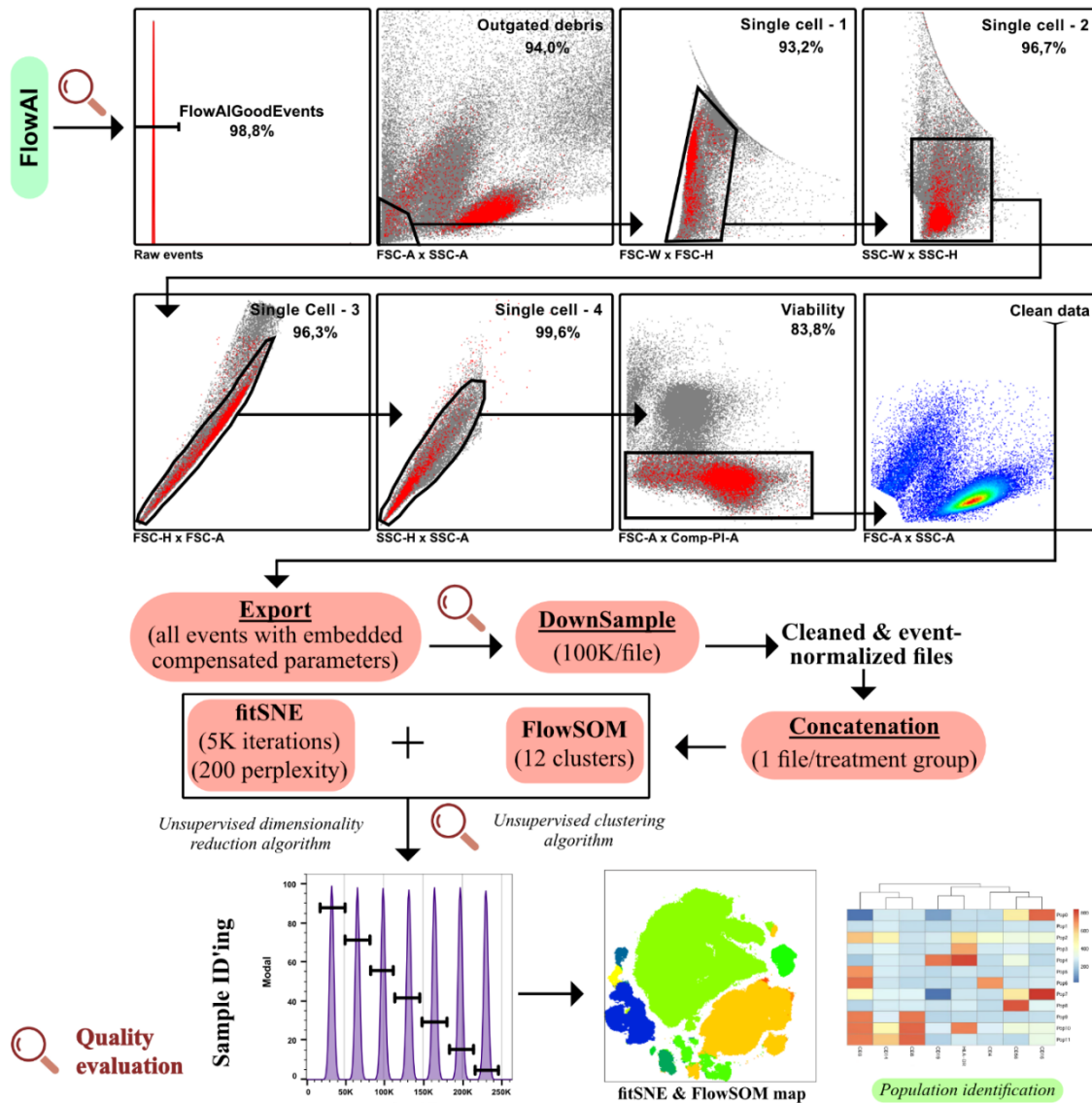


Figure 35. Flow cytometry gating and analysis workflow.

Raw events were cleaned using FlowAI to remove outliers caused by flow rate fluctuations, signal instability, or reduced dynamic range, yielding "GoodEvents." Subsequent gating removed debris, doublets, and dead cells, resulting in clean and viable single-cell data. Cleaned files were normalized to 100,000 single viable cells per sample using the DownSample plugin. Each donor's files were concatenated, forming a single file for each donor. This meta-file was used for dimensionality reduction (fitSNE) and unsupervised clustering (FlowSOM) analyses. fitSNE was used for visualizing PBMC subsets across samples, while FlowSOM identified 12 meta-clusters to assess marker behaviour and detect distinct cell subsets. "Sample ID" annotation enabled treatment-specific identification in the meta-file. Data quality was evaluated to ensure artifact-free processing and robust downstream analysis. The FlowSOM PopHm dataset was used for population identification based on marker expression.

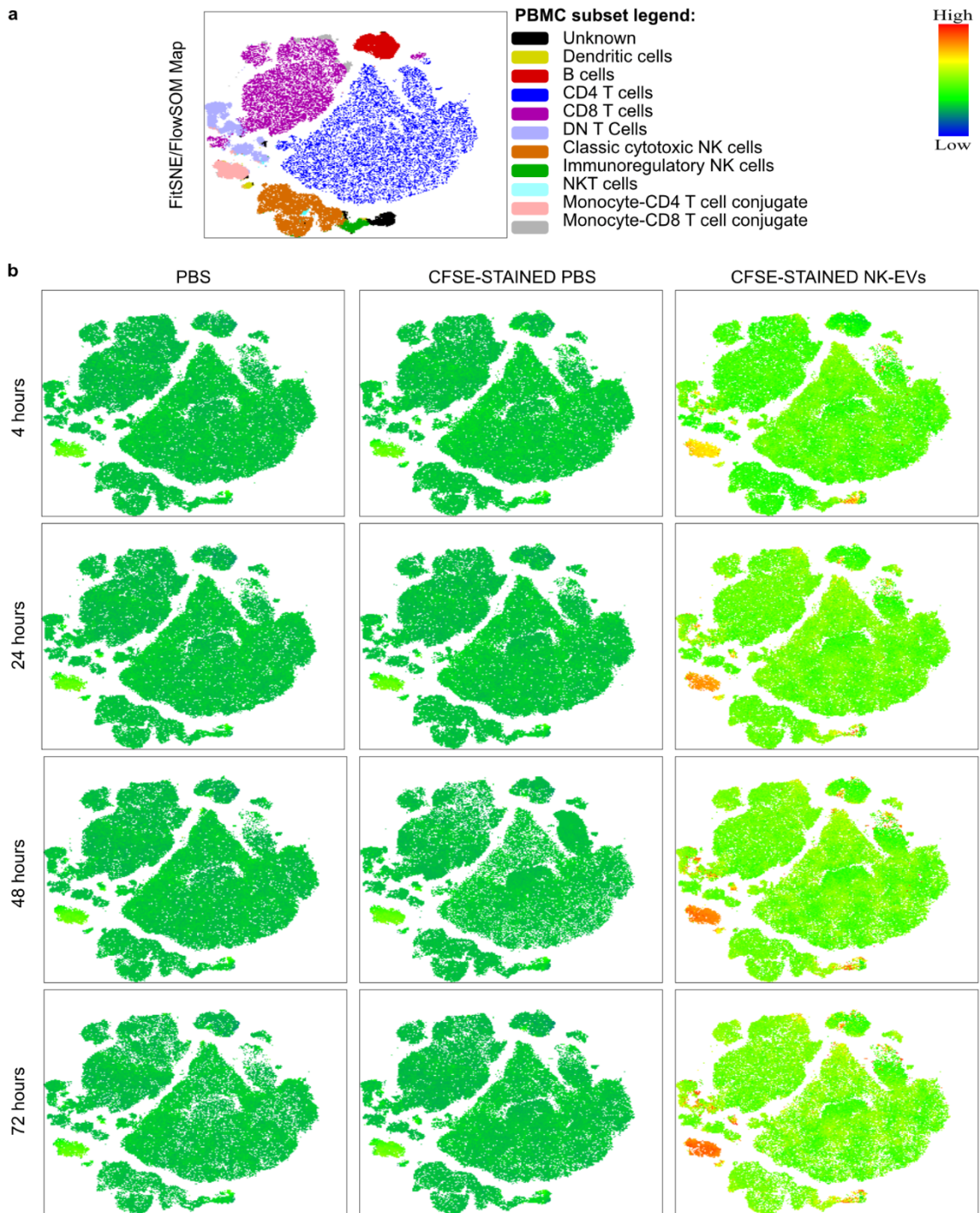


Figure 36. CFSE-stained NK-EV uptake in PBMCs by flow cytometry using healthy donor #1.
A) FitSNE & FlowSOM map legend. B) fitSNE & FlowSOM for all dose and time conditions tested, showing PBS control, CFSE-stained PBS control and CFSE-stained NK-EVs over several time points (4h, 24h, 48h and 72h).

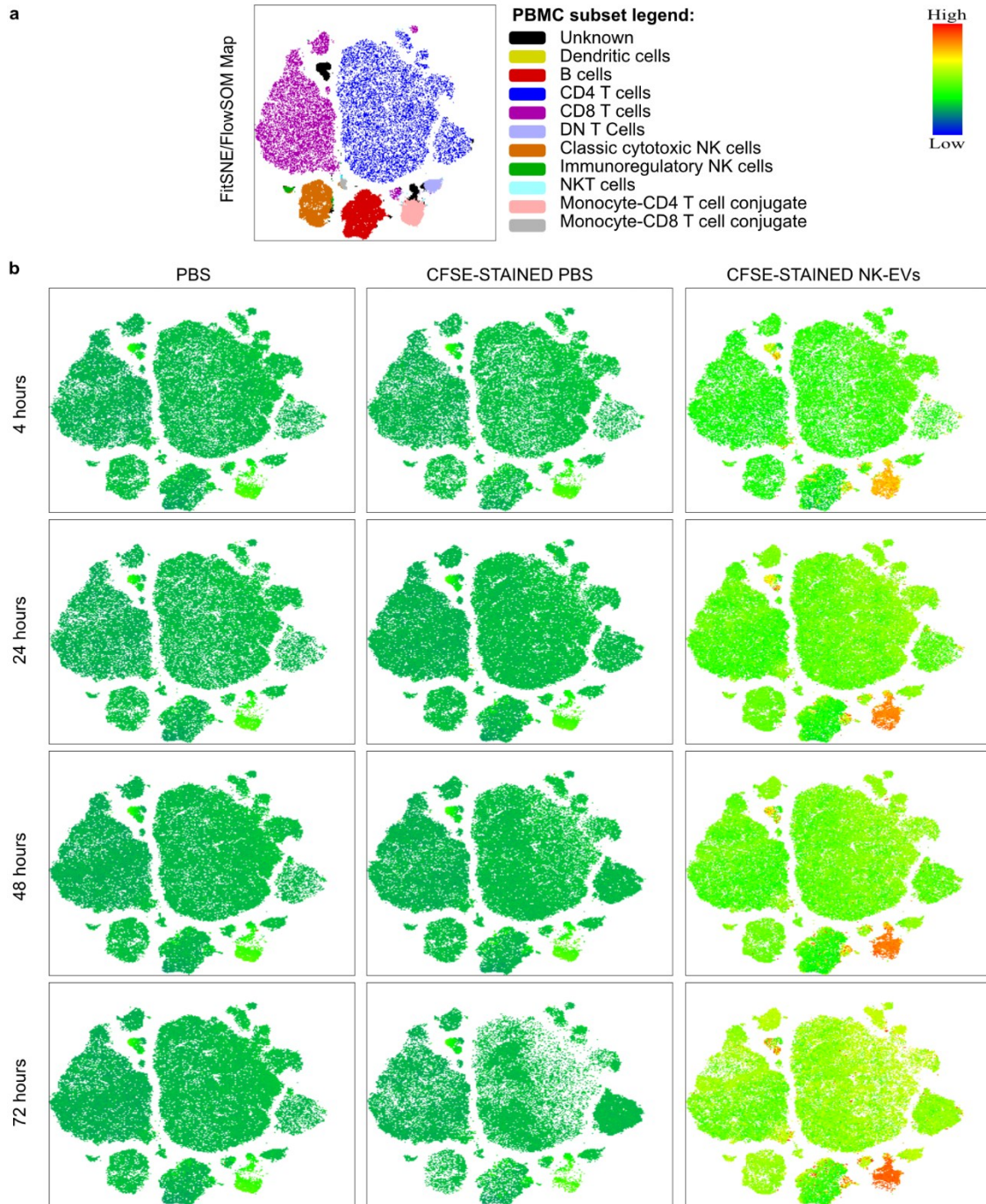


Figure 37. CFSE-stained NK-EV uptake in PBMCs by flow cytometry using healthy donor #2.
A) FitSNE & FlowSOM map legend. B) fitSNE & FlowSOM for all dose and time conditions tested, showing PBS control, CFSE-stained PBS control and CFSE-stained NK-EVs over several time points (4h, 24h, 48h and 72h).

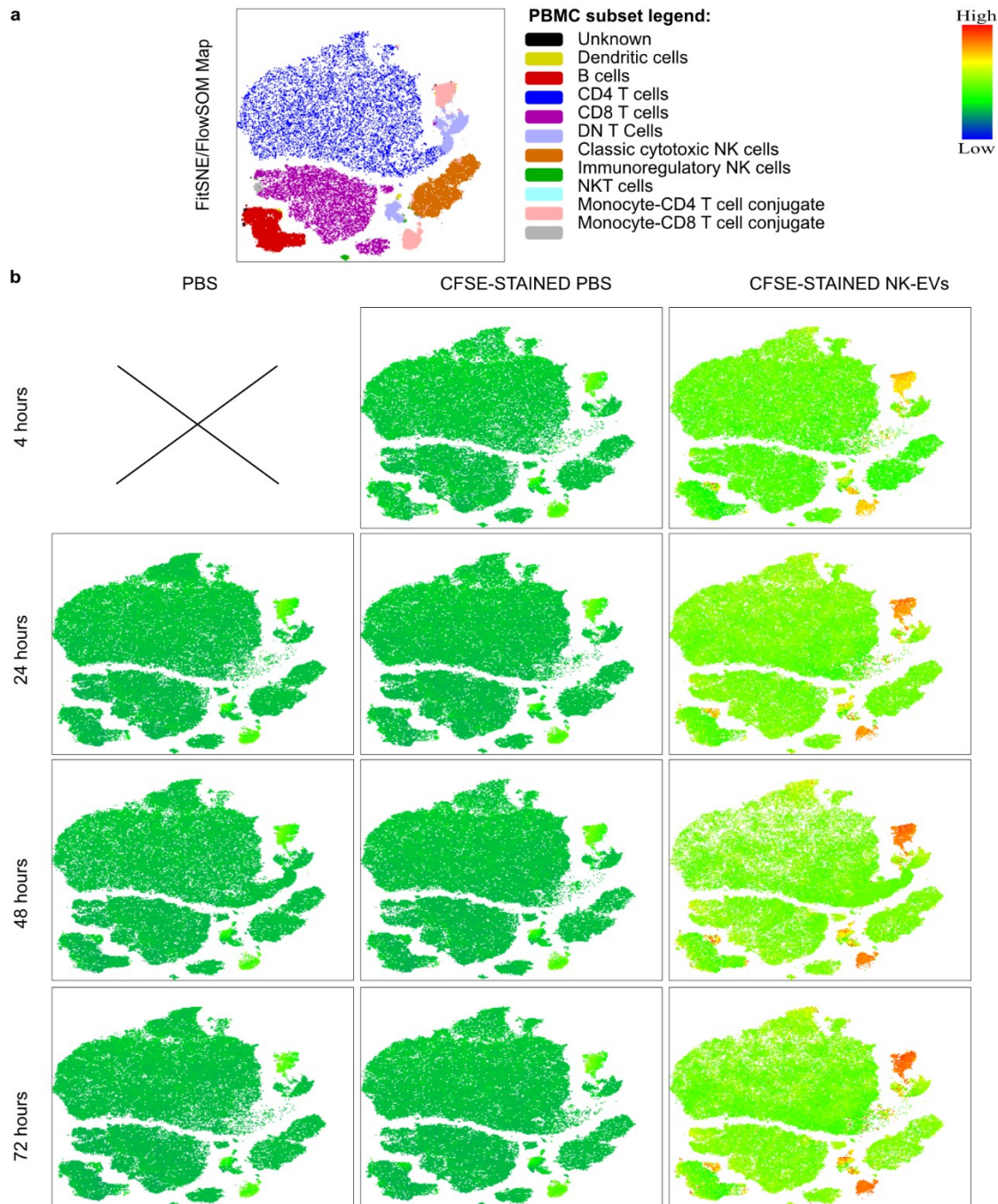


Figure 38. CFSE-stained NK-EV uptake in PBMCs by flow cytometry using healthy donor #3.
A) FitSNE & FlowSOM map legend. B) fitSNE & FlowSOM for all dose and time conditions tested, showing PBS control, CFSE-stained PBS control and CFSE-stained NK-EVs over several time points (4h, 24h, 48h and 72h).

NK-EVs boost PBMC anti-cancer function

Building on the finding that NK-EVs associate with specific immune cell subsets, functional reprogramming was evaluated following NK-EV stimulation of PBMCs. PBMCs derived from healthy donors (n=3) and TNBC patients (n=3) were stimulated with NK-EVs for 72 hours and subsequently used in a live-cell imaging assay against human MDA-MB-231 TNBC cells and MCF-7 breast cancer cells. Unstimulated (MOCK) and NK-EV-stimulated PBMCs (NK-EV) were co-cultured with RFP-tagged breast cancer cells, and tumour cell confluence/viability (RFP⁺ cells in the red channel) and apoptosis (Annexin-V staining in the green channel) were monitored over 96 hours (**Figure 39A**).

