RESEARCH PAPER

Gastric cancer-derived nanovesicles affect the viability and effector function of an NK cell line

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ABSTRACT

Objective(s): Gastric cancer is characterized by high mortality rates due to its aggressive progression and immune-evasion strategies. Tumor-derived exosomes (TDEs) are nanovesicles mainly known for their role in modulating the tumor microenvironment, including the suppression of immune effector cells such as NK cells.

Materials and Methods: This study aimed to assess the impact of exosomes released from the human AGS GC cell line on the viability and effector function of the NK-92 cell line. Exosomes were characterized using FE-SEM, DLS, and flow cytometry, confirming their size (approximately 109 nm) and expression of the CD63 marker.

Results: Co-culture of NK-92 cells with exosomes at 30 µg/ml for 48 hours revealed a significant reduction in cell viability in the MTT assay. Quantitative real-time PCR showed increased expression of suppression markers (NKG2A, TGF-β1, and LAG-3), while ELISA indicated decreased IFN-γ production. Flow cytometry-based cell cycle analysis demonstrated increases in the sub-G1 (apoptotic) and G0/G1 phases.

Conclusion: These results suggest that GC-derived exosomes may impair NK cells, potentially contributing to immune evasion and tumor progression. However, these findings were obtained using immortalized AGS and NK-92 cell lines and require validation in primary cells and in vivo models. Insights from this study could inform novel immunotherapeutic approaches targeting exosomal pathways in GC.

Keywords: *Gastric cancer; Tumor-derived exosomes; Natural killer cells; Immune evasion; Cytotoxicity; Cell viability*

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INTRODUCTION

Gastric cancer (GC) represents an important health problem worldwide. Despite the increasing diversity of available treatment approaches for gastric cancer, there has been only minimal improvement in outcomes among patients with advanced disease. Consequently, the identification of noninvasive biomarkers with high specificity, the investigation of cellular processes underlying GC progression, and the development of novel therapeutic strategies remain crucial for improving diagnostic precision, treatment effectiveness, and patient outcomes in GC (2).

Exosomes are nanometer-sized vesicles secreted by living cells into the extracellular environment and play important roles in cell-to-cell communication through paracrine signaling and other mechanisms (3, 4). EVs have been associated with cancer growth, heart disease, neurological conditions, and metabolic disorders (5-7). These vesicles have potential applications as screening indicators, therapeutic agents, therapeutic targets,

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and pharmaceutical carriers (8-11). The diversity of EVs reflects the variety of cell types and the different conditions from which they originate, together with the numerous physiological and biological processes in which they are involved. Therefore, EVs can directly reflect cellular conditions, and changes in exosomal cargo can induce specific physiological alterations in target cells, resulting in biological heterogeneity (12). Different types of EVs have distinct biological functions because of the diverse molecules they contain. Immune cells produce EVs containing molecules that can influence immunity within the cellular environment and exert effects against tumor cells (13)(14). Research indicates that tumor-derived exosomes can substantially modify the immune characteristics of the tumor microenvironment by suppressing specific T-lymphocyte immune responses and directing cells of the innate immune system toward a tumor-promoting phenotype (15-17). A major mechanism involves reducing immune surveillance of primary cancers by establishing a permissive environment conducive to the formation of a distant niche (18). Tumor evasion of the human immune system is considered a significant barrier to biotherapy.

Natural killer (NK) cells are capable of responding to infections and tumors without the need for antigen-specific recognition and stimulation (19). However, accumulating evidence indicates that tumors can alter the functionality of tumor-infiltrating NK cells, rendering these cells ineffective and promoting an immunosuppressive state. NK cell dysfunction can result from multiple factors, including a reduced capacity for effector secretion, impaired metabolic processes, secretion of suppressive inflammatory mediators, reduced expression levels of activating signaling receptors, or enhanced expression of suppressive receptors, thereby creating an imbalance between activating and inhibitory signals (20, 21). In patients with GC, prognosis, lymphatic and vascular dissemination, lymph node metastases, and clinical stage are all strongly correlated with NK cell activity (22). Studies have shown that, in patients with gastric cancer, peripheral blood NK cells exhibit decreased expression levels of NKp46, NKG2D, NKp30, and DNAM-1, changes that are associated with tumor growth (23). These findings highlight the important connection between GC progression and NK cell recruitment. Nonetheless, the factors contributing to systemic alterations in NK cell functionality in GC remain unclear.

