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ORIGINAL ARTICLE

Regulation of the PD-1/PD-L1 Axis and NK Cell Dysfunction by Exosomal miR-552-5p in Gastric Cancer

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Abstract

Objective NK cells play a vital role in tumor immune resistance. Various factors affect NK cell activity. While NK cell dysfunction has been observed in numerous malignancies, the underlying mechanisms in gastric cancer remain unclear.

Method Flow cytometry was used to identify the phenotypic distribution and expression of activated receptors on NK cells. ELISA was used to determine the expression of cytokines. We examined the expression of NK cell-related genes and explored their association with survival and prognosis. Additionally, we conducted PCR detection of miR-552-5p expression levels in plasma exosomes of patients and investigated its correlation with phenotypic distribution and activated receptors. We used flow cytometry and ELISA to verify the role of miR-552-5p in NK cell dysfunction. Furthermore, we investigated the potential role of PD-1/PD-L1 in regulating NK cell dysfunction in patients' cells.

Results We observed a significant decrease in the percentage of NKG2D and NKp30 and IFN-γ and TNF-α in patients than in healthy volunteers. Patients with low levels of CD56, CD16, NKG2D, and NKP46 exhibited poorer survival prognoses. Moreover, increased expression levels of plasma exosomal miR-552-5p in patients were negatively associated with NK cell phenotypic distribution and activated receptor expression. MiR-552-5p downregulated the secretion of perforin, granzyme, and IFN-γ as well as the expression of NKp30, NKp46, and NKG2D. Additionally, it suppressed the cytotoxicity of NK cells. The inhibitory effect of miR-552-5p, on NK cell function was reversed when anti-PD-L1 antibodies were used.

Conclusion Exosomal miR-552-5p targets the PD-1/PD-L1 axis, leading to impaired NK cell function.

Keywords Gastric cancer · Natural killer cell · Exosome · miR-552-5p · Dysfunction

Abbreviations

GC	Gastric cancer
TME	The tumor microenvironment
NK cell	Natural killer cell
TCGA	The cancer genome atlas
GTEx	The genotype-tissue expression
mRNA	Messenger ribonucleic acid
miRNA	MicroRNA
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
FBS	Fetal bovine serum
RPMI	Roswell Park memorial institute
DMEM	DuIbecco's modified EagIe's
PBS	Phosphate buffered solution
EDTA	Ethylenediaminetetraacetic acid
PBMC	Peripheral blood mononuclear cell
FCM	Flow cytometry
FSC	Forward scatter
SSC	Side scatter
APC	Allophycocyanin
PE-Cy7	Phycoerythrin-cyanine7
FITC	Fluorescein Isothiocyanate
ELISA	Enzyme-linked immunosorbent assay
HRP	Horseradish peroxidase
IFN-γ	Interferon γ
TNF-α	Tumornecrosis factor-α
GZMS-B	Granzyme B
WB	Western blot
SDS	Sodium dodecyl sulfonate
PAGE	Polyacrylamidegelelectrophoresis

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TEMED	Tetramethylethylenediamine
PMSF	Phenylmethanesulfonylfluoride
PVDF	Polyvinylidene fluoride
TEM	Transmission electron microscopy
DMSO	DimethylSulfoxide
LV	Lentiviral vector
CCK-8	Cell counting kit 8
PD-1	Programmed death 1
PD-L1	Programmed cell death-ligand 1
PE	Phycoerythrin

Introduction

Natural killer (NK) cells are innate lymphocytes that can act against viruses and malignancies without antigen-specific identification and amplification [1]. However, there were accumulating evidence suggests that the tumor microenvironment can alter the function of tumor-infiltrating. NK cells, rendering them dysfunctional and promoting immunosuppression. NK cell dysfunction can arise from various factors, including a diminished ability of NK cells to secrete their effectors, impaired cellular metabolism, the release of inhibitory inflammatory factors, downregulation of NK cell-activating receptor expression, or upregulation of inhibitory receptors, leading to an imbalance between activated and inhibited receptors [2, 3]. The involvement of activating receptors such as NKG2D and natural cytotoxic receptors such as NKp30, NKp44, and NKp46 is crucial for NK cell activation and killing capacity [4, 5]. Consequently, the characteristics of NK cell surface receptors and effector expression can serve as valuable indicators of their functionality. A comprehensive examination of the phenotypes and functions of NK cells can enhance our understanding of their role in tumor resistance.