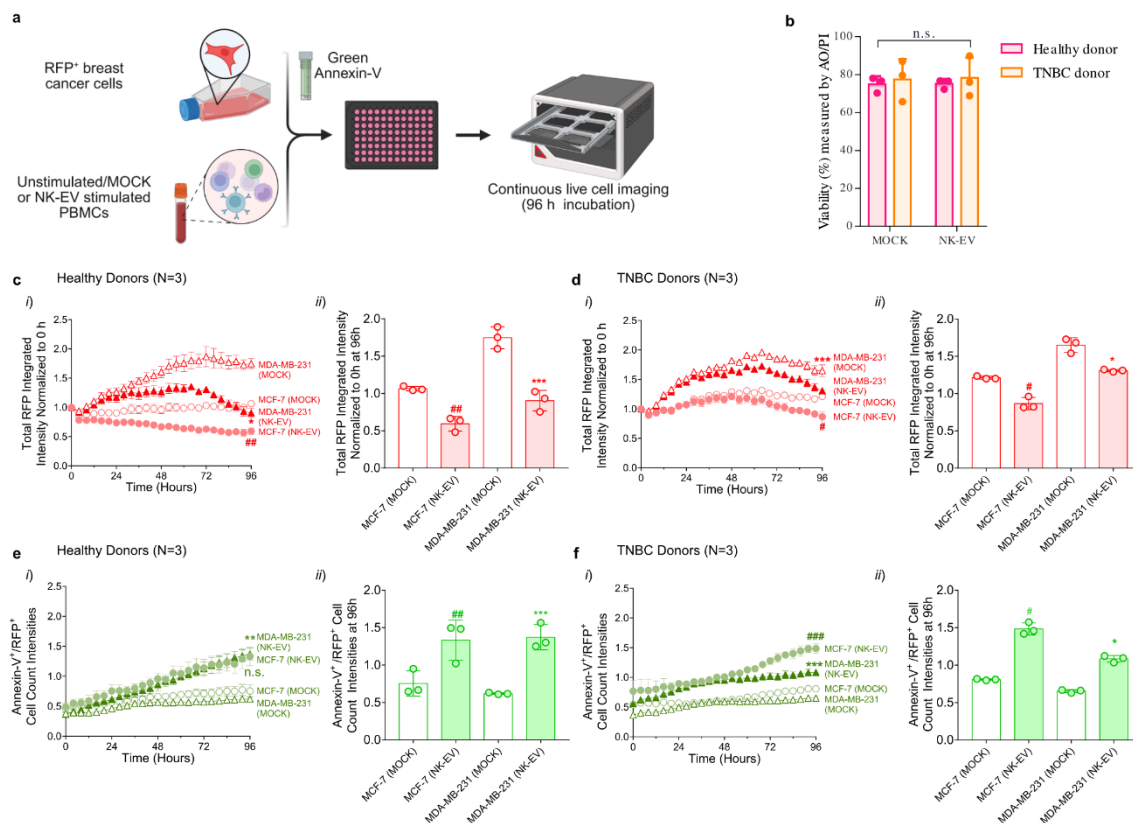


Figure 39. NK-EVs boost human PBMC anti-cancer function in a live-cell imaging assay.

A) Experimental design of human MDA-MB-231 and MCF-7 breast cancer cell lines constitutively expressing RFP to track confluence (health and viability surrogate), co-cultured with unstimulated PBMCs (MOCK) or NK-EVs-stimulated PBMCs (NK-EVs; 72 hours of stimulation) to achieve a 2:1 effector-to-target (PBMC: cancer) cell ratio. B) PBMC viability measured by AO/PI staining (mean $\pm$ S.E.M., N=3 biological replicates with technical duplicates). C) Healthy PBMC donors' RFP expression was used to track confluence (health and viability surrogate), where *i)* over time and *ii)*

endpoint (t=96 h). D) TNBC PBMC donors' RFP expression was used to track confluence (health and viability surrogate), where *i*) over time and *ii*) endpoint (t=96 h) were recorded or analyzed; E) Healthy PBMC donors' apoptotic marker Annexin-V, normalized to the RFP-positive cell count, where *i*) over time and *ii*) endpoint (t=96 h) were recorded or analyzed. F) TNBC PBMC donors' apoptotic marker Annexin-V, normalized to the RFP-positive cell count, where *i*) over time and *ii*) endpoint (t=96 h) were analyzed (mean $\pm$ S.E.M., N=3 biological replicates with technical quadruplets). Two-way ANOVA followed by post hoc Tukey's multiple comparison was performed on the entire timeline panels (*i*), where n.s., non significant; * (MDA) or # (MCF), $P < 0.05$; ** (MDA) or ## (MCF), $P < 0.01$; *** (MDA) or ### (MCF), $P < 0.001$. Welch's t-test was performed for endpoint panels (*ii*), where n.s., non significant; * (MDA) or # (MCF), $P < 0.05$; ** (MDA) or ## (MCF), $P < 0.01$; *** (MDA) or ### (MCF), $P < 0.001$.

Importantly, NK-EV treatment did not affect PBMC viability, as assessed by AO/PI cell counting across healthy and TNBC donors (n=6) prior to co-culture (**Figure 39B**), confirming that NK-EVs do not compromise survival of normal immune cells[105]. In contrast, NK-EV-stimulated PMBCs induced a robust reduction in the growth of both MDA-MB-231 and MCF-7 breast cancer cell lines compared with MOCK PBMCs (**Figure 39C-D**). Moreover, when normalized to RFP⁺ cell count, Annexin-V expression was significantly higher in breast cancer cells co-cultured with NK-EV-stimulated PBMCs than with MOCK PBMCs, in both healthy and TNBC donor groups (**Figure 39E-F**). Donor-specific data are provided in **Figures 40-41**. Together, these findings suggest that NK-EV stimulation reprograms PBMCs to enhance their anti-cancer activities.

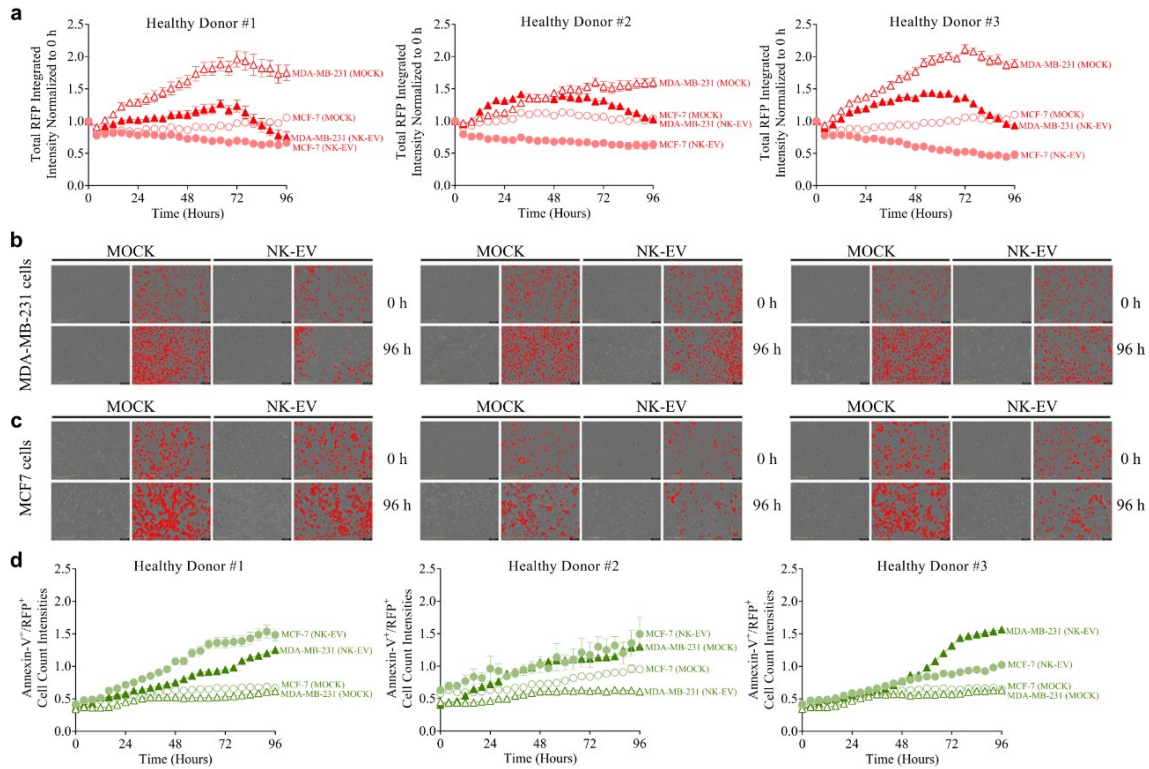


Figure 40. Live-cell imaging analysis of PBMCs from 3 healthy donors co-cultured with human breast cancer cell lines.

Human MDA-MB-231 and MCF-7 breast cancer cell lines constitutively expressing RFP, co-cultured with unstimulated PBMCs (MOCK) or NK-EVs-stimulated PBMCs (NK-EVs; 72 hours of stimulation) to achieve a 2:1 effector-to-target cell ratio. A) RFP expression was used to track confluence (health and viability surrogate); Representative images (before and after) in brightfield and red channels capturing the RFP signal illustrate the confluence (health and viability surrogate) of MDA-MB-231 (B) and MCF-7 (C) human breast cancer cells. D) Early apoptotic marker Annexin-V was tracked in the green channel over time and normalized to the RFP-positive breast cancer cell count (mean $\pm$ S.E.M., technical quadruplets).

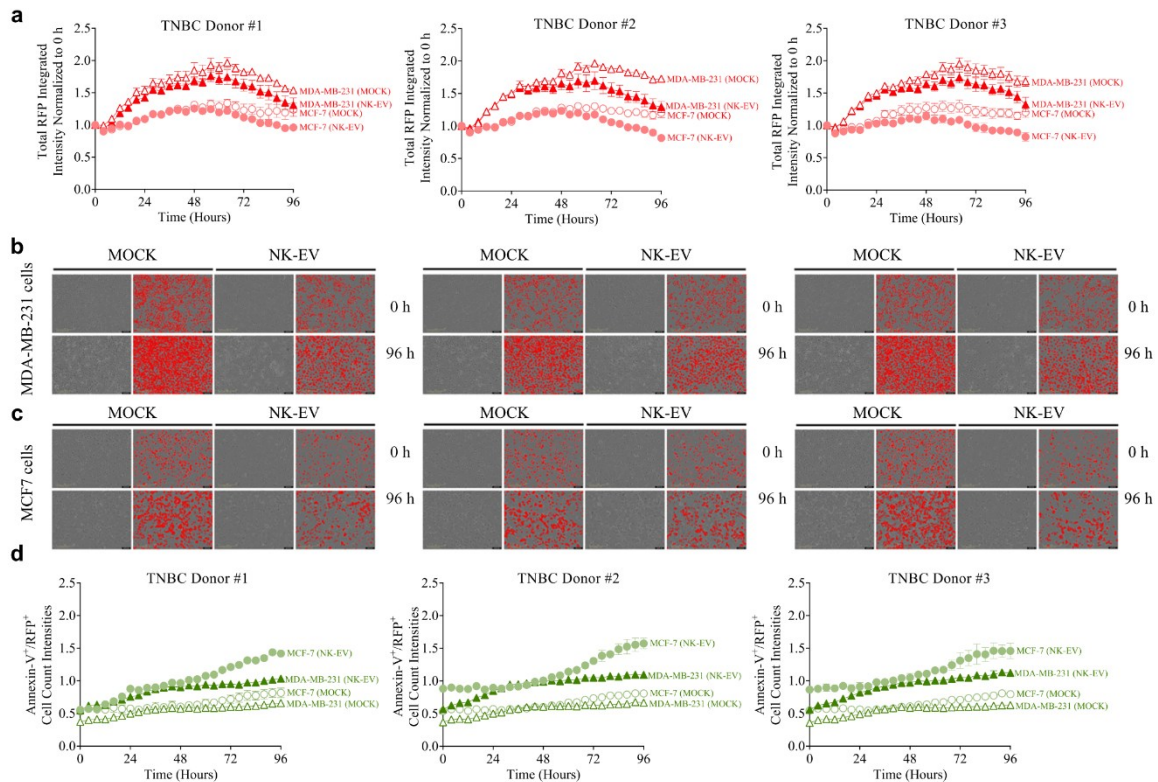


Figure 41. Live-cell imaging analysis of PBMCs from 3 TNBC donors co-cultured with human breast cancer cell lines.

Human MDA-MB-231 and MCF-7 breast cancer cell lines constitutively expressing RFP, co-cultured with unstimulated PBMCs (MOCK) or NK-EVs-stimulated PBMCs (NK-EVs; 72 hours of stimulation) to achieve a 2:1 effector-to-target cell ratio. A) RFP expression was used to track confluence (health and viability surrogate); Representative images (before and after) in brightfield and red channels illustrate the confluence (health and viability surrogate) of MDA-MB-231 (B) and MCF-7 (C) human breast cancer cells. D) Early apoptotic marker Annexin-V was tracked in the green channel over time and normalized to the RFP-positive breast cancer cell count (mean $\pm$ S.E.M., technical quadruplets).

NK-EVs reprogram immune transcriptome

Given the enhanced anti-cancer activity observed in NK-EV-treated PBMCs at the cellular level, PBMC from healthy donors and TNBC patients were treated with NK-EV (72 hours) and processed for single-cell RNA sequencing (scRNA-seq) was performed to characterize the transcriptomic changes (**Figure 42A**) [231, 232].

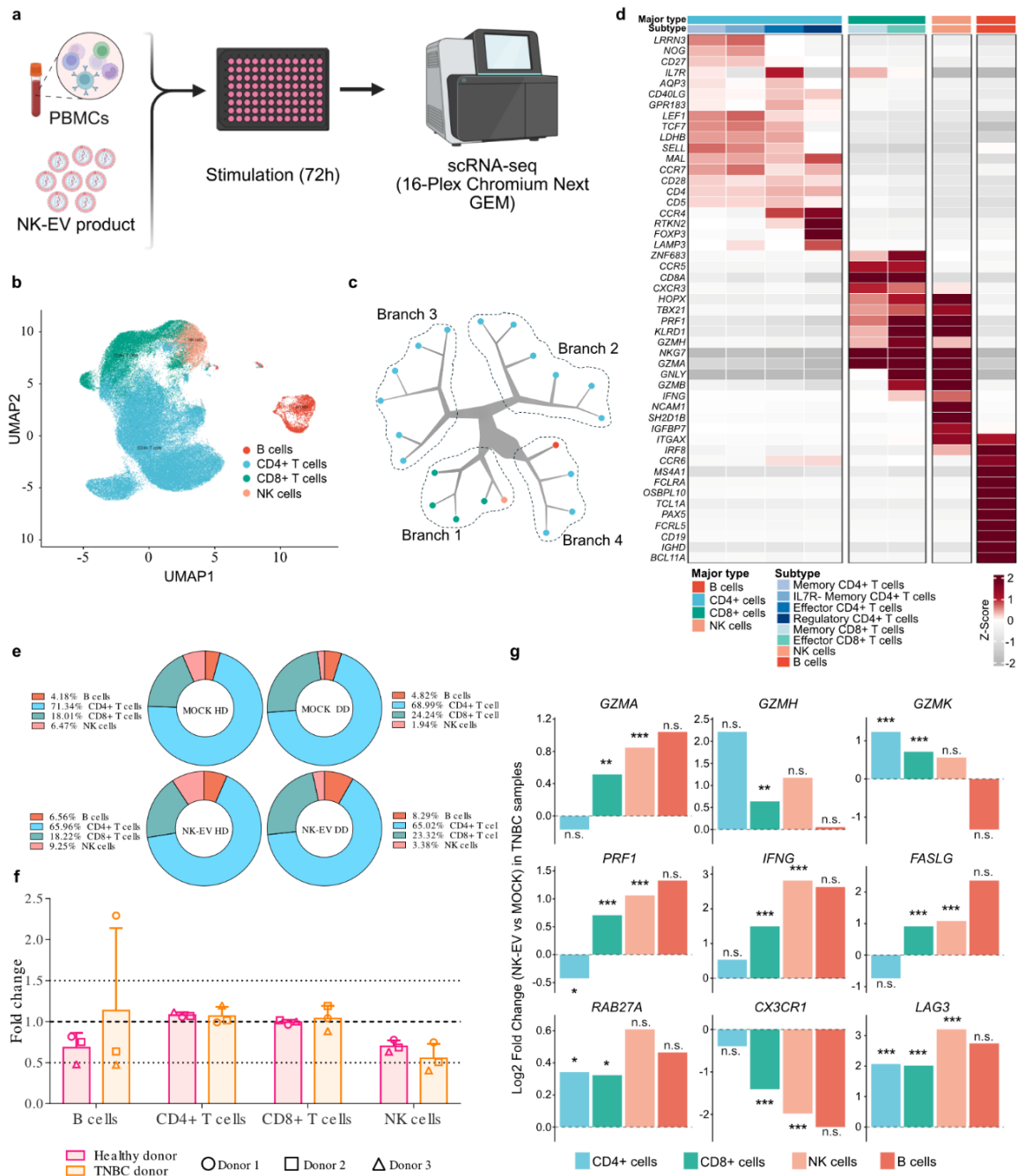


Figure 42. Single-cell RNA-seq (scRNA-seq) shows NK-EVs reprogram and activate distinct human PBMC immune cell subsets.

A) Experimental design of PBMCs (from healthy and TNBC-diseased donors) incubated with NK-EVs (1×10^{11} EVs/mL) for 72 hours. B) UMAP representation (i) and TooManyCells representation (ii) of 88,213 quality-filtered PBMCs from healthy and TNBC donors, showing five major immune populations and four distinct cellular branches. C) Heatmap displaying top differentially expressed marker genes for each annotated cell type, with expression level z-scored and normalized (red indicates higher than average expression, blue indicates lower than average relative to all cell types), and hierarchically clustered. D) PBMC subtype composition analysis showing (i) pie charts of major immune populations distribution and (ii) subtype population fold change following NK-EV treatment. A two-way ANOVA followed by post hoc Tukey's multiple comparison test was performed to assess PBMC subtype composition. E) Cytotoxic effector gene expression analysis

showing log fold changes (NK-EV vs PBS control) across cell types and among TNBC samples (N=3 biological replicates). Seq data represent the DESeq2 pseudobulk analysis, with paired donor design using Wald test, followed by post hoc Benjamini-Hochberg multiple testing correction, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (adjusted p-values); n.s., non significant.

Untreated PBMCs served as controls to establish a baseline profile. Sample processing, including fixation and recovery, was successful (**Figure 43**), and quality control metrics confirmed high data quality, with appropriate feature-count relationships and mitochondrial gene percentages across all samples (**Figure 44**).

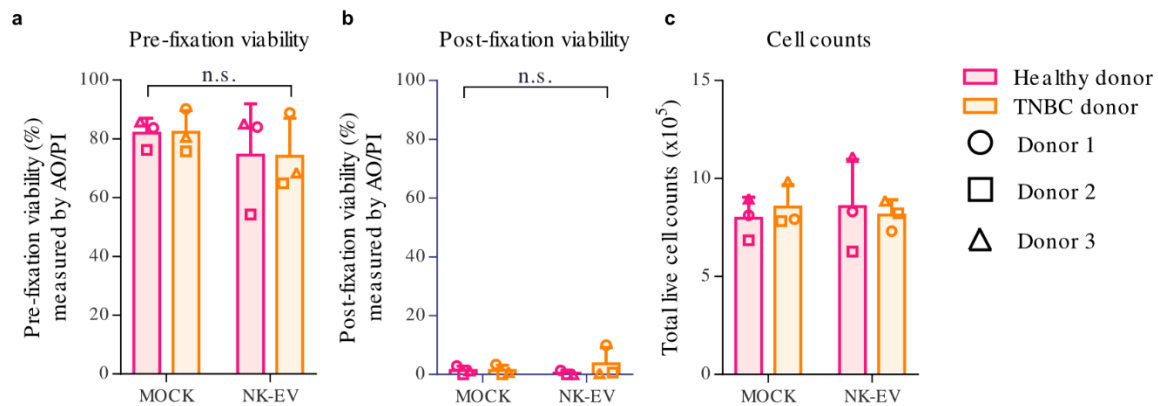


Figure 43. PBMC counts by AO/PI staining of healthy and TNBC donors during scRNA-sequencing processing.