The selection of the AGS and NK-92 cell lines for investigating the influence of exosomes derived from AGS gastric cancer cells on NK cells was based on their well-established biological characteristics and their relevance to the objectives of this study. AGS cells constitute a frequently used human gastric adenocarcinoma cell line that consistently produces exosomes, making this cell line a suitable model for investigating cancer-derived exosome biology. In contrast, NK-92 cells are a well-characterized human natural killer (NK) cell line that retains functional immune properties and is readily amenable to in vitro manipulation, making this cell line suitable for assessing the immunomodulatory effects of exosomes. Taken together, these cell lines provide a robust experimental system for investigating the interplay between cancer-released exosomes and host immune cells and for gaining insight into the mechanisms underlying tumor-immune interactions. The present study aimed to explore the influence of exosomes isolated from the human AGS GC cell line on the viability, effector function, and molecular characteristics of the NK-92 cell line, thereby providing insights into potential immunotherapeutic targets for GC.

MATERIALS AND METHODS

Cell culture

The human gastric cancer AGS and natural killer NK-92 cell lines were acquired from Iran's National Cellular Bank (Pasteur, Iran). Both cell lines were maintained in RPMI 1640 culture medium (Gibco Inc, El-Paso, TX) supplemented with 10 percent fetal bovine serum (FBS; Gibco Inc.) and 100 units/ml penicillin. Cultured cells were maintained at 37°C in a 5% CO2 incubator. To support maintenance of the NK-92 cell line, 200 IU/ml of IL-2 (Sigma-Aldrich) was added.

Exosome isolation

Exosomes were isolated from the supernatant of AGS cells. Initially, cells were washed with RPMI 1640 medium and then incubated in RPMI 1640 supplemented with 5% exosome-free FBS for 24 hours. The collected supernatant was centrifuged at 4000 rpm for 15 minutes (Hettich 46R, Germany) to remove cells and debris. Following filtration through a 0.22 µm membrane, the supernatant was ultracentrifuged at 100,000g for 1 hour at 4°C (TLA-100.3, Indiana, US) to pellet the exosomes. The pellet was resuspended in phosphate-buffered saline (PBS), vortexed three times for 30 seconds to ensure homogeneity, and stored at -20°C for less than one month until further experiments. Exosome purity was not independently assessed; therefore, the exosomal protein concentration was determined using a bicinchoninic acid (BCA) kit

(Parsgene, Iran). This concentration was used as the criterion for subsequent analyses.

Characterization of isolated exosomes

Field-Emission Scanning Electron Microscopy (FE-SEM)

To evaluate exosome morphology and size, a diluted sample was applied to an aluminum substrate, spread evenly, and air-dried. Imaging was performed using an FE-SEM device (MIRA3, CZ) at 15 kV, and images were captured at multiple magnifications.

Dynamic Light Scattering (DLS)

Exosome size distribution and the polydispersity index (PDI) were assessed by transferring 1 mL of the suspension to a cuvette and analyzing it with a DLS instrument (Malvern, UK). The system used laser illumination to detect scattered light and determine particle dimensions.

Flow cytometry analysis

Exosome identity was confirmed by flow cytometry using the CD63 marker. Samples (100 µl) were divided into an unstained control tube and a test tube. The test tube received 3 µl of CD63-FITC antibody (BioLegend) and was incubated for 30 min at 4°C. Analysis involved 100-nanometer beads for size gating, with initial runs of unstained and single color controls followed by the labeled sample.