According to global cancer statistics in 2020, gastric cancer (GC) ranks fifth in incidence and fourth in fatality, making it one of the most prevalent malignant tumors worldwide [6]. Despite major advancements in diagnostic techniques, surgical treatments, and chemotherapy, the prognosis for GC patients survival prognosis remains poor due to advanced disease, recurrence, metastasis, and adverse medication effects [7]. GC progression is often influenced by the interplay between tumor cells and host immune responses [8]. The activity of NK cells is closely associated with clinical staging, lymphatic and vascular invasion, lymph node metastasis, and prognosis in GC patients [9]. Reports indicate that the expression of NKp30, NKp46, NKG2D, and DNAM-1 in peripheral blood NK cells of GC patients are downregulated and correlated with tumor progression [10]. These findings underscore the strong correlation between GC development and NK cell infiltration. However, several aspects regarding the variables contributing to systemic changes in NK cell function in GC remain unknown.

Exosomal miRNAs play a vital role in reshaping the tumor microenvironment by serving as communication mediators between cells. They can convert normal cells into tumor cells, inhibit the immune cell response to tumor cells, promote angiogenesis, induce EMT, and create a microenvironment conducive to tumor growth, metastasis, immune evasion, and chemoresistance [11]. miR-552, a small non-coding RNA located on chromosome 1p34.3, is highly expressed in various cancers, including GC [12]. It promotes cell proliferation, invasion, migration, and drug resistance, thereby accelerating tumor progression [13]. Additionally, miR-552 expression has been found to correlate with the prognosis of GC patients in related studies [14]. In our previous study on exosomal miRNAs in GC, we discovered that GC cell-derived exosomal miR-552-5p promotes GC progression by inhibiting PTEN/TOB1 expression and contributing to tumor angiogenesis, invasion, and migration [15]. We thus hypothesize that exosomal miR-552-5p may be associated with immune effects in GC, leading to NK cell dysfunction in the GC microenvironment and subsequent immunosuppression.

In this study, we characterized the phenotype and expression of surface-activating receptors on peripheral blood NK cells in GC patients. We found that compared with that in healthy volunteers, the expression of NKG2D, NKp30, IFN-γ, and TNF-α was significantly downregulated in peripheral blood NK cells of GC patients, resulting in inhibited NK cell function and a poorer prognosis. Additionally, exosomal miR-552-5p expression levels in the blood of GC patients were found to be negatively correlated with the distribution of NK cell subsets CD3−/CD16+ and CD3−/CD56+ as well as the expression levels of the NK cell activating receptors NKG2D, NKp30, and NKp46. Furthermore, we identified that GC cell-derived exosomal miR-552-5p induced NK cell dysfunction by targeting the PD-1/PD-L1 axis, promoting immunosuppression in GC. Therefore, comprehending the changes in NK cell-related phenotypes, activating receptors, and cytokine expression levels holds clinical value in terms of immunosurveillance for GC. miR-552-5p and PD-1/PD-L1 represent promising immunotherapeutic targets for GC patients.

Material and Methods

Patients and Samples Collection

In total, 33 peripheral blood specimens were collected from GC patients between October 2021 and May 2022 at the Affiliated Cancer Hospital of Guangxi Medical University and First Affiliated Hospital of Guangxi Medical University.