A) Pre-fixation cell count viability. B) Post-fixation cell count viability. C) Total live cell counts recovered for scRNA-sequencing (mean ± S.E.M., N=3 biological replicates). Two-way ANOVA followed by post hoc Tukey's multiple comparison was performed, where n.s., non significant.

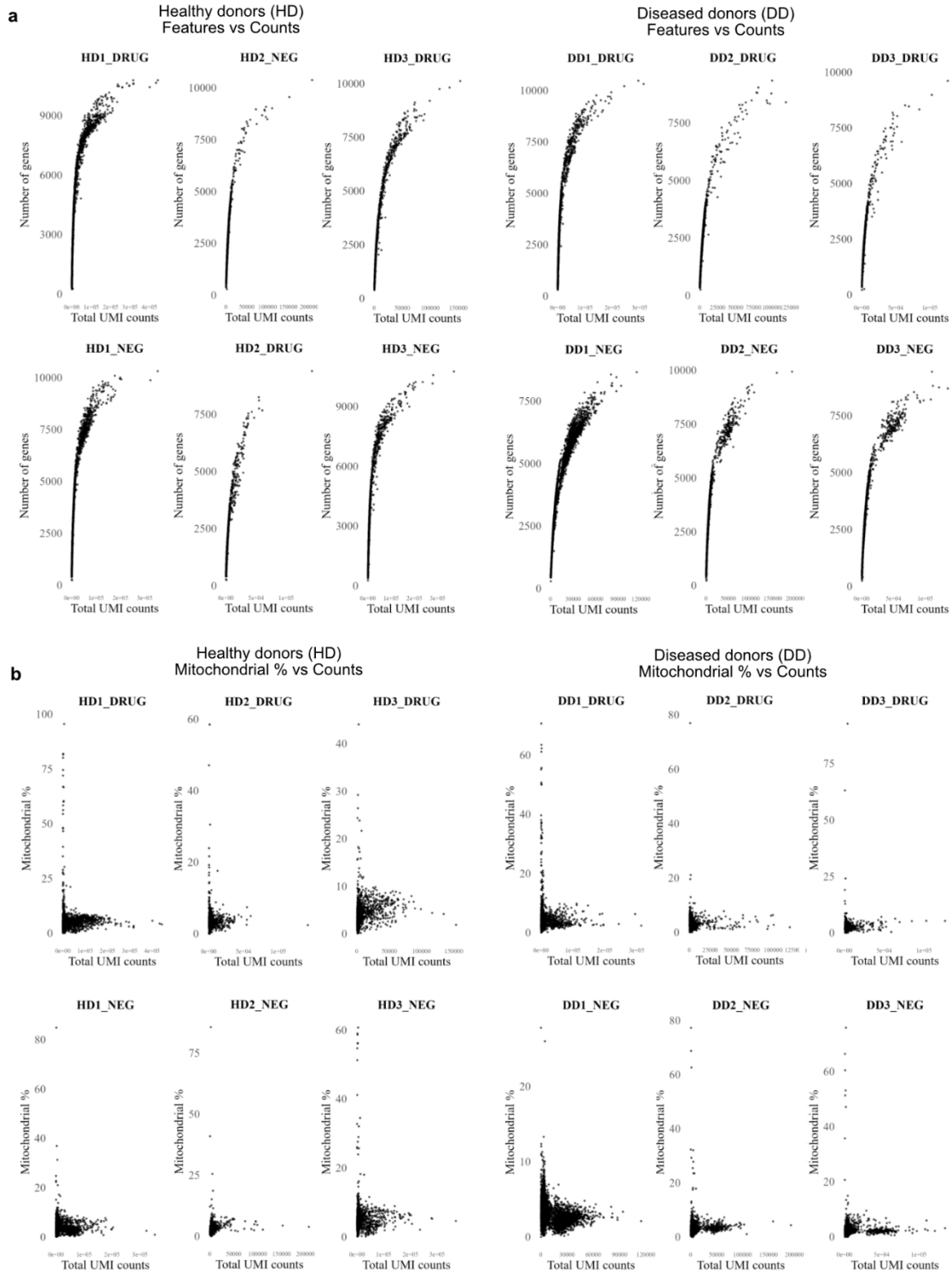


Figure 44. scRNA-sequencing data quality control metrics for healthy and TNBC-patient PBMC samples.

A) Features vs counts scatter plots showing the relationship between detected genes and total UMI counts per cell across all samples. B) Mitochondrial gene percentage vs total UMI counts, demonstrating mitochondrial content distribution for quality assessment. Data shown for healthy donors (HD1-HD3) and TNBC patients (DD1-DD3) under different conditions (DRUG-NK-EVs, NEG-MOCK, POS-TCR stimulation).

The scRNA-seq profiling of ~90K high-quality PBMCs from both donor groups (n=6, biological replicates) revealed four major immune cell subtypes (**Figure 42B**). UMAP visualization identified distinct populations, including B cells, CD4⁺ T cells, CD8⁺ T cells and NK cells. Similarly, TooManyCells hierarchical clustering resolved four distinct transcriptomic-cellular branches (**Figure 42C** and **Table 12**) [233]. Branch 1 comprises CD8⁺ effector/memory T cells and NK cells, representing the principal cytotoxic effector compartment. Branches 2 and 3 consisted exclusively of CD4⁺ T cells, with branch 2 enriched for a stem-like population and branch 3 representing more differentiated subsets. Branch 4 encompassed immunoregulatory cells such as B cells, IL-7R- memory CD4⁺ T cells, and regulatory CD4⁺ T cells. Cell type classification was validated by canonical marker gene expression (**Figure 42D**).

Table 12. TooManyCells hierarchical clustering identified four distinct cellular branches.

TooManyCells Branches	Cell group	Cell type
Branch1	Cytotoxic cells	Memory CD8+ T cells
		Effector CD8+ T cells
		Effector CD8+ T cells (NK-like)
		Cytotoxic NK cells
Branch2	Stem-like CD4+ T cells	Naive CD4+ T cells
		Activated CD4+ T cells
		Memory CD4+ T cells
		Helper CD4+ T cells
Branch 3	Mature CD4+ T cells	Memory CD4+ T cells
		Helper CD4+ T cells
Branch 4	Immunoregulatory cells	IL7R- memory CD4+ T cells
		Regulatory CD4+ T cells
		B cells

Immune cell composition doesn't change following NK-EV treatment ($\alpha \geq 0.05$, **Figure 42E-F** and **Figure 45**): B cells (healthy donor: 5.37 ± 2.88 % vs TNBC donor: 6.56 ± 5.21 %), CD4⁺ T cells (healthy donor: 68.65 ± 5.11 % vs TNBC donor: 67.01 ± 7.14 %), CD8⁺ T cells (healthy donor: 18.12 ± 5.68 % vs TNBC donor: 23.78 ± 3.27 %), and NK cells (healthy donor: 7.86 ± 1.65 % vs TNBC donor: 2.66 ± 1.26 %). The reduced NK cell representation in TNBC donor PBMCs is consistent with systemic immune suppression in cancer patients. Conversely, it is interesting to

note that B cell and NK cell levels increased following NK-EV treatment, independently of disease status.

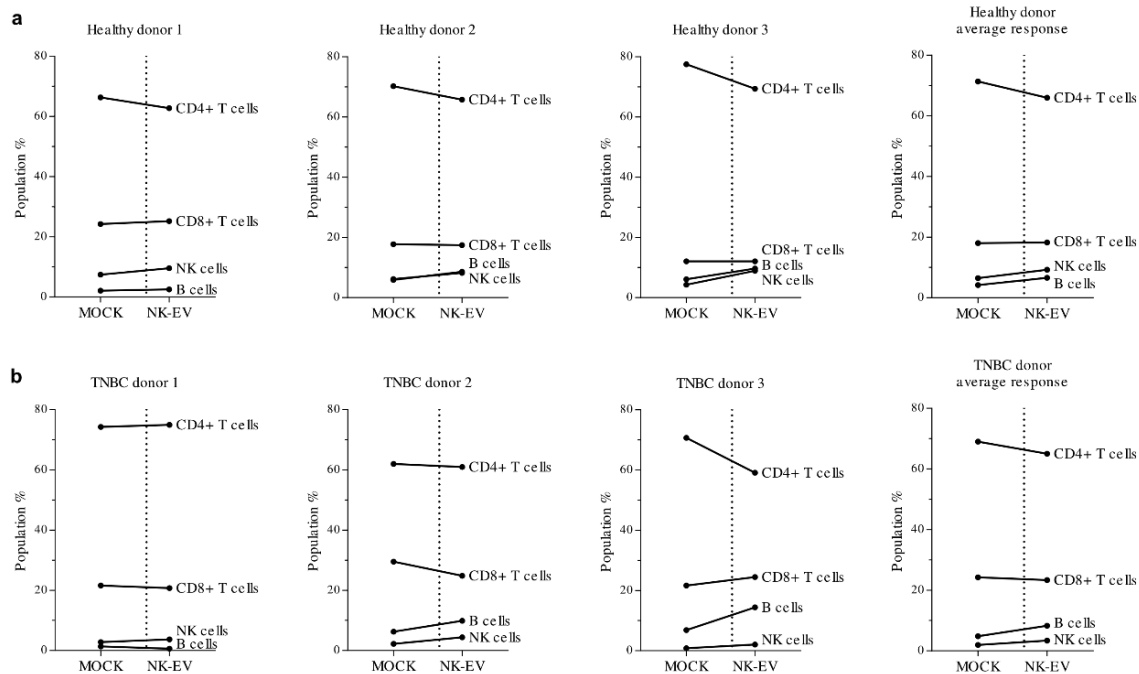


Figure 45. Before-and-after subtype population percentage plots of PBMC donors treated with NK-EVs.

A) Healthy donors (N=3 biological replicates). B) TNBC donors (N=3 biological replicates).

Conversely, at the transcriptomic level, NK-EV treatment induced diverse responses across immune cell subsets, with the most pronounced transcriptional changes observed in cytotoxic populations, such as those comprising Branch 1 (**Figure 42G** and **Figure 46**). In CD8⁺ T cells from TNBC donors, NK-EVs markedly upregulated the granzyme gene family (*GZMA*, *GZMB*, *GZMK*, and *GZMH*), along with *PRF1*, *FASLG*, and *IFNG*, while concurrently downregulating *CX3CR1*, a receptor essential for T cell migration and persistence within tissues [234]. NK cells exhibited similar upregulations of primary cytotoxic genes, including *GZMA*, *GZMB*, *PRF1*, *FASLG*, and *IFNG*, again accompanied by *CX3CR1* downregulation [235]. The downregulation of *CX3CR1* in both populations may favour tissue residency, as *CX3CR1* expression typically marks peripheral circulating rather than tissue-infiltrating lymphocytes [236, 237]. The observed upregulation of

cytotoxic pathways aligns with granzyme-perforin-mediated killing and Fas-FasL-induced apoptosis, indicating an enhanced killing potential following NK-EV stimulation [238, 239].

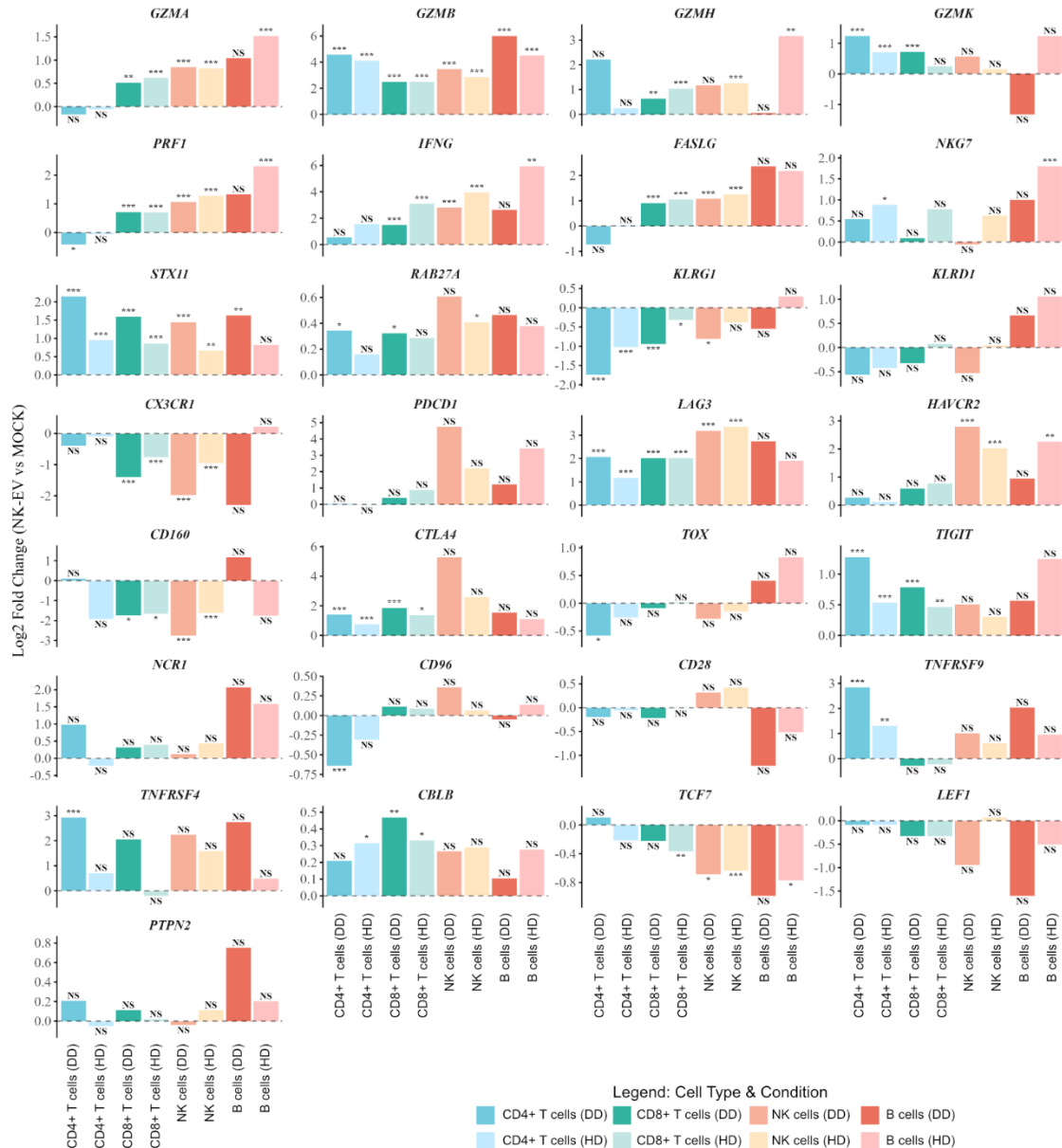


Figure 46. Cytotoxic effector and exhaustion-associated genes in healthy (HD) and TNBC-diseased (DD) PBMC donors following NK-EV stimulation.

A) Log fold change (NK-EVs vs MOCK) shown for cytotoxic effector genes (*GZMA*, *GZMB*, *GZMH*, *GZMK*, *PRF1*, *IFNG*, *FASLG*, *NKG7*, *STX11*, *RAB27A*, *KLRG1* and *CX3CR1*), exhaustion-associated genes (*PDCD1*, *LAG3*, *HAVCR2*, *CD160*, *CTLA4*, *TOX*, *TIGIT*, *KLRG1*) and NK cell receptors (*NKG2A*, *NKG2D*, *NCR1* and *CD96*). HD = healthy donors (N=3 biological replicates) and DD = diseased donors (N=3 biological replicates). Data represent the DESeq2 pseudobulk analysis, with paired donor design using Wald test, followed by post hoc Benjamini-Hochberg multiple testing correction, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (adjusted p-values); n.s., non significant.

Furthermore, NK cells exhibited significant *CD160* downregulation, a marker often associated with activation or exhaustion, whereas $CD8^+$ T cells showed no significant changes, suggesting population-specific responses (**Figure 46**). In NK cells, *CD160* functions as an activating co-receptor that enhances cytotoxicity and IFN- γ production through engagement with HLA-C molecules [240]. However, *CD160* can also denote highly activated NK cells approaching functional exhaustion. Thus, its downregulation in functionally competent NK cells may reflect a shift away from *CD160*-dependent activation toward alternative cytotoxic mechanisms [241].

Concurrent *LAG3* upregulation was observed across multiple populations, including $CD8^+$ T cells, NK cells, and memory $CD4^+$ T cells (**Figure 42G**). This pattern likely reflects activation-induced checkpoint expression rather than exhaustion, given the concurrent enhancement of effector functions [242, 243]. Notably, effector $CD4^+$ T cells showed marked *GZMB* upregulation alongside significantly decreased *IL2* expression, suggesting a shift toward cytotoxic functions rather than helper functions (**Figure 46**). Expression of key immunomodulatory cytokines, such as *IL10* and *TGFB1*, remained unchanged across populations (**Figure 46**), suggesting that NK-EVs specifically potentiate cytotoxic pathways rather than broadly modulating immune regulatory networks [244]. Collectively, these findings identify NK-EVs as potent enhancers of cytotoxic effector activity, particularly within immune compartments critical for tumour elimination [245].

Branch 1 cells modulate reprogramming

To dissect the contribution of specific immune subsets to NK-EV-mediated immunomodulation, $CD4^+$ T cells, $CD8^+$ T cells, or $CD56^+$ NK cells were selectively depleted from healthy donor PBMCs using immunomagnetic separation (**Figure 47A-i**). Flow cytometry confirmed the efficient removal of $CD4^+$ (94.45 ± 5.47 %), $CD8^+$ (94.82 ± 3.037 %), and $CD56^+$ populations (88.63 ± 1.24 %) (**Figure 47A-ii to -iv**).