MTT assay

To determine an appropriate exosome dose for treating NK-92 cells, an MTT assay was performed. NK-92 cells (1×10^4) were seeded in a 96-well plate and treated with varying concentrations of AGS-released exosomes (1 to 50 µg/ml) for 48 hours in triplicate. MTT solution (5 mg/ml) was added to each well, and the plate was incubated for 4 hours at 37°C. Formazan crystals were then dissolved using DMSO, and the absorbance was measured at 580 nm with a plate reader (Tecan/Sunrise).

Co-culture of NK-92 cells with exosomes

To evaluate the effects of GC-EXOs on NK-92 cells, 1×10^5 NK-92 cells were distributed into 24-well plates and allowed to stabilize overnight. Exosomes were then added at a concentration of 30 µg/ml for 48 h. This concentration was selected based on the MTT assay results and previous studies investigating exosome–immune cell interactions (24). Control groups consisted of untreated cells and vehicle-control cells treated with a volume of PBS equivalent to the volume used for exosome resuspension. After the incubation period, cells were collected for RNA extraction, and culture supernatants were collected for the enzyme-linked immunosorbent assay (ELISA).

ELISA

The IFN-γ concentration in culture supernatants from NK-92 cells was determined using a human IFN-γ ELISA kit (Parsgene, Iran) according to the manufacturer's instructions. The contents of each well were collected, centrifuged at 800 rpm for 5 min to remove cell debris, and stored at –80 degrees until testing. Standards and test samples were added to the pre-coated ELISA plates, followed by incubation and washing; detection was performed using HRP-conjugated secondary antibodies and TMB substrate. Sulfuric acid was used to stop the reaction, and absorbance was measured at 470 nm using a microplate reader. IFN-γ concentrations were then determined using a standard curve and expressed in pg/mL. All samples were analyzed in duplicate, and results were normalized to cell counts to ensure accurate comparisons.

Total RNA isolation

Total RNA was extracted from NK-92 cells in all groups using RiboEX reagent (Gene All Biotechnology, South Korea) in accordance with the manufacturer's protocol. A NanoDrop spectrophotometer (NanoDrop Technologies, USA) was used to assess the quality of the extracted RNA, and the A260/280 ratios for all samples ranged from 1.7 to 1.8. The RNA concentrations of all samples were recorded, and the samples were diluted as needed to obtain equal concentrations for use in RT-PCR.

cDNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The Sinaclon cDNA synthesis kit (Tehran, Iran) was used for cDNA synthesis in accordance with the protocols provided by the manufacturer. Quantitative real-time PCR was performed using a SYBR Green master mix on a Light Cycler 96 system (Roche Diagnostics, Mannheim, Germany) to assess the levels of gene expression. RT-PCR was performed in three steps: in the first step, initial denaturation (95 °C, 2 min); in the second step, 40 cycles consisting of denaturation (95°C, 30s), annealing (60°C, 30s), and extension (72°C, 30s); and in the third step, final extension (72°C, 5 min). GAPDH was used as the endogenous control. Relative mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method. Primers were designed using Primer Express 3.0 (Applied Biosystems, CA) (Table 1).

Table 1. Characteristics of primers for the studied genes

Gene Symbol	Forward Primer Sequence (5' to 3')	Reverse Primer Sequence (5' to 3')	Tm (°C)
LAG3	GCAGTGTACTTCACAGAGCTGTC	AAGCCAAAGGCTCCAGTCACCA	61
KLRC1	GCCTCTGTGGTAACGATAGTTGT	ATCCACTCCTCAGGACAATGGC	59.5
TGFB1	TACCTGAACCCGTGTTGCTCTC	GTTGCTGAGGTATCGCCAGGAA	60
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA	60

Flow cytometry for cell cycle analysis

To assess the effect on NK-92 cell cycle progression, cells were harvested after 48 hours of co-culture with exosomes (30 µg/ml), washed in PBS, and fixed using 70 percent ethanol overnight at -20°C. Following this step, the fixed cells were stained with DAPI (Sigma-Aldrich, USA) at 1 µg/ml in PBS containing 0.1% Triton X-100 and 100 µg/ml RNase A for 30 min at 25°C in the dark. The distribution of cells across the cell cycle phases (sub-G1, G0/G1, S, and G2/M) was measured using a FACSCalibur flow cytometer (BD Biosciences, USA) and FlowJo software (v 10).