Additionally, from December 2021 to May 2022, 22 peripheral blood specimens were collected from healthy donors at the Cancer Hospital of Guangxi Medical University. All specimens were collected with the approval of the ethics committee of the First Affiliated Hospital of Guangxi Medical University and Affiliated Cancer Hospital of Guangxi Medical University (Approval Number: LW2024063), per the principles of medical ethics and informed consent. The inclusion criteria for GC patients were as follows: first diagnosis of GC, no prior surgical treatment or radiation, and no autoimmune illnesses, multisite tumors, or substantial infections. Healthy donors were individuals who were in good health, free of major physical and mental illnesses, and routinely underwent medical examinations. The RNA sequencing data of tumor and normal tissues of GC patients, along with corresponding clinical information, were obtained from the UCSC XENA (https://xenabrowser.net/datapages/) database. As these data are publicly available, ethical approval was not required. Table 1 presents the clinical characteristics of the GC patients included in the study.

Cell Culture

SGC-7901 and AGS cell lines were obtained from the ATCC cell bank of the Chinese Academy of Sciences and cultured in RPMI 1640 medium (Gibco, USA) or Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Biological Industries, Israel) and 1% penicillin–streptomycin. The NK-92 cell line (derived from NK cells of human malignant non-Hodgkin's lymphoma patients and purchased from Procell Life Sciences Ltd.) was cultured in RPMI-1640 (Gibco) supplemented with 20% FBS and 100 IU/mL recombinant human interleukin-2 (IL-2; PeproTech Human IL-2, Shanghai, China) or NK cell-specific medium (PeproTech). All cell lines were cultured at 37 °C in a 5% CO2 incubator.

Table 1 The following table presents baseline clinical information obtained from patients diagnosed with gastric cancer

Characteristics	Levels	Overall
n		33
Age group, n (%)	≤60	16 (48.5%)
	>65	17 (51.5%)
Gender, n (%)	Female	11 (33.3%)
	Male	22 (66.7%)
Clinical stage, n (%)	I	6 (18.2%)
	II	3 (9.1%)
	III	7 (21.2%)
	IV	17 (51.5%)
T, n (%)	T1	4 (12.1%)
	T2	4 (12.1%)
	T3	10 (30.3%)
	T4	15 (45.5%)
N, n (%)	N0	3 (9.4%)
	N1	4 (12.5%)
	N2	10 (31.2%)
	N3	15 (46.9%)
M, n (%)	M0	16 (48.5%)
	M1	17 (51.5%)
Age, median (IQR)		60 (51, 67)

Antibodies and Flow Cytometry

Single-cell suspensions were stained using the following antibodies: anti-human CD3 PE (BioGems, USA), anti-human CD16 PE-Cy7 (BioGems), anti-human CD56 APC (BioGems), anti-human NKG2D (CD314) PE (BioGems), anti-human NKp30(CD337) PE-Cy7 (BD, USA), anti-human NKp46(CD335) APC (BD), and PD-L1 PE (CST, USA). Cells were stained with the antibodies ed according to the manufacturer's instructions and analyzed using flow cytometry. Data analysis was performed using the FlowJo software (BD Biosciences).

Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma samples from GC patients and healthy volunteers were collected using standard protocols. The concentration of IFN-γ and TNF-α in the plasma was quantified using ELISA kits (China Lianke Biotech) following the manufacturer's instructions. Cell culture supernatants were also collected, and the concentrations of IFN-γ, perforin, and granzyme B were determined using ELISA kits (China Lianke Biotech for IFN-γ and Shanghai Enzyme-linked Biotechnology perforin and granzyme B) according to the manufacturer's instructions.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

RNA was extracted from plasma samples using the miRNeasy serum/plasma kit (Qiagen, Germany) following the manufacturer's instructions. Exosomal RNA was purified using the exoRNeasy serum/plasma Midi kit (Qiagen). Reverse transcription was performed using the PrimeScript RT kit (TaKaRa, Japan) or Mir-X miRNA first strand synthesis kit (TaKaRa) for miRNA. qRT-PCR was performed using TBGreen® Premix Ex Taq (TaKaRa) with the following protocol: pre-denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 30 s. U6 was used as an internal control to normalize the miRNA expression levels. The relative expression was determined using the 2-ΔΔCT method.