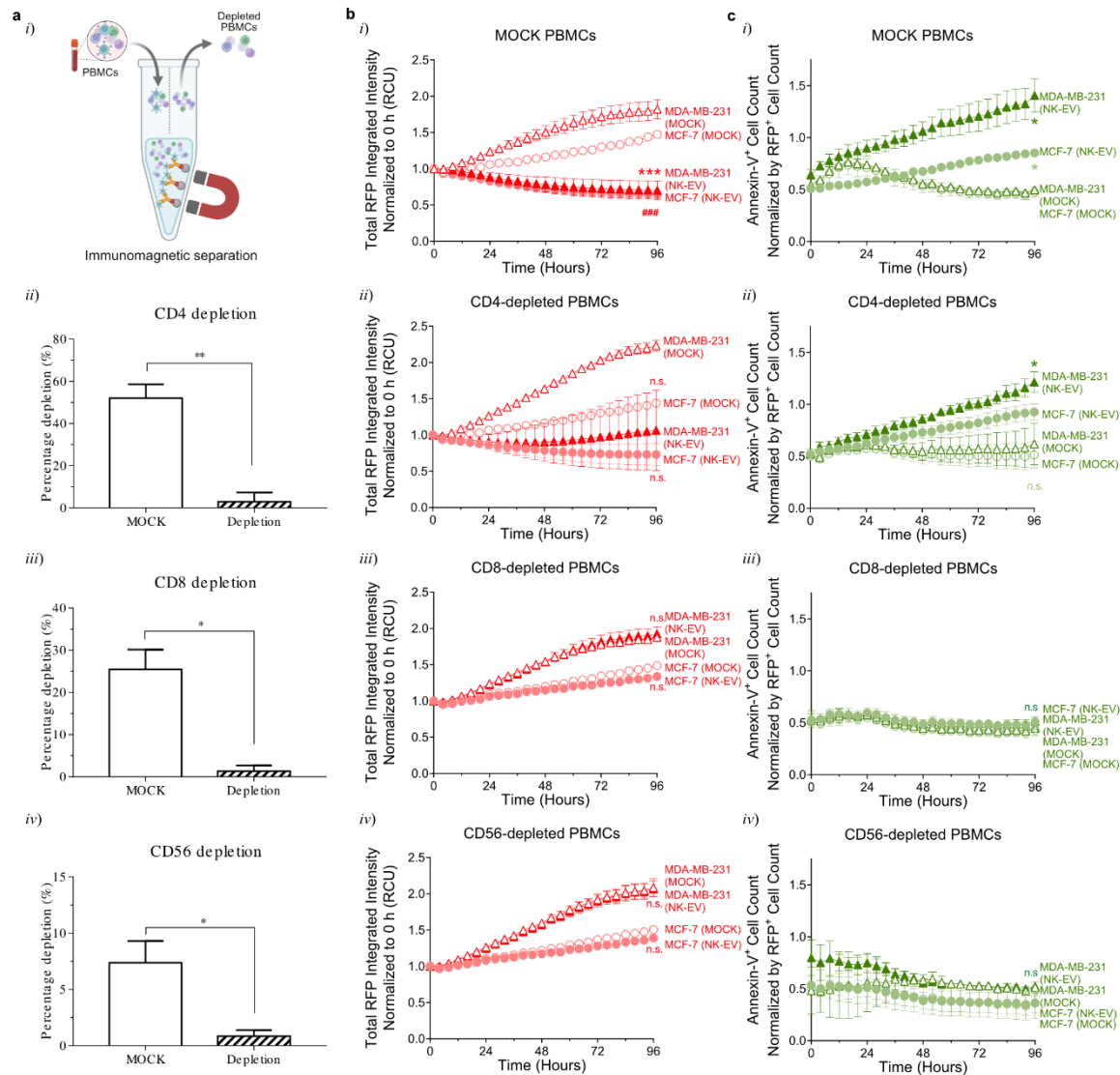


Figure 47. PBMC depletion of CD4⁺ T cells, CD8⁺ T cells, or CD56⁺ NK cells impairs NK-EV reprogramming in a live-cell imaging assay.

Unstimulated healthy PBMCs (MOCK) or healthy PBMCs stimulated by NK-EVs (NK-EV; 72 hours of stimulation) depleted of either CD4 cells, CD8 cells, or CD56 were co-cultured with human MDA-MB-231 and MCF-7 breast cancer cell lines constitutively expressing RFP in a live-cell imaging assay. A) Schematic of the immunomagnetic-depletion (i) with flow cytometry confirmation of (ii) CD4-depleted PBMCs, (iii) CD8-depleted, (iv) CD56-depleted PBMCs (mean $\pm$ S.E.M., N=3 biological replicates). Paired t-test was performed, where $p < 0.05$ (*), $p < 0.01$ (**), n.s., non significant. B) RFP expression was used to track confluence (health and viability surrogate) of (i) whole PBMCs, (ii) CD4-depleted, (iii) CD8-depleted, (iv) CD56-depleted PBMCs. C) Early apoptotic marker Annexin-V was tracked in the green channel over time and normalized to the RFP-positive breast cancer cell count (i) whole PBMCs, (ii) CD4-depleted, (iii) CD8-depleted, (iv) CD56-depleted PBMCs (mean $\pm$ S.E.M., N=3 biological replicates). Two-way ANOVA followed by post hoc Tukey's multiple comparison was performed, where n.s., non significant; * (MDA) or # (MCF), $P < 0.05$; ** (MDA) or ## (MCF), $P < 0.01$; *** (MDA) or ### (MCF), $P < 0.001$.

Following depletion, PBMCs were stimulated with NK-EVs for 72 hours and their cytotoxicity was assessed by live-cell imaging over 96 hours, as previously demonstrated (**Figure 47B-C**). As previously demonstrated, the NK-EV boost the cytotoxicity profile of MOCK PBMCs (without depletion) against cancer models (**Figures 47B-i** and **47C-i**). Interestingly, NK-EV-stimulated PBMCs depleted of CD4⁺ cells retained a cytotoxicity profile comparable to MOCK PBMCs, though slightly altered, in contrast to unstimulated controls (**Figures 47B-ii** and **47C-ii**). By comparison, NK-EV-stimulated PBMCs depleted of CD8⁺ (**Figures 47B-iii** and **47C-iii**) or CD56⁺ (**Figures 47B-iv** and **47C-iv**) subsets exhibited profiles similar to unstimulated controls, with the enhanced cytotoxic response abolished. These findings suggest that CD8⁺ T cells and NK cells are primary contributors to NK-EV-driven immunomodulation, whereas CD4⁺ T cells play a comparatively minor role. Donor-specific depletion trends are provided in **Figures 48-53**.

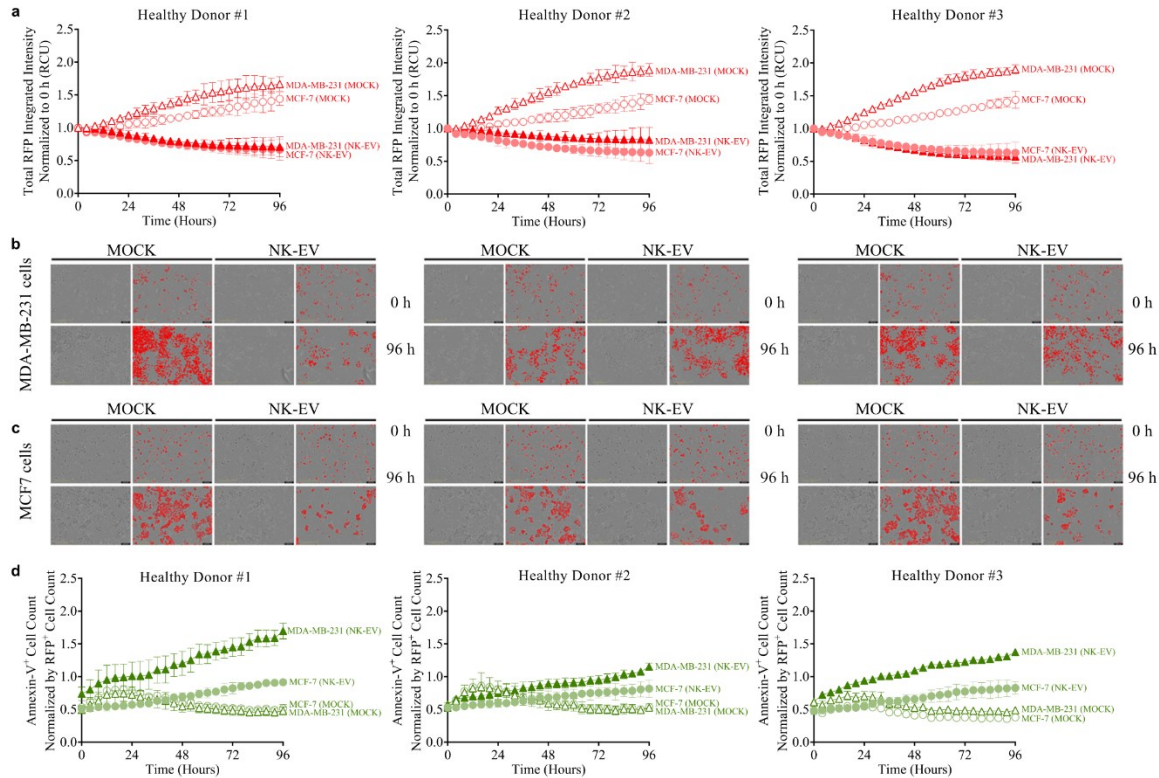


Figure 48. Live cell imaging analysis of healthy donor whole PBMCs (no depletion) co-cultured with human breast cancer cell lines.

Human MDA-MB-231 and MCF-7 breast cancer cell lines constitutively expressing RFP, co-cultured with unstimulated PBMCs (MOCK) or NK-EVs-stimulated PBMCs (NK-EVs; 72 hours of stimulation) to achieve a 2:1 effector-to-target cell ratio. A) RFP expression was used to track confluence (health and viability surrogate); Representative images (before and after) in brightfield and red channels illustrate the health and viability of MDA-MB-231 (B) and MCF-7 (C) human breast cancer cells. D) Early apoptotic marker Annexin-V was tracked in the green channel over time and normalized to the RFP-positive breast cancer cell count (mean $\pm$ S.E.M., technical triplicates).

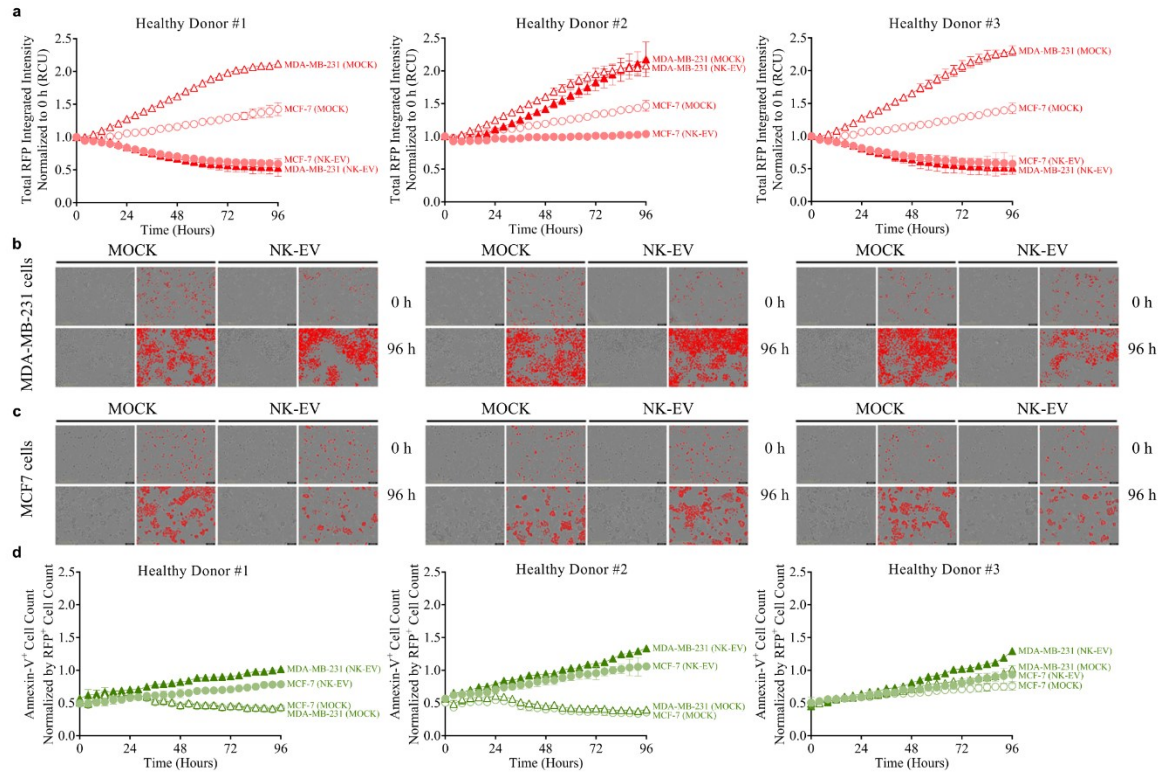


Figure 49. Live cell imaging analysis of healthy donor CD4-depleted PBMCs co-cultured with human breast cancer cell lines.

Human MDA-MB-231 and MCF-7 breast cancer cell lines constitutively expressing RFP, co-cultured with unstimulated PBMCs (MOCK) or NK-EVs-stimulated PBMCs (NK-EVs; 72 hours of stimulation) to achieve a 2:1 effector-to-target cell ratio. A) RFP expression was used to track confluence (health and viability surrogate); Representative images (before and after) in brightfield and red channels illustrate the health and viability of MDA-MB-231 (B) and MCF-7 (C) human breast cancer cells. D) Early apoptotic marker Annexin-V was tracked in the green channel over time and normalized to the RFP-positive breast cancer cell count (mean $\pm$ S.E.M., technical triplicates).

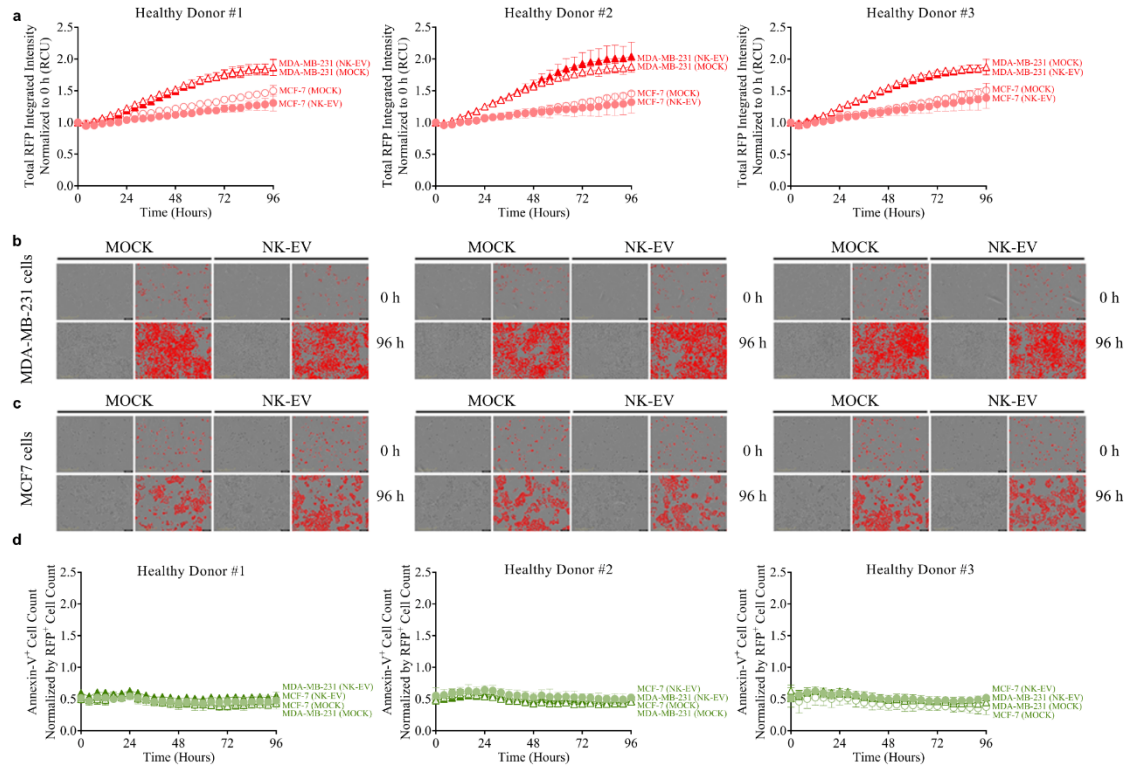


Figure 50. Live cell imaging analysis of healthy donor CD8-depleted PBMCs co-cultured with human breast cancer cell lines.

Human MDA-MB-231 and MCF-7 breast cancer cell lines constitutively expressing RFP, co-cultured with unstimulated PBMCs (MOCK) or NK-EVs-stimulated PBMCs (NK-EVs; 72 hours of stimulation) to achieve a 2:1 effector-to-target cell ratio. A) RFP expression was used to track confluence (health and viability surrogate); Representative images (before and after) in brightfield and red channels illustrate the health and viability of MDA-MB-231 (B) and MCF-7 (C) human breast cancer cells. D) Early apoptotic marker Annexin-V was tracked in the green channel over time and normalized to the RFP-positive breast cancer cell count (mean $\pm$ S.E.M., technical triplicates).

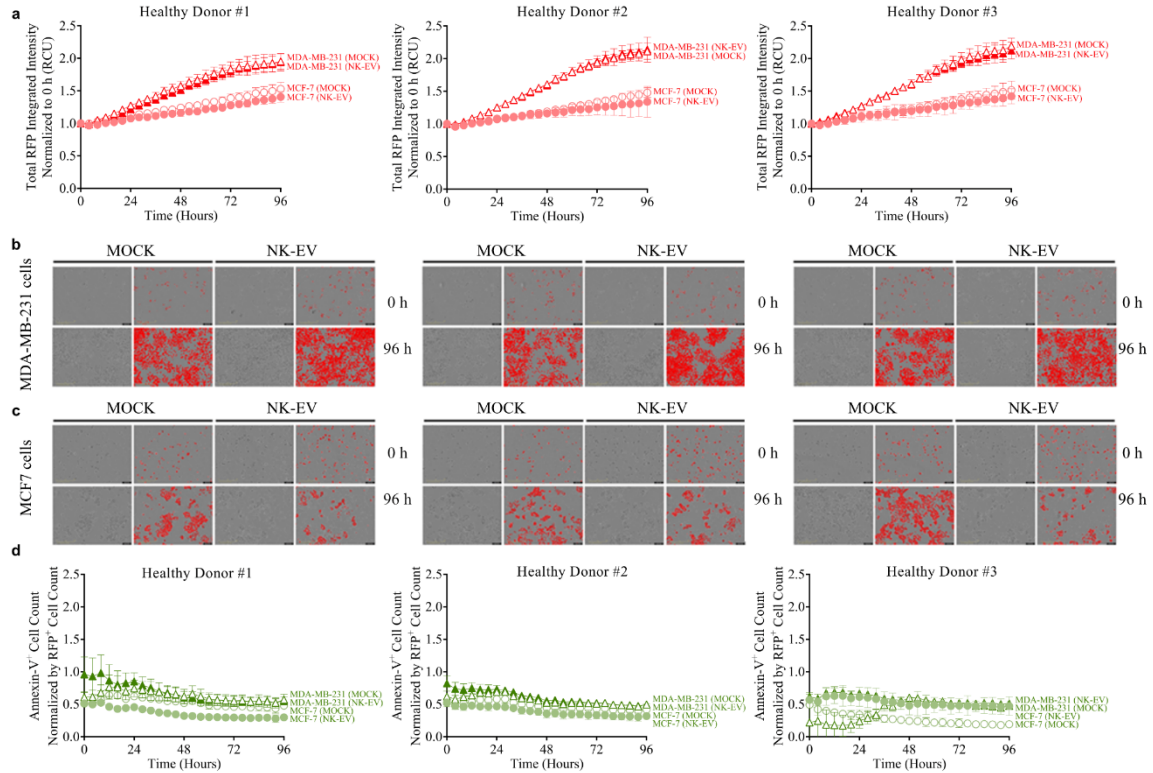


Figure 51. Live cell imaging analysis of healthy donor CD56-depleted PBMCs co-cultured with human breast cancer cell lines.