Statistical analysis

GraphPad software v 10.1 (GraphPad Software, San Diego, CA) was used to analyze the data. Data normality was assessed using the Shapiro-Wilk test. Then, differences among groups were evaluated using analysis of variance. Multiple comparisons were performed using the Dunnett test. Results are presented as mean ± SD, with $P < 0.05$ considered statistically significant.

RESULTS

Exosome characterization

The isolated exosomes from AGS cells were characterized by flow cytometry, FE-SEM, and DLS. Flow cytometry confirmed exosome identity, showing 78.5% positivity for the CD63 marker (Fig. 1A). FE-SEM revealed spherical vesicles with a uniform distribution (Fig. 1B). DLS analysis indicated an average particle size of approximately 109 nm with a polydispersity index (PDI) of 0.23, suggesting a relatively homogeneous vesicle population (Fig. 1C).

MTT results

The MTT assay demonstrated a dose-dependent reduction in NK-92 cell viability upon exposure to AGS-derived exosomes. Viability, measured as optical density (OD) values, showed a significant decline compared with controls beginning at 30 µg/ml ($P < 0.05$, one-way ANOVA, Dunnett) (Fig. 2).

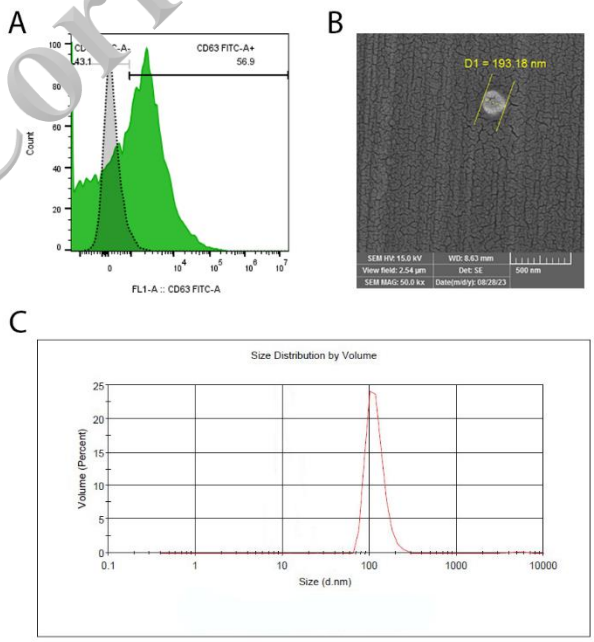

Fig. 1. Characteristics of AGS cell-derived EVs. (A) Flow cytometry confirmed the expression of the exosomal marker CD63; (B) FE-SEM was used to evaluate the size and shape of the isolated exosomes; (C) DLS showed that their average size was approximately 109 nm (PDI = 0.23).

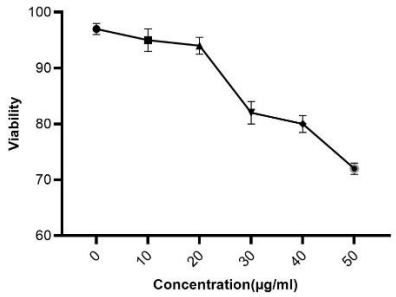

Fig. 2. Effect of different concentrations of exosomes derived from the AGS cell line on the viability of NK-92 cells. The MTT assay showed a decrease in cell viability with increasing exosome concentration. The first substantial decrease was observed at a concentration of 30 µg/ml.