The following primers were used:

has-miR-552-5p forward, 5′-ATTTAACCTTTTGCCTGT TGGAA-3′;
U6 forward, 5′-GGAACGATACAGAGAAGATTAGC-3′;
U6 reverse, 5′-TGGAACGCTTCACGAATTTGCG-3′.

Exosome Extraction

Exosome-free FBS was obtained by centrifugation at 100,000×*g* at 4 °C for 16 h [16]. SGC-7901 cells were cultured for 48 h in DMEM until they reached approximately 80% confluence, after which their medium was replaced with an exosome-free conditioned medium. Following a 48 h incubation, the culture medium was harvested and subjected to centrifugation at 300×*g* at 4 °C for 10 min and then at 2000×*g* at 4 °C for 20 min to remove dead cells and cell debris. The larger vesicles were subsequently removed by centrifugation at 10,000×*g* at 4 °C for 30 min. The supernatant was filtered through a 0.22 μm filter (Millipore, USA), and the exosomes were pelleted by centrifugation at 100,000×*g* at 4 °C for 70 min. The resulting pellet was diluted with PBS and centrifuged at 100,000×*g* at 4 °C for 70 min. Finally, the exosome pellets were resuspended in PBS and stored at − 80 °C.

Transmission Electron Microscopy (TEM)

The resuspended exosomes were thoroughly mixed, and 5 μL of the exosome suspension was applied onto a formvar carbon-coated copper grid, allowing it to adsorb for 5 min. The suspension was then negatively stained using a 2% uranyl acetate solution, and the density and size of the exosomes were determined under an electron microscope (Hitachi, Japan).

Western Blot Analysis

Exosomes were lysed in radioimmunoprecipitation lysis buffer (RIPA) containing PMSF (100:1 dilution), and the total protein concentration was determined using the BCA protein quantification kit. Protein samples were separated using polyacrylamide gels containing 10% SDS (SDS-PAGE) and then transferred to 0.22 μm polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked in TBST containing 5% skim milk for 1.5 h. After washing three times with PBS, PVDF membranes were incubated overnight at 4 °C with antibodies against the following proteins: TSG101, CD9, and ALIX (ImmunoWay). Following three washes with PBS, the membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence or immunofluorescence.

Lentiviral Transfection

Lentiviral plasmids were synthesized by Genechem (Shanghai, China) to overexpress miR-552-5p ("Lv-miR-552-5p"), and the corresponding negative controls were labeled as "Lv-NC." Cells were seeded in a 6-well plate (5 × 104 cells/mL) and transfected with the lentiviral plasmids. After 3 days of culture, cells were selected in 1.5 μg/mL puromycin. The expression levels of miR-552-5p were verified using quantitative PCR.

CCK8

GC cells treated with lentivirus transfection were co-cultured with NK-92 cells for 24 h, The NK-92 cells were then removed from the lower chamber and resuspended in a complete medium. The cell suspension was inoculated in 96-well plates at 100 μL/well with 5000 NK-92 cells/well, and each group had five replicate wells. The plates were shaken well to ensure even distribution. Thereafter, 10 μL CCK-8 was added to each well, and the plates were incubated in a 5% CO2 incubator at 37 °C for 3 h. After incubation, the plates were removed from the cell incubator, and the absorbance values at 450 nm were measured.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9.0.0 for Windows, GraphPad Software. Comparisons between the two groups were made using the independent samples t-test for parametric data and the Mann–Whitney *U* test for nonparametric data. The results are expressed as mean ± standard deviation for parametric data and median (or quartiles) for nonparametric data. One-way analysis of variance was used to compare samples among multiple groups. Correlation analysis between different groups was conducted using Pearson or Spearman's correlation test. Results were considered statistically significant at $P < 0.05$.