Human MDA-MB-231 and MCF-7 breast cancer cell lines constitutively expressing RFP, co-cultured with unstimulated PBMCs (MOCK) or NK-EVs-stimulated PBMCs (NK-EVs; 72 hours of stimulation) to achieve a 2:1 effector-to-target cell ratio. A) RFP expression was used to track confluence (health and viability surrogate); Representative images (before and after) in brightfield and red channels illustrate the health and viability of MDA-MB-231 (B) and MCF-7 (C) human breast cancer cells. D) Early apoptotic marker Annexin-V was tracked in the green channel over time and normalized to the RFP-positive breast cancer cell count (mean $\pm$ S.E.M., technical triplicates).

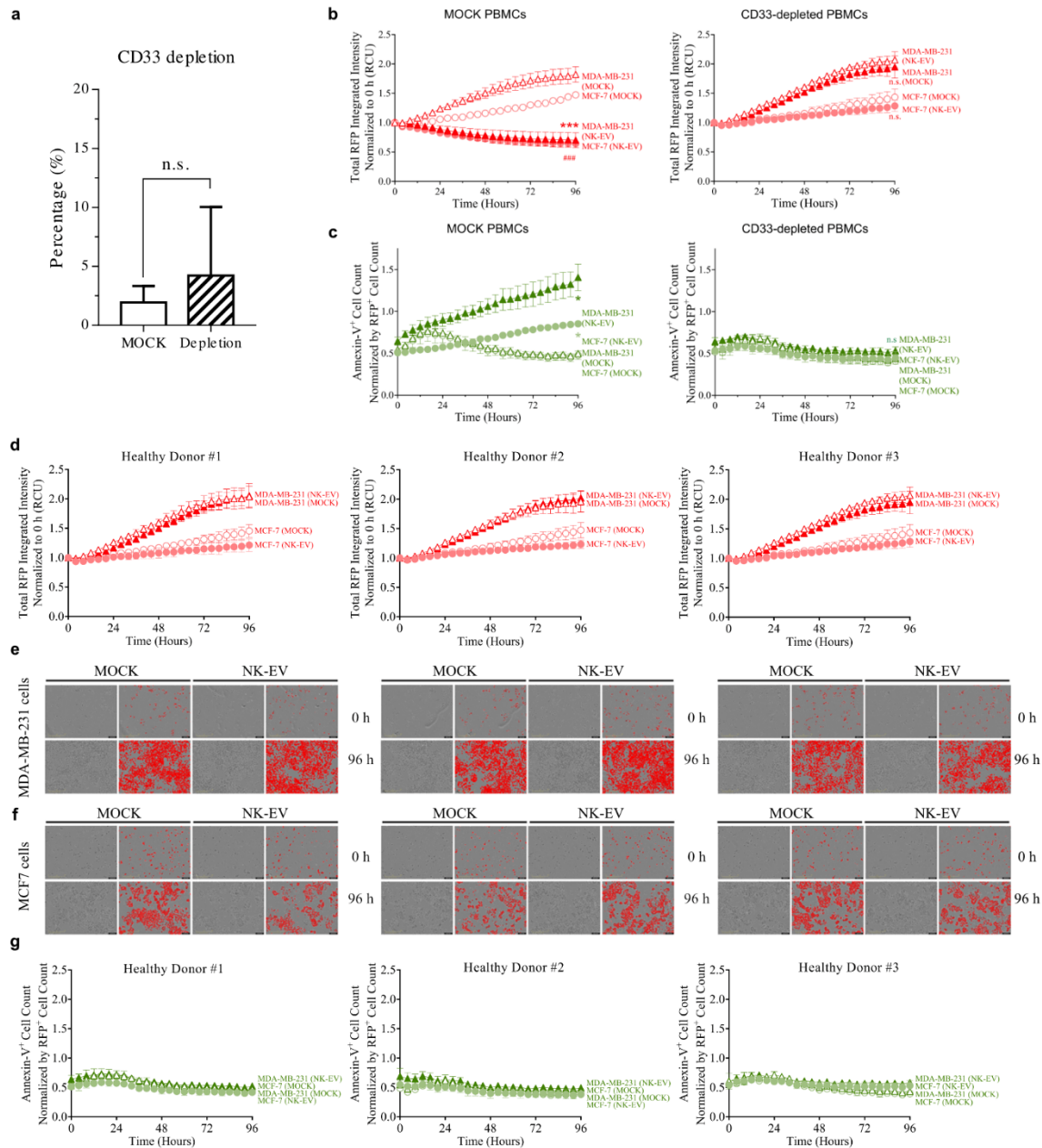


Figure 52. Live cell imaging analysis of healthy donor CD33-depleted PBMCs co-cultured with human breast cancer cell lines.

A) Immunomagnetic-depletion with flow cytometry confirmation of CD33-depleted PBMCs (mean $\pm$ S.E.M., N=3 biological replicates). Paired t-test was performed, where $p < 0.05$ (*), $p < 0.01$ (**), n.s., non significant. Human MDA-MB-231 and MCF-7 breast cancer cell lines constitutively expressing RFP, co-cultured with unstimulated PBMCs (MOCK) or NK-EVs-stimulated PBMCs (NK-EVs; 72 hours of stimulation) to achieve a 2:1 effector-to-target cell ratio. B) RFP expression was used to track confluence (health and viability surrogate) of whole PBMCs and CD33-depleted PBMCs (mean $\pm$ S.E.M., N=3 biological replicates). C) Early apoptotic marker Annexin-V was tracked in the green channel over time and normalized to the RFP-positive breast cancer cell count of whole PBMCs and CD33-depleted PBMCs (mean $\pm$ S.E.M., N=3 biological replicates). Two-way ANOVA followed by post hoc Tukey's multiple comparison was performed, where n.s., non significant; * (MDA) or # (MCF), $P < 0.05$; ** (MDA) or ## (MCF), $P < 0.01$; *** (MDA) or ### (MCF), $P < 0.001$.

P<0.001. D) Single donor RFP expression. Representative images (before and after) in brightfield and red channels illustrate the health and viability of MDA-MB-231 (E) and MCF-7 (F) human breast cancer cells. G) Early apoptotic marker Annexin-V.

CD33⁺ depletion was also attempted, but could not be validated due to the lack of a detection antibody, leaving myeloid cell removal unconfirmed (**Figure 52A**). However, their cytotoxicity profile was similar to that of PBMCs depleted of CD8⁺ or CD56⁺ subsets, potentially implicating myeloid cells in the immune cascade (**Figure 52B-C**). Endpoint imaging of tumour cell density and early apoptosis for all depletion conditions corroborates these observations (**Figure 53**).

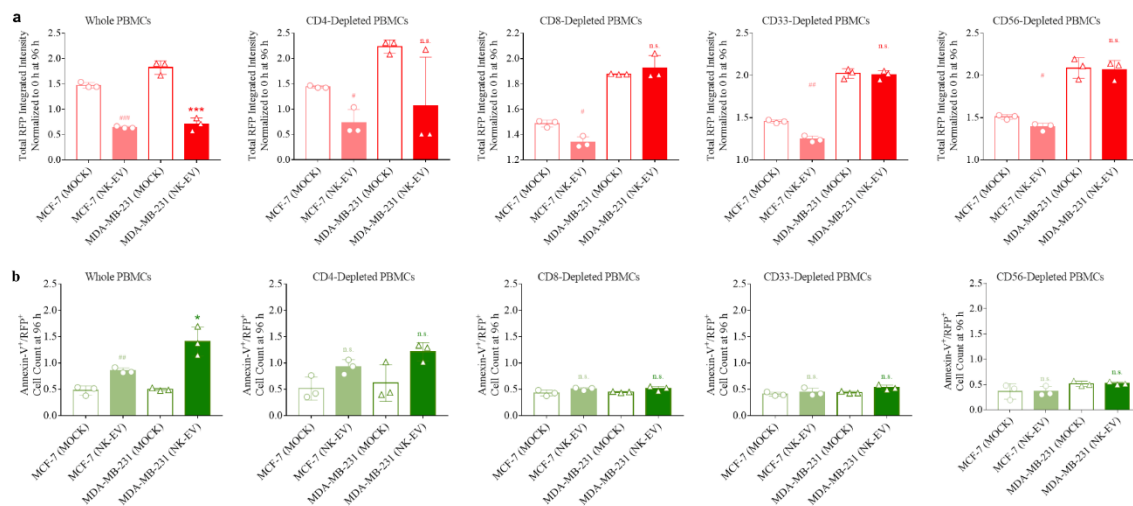


Figure 53. Endpoint analysis of live cell imaging of healthy patient donor PBMCs co-cultured with human breast cancer cell lines.

Human MDA-MB-231 and MCF-7 breast cancer cell lines constitutively expressing RFP, co-cultured with unstimulated PBMCs (MOCK) or NK-EVs-stimulated PBMCs (NK-EVs; 72 hours of stimulation) to achieve a 2:1 effector-to-target cell ratio. A) RFP expression was assessed at the 96-hour time point. B) Early apoptotic marker Annexin-V expression relative to RFP expression was assessed at the 96-hour time point (mean $\pm$ S.D., N=3 biological replicates). Welch's t-test was performed, where n.s., non significant; * (MDA) or # (MCF), P<0.05; ** (MDA) or ## (MCF), P<0.01; *** (MDA) or ### (MCF), P<0.001.

Discussion

This study demonstrates that NK-EVs, produced using a clinically relevant biomanufacturing workflow, selectively associate with T cells, NK cells, monocytes, and dendritic cells, and significantly reprogram their transcriptomes, thereby enhancing cytotoxicity against cancer cells. Notably, cytotoxic subtypes exhibited pronounced upregulation of cytotoxicity-associated genes and downregulation of exhaustion-associated genes. Live-cell imaging confirmed enhanced killing capacity of PBMCs against tumour targets, supporting the potential of NK-EVs as novel cancer immunotherapeutics capable of priming PBMCs. While the precise immunomodulatory cascade of NK-EV stimulation upon PBMCs, potentially involving a complex series of interactions between cell populations, remains unresolved, this study strongly implicates CD8⁺ T cells, CD56⁺ NK cells, and potentially CD33⁺ myeloid cells, as key mediators of these effects, which were remarkably consistent across donors. Conversely, CD4⁺ T cells appear to play a relatively minor role in the immunomodulatory cascade of NK-EVs.

Transcriptomic (scRNA-seq.) analysis and depletion experiments suggest that NK-EVs engage the lymphoid and myeloid arms of the immune system, aligning with previous reports implicating monocyte-T cell crosstalk and APC licensing by NK-EVs [62, 246, 247]. NK-EV treatment remodels the transcriptomic landscape of cytotoxic immune cells identified in Branch 1 (CD8⁺ T cells and NK cells) [248]. This clustering pattern highlights the shared transcriptional programs between these cytotoxic populations and their coordinated response to NK-EV stimulation [249]. Noticeably, *PRF1* upregulation indicates enhanced target cell membrane disruption capacity [250], *RAB27A* upregulation suggests improved cytotoxic granule delivery efficiency [251, 252], and *FASLG* upregulation indicates activation of alternative Fas-mediated death pathways [253]. Likewise, *IFNG* upregulation, on the other hand, suggests enhanced anti-tumour immunity and immune coordination capabilities [254, 255]. Notably, *CX3CR1* downregulation suggests reduced tissue residency, potentially improving tumour trafficking and systemic surveillance [256, 257].

By flow cytometry, NK-EVs induce CD4⁺/CD8⁺-monocyte conjugates, suggesting EV-driven cell-cell bridging and immune coordination. This supports a model in which NK-EVs could

promote immune-synapse formation and functional coordination between immune subsets [258]. Diversification of cytotoxic subsets, including NKT-like populations with dual regulatory and cytotoxic profiles, were also noted. These findings align with Dosil *et al.*, who reported that NK-EVs stimulate IL-2 production, suppress Treg-associated IL-10 secretion, and enhance monocyte-to-dendritic cell differentiation, driving Th1-type polarization [132]. Furthermore, cytokine upregulation is consistent with previous findings, where NK-EVs stimulating NK cells cause increased production of IFN γ and TNF α [210].

These findings establish NK-EVs as powerful immunotherapeutics capable of reprogramming immune responses across multiple effector axes. Clinically, NK-EVs deliver both cytotoxic and immunomodulatory molecules, enabling broad engagement of immune pathways within the tumour microenvironment [134]. By amplifying immune responses while remaining effective in immunosuppressive settings, NK-EVs offer a promising complement or potential alternative to cell-based immunotherapies. They may be particularly valuable in the context of “cold” tumours, such as TNBC, where immune evasion and exhaustion are prevalent. NK-EVs could also synergize with other immune-priming strategies. Whether NK-EV therapy could serve as a standalone modality remains an open and important question.

Future work should explore how individual donor immune profiles impact NK-EV efficacy, informing the development of personalized and tailored therapies. Additional work should also generate knockout-specific models of the producer NK cell line to fully elucidate the key receptors/ligands involved in the immunoregulatory cascade.

In conclusion, this is the first study to integrate scRNA-sequencing with functional live-cell imaging to demonstrate the immunomodulatory effects of clinical-grade NK-EVs in both healthy and cancer-derived immune systems. As a scalable platform, NK-EVs hold promise for expanding the therapeutic arsenal against aggressive and treatment-refractory cancers.

Chapter 5. Project Summary & Conclusion

Thesis highlight

This thesis addresses several key challenges limiting the clinical translation of EV therapeutics, including scalable manufacturing, standardized potency assessment, and mechanistic understanding of immune modulation. This thesis presents a comprehensive investigation into the scalable biomanufacturing, characterization, and functional evaluation of NK-EVs as a next-generation platform for cancer immunotherapy, with a focus on TNBC. A review article covering the general application of EVs in TNBC is presented in Appendix A. The cumulative findings address several of the major translational bottlenecks in the field, spanning from upstream production and purification to downstream functional and mechanistic validation.

In Chapter 2 (NK-EV biomanufacturing and product characterization), an optimized, clinically relevant, large-scale biomanufacturing workflow was developed to produce NK cells and NK-EVs under XF/SF/FF and antibiotic-free conditions using a closed-loop hollow-fibre bioreactor (HFB) system (published in *JEV*, 2023). This process enabled continuous, reproducible production of EVs suitable for pre-clinical and clinical-grade applications. Through rigorous characterization in accordance with MISEV guidelines, the study established a foundation for scalable NK-EV production in compliance with GMP standards. The accompanying *J. Vis. Exp* publication (2024) provides a detailed procedural framework that contributes to field-wide reproducibility and standardization (Appendix B). Additional discussion points absent from the chapter content: A central challenge in the development of EV-based therapeutics is producing sufficient vesicles with consistent quality and biological activity. While EVs can be isolated from a variety of biological sources, cell culture systems remain the most practical approach for controlled large-scale production. However, conventional two-dimensional culture systems are limited in their ability to generate clinically relevant quantities of EVs. Bioreactor-based systems, particularly HFB and other three-dimensional culture platforms, offer important advantages by increasing cell density, enabling continuous media perfusion, and improving EV harvest efficiency. These systems are therefore increasingly viewed as a critical component of scalable EV manufacturing strategies. The HFB

system used in this work was selected for several practical and biological advantages relevant to EV biomanufacturing. Compared to conventional flask-based culture systems, HFB provide a high-surface-area three-dimensional environment that more closely resembles physiological tissue architecture while enabling continuous perfusion and efficient recovery of conditioned media. These systems are also relatively affordable, scalable, and compatible with closed-loop workflows suitable for GMP-oriented manufacturing. Although this study did not experimentally evaluate specific environmental stimulation strategies, such as hypoxia or cytokine preconditioning, the confined three-dimensional culture environment and continuous media exchange may already provide a degree of physiological stimulation that supports EV secretion. In addition, the use of NK92-MI cells, which are genetically modified to produce IL-2 autonomously, eliminated the need for exogenous cytokine supplementation during large-scale culture. This feature simplifies the manufacturing workflow and reduces reliance on costly GMP-grade cytokines, which represent a significant barrier for large-scale production of immune cell-derived EVs. While the NK92MI cell line represents a practical and scalable platform for NK-EV production, translating these findings to primary NK cell-derived EVs remains an important area for future investigation. Primary NK cells present additional manufacturing challenges, including donor variability, limited expansion potential, and the requirement for a feeder system (using cancerous cells) and/or exogenous cytokines supplementation, such as IL-2 or IL-15, to maintain viability and functional activity. These cytokines, particularly in GMP-grade formulations, can significantly increase manufacturing costs and process complexity. Future work may explore engineering solutions to support large-scale primary NK cell cultures within HFB, including biomaterial coatings or feeder-free expansion strategies designed to enhance NK cell persistence and EV secretion. Addressing these challenges will be important for determining whether primary NK-EVs offer functional advantages over immortalized cell-derived products in clinical applications. Beyond biological efficacy, the successful clinical translation of EV-based therapeutics will depend on robust manufacturing infrastructure, regulatory frameworks, and sustainable commercialization strategies. Recent developments in the EV biotechnology sector illustrate both the promise and the challenges of advancing EV therapeutics toward the clinic. Large-scale production, standardized characterization

methodologies, and validated potency assays remain essential prerequisites for regulatory approval. Moreover, the financial and operational demands of building EV manufacturing pipelines underscore the importance of realistic development strategies and robust translational infrastructure. The scalable production workflows and functional potency assays developed in this thesis represent important steps toward addressing these challenges. Still, continued collaboration among academic laboratories, industry partners, regulatory agencies, and government will be required to realize the clinical potential of EV-based products fully. Beyond manufacturing and purification, the long-term preservation of EV products represents an additional translational bottleneck. Current storage strategies typically rely on ultra-low temperature conditions (-80°C) to maintain vesicle integrity and bioactivity, which may complicate large-scale distribution and clinical deployment. Consequently, improved preservation methods, including lyophilization-based approaches and optimized cryoprotectant formulations, are actively being explored. The establishment of robust quality control frameworks is another essential requirement for the clinical translation of EV therapeutics. Like other biological products, EV-based therapies must be evaluated according to well-defined critical quality attributes (CQAs), including particle concentration, size distribution, vesicle imaging, purity assessment, molecular composition, and functional potency. In addition, rigorous testing for microbial contamination, endotoxin levels, and batch-to-batch consistency is required to meet regulatory standards. Importantly, functional potency assays that reliably quantify EV biological activity are a key component of this quality-by-design framework. The development of sensitive and reproducible assays, therefore, plays a critical role in enabling standardized product release criteria for EV therapeutics.