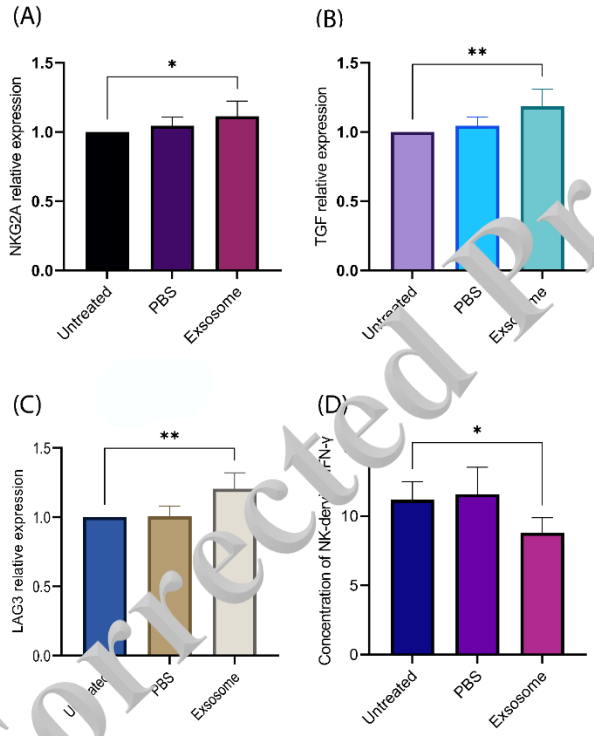

Fig. 3. Comparison of mRNA fold change (FC) in the expression levels of suppression markers and IFN-γ production between AGS cell line-derived exosome-treated and untreated groups (mean ± SD). (A-C) The expression levels of three candidate genes (NKG2A, TGF-β1, and LAG-3) were significantly increased in the AGS cell line-derived exosome-treated group compared with control groups (**P < 0.01, ***P < 0.001). (D) IFN-γ concentrations measured by ELISA were significantly decreased in the treated group (***P < 0.001).

Expression levels of suppression markers and IFN-γ production

The expression of suppression markers, including KLRC1 (NKG2A), TGFB1 (TGF-β1), and LAG3 (LAG-3), in exosome-treated NK-92 cells was significantly increased compared with untreated controls (P = 0.0015, P = 0.0021, and P = 0.0008, respectively) (Fig. 3A-C). Additionally, IFN-γ concentrations in culture supernatants, as measured by ELISA, were markedly reduced in the exosome-treated group relative to controls (P < 0.0001) (Fig. 3D). No significant differences were found between untreated and PBS controls (P > 0.05), whereas exosome treatment significantly altered marker expression and IFN-γ production compared with both control groups (**P < 0.01, ***P < 0.001).

Cell cycle analysis

Flow cytometric analysis of DAPI-stained NK-92 cells revealed alterations in cell cycle distribution following exosome co-culture. The exosome-treated group exhibited an increase in the sub-G1 phase (indicative of apoptosis) and G0/G1 arrest compared with controls, with corresponding decreases in the S and G2/M phases (Fig. 4). No differences were observed between untreated and PBS groups.

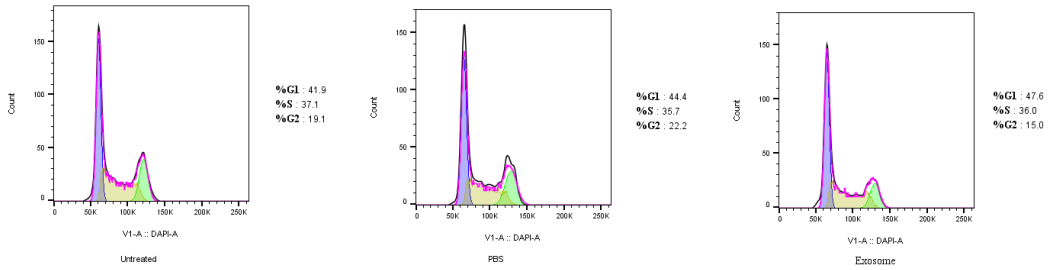

Fig. 4. Flow cytometry results of cell cycle analysis in NK-92 cells after co-culture with exosomes. The exosome-treated group showed increased sub-G1 and G0/G1 populations and reduced S and G2/M phases compared with control groups.