Results

Downregulation of NKG2D and NKp30 Receptors and IFN-γ and TNF-α Cytokines in GC

Initially, we characterized the distribution of CD3−/CD56+ and CD3−/CD16+ peripheral blood NK cell subsets in GC patients. Figure 1A–C show lymphocytes circled based on FSC and SSC characteristics and highlight the CD3−/CD56+ and CD3−/CD16+ cell populations. Statistical analysis revealed no significant difference in the distribution of peripheral blood NK cell subsets between GC patients and healthy donors (Fig. 1D, E).

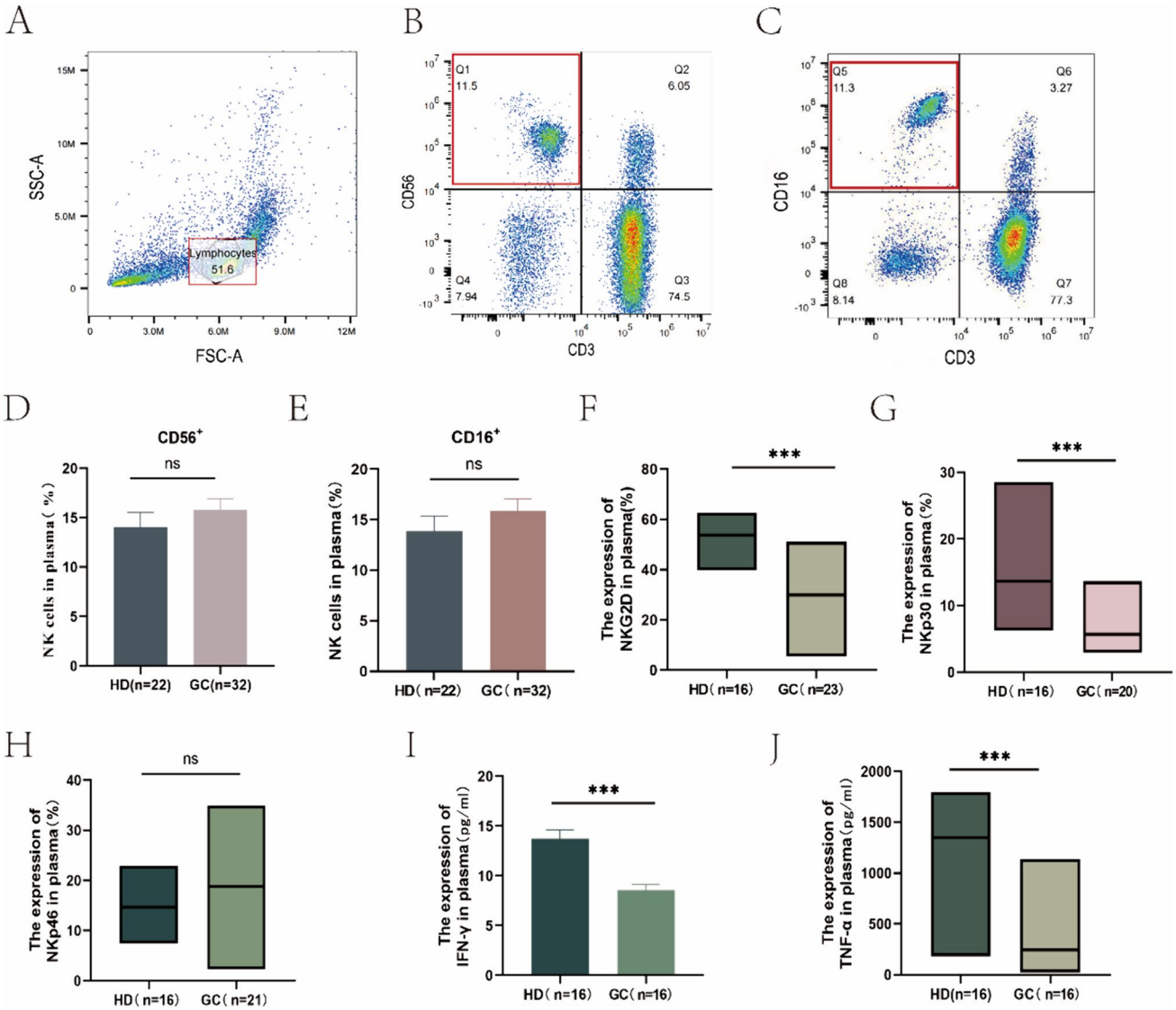

Fig. 1 Downregulation of peripheral blood NK cell-activating receptors NKG2D and NKp30 and cytokine factors IFN-γ and TNF-α in gastric cancer. **A**–**C** NK cell gating strategy. **D** and **E** Flow cytometry analysis comparing the expression of NK cell subsets CD3−/CD56+ and CD3−/CD16+ in GC and HD peripheral blood. **F**–**H** Flow cytometry analysis comparing the expression of NK cell receptors NKG2D, NKp30, and NKp46 in GC and HD peripheral blood. **I** and **J** ELISA detection of cytokine IFN-γ and TNF-α expression in GC (n = 16) and HD (n = 16) plasma. *P < 0.05; **P < 0.01; ***P < 0.001; *ns* not significant

Further investigation showed considerably lower expression levels of NKG2D and NKp30 receptors on peripheral blood NK cells in GC patients compared with those in healthy volunteers (Fig. 1F, G). However, no substantial difference was observed in the expression of the NK cell-activating receptor NKp46 between GC patients and healthy volunteers (Fig. 1H). Additionally, we examined the expression of the cytokines IFN-γ and TNF-α in the plasma of GC patients and healthy volunteers. Our findings demonstrated that the expression of IFN-γ and TNF-α in the plasma of GC patients was lower than that in healthy volunteers (Fig. 1I and J). Collectively, our results indicate that expression of NK cell-activating receptors NKG2D and NKp30 and associated cytokines IFN-γ and TNF-α are reduced, and NK cells are inhibited in the plasma of GC patients.

Correlation of Patient Survival Prognosis with Downregulation of NK Cell-Related Genes CD56 and NKG2D

To gain further insight into the functional role of NK cells in GC, we analyzed RNAseq data from TCGA and GTEx using UCSC XENA (https://xenabrowser.net/datapages/). The analysis was performed using the Toil process and