Building upon the work established in Chapter 2, Chapter 3 (NK-EV cytotoxicity assessment) focused on the development and validation of a highly sensitive cytotoxicity potency assay for NK-EVs using the resazurin phenoxazine dye (published in *JExBio*, 2024). This work addressed a critical gap in the quantitative assessment of NK-EV bioactivity by demonstrating that the resazurin-based assay provides superior sensitivity, linearity, and dynamic range compared to several other conventional cell viability assays. Additionally, this assay was shown to be stability-indicating, a crucial requirement for NK-EV therapeutic assessment. This study's findings lay the

groundwork for standardizing potency measurements for NK-EV-based therapeutics and for establishing reliable lot-release criteria for manufacturing and regulatory evaluation. An accompanying manuscript (Appendix C) further assesses NK-EVs' cytotoxicity using clinically relevant TNBC models and provides additional mechanistic insight into their direct killing mechanism. Additional discussion points absent from the chapter content: An important conceptual distinction between NK cells and NK-EVs concerns the nature of their cytotoxic capacity. While NK cells can perform serial killing through repeated formation of immunological synapses and release of cytotoxic granules, EVs are finite biological packages containing limited functional cargo. Each vesicle delivers a discrete payload of proteins, lipids, and nucleic acids that can initiate cytotoxic signaling within target cells. Still, unlike living immune cells, EVs cannot replenish or regenerate these components. This limitation is reflected in the relatively high particle-to-cell ratios required to achieve measurable cytotoxic effects *in vitro*. While this does not preclude therapeutic application, it highlights the importance of scalable biomanufacturing and efficient delivery strategies. Importantly, the nanoscale nature of EVs allows for higher particle numbers than cell-based therapies, suggesting that dosing strategies may compensate for the finite payload of individual vesicles. While this may require repeated EV dosing to maintain effective therapeutic concentrations, it may also offer an important clinical advantage by providing physicians with greater control over treatment exposure. Unlike living cell therapies, EVs represent finite biological packages whose activity diminishes over time, potentially reducing the risk of uncontrolled immune hyperactivation. This pharmacological controllability may be particularly valuable given the immune-related toxicities historically observed during early development of potent immunotherapies such as IL-2 treatment and CAR-T cell therapy. Furthermore, the detection of cytokines such as IL-6 and IL-10 within the NK-EV cargo represents an intriguing and somewhat unexpected observation that warrants further investigation. These cytokines are commonly associated with immunoregulatory or anti-inflammatory functions, raising questions about their role within NK-EV-mediated immune responses. One possibility is that the presence of these molecules contributes to a balanced immune-modulatory profile that prevents excessive inflammatory responses while still promoting cytotoxic immune activation. Alternatively, these cytokines may

reflect broader regulatory programs present within NK cells themselves, which can produce both pro-inflammatory and immunomodulatory signals depending on activation state and environmental context. Determining whether these cytokines actively contribute to NK-EV function (cytotoxic or immunomodulatory) or serve as passive cargo components will require future mechanistic studies using targeted depletion or neutralization strategies. Additionally, future studies should perform more comprehensive profiling of cytokines within NK-EVs and assess whether upstream influences (*e.g.*, xyz stimulation) can modulate the NK-EVs' biomolecular profile to regulate downstream function. Such investigations may help clarify the signaling networks through which NK-EVs influence immune cell populations in the blood circulation or within the tumour microenvironment. Although the resazurin-based cytotoxicity assay developed in this work demonstrated strong sensitivity and reproducibility within a single laboratory setting, broader validation will be required to support its adoption as a standardized potency assay for NK-EV therapeutics. Inter-laboratory variability represents a well-recognized challenge in EV research due to differences in instrumentation, cell culture conditions, and EV quantification methods. Future studies should therefore aim to evaluate the assay across multiple laboratories and experimental platforms to establish reproducibility under diverse conditions. The adoption of EC₅₀-based reporting may further facilitate cross-study comparisons by providing a normalized metric of NK-EV potency that is less dependent on absolute particle counts or experimental setup. Such standardization will be important for enabling regulatory evaluation and ensuring consistent product characterization across manufacturing sites.

Building upon the methods established in Chapter 2, Chapter 4 (NK-EV immunomodulation assessment) explored the immunomodulatory capacity of NK-EVs through co-culture assays with human peripheral blood mononuclear cells (PBMCs) and tumour targets. Comprehensive flow cytometry, live-cell imaging assays (with/without depletion/enrichment conditions), and transcriptomic analyses by single-cell RNA sequencing (scRNA-seq) revealed that NK-EVs reprogrammed immune responses by enhancing NK cell and CD8⁺ T cell activation and increasing tumour cytotoxicity. Depletion experiments, combined with live cell imaging assays, further suggest that CD33⁺ myeloid populations may act as early intermediaries in this process, potentially

amplifying NK-EV signals and coordinating downstream activation of cytotoxic lymphocytes. These findings demonstrated that NK-EVs possess intrinsic tumouricidal properties and act as immunological adjuvants, modulating the tumour microenvironment. Together, these results highlight NK-EVs as multifaceted biological effectors that bridge innate and adaptive immunity. Additional discussion points absent from the chapter content: Although these findings clearly demonstrate the immunomodulatory capacity of NK-EVs, the precise molecular mechanisms governing immune reprogramming remain incompletely defined and likely involve a complex cascade of receptor-ligand interactions and cytokine-mediated signaling between multiple immune cell populations. Interestingly, NK-EV treatment was associated with an increased frequency of CD4⁺/CD8⁺ T cell-monocyte conjugates, suggesting that EV-mediated signalling may influence immune cell interactions and coordination. However, the functional significance of these observations remains unclear. It will require further investigation to determine whether NK-EVs actively promote immune cell bridging or alter activation states that increase transient cell-cell contacts. Future studies using humanized mouse models will therefore be essential to evaluate these interactions *in vivo* and determine how NK-EV-mediated immune reprogramming contributes to tumour control within a physiologically relevant tumour microenvironment. Future studies should also employ targeted transcriptomic perturbation approaches targeting candidate signaling pathways to identify the molecular regulators responsible for NK-EV-mediated immune reprogramming. Such experiments would enable systematic dissection of receptor-ligand interactions and downstream signaling cascades that drive activation of cytotoxic lymphocytes and myeloid intermediates. Complementary studies further strengthened this thesis's contribution to EV science. These methodological advancements offer tools for broader EV research, enabling more accurate structure-function correlations and improved reproducibility across laboratories. Work on the EV protein corona, an emerging topic in the field at the time of writing, elucidated how different purification methods, such as ultrafiltration and ultracentrifugation, differentially affect corona integrity, payload retention, and biological activity, advancing understanding of how bioprocessing parameters influence therapeutic potency (Appendix D). The findings regarding the EV biomolecular corona further emphasize how upstream and downstream processing steps can

influence EV therapeutic function. Like synthetic nanoparticles, EVs interact dynamically with proteins present in biological fluids, forming a biomolecular corona composed of both tightly bound "hard corona" proteins and more transient "soft corona" layers. These interactions may alter EV surface properties, receptor engagement, biodistribution, and ultimately biological activity. The observation that ultracentrifugation-based purification disrupted corona-associated proteins and abrogated NK-EV function highlights the importance of preserving native EV surface composition during manufacturing. In contrast, gentler purification approaches, such as TFF and SEC, appear to preserve corona integrity and functional activity better, and are also more scalable than ultracentrifugation. Furthermore, purification strategies must balance EV yield, purity, and preservation of functional integrity. While ultracentrifugation remains widely used in research settings, scalable technologies such as TFF are increasingly favoured for industrial production due to their linear scalability, compatibility with automation, and reduced mechanical stress on vesicles. Future studies should investigate how EV coronas evolve following systemic administration, particularly in plasma environments rich in albumin and immunoglobulins, as these interactions may strongly influence pharmacokinetics and tissue targeting.

Moreover, work on a robust imaging standard for EV visualization, improving morphological assessment, demonstrated that an optimized positive-staining TEM protocol using Uranylless and lead citrate was optimized, while minimizing artifacts (Appendix E). Because EVs inherently have low electron density, their visualization by TEM often requires contrast-enhancing staining. Positive staining directly binds heavy metal salts to vesicle structures, enhancing membrane contrast and facilitating reliable identification of nanoscale vesicles while preserving structural integrity. Establishing reproducible staining protocols is therefore critical for accurate EV morphological characterization and helps reduce variability in TEM-based EV analysis across laboratories.

Additional ongoing studies, at the time of writing, are addressing several additional aspects of NK-EV biology, including: 1) stability (*i.e.*, how long do they remain stable?), 2) NK-EV therapeutic potential with other drugs (*i.e.*, do they synergize with other cancer therapeutics?), 3)

NK-EV therapeutic potential across various cancer models (*i.e.*, platform versatility) and 4) upstream manufacturing modifications to modulate the NK-EV biomolecular profile.

NK-EV therapeutics may ultimately be best positioned within combinatorial treatment strategies rather than as standalone monotherapies. Modern oncology increasingly relies on multi-modal therapeutic approaches combining surgery, chemotherapy, radiation therapy, and immunotherapy to maximize tumour control while minimizing resistance mechanisms [1]. Within this landscape, NK-EVs could serve as a versatile immunological adjunct, enhancing anti-tumour immune responses while directly targeting malignant cells. Their intrinsic cytotoxic cargo and immune-modulatory capacity suggest potential synergy with other immunotherapies. By enhancing NK cell and CD8⁺ T cell activity, NK-EVs may help overcome immune suppression within the tumour microenvironment and improve responses to checkpoint blockade. For example, NK-EVs could complement adoptive cellular therapies such as CAR-T/CAR-NK, or tumour-infiltrating lymphocyte (TIL) therapies by priming tumour cells for immune-mediated killing or by modulating surrounding immune populations. From a clinical positioning standpoint, NK-EVs may therefore be explored both as first-line immunomodulatory agents to enhance anti-tumour immunity and as second-line adjuvants to overcome resistance to established therapies. Future clinical trial designs should evaluate both contexts to determine where NK-EV therapy provides maximal benefit.

While this thesis focused primarily on proteomic characterization and functional assessment of NK-EVs, additional molecular layers, such as lipidomic, metabolomic, and transcriptomic profiling, were not explored and represent important avenues for future work better to understand the full biological complexity of NK-EV cargo. Collectively, this body of work establishes a robust framework for the clinical translation of NK-EV-based therapeutics, combining scientific and engineering rigour, functional validation, and mechanistic insight. By integrating biomanufacturing scalability with molecular and immunological evaluations, this thesis bridges the gap between fundamental EV biology and translational applications in cancer immunotherapy.

Significance and future directions

The findings presented in this thesis hold significant implications for the future of cell-free immunotherapy and EV-based biotherapeutics. The developed workflow and characterization methodologies provide a reproducible, GMP/MISEV-aligned foundation that can accelerate the clinical deployment of NK-EVs and other immune-derived EV products. Importantly, integrating bioprocess optimization with functional and immunological assays establishes a comprehensive quality-by-design framework that can be extended to other EV-producing cell types, including T cells and mesenchymal stem cells (MSCs).

The demonstration of dual NK-EV activity, direct cytotoxicity and immune reprogramming positions these vesicles as promising off-the-shelf therapeutics capable of complementing or replacing traditional NK cell therapies. Their nanoscale size, stability, and intrinsic safety profile further support their potential to overcome the limitations of cell-based products, such as manufacturing variability, storage constraints, immune rejection, and patient burden (if applicable, *i.e.*, lymphodepletion is not required for EV-based treatment). Future work should aim to translate these preclinical findings into *in vivo* and clinical contexts, focusing on three main axes:

1. Mechanistic elucidation: employing multi-omics and proteomic profiling to uncover the precise signaling networks and EV cargo responsible for immune modulation and tumour cytotoxicity. Now, there is sufficient evidence to support the clinical trial phase I requirements for mechanistic understanding of this therapeutic approach (*i.e.*, induction of apoptosis in cancerous cells and immunoregulation of cytotoxic immune cells). Still, the precise mechanism remains to be elucidated.
2. Pre-clinical assessment using the appropriate genetic background: While NK-EVs demonstrated potent function *in vitro*, a preclinical comprehensive evaluation remains. Considering the profound immunodeficiency of NSG mice, which lack functional T, B, and NK cells, as well as defective macrophage and dendritic cell activity, such a model would provide limited biological context and may therefore be insufficient for assessing part of the NK-EV's function. Future studies should employ humanized mouse models

capable of partially reconstituting human immune components, including T and NK cells, cytokine networks, and tumour-associated immune populations. Such systems would enable clinically-relevant mechanistic assessment of NK-EV immunoregulatory functions and their contribution to long-term tumour control beyond direct cytotoxicity. Beyond immune system reconstruction, future *in vivo* studies should also consider patient-derived xenograft (PDX) tumour models in combination with humanized immune systems. Humanized PDX models preserve tumour heterogeneity and clinically relevant tumour microenvironment features while enabling interactions with human immune cells. Such systems may provide a more accurate representation of how NK-EVs influence tumour-immune dynamics *in vivo*. In addition, biodistribution and pharmacokinetic studies remain an important area of investigation. Although EV accumulation in tumours has been observed in several preclinical models through mechanisms such as the EPR effect, the proportion of injected vesicles that ultimately reach tumour tissue and the resulting biodistribution dynamics remain poorly defined. Quantifying EV delivery efficiency and per-cell uptake *in vivo* will be essential for rational dose design and clinical translation.

3. Clinical integration and combinatorial strategies: evaluating NK-EVs as an adjuvant to other therapeutic modalities, such as ICIs, CAR-T/CAR-NK or TILs therapies, and chemotherapeutic regimens, with emphasis on hard-to-treat cancers such as TNBC.

The clinical translation of EV-based therapeutics will ultimately depend not only on biological efficacy but also on robust manufacturing infrastructure and regulatory readiness. Recent developments in the EV biotechnology sector have demonstrated both the promise and the challenges of developing EV-based therapeutics at an industrial scale. Ensuring reproducible large-scale production, standardized potency assays, and well-defined product characterization will be critical for regulatory approval and clinical adoption.

In conclusion, this thesis advances both the scientific understanding and translational readiness of NK-EVs as cancer biotherapeutics. By integrating scalable, GMP-aligned biomanufacturing, validated functional potency assessment, and mechanistic immune profiling, this work demonstrates how EV-based therapies can be developed within a rigorous translational framework. Collectively, these findings support positioning NK-EVs as a credible and clinically relevant class of cell-free immunotherapeutics for cancer treatment.

Chapter 6. Appendix

Several complementary studies were conducted during this doctoral research. While these investigations contributed to the broader scientific context of this work or were initiated later because of insights and methodological advances generated from the studies presented in the preceding chapters, they are not central to the primary objectives addressed in the main chapters of this thesis. To maintain clarity and focus on the core findings presented herein, these studies are not discussed in detail in the main text. They are instead provided in the appendices for reference. The appendix includes only studies that were published or under consideration for publication at the time of writing. Other studies that remain ongoing or in preparation at the time of writing are therefore not included.

Appendix list:

- Appendix A: Review Article - Applications of Extracellular Vesicles in Triple-Negative Breast Cancer.
- Appendix B: Original Research Article - Scalable Biomanufacturing Workflow to Produce and Isolate Natural Killer Cell-derived Extracellular Vesicle-based Cancer Biotherapeutics.
- Appendix C: Original Research Article - Natural Killer Cell-Derived Extracellular Vesicles Exhibit Cytotoxicity Against Bulk Tumour Cells and Cancer Stem Cells in Triple-Negative Breast Cancer.
- Appendix D: Original Research Article - Ultracentrifugation and ultrafiltration differentially alter the composition and functionality of the extracellular vesicles' biomolecular corona.
- Appendix E: Original Research Article - An Optimized Positive Staining Protocol Method for Clear Visualization of Extracellular Vesicles by TEM Using Uranylless and Lead Citrate.

Appendix A: Review Article - Applications of Extracellular Vesicles in Triple-Negative Breast Cancer

Preface: This manuscript represents my early work when I began my thesis and is my first-author publication.

Citation: St-Denis-Bissonnette, Frederic et al. "Applications of Extracellular Vesicles in Triple-Negative Breast Cancer." *Cancers* vol. 14,2 451. 17 Jan. 2022, doi:[10.3390/cancers14020451](https://doi.org/10.3390/cancers14020451).

Abstract: Triple-negative breast cancer (TNBC) is the most aggressive and refractory subtype of breast cancer, often occurring in younger patients with poor clinical prognosis. Given the current lack of specific targets for effective intervention, the development of better treatment strategies remains an unmet medical need. Over the last decade, the field of extracellular vesicles (EVs) has grown tremendously, offering immense potential for clinical diagnosis/prognosis and therapeutic applications. While TNBC-EVs have been shown to play important roles in tumourigenesis, chemoresistance, and metastasis, they can be repurposed as biomarkers for TNBC diagnosis and prognosis. Furthermore, EVs from various cell types can be utilized as nanoscale drug delivery systems (NDDS) for TNBC treatment. Remarkably, EVs generated from specific immune cell subsets have been shown to delay solid tumour growth and reduce tumour burden, suggesting a new immunotherapy approach for TNBC. Intrinsically, EVs can cross the blood-brain barrier (BBB), which holds great potential for treating brain metastases diagnosed in one-third of TNBC patients, a substantial clinical challenge. In this review, we present the most recent applications of EVs in TNBC as diagnostic/prognostic biomarkers, nanoscale drug delivery systems and immunotherapeutic agents, as well as discuss the associated challenges and future directions of EVs in cancer immunotherapy.

Appendix B: Original Research Article - Scalable Biomanufacturing Workflow to Produce and Isolate Natural Killer Cell-derived Extracellular Vesicle-based Cancer Biotherapeutics

Preface: Due to the significant interest in the biomanufacturing publication [105]. A protocol-based study (video) was subsequently published in the Journal of Visualized Experiments. The following article is a protocol-based study that illustrates a scalable biomanufacturing workflow and demonstrates the methodology for isolating NK-EVs, as discussed in **Chapter 2**, for which I am the primary author. This article was published in the *J. Vis. Exp* in 2024 and complements the manuscript detailed in **Chapter 2**, showcasing the isolation workflow on NK-EVs in great detail.