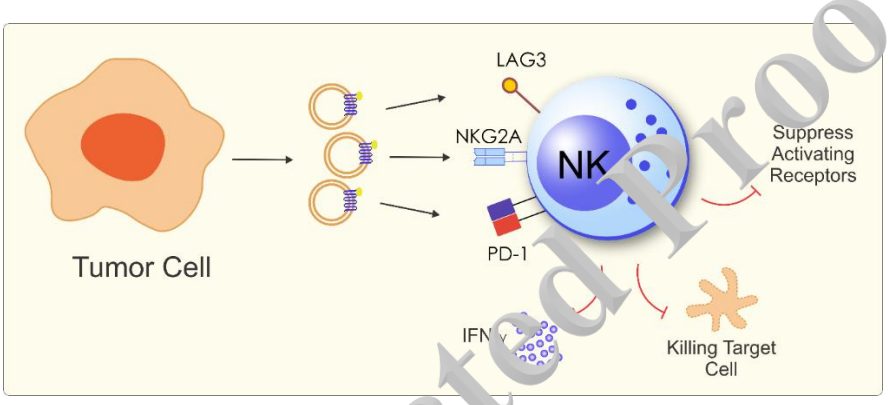

Fig. 5. The effect of exosomes derived from gastric tumor cells on NK cell function.

DISCUSSION

The TME is important in cancer progression because tumor cells actively modify their surroundings to promote growth, invasion, and immune escape. Recent findings indicate that exosomes contribute to this process, especially in GC, by promoting intercellular communication through the transfer of immunosuppressive components, including microRNAs, proteins, and cytokines. In our study, exosomes derived from the AGS GC cell line significantly impaired the viability and effector function of NK-92 cells, as evidenced by reduced cell survival in MTT assays, increased expression of inhibitory markers (NKG2A, TGF-β1, and LAG-3), decreased IFN-γ production, and altered cell cycle dynamics with increased apoptosis (sub-G1 phase) and G0/G1 arrest. These findings support the immunosuppressive potential of GC-derived exosomes in NK cells and are consistent with broader research on exosome-driven tumor immune escape (Fig. 5).

The findings showed a dose-dependent reduction in NK-92 viability following exposure to AGS exosomes, with a significant decrease beginning at 30 µg/ml, suggesting that exosomes may induce cytotoxic stress or inhibitory signaling in NK cells. This is consistent with previous studies indicating that TDEs can directly impair NK cell function. In GC models, Liu et al. demonstrated that exosomes derived from GC cells, specifically the MKN-45 and MKN-28 lines, reprogrammed CD8+ T cells and NK cells, leading to decreased cytokine production and increased tumor metastasis to the lungs. Their study highlighted the role of exosomes in establishing an immunosuppressed tumor microenvironment by inhibiting NK cell activation (24), which is consistent with our finding of reduced IFN-γ levels, an essential cytokine for NK-mediated effector function. In our ELISA assays, IFN-γ concentrations were significantly reduced in supernatants from exosome-treated NK-92 cells, suggesting impaired effector function that may facilitate GC progression by diminishing innate immune surveillance.

The upregulation of suppression markers in exosome-treated NK-92 cells provides mechanistic insight into NK cell dysfunction at the molecular level. Significant increases were observed in NKG2A, an inhibitory receptor that attenuates NK activation through HLA-E binding; TGF-β1, a cytokine that facilitates immune tolerance; and LAG-3, an exhaustion marker linked to diminished cytolytic activity. The observed changes were associated with cell cycle arrest and apoptosis, as