focused on comparing the expression of CD56 and NKG2D between GC and normal tissues. The expression of CD56 and NKG2D in GC tumor tissues was significantly lower than that in normal tissues (Fig. 2A and B). Furthermore, we investigated the association between NK cell-related genes and survival prognosis in GC patients, using the Kaplan–Meier Plotter online database (http://kmplot.com/analysis/index.php?p=background). We analyzed mRNA gene sequencing data from GC datasets (GES14210, GES15459, GES22377, GES29272, and GES51105) corresponding to the coding genes FCGR3, NCAM, KLRK1, NCR3, and NCR1, which correspond to CD16, CD56, NKG2D, NKp30, and NKp46, respectively. Our results demonstrated a significant association between the expression of CD56, CD16, NKG2D, and NKp46 and the survival prognosis of GC patients. However, the expression of NKp30 was not significantly different between the groups (Fig. 3A–E). These findings provide insights into the potential prognostic role of NK cells and associated markers in GC.

Associations of Increased Plasma Exosomal miR-552-5p Expression with NK Cell Phenotype and Activated Receptor Expression

In our previous study, we found increased expression of plasma exosomal miR-552-5p expression in GC patients, which was associated with tumor infiltration depth, lymph node metastases, and TNM stage [15]. To investigate the potential link between the systemic downregulation of NK cell function and the expression of plasma exosomal miR-552-5p, we examined its expression in GC patients and its correlation with the distribution of peripheral blood NK cell subpopulations and activation receptors. We observed a negative correlation between plasma exosomal miR-552-5p expression levels and the distribution of NK cell subpopulations (Fig. 4A and B). Additionally, an inverse linear relationship was observed between the expression of plasma exosomal miR-552-5p and the positive expression of NK cell-activated receptors (Fig. 4C–E). These findings imply a potential association between the systemic downregulation of NK cell activity and high levels of GC plasma exosomal miR-552-5p.