Citation: St-Denis-Bissonnette, F., Kirkby, M., Wang, L., Lavoie, J. R. (2024). Scalable Biomanufacturing Workflow to Produce and Isolate Natural Killer Cell-derived Extracellular Vesicle-based Cancer Biotherapeutics. *J. Vis. Exp.*, 210, e67227, <https://doi.org/10.3791/67227>.

Contributions: All authors conceived the idea. FSTDB performed the Hollow-Fibre Bioreactor EV production and cell expansion. FSTDB and MK performed the NTA experiments and analyses. FSTDB performed the EV cytotoxicity assay, and all authors contributed to the cytotoxicity analyses. FSTDB performed the statistical analyses. All authors contributed general intellectual input to the experimental design, participated in the data analysis, and discussed the results. All authors wrote the manuscript and assembled the figures. All authors reviewed and edited the manuscript and approved its final version.

Abstract: Natural killer cell-derived extracellular vesicles (NK-EVs) are being investigated as cancer biotherapeutics. They possess unique properties as cytotoxic nanovesicles targeting cancer cells and as immunomodulatory communicators. A scalable biomanufacturing workflow enables the production of large quantities of high-purity NK-EVs to meet the preclinical and clinical demands. The workflow employs a closed-loop hollow-fibre bioreactor, enabling continuous production of NK-EVs from the NK92-M1 cell line under serum-free, xeno-free, feeder-free, and antibiotic-free conditions, in compliance with Good Manufacturing Practices. This protocol-driven

study outlines the biomanufacturing workflow for isolating NK-EVs using size-exclusion chromatography, ultrafiltration, and filter-based sterilization. Essential NK-EV product characterization is performed via nanoparticle tracking analysis, and their functionality is assessed through a validated cell viability-based potency assay against cancer cells. This scalable biomanufacturing process holds significant potential to advance the clinical translation of NK-EV-based cancer biotherapeutics by adhering to best practices and ensuring reproducibility.

Appendix C: Original Research Article - Natural Killer Cell-Derived Extracellular Vesicles Exhibit Cytotoxicity Against Bulk Tumour Cells and Cancer Stem Cells in Triple-Negative Breast Cancer

Preface: The following article was conducted to investigate the therapeutic efficacy of NK-EVs in TNBC, for which I am a co-first author. It evaluates the cytotoxic and mechanistic effects of NK-EVs across clinically relevant TNBC models, including patient-derived xenografts, with emphasis on targeting both bulk tumour cells and cancer stem cells. This study provides key translational insights into NK-EV-based immunotherapeutics and complements the mechanistic work presented in **Chapter 3**.

Citation: Kirkby, M. and St-Denis-Bissonnette, F., Mediratta, K., Diab, M., Korobkow, A., Humber, J., Han, P., Muradia, G., Ardolino, M., Lee, S.-H., Gibbins, D., Burger, D., Wang, L., Lavoie, J. R. Natural Killer Cell-Derived Extracellular Vesicles Show Cytotoxicity Against Bulk Tumour Cells and Cancer Stem Cells Found in Triple-Negative Breast Cancer. Under review in an undisclosed journal.

Contributions: MK, FSTDB, LW and JRL conceptualized the project. MK and FSTDB designed the experiments. FSTDB and GM generated the NK-EV material. MK and FSTDB performed all cell line-based experiments, with assistance from MD, AK, and JH. MK performed all patient-derived xenograft (PDX) experiments, with assistance from MD, AK, and PH. MK and FSTDB performed data visualization. KM performed Incucyte experiments. MK, FSTDB, LW and JRL managed the project. MK, FSTDB, LW and JRL wrote the manuscript. All authors read and approved the manuscript.

Abstract: Triple-negative breast cancer (TNBC) remains a significant challenge in oncology, contributing to a significant portion of cancer-related deaths among women. Current therapeutic options exhibit limited efficacy, necessitating the exploration of innovative treatment modalities. The frequent emergence of drug resistance and the persistence of cancer stem cells (CSCs), a subpopulation of cancer cells associated with tumour relapse and metastasis, further

complicate disease management. In this context, natural killer cell-derived extracellular vesicles (NK-EVs) have emerged as a promising cell-free, off-the-shelf therapeutic approach with the potential to overcome these limitations. NK-EVs were produced using a bioreactor-based workflow. The efficacy of NK-EVs as a therapeutic strategy for TNBC was assessed using TNBC cell lines and clinically relevant TNBC patient-derived xenograft (PDX) organotypic slice cultures. NK-EV cytotoxicity was evaluated using a resazurin-based cell viability assay, alongside mechanistic assays. NK-EV-treated CSC populations were evaluated using tumour sphere formation assays, RT-qPCR, and live-cell imaging. NK-EV treatment rapidly induced a dose-dependent cytotoxic response in TNBC cells, characterized by apoptotic cell death via canonical caspase activation and concurrent necrotic events. These cytotoxic effects were further validated in three-dimensional TNBC PDX organotypic slice cultures. Moreover, NK-EVs suppressed tumorsphere formation and Hippo-YAP gene expression in TNBC cell lines. Consistently, NK-EV treatment increased the expression of apoptosis-associated markers in both CD44^{high}/CD24^{low} CSCs and non-CSCs populations. These findings highlight the therapeutic efficacy and translational potential of NK-EV-based interventions in TNBC and offer valuable insights for the rational design of next-generation immunotherapeutic strategies against this aggressive breast cancer subtype.

Appendix D: Original Research Article - Ultracentrifugation and ultrafiltration differentially alter the composition and functionality of the extracellular vesicles' biomolecular corona

Preface: The following article was conducted to investigate how purification methods influence the structural and functional integrity of NK-EVs following enzymatic de-coronization, for which I am the primary author. This article is under revision by the Journal of Extracellular Biology (JExBio) and complements the manuscript detailed in Chapters 2 and 3.

Citation: St-Denis-Bissonnette, F., Kirkby, Halabi, H., Creskey, M., Zhang, X., Burger, D., Wang, L., Lavoie, J. R. Ultracentrifugation and ultrafiltration differentially alter the composition and functionality of the extracellular vesicles' biomolecular corona. Accepted and under publication by JExBio (Impact Factor 4.0).

Contributions: FSTDB, LW and JRL conceptualized the project. FSTDB designed the experiments. FSTDB generated the NK-EV material and did all experiments. HH assisted with the MACSPLEX experiment. HH and MC performed the mass spectrometry experiment and data analyses. FSTDB performed data visualization. FSTDB, LW and JRL managed the project. FSTDB, LW and JRL wrote the manuscript. All authors read and approved the manuscript.

Abstract: Extracellular vesicles (EVs) are increasingly recognized for their diagnostic and therapeutic potential. An emerging important concept in EV biology is the EV biomolecular corona, a dynamic layer of absorbed biomolecules, such as proteins, to the EV membrane that influences EV behaviour, cellular uptake, biodistribution and function. A key challenge involves identifying effective purification methods to selectively and reliably remove enzymatically digested surface proteins in de-coronization studies. This study assesses the effectiveness of ultracentrifugation (UC) and ultrafiltration (UF) purification techniques following proteinase K (PK) treatment of pre-isolated natural killer cell-derived EVs (NK-EVs). The efficiency and specificity/selectivity of these purification methods in removing cleaved proteins while preserving EV physical properties (size), biomolecular profile (surface immunophenotyping, cytokine and total protein profiling) and

cytotoxicity against cancer cells were evaluated. While both methods achieve comparable NK-EV particle recovery, only UF purification selectively removed PK-cleaved proteins, as indicated by the protein-to-particle ratio, biomolecular profiling, and NK-EV cytotoxicity after PK treatment. In contrast, UC purification caused nonspecific disruption of the corona, dramatically reducing cytokine payloads and NK-EV cytotoxicity. Interestingly, NTA and TEM (positive staining) analyses showed that de-coronized EVs were smaller than their untreated counterpart. This finding suggests that PK treatment affects surface-associated proteins with varying sensitivity to proteolysis, an effect that remained consistent regardless of the subsequent purification methods. Collectively, these results highlight UF as the purification method of choice for controlled de-coronization studies, potentially supporting advances in EV research across multi-omics, investigative, and preclinical applications.

Appendix E: Original Research Article - An Optimized Positive Staining Protocol Method for Clear Visualization of Extracellular Vesicles by TEM Using Uranylless and Lead Citrate

Preface: The following article was conducted to refine imaging methodologies supporting the structural characterization of EVs, for which I am the primary author. It presents an optimized positive-staining protocol for transmission electron microscopy (TEM) that enhances image clarity, reproducibility, and interpretability across EV research applications. Published by the *Journal of Langmuir* and complements the manuscript detailed in **Chapter 2**.

Citation: St-Denis-Bissonnette, F., Ngo, B., Halabi, H., Korobkow, A., Wang, L., Lavoie, J. R. An Optimized Positive Staining Protocol Method for Clear Visualization of Extracellular Vesicles by TEM Using Uranylless and Lead Citrate. *Langmuir* 2026 42 (1), 313-321. DOI: [10.1021/acs.langmuir.5c03977](https://doi.org/10.1021/acs.langmuir.5c03977).

Contributions: F. St-Denis-Bissonnette, L. Wang and J.R. Lavoie conceptualized the project. F. St-Denis-Bissonnette designed the experiments. F. St-Denis-Bissonnette generated the NK-EV material. B. Ngo, H. Halabi, A. Korobkow and J. Wang performed the TEM experiments. F. St-Denis-Bissonnette, B. Ngo, H. Halabi, and A. Korobkow performed data visualization. F. St-Denis-Bissonnette, L. Wang and J.R. Lavoie managed the project. F. St-Denis-Bissonnette, B. Ngo, H. Halabi, A. Korobkow, L. Wang and J.R. Lavoie wrote the manuscript. All authors read and approved the manuscript.

Abstract: Transmission electron microscopy (TEM) is widely used for visualizing extracellular vesicles (EVs) due to its nanometer-scale resolution. However, existing sample preparation protocols often lack sufficient methodological detail, requiring researchers to rely on trial-and-error approaches to achieve high-quality images. Here, we report an optimized positive-staining method that utilizes the sequential application of Uranylless and lead citrate with an intermediate buffered wash. This approach yields high image contrast, preserves EV morphology, and significantly reduces background artifacts. Staining specificity and elemental localization were

confirmed using Energy-Dispersive X-ray Spectroscopy (EDS). The protocol is reproducible and robust, offering a reliable framework for EV structural analysis and potentially serving as a reference standard for TEM-based EV imaging.

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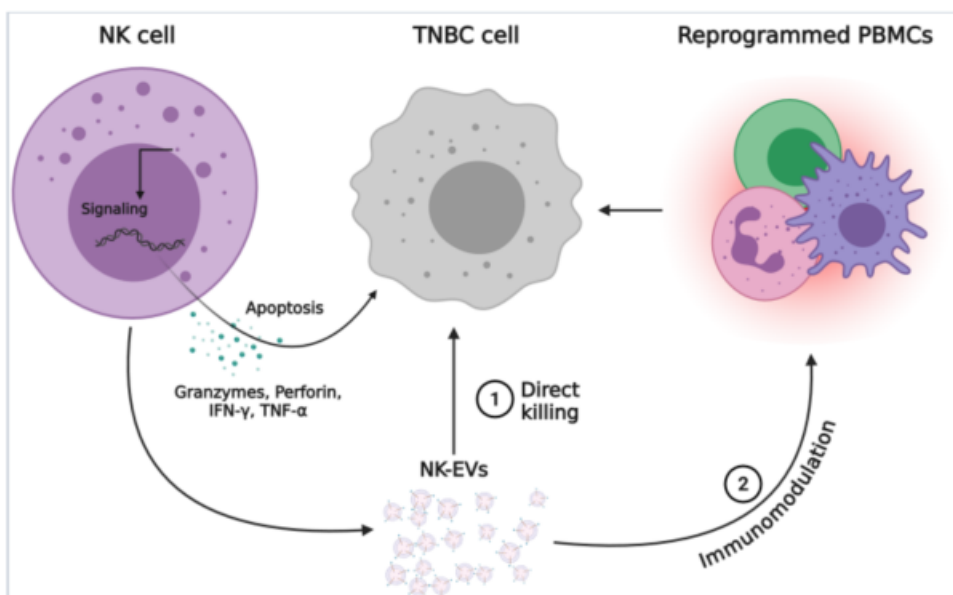
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Chapter 8. Glossary

Adaptive Immunity: Immune response characterized by antigen specificity and memory, mediated by T and B lymphocytes.

Apoptosis: A form of programmed cell death that occurs in multicellular organisms, characterized by cell shrinkage, chromatin condensation, and DNA fragmentation. It is a controlled mechanism often triggered by damage or stress.

Apoptotic Bodies: A subtype of extracellular vesicles (1-5 μm) released during programmed cell death (apoptosis), containing fragments of cytoplasm, organelles, and nuclear material.

Benign: An adjective used to describe growths which do not infiltrate into surrounding tissues.

Biomanufacturing: The process of producing biological products such as cells, proteins, or extracellular vesicles using controlled, scalable systems under defined quality and regulatory standards.

Bioreactor: A device or system that supports a biologically active environment, typically used for growing cells or tissues in the context of cell culture, tissue engineering, or biochemical processes.

Blood-Brain Barrier: A selective barrier formed by endothelial cells that protects the brain from circulating toxins and pathogens while regulating the transport of molecules. It often limits drug delivery to the brain.

Breast cancer: A type of cancer that originates in the cells of the breast. It is the most common cancer in women worldwide and can occur in both men and women, although it is rare in men.

Cancer: A group of diseases characterized by uncontrolled cell growth and the potential to invade or spread to other parts of the body.

Cancer stem cells (CSCs): A subpopulation of cancer cells with the ability to self-renew, differentiate, and sustain the growth of the tumour. They are thought to be responsible for cancer recurrence and resistance to conventional therapies.

Cell culture media: A nutrient-rich solution used to support the growth and maintenance of cells *in vitro*. It typically contains a mixture of salts, amino acids, vitamins, glucose, and serum.

Conditioned cell culture media: Cell culture media that have been used to grow cells and subsequently contain cell-secreted factors, including extracellular vesicles, which can influence the behaviour of other cells.

Cytokine: Small signaling proteins secreted by immune and other cells that regulate inflammation, immunity, and cell communication. Examples include interferons, interleukins, and tumour necrosis factors.

Cytotoxicity: The ability to kill cells, commonly used to assess the therapeutic potential of cancer treatments.

Cytolytic Granules: Specialized secretory vesicles in cytotoxic lymphocytes, such as NK and CD8⁺ T cells, containing perforin and granzymes that induce apoptosis in target cells.

Dendritic cells: Immune cells that function as antigen-presenting cells. They process antigen material and present it on their surface to T cells, initiating an immune response.

Ectosomes (Microvesicles): A subtype of extracellular vesicles (100-1000 nm) formed by outward budding of the plasma membrane. They differ from exosomes in their biogenesis pathway.

Enhanced Permeability and Retention Effect: A phenomenon in which nanoparticles and vesicles preferentially accumulate in tumour tissues due to leaky vasculature and poor lymphatic drainage.

Extracellular vesicles: Small membrane-bound particles released from cells that facilitate intercellular communication by transferring proteins, lipids, and nucleic acids between cells.

Exosomes: A subtype of extracellular vesicles (30-150 nm in diameter) formed within endosomal compartments and released upon fusion with the plasma membrane.

Good Manufacturing Practice: A system of regulations and guidelines that ensure the quality, safety, and consistency of pharmaceutical and biotechnological products during production.

Granzymes: Serine proteases released by cytotoxic lymphocytes that enter target cells through perforin pores and induce apoptosis.

Immunomodulation: The alteration of the immune response through stimulation or suppression, often achieved by therapeutic agents.

Innate Immunity: First line of defence involving non-specific immune cells like NK cells and macrophages.

Interferon-Gamma: A cytokine produced mainly by NK and T cells that activates macrophages, enhances antigen presentation, and promotes anti-tumour immunity.

Liquid tumour (hematological cancer): A type of cancer that affects the blood, bone marrow, or lymphatic system, such as leukemia or lymphoma.

Malignant: An adjective used to describe a tumour which grows progressively and invades other tissues, often leading to metastasis and posing a significant health risk.

Metastasis: The movement and colonization by tumour cells to sites distant from the primary site of tumour development, resulting in the formation of secondary tumours.

Mutation: A change in the DNA sequence of a gene, which can lead to alterations in the function of the encoded protein and potentially contribute to cancer development.

Natural Killer cells: A type of immune cell that plays a crucial role in the body's defence against tumours and virally infected cells by recognizing and destroying abnormal cells.

Oncogene: The mutated version of a proto-oncogene, usually characterized by overactivity or overexpression, which can contribute to tumour development.

Oncolytic Virus Therapy: A type of cancer treatment that uses genetically engineered or naturally occurring viruses to selectively infect, replicate in, and destroy cancer cells while stimulating anti-tumour immunity.

Perforin: A pore-forming protein secreted by cytotoxic immune cells that facilitates the entry of granzymes into target cells to trigger apoptosis.

Prognosis: The likely course and outcome of a disease, often based on the individual's specific condition, including factors such as the type and stage of cancer.

Resazurin-Based Assay: A cell viability assay that measures metabolic reduction of resazurin to resorufin as an indicator of cell health, proliferation, and cytotoxicity.

Solid tumour: A mass of cancer cells that arises from tissues such as the bone, muscle, or organs, as opposed to liquid tumours, which affect the blood or lymphatic system.

Single-Cell Transcriptomics: A method for profiling gene expression at the single-cell level, allowing the study of cellular heterogeneity within complex tissues.

Triple Negative Breast Cancer: A subtype of breast cancer that does not express the genes for estrogen receptor (ER), progesterone receptor (PR), or HER2. It is often more aggressive and difficult-to-treat than other breast cancer subtypes.

Tumour Microenvironment: The complex environment surrounding a tumour, including stromal cells, immune cells, blood vessels, and extracellular matrix, which influences tumour growth, immune evasion, and treatment response.

Tumour suppressor gene: Genes that encode proteins that limit cell growth, division, differentiation, and proliferation, normally preventing tumour formation. Inactivation or loss of function of these genes requires a double mutation event.

Potency Assay: A quantitative laboratory test used to measure the biological activity of a therapeutic product and to ensure consistency, quality, and efficacy in biomanufacturing.

Proto-oncogene: Genes that encode proteins which normally regulate processes such as cell growth, division, differentiation, and survival. When mutated, they can become oncogenes, leading to cancer. Activation requires a single mutation event.

Xeno-Free: Refers to cell culture or biomanufacturing conditions that exclude animal-derived components, reducing the risk of zoonotic contamination and improving clinical safety.