flow cytometry indicated a shift toward the sub-G1 and G0/G1 phases, which may reflect exosome-induced quiescence or programmed cell death in NK cells. Similar mechanisms have been reported in studies of exosomal microRNAs in GC. Tang et al. demonstrated that exosomal miR-552-5p derived from GC cells targets the PD-1/PD-L1 axis, diminishing NK cell effector function by downregulating perforin, granzyme, and IFN-γ and suppressing activating receptors such as NKp30, NKp46, and NKG2D (25). Qin et al. demonstrated that miR-552-5p promotes epithelial-mesenchymal transition (EMT) in GC by inhibiting NK cell activity through PD-1/PD-L1 interactions. In vivo evidence indicated a reduction in NK cell percentages and decreased PD-L1 expression in peripheral blood following modulation of miR-552-5p (26). Although our study did not directly evaluate miRNA content, the shared pattern of NK suppression suggests that analogous cargo, such as miR-552-5p or TGF-β-related factors, could be involved in AGS exosomes, warranting future cargo profiling.

Beyond GC, similar mechanisms have been observed in other malignancies in which TDEs circumvent NK-mediated immunity. Huang et al. demonstrated that exosomal lncRNA SNHG10 upregulates INHBC in NK cells, suppressing their cytotoxicity and promoting immune escape (27). This is consistent with our findings of increased inhibitory gene expression and decreased viability. In acute myeloid leukemia (AML), Wang et al. demonstrated that AML-derived exosomes containing PD-L1 suppress NK cell activation and cytotoxicity via the PD-1/PD-L1 pathway (28). Blockade of this pathway mitigates immunosuppression, suggesting a potential strategy for exploration in GC based on our findings. Pace et al. emphasized the role of DNAM1 receptors on NK exosomes in facilitating cytotoxicity against tumors. However, they observed that tumor exosomes can reduce this effect by downregulating DNAM1, resulting in diminished NK-tumor interactions—a possible complementary mechanism to the suppression observed in our study (29).

While these studies collectively support the role of TDEs in NK cell impairment across cancers, our study extends these observations to GC using the NK-92 model and highlights cell cycle analysis as an additional perspective. The increased sub-G1 fraction in our flow cytometry data is consistent with exosome-induced apoptosis, which may interact with exhaustion pathways such as LAG-3 upregulation to sustain long-term NK dysfunction. However, several limitations should be acknowledged. Our in vitro approach using immortalized cell lines (AGS and NK-92) may not fully recapitulate the complexity of primary NK cells or the in vivo TME, where additional factors such as hypoxia or stromal interactions could amplify exosomal effects. Furthermore, we did not examine specific exosomal cargo or functional assays beyond gene expression and ELISA, such as direct cytotoxicity against target cells. Future studies could incorporate in vivo models, exosome cargo sequencing, or PD-1/PD-L1 blockade to validate therapeutic potential, as suggested by the reversal of suppression in AML and GC models.

CONCLUSION

Our examination of exosomes derived from the AGS gastric cancer cell line reveals their influence on NK-92 cell viability and cytotoxic function, highlighting a mechanism of tumor-induced immunosuppression. By inducing upregulation of inhibitory markers such as NKG2A, TGF-β1, and LAG-3, along with reduced IFN-γ secretion and cell cycle perturbations favoring cell proliferation arrest, these exosomes effectively blunt NK cell responses and may facilitate gastric cancer progression. This work builds on existing evidence regarding the role of exosomes in immune modulation and offers a clearer understanding of how tumor cells exploit exosomes to evade innate immunity. Although our in vitro model provides molecular insights, future studies incorporating primary NK cells, in vivo validation, and exosomal cargo analyses could further elucidate therapeutic targets, such as blocking immunosuppressive pathways to restore NK activity. Ultimately, targeting GC-derived exosomes may enhance immunotherapy efficacy and improve outcomes for patients with this disease.

ETHICS CONSIDERATIONS

Not applicable

DATA AVAILABILITY

Datasets generated and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Not applicable

AUTHOR CONTRIBUTIONS

AA, SM, and AM, Investigation, Writing - Original Draft; JB, HAN, AB, and HA, Writing - Review & Editing, Validation;
MBA, Supervision, Project administration.

ARTIFICIAL INTELLIGENCE (AI)

AI tools were used only for grammatical improvements and language editing.

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