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SUMMARY OF QUALIFICATIONS

- **Biotech Startup Founder:** pioneering innovative applications of extracellular vesicles.
- **Experienced Researcher** with 8+ years in cancer immunotherapy, specializing in cell-based therapeutics, including adoptive cell transfer (*i.e.*, CAR-T, CAR-NK cells) and extracellular vesicles (EVs) therapeutics.
- **Highly Published:** multiple first-author publications in high-impact journals and several citations
- **Industry skills:** experienced with ISO (ISO/IEC 17025 and 9001), GxP (GLP, GDP and GMP) and QA/QC.
- **Regulatory knowledge:** familiar with industry standards, regulatory guidelines and quality management.
- **Technical expertise:** various basic and advanced techniques commonly performed in scientific research.
- **Leadership and collaboration:** led several research projects and collaborated closely with cross-disciplinary teams. Extensive experience working for large and diverse organizations (*i.e.*, Health Canada) and mentoring students or managing technicians. Proven track record of participation in scientific societies.

CAREER OBJECTIVE

Career Objective: To advance the discovery and commercialization of next-generation biotechnologies that maximize human healthspan, and consequently lifespan, by combining scientific rigour with leadership that enables scalable innovation and global impact.

EDUCATION AND TRAINING

Ph.D. Biochemistry (fast-tracked)

University of Ottawa, Ontario, Canada

- Conducted cutting-edge research on triple-negative breast cancer, investigating natural killer cell-derived extracellular vesicles as an immunotherapeutic strategy. 2021-2026
- Received an admission scholarship with a GPA of 10/10.
- Published numerous first-author peer-reviewed articles in top-tier journals.

BSc. Biochemistry with a double major in Immunology and Microbiology + COOP

University of Ottawa, Ontario, Canada

2016-
2021

- Scholarship partially funded with a GPA of 8/10.

PROFESSIONAL EXPERIENCE

Founder and CEO

BioThera Solutions Inc., Ontario, Canada

2024-present

- A biotech startup pioneering innovative applications of extracellular vesicles.

Doctoral Student Researcher

Health Canada, Ontario, Canada

- Conducted cutting-edge research on triple-negative breast cancer, investigating natural killer cell-derived extracellular vesicles as an immunotherapeutic strategy.
- Conducted work under GxP (GLP, GDP and GMP) conditions, ISO practices (ISO/IEC 9001) and under field-specific guidelines (*i.e.*, MISEV).
- Produced several high-quality, peer-reviewed research papers with significant impact.

2021-2026

Standardized Patient, on-call

University of Ottawa, Ontario, Canada

- Accurately portrays patients with specific medical conditions, emphasizing empathy, confidentiality, and effective communication to enhance medical students' learning experiences.

2023-2026

Teacher Assistant and Proctor, on-call

University of Ottawa, Ontario, Canada

- Assisted professors in properly executing university courses (supervision, mentoring).

2022-2026

Junior Technician

Ottawa Hospital Research Institute, Ontario, Canada

- Collaborated closely with a large team of researchers to research SARS-CoV-2-acquired immunity in diverse populations. Performed biological sample collection and analyses.

2020-2021

Undergraduate student researcher (COOP intern)

Health Canada, Ontario, Canada

- Engaged in developing new protocols and techniques for characterizing therapeutic extracellular vesicle products.

2019-2020

Risk evaluator assistant (COOP intern)

Health Canada, Ontario, Canada

Summer of
2019

- Developed a knowledge base in the Canadian Environmental Protection Act 1999 regulatory framework and delivered comprehensive risk assessments of pathogenic microorganisms.

Laboratory technician at ROPEC laboratory

City of Ottawa, Ontario, Canada

Summer of
2018

- Performed sample analysis with high accuracy by adhering to standard operating procedures (SOPs) and ISO best practices (ISO/IEC 17025).

Enrichment Tutor

Various organizations (various school board + freelancing), Ontario, Canada

2016-2019,
2024-2025

- Adapted teaching methods to meet diverse student needs, demonstrating patience and creativity to enhance engagement and build strong relationships across all education levels.

LABORATORY SKILLS

- Highly proficient in flow cytometry sample processing, analysis, and sorting, including plugins (*i.e.*, t-SNE, fitSNE, FlowSOM). Also, skilled and knowledgeable with OMICS (mass spectrometry and scRNA-sequencing) processing and data analysis.
- Experienced in chromatography-based purification techniques, including affinity column, size exclusion and tangential flow filtration methods for the scalable production of various biological products (*e.g.*, extracellular vesicles, nucleic acids, and proteins).
- Skilled in cell culture procedures (*e.g.*, microscopy, cell counting, cell expansion, and banking) and various assays to characterize samples (ELISA, Western blotting, TEM) and macromolecule quantification.
- Experienced in genetic manipulation techniques (sub-cloning, PCR, gel electrophoresis, CRISPR-Cas9) and sequencing analysis.
- Trained in working with immune-compromised mice for injections, surgeries, and breeding.

PEER-REVIEWED PUBLICATIONS

- **Frederic St-Denis-Bissonnette**, Karan Mediratta, Nicolas Ho, Melanie Doan Kirkby, Andrew Stalker, Michele Ardolino, Seung-Hwan Lee, Dylan Burger, Arvind Mer, Lisheng Wang and Jessie R. Lavoie. Natural Killer Cell-derived Extracellular Vesicles boost immune cells' cancer killing function: Insights from Single-cell RNA Sequencing and Live-cell Cytotoxicity Assay. Under revision by Molecular Therapy Oncology (Impact Factor 5.3) – *expecting publication in 2026*.

- Melanie Doan Kirkby and **Frederic St-Denis-Bissonnette**, Karan Mediratta, Marena Diab, Anna Korobkow, Justin Humber, Peter Han, Gauri Muradia, Michele Ardolino, Seung-Hwan Lee, Derek Gibbins, Dylan Burger, Lisheng Wang, and Jessie R. Lavoie. Natural Killer Cell-Derived Extracellular Vesicles Show Cytotoxicity Against Bulk Tumour Cells and Cancer Stem Cells Found in Triple-Negative Breast Cancer. Under revision by Breast Cancer Research journal (Impact Factor 5.6) – *expecting publication in 2026*.
- **Frédéric St-Denis-Bissonnette**, Barry Ngo, Hala Halabi, Anna Korobkow, Jianqun Wang, Lisheng Wang and Jessie R. Lavoie. An Optimized Positive Staining Protocol Method for Clear Visualization of Extracellular Vesicles by TEM Using Uranylless and Lead Citrate. *Langmuir Journal* (Impact Factor of 3.9). Website: <https://doi/10.1021/acs.langmuir.5c03977>.
- **Frederic St-Denis-Bissonnette**, Hala Halabi, Marybeth Creskey, Melanie Doan Kirkby, Xu Zhang, Dylan Burger, Lisheng Wang and Jessie R. Lavoie. Ultracentrifugation and ultrafiltration differentially alter the composition and functionality of the extracellular vesicles' biomolecular corona. Accepted in the *Journal of Extracellular Biology* (Impact Factor of 4.0) – *expecting publication in 2026*.
- **Frederic St-Denis-Bissonnette**, Melanie Doan Kirkby, Lisheng Wang and Jessie R. Lavoie. A Scalable Biomanufacturing Workflow to Produce and Isolate Natural Killer Cell-derived Extracellular Vesicle-based Cancer Biotherapeutics. *Journal of Visualized Experiments* (Impact Factor 1.2). 2024. Website: <https://dx.doi.org/10.3791/67227> (1 citations).
- **Frederic St-Denis-Bissonnette**, Shirley Qiu, Sarah E. Cummings, Melanie Doan Kirkby, Yohannes Haile, Sarah Wassmer, Gauri Muradia, Jelica Mehic, Andrew Stalker, Amit Shrestha, Michele Ardolino, Seung-Hwan Lee, Dylan Burger, Lisheng Wang and Jessie R. Lavoie. Evaluation of resazurin phenoxazine dye as a Highly Sensitive Cell Viability Potency Assay for Natural Killer Cell-derived Extracellular Vesicle-based Cancer Biotherapeutics. *Journal of Extracellular Biology* (Impact Factor 4.5). 2023. Website: <https://doi.org/10.1002/jex2.166> (3 citations).
- Welsh et **MISEV Consortium**. Minimal information for studies of extracellular vesicles (MISEV2023): from basic to advanced approaches. *Journal of Extracellular Vesicles* (Impact Factor of 13.6). 2024. Weblink: <https://doi.org/10.1002/jev2.12404> (459 citations).

- Sarah Cummings, Sean Delaney, **Frederic St-Denis-Bissonnette**, Andrew Stalker, Gauri Muradia, Jelica Mehic, Tyson Graber, Tommy Alain and Jessie R. Lavoie. SARS-CoV-2 Antigen-Carrying Extracellular Vesicles Activate T Cell Responses in Human Immunogenicity Model. *iScience* (Impact Factor 4.2). 2023. Weblink: <https://doi.org/10.1016/j.isci.2023.108708> (2 citation).
- **Frederic St-Denis-Bissonnette**, Sarah E. Cummings, Shirley Qiu, Andrew Stalker, Gauri Muradia, Jelica Mehic, Shelby Kaczmarek, Karan Mediratta, Dylan Burger, Seung-Hwan Lee, Lisheng Wang and Jessie R. Lavoie. A clinically relevant biomanufacturing workflow to produce Natural Killer cells and Natural Killer cell-derived extracellular vesicles for cancer immunotherapy. *Journal of Extracellular Vesicles* (Impact Factor of 13.6). 2023. Weblink: <https://doi.org/10.1002/jev2.12387> (4 citations).
- Erin Collins, Yannick Galipeau, Corey Arnold, Cameron Bosveld, Alisa Heiskanen, Alexa Keeshan, Kiran Nakka, Khatereh Shir-Mohammadi, **Frederic St-Denis-Bissonnette**, Laura Tamblyn, Agatha Vranjkovic, Leah C Wood, Ronald Booth, C Arianne Buchan, Angela M Crawley, Julian Little, Michaeline McGuinty, Raphael Saginur, Marc-André Langlois, Curtis L Cooper. Cohort profile: Stop the Spread Ottawa (SSO)—a community-based prospective cohort study on antibody responses, antibody neutralization efficiency and cellular immunity to SARS-CoV-2 infection and vaccination. *BMJ Open* (Impact Factor 2.4). 2022. Weblink: <https://doi.org/10.1136/bmjopen-2022-062187> (17 citations).
- **Frédéric St-Denis-Bissonnette**, Rachil Khoury, Karan Mediratta, Sara El-Sahli, Lisheng Wang and Jessie R. Lavoie. Applications of Extracellular Vesicles in Triple-Negative Breast Cancer. *Cancers MDPI* (Impact Factor 6.6). 2022. Weblink: <https://doi.org/10.3390/cancers14020451> (29 citations).
- Jonathan Gobin, Gauri Muradia, Jelica Mehic, Carole Westwood, Lauren Couvrette, Andrew Stalker, Stewart Bigelow, Christian C. Luebbert, **Frederic St-Denis-Bissonnette**, Michael J. W. Johnston, Simon Sauvé, Roger Y. Tam, Lisheng Wang, Michael Rosu-Myles & Jessie R. Lavoie. Hollow-fiber bioreactor production of extracellular vesicles from human bone marrow mesenchymal stromal cells yields nanovesicles that mirrors the immuno-modulatory antigenic signature of the producer cell. *Stem Cell Research & Therapy* (Impact Factor 4.5). 2021. Weblink: <https://doi.org/10.1186/s13287-021-02190-3> (101 citations).

*Note: Patents or other legal derivatives are not included in this CV.

PRESENTATION EXPERIENCE

Natural Killer Cell-derived Extracellular Vesicles boost immune cells' cancer killing function: Insights from Single-cell RNA Sequencing and Live-cell Cytotoxicity Assay	2025
Oral presentation: Gene Therapy Coffee Club at Health Canada	
Investigating natural killer cell-derived extracellular vesicles in a clinically relevant TNBC PDX model	2025
Oral presentation: Canadian Society Extracellular Vesicle 2025 Symposium	
Evaluation of resazurin phenoxazine dye as a Highly Sensitive Cell Viability Potency Assay for Natural Killer Cell-derived Extracellular Vesicle-based Cancer Biotherapeutics.	2023-
Oral presentation: University of Ottawa Research Day 2024 and Health Canada Science Forum 2025	2024
Poster: 5th Annual Quebec Extracellular Vesicle Workshop and Health Canada Science Forum	
A clinically relevant biomanufacturing workflow to produce Natural Killer cells and Natural Killer cell-derived extracellular vesicles for cancer immunotherapy.	
Oral presentation: Gene Therapy Coffee Club at Health Canada, CORR-RRD Research Seminar Series at Health Canada, ISEV2023—International conference (invited to present a 15-minute talk but could not attend due to the financial restrictions imposed by Health Canada).	2022 2023-
Poster presentation: University of Ottawa Research Day 2022 and 2023, Health Canada Science Forum 2023 and BioCanRx Summit4CI 2022	

LEADERSHIP AND VOLUNTEER EXPERIENCE

Student Network on Extracellular Vesicles (SNEV) – Ambassador for CanSEV	2026-
Official SNEV/ISEV-designated ambassador for CanSEV.	present
Student supervision	
Supervised undergraduate, COOP and graduate students' academic progress, research, and projects while providing guidance, feedback and fostering professional development.	2023- present
Canadian Society of Extracellular Vesicles (CanSEV) - Board Member, Founding member and Junior Member	2022- present

- Founding member of CanSEV, organized symposiums, managed website, initiated quarterly newsletters, and moderated events.

International Society of Extracellular Vesicles (ISEV) - Junior Member 2022-

- Contributed to the revision and publication of the MISEV2024 guidance document. present

IgNITE Medical Case Competition Vice President Of Development 2021-

- Led competition development, co-authored case packages, and coordinated workshops. 2024

BioCanRx Highly Qualified Personnel 2021-

- Organized and participated in learning institutes and contributed to educational materials. 2024

IgNITE Medical Case Competition mentor 2021-

- Mentored students in medical case competitions. 2022

StemCellTalks symposium organizer 2021-

- Organized events to promote stem cell research. 2022

HEO Hockey assistant coach 2016-

- Assisted with player evaluations, practices, and conditioning camps. 2018

Founder and Organizer of a Fundraiser event for the CHEO Foundation 2016-

- Raised funds (\$12K), managed event logistics and community engagement. 2019

AWARDS AND HONORIFIC MENTION

Fulbright Canada Short-term Entrepreneurship Award (\$10K USD), Massachusetts Institute of Technology (MIT), Cambridge, Massachusetts, United States of America 2026

Enactus 2025-2026 Student Entrepreneur National Competition (Nominated; ongoing process), University of Ottawa, Ontario, Canada 2025

Pitch Award (1st prize - \$15K CAD) - Startup Garage Rally Day, University of Ottawa, Ontario, Canada 2025

Pitch Award (1st prize - \$1K CAD) - Startup Garage Revup Circuit, University of Ottawa, Ontario, Canada 2025

Ontario Graduate Scholarship (\$15K CAD), University of Ottawa, Ontario, Canada 2025

Judith E. Raymond Cancer Scholarship (\$5K CAD), University of Ottawa, Ontario, Canada 2025

Barbara Berthon Scholarship (\$10K CAD) , University of Ottawa, Ontario, Canada	2025
Walter Faigan Club Kiwanis Scholarship (\$5K CAD) , University of Ottawa, Ontario, Canada	2025
Queen Elizabeth II Graduate Scholarship (\$15K CAD) , University of Ottawa, Ontario, Canada	2023
Summit4CI-BioCanRx travel award (\$1K CAD) , Montreal, Quebec, Canada	2022
Doctoral Admission Scholarship (\$36K CAD) , University of Ottawa, Ontario, Canada	2022-2026
Health Law, Policy and Ethics award (\$1K CAD) , University of Ottawa, Ontario, Canada	2022
Master Admission Scholarship (\$7.5K CAD) , University of Ottawa, Ontario, Canada	2021-2022
Dr. Hubert Patenaude Memorial Scholarship (\$1.5K CAD) , University of Ottawa, Ontario, Canada	2021
Merit Scholarship (\$5K CAD) , University of Ottawa, Ontario, Canada	2017-2021
Dean's Honour List - Bachelor , University of Ottawa, Ontario, Canada	2017-2021
Bachelor Admission Scholarship (\$3K CAD) , University of Ottawa, Ontario, Canada	2016
Perseverance Scholarship (\$1K CAD) , University of Ottawa, Ontario, Canada	2016
Governor-General Medal , Ontario, Canada	2016
Academic excellence medal (honorific mention) , CEPEO, Ontario, Canada	2016

ADDITIONAL INFORMATION

Certifications

- **Health and Safety:** WHMIS (Workplace Hazardous Materials Information System) [since 2014], MSDS (Material Safety Data Sheet) [since 2014] and First Aid CPR [since 2012].
- **Entrepreneurship/startup and industry:** Lab2Market Launch program (with \$10K CAD award) [2025], Lab2Market Validate program (with \$10K CAD award) [2025], Startup Garage (Exploration circuit; with \$5K CAD award) [2025], adMareBio Academy BioInnovation Scientist Program (BSI)—Foundations: Introduce early-career scientists to therapeutic discovery, development, and commercialization [2024], Lab2Market Validate Foundations program [2024], University of Ottawa Entrepreneurial Mindset Certificate [2024], BioTalent Canada industry-specific courses (Essential and Technical Skills Fundamentals) [2022], University of Ottawa Summer Institute in Health Law, Policy and Ethics on artificial intelligence in healthcare [2022], BioCanRx/Algonquin College's GxP Knowledge and Principals Micro-certification

[2022], Hospital Centre for Journalology courses accreditation: Data Sharing and Copyright, Managing and Sharing Tabular Data, FAIR Principles and Data Storage and Privacy [2021].

Other Skills and Interests

- Bilingual Professional, enabling effective communication and collaboration with small-to-big teams.
- Excellent problem-solving skills and proficiency with all Office Suites software (*i.e.*, Word, Excel, PowerPoint) or local AI interfacing
- Strong critical and analytical thinking. Excel at planning and forecasting project needs.
- Research & Reading: Passionate about staying current with the latest scientific advancements, which enhances critical thinking and a solid knowledge base.
- Physical exercise and team sports like hockey foster discipline, teamwork, and communication skills.
- Mechanical Projects: Skilled in car repairs, which sharpens problem-solving abilities and attention to detail.

External links

- LinkedIn: <https://www.linkedin.com/in/frederic-st-denis-bissonnette/>
- ORCID: <https://orcid.org/0000-0003-3355-4950>
- Research Gate: <https://www.researchgate.net/profile/Frederic-St-Denis-Bissonnette-2>
- Google Scholar: <https://scholar.google.ca/citations?user=VM9rvnYAAAAJ&hl=en&authuser=4>