Induction of NK Cell Dysfunction by GC Cell-Derived Exosomes

To investigate the impact of GC cell-derived exosomes on NK cell function, we extracted exosomes from the supernatants of SGC-7901 and AGS GC cells, using a gradient centrifugation approach. Western blot analysis confirmed the presence of exosomal protein markers ALIX, TSG101, and CD9 (Fig. 5A). Electron transmission microscopy revealed the characteristic vesicle structure of exosomes, ranging from 40 to 160 nm in diameter and exhibiting a near hemispherical pattern (Fig. 5B and C). To determine the influence of GC cell-derived exosomes on NK cells, we co-cultured NK-92 cells with SGC-7901 and AGS cells in a 1:1 ratio for 24 h (Fig. 5D). Compared with NK-92 cells cultured alone, NK-92 cells co-cultured with SGC-7901 and AGS cells exhibited reduced expression levels of NKG2D, NKp30, and NKp46 as well as impaired secretion of IFN-γ (Fig. 5E–G). In contrast, when SGC-7901 and AGS cells were co-cultured with NK-92 cells for 24 h in the presence of the exosome inhibitor GW4869, the expression of NKG2D, NKp30, and NKp46 in NK-92 cells was restored compared with that in the group without GW4869 (Fig. 5H and I). Additionally, the level of IFN-γ secretion by NK-92 cells increased (Fig. 5J and K). These results suggest that GC cell-derived exosomes contribute to NK cell dysfunction.

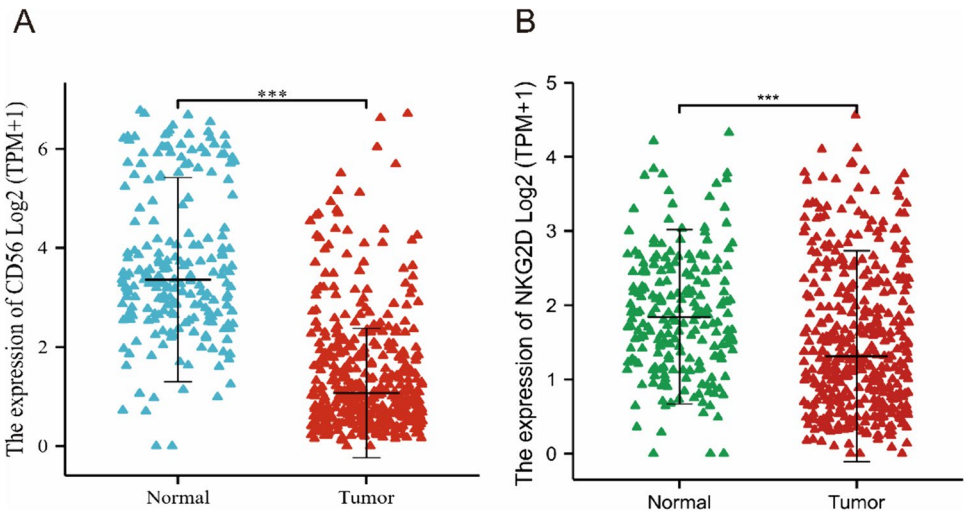

Fig. 2 Downregulation of NK cell-related genes CD56 and NKG2D in gastric cancer tissues. **A** Downregulation of CD56 in paired GC cancer tissues (n = 414) compared with that in normal tissues (n = 210), using a combination of TCGA and GTEx data. **B** Downregulation of NKG2D in paired GC cancer tissues (n = 414) compared with that in normal tissues (n = 210), using a combination of TCGA and GTEx data. *P < 0.05; **P < 0.01; ***P < 0.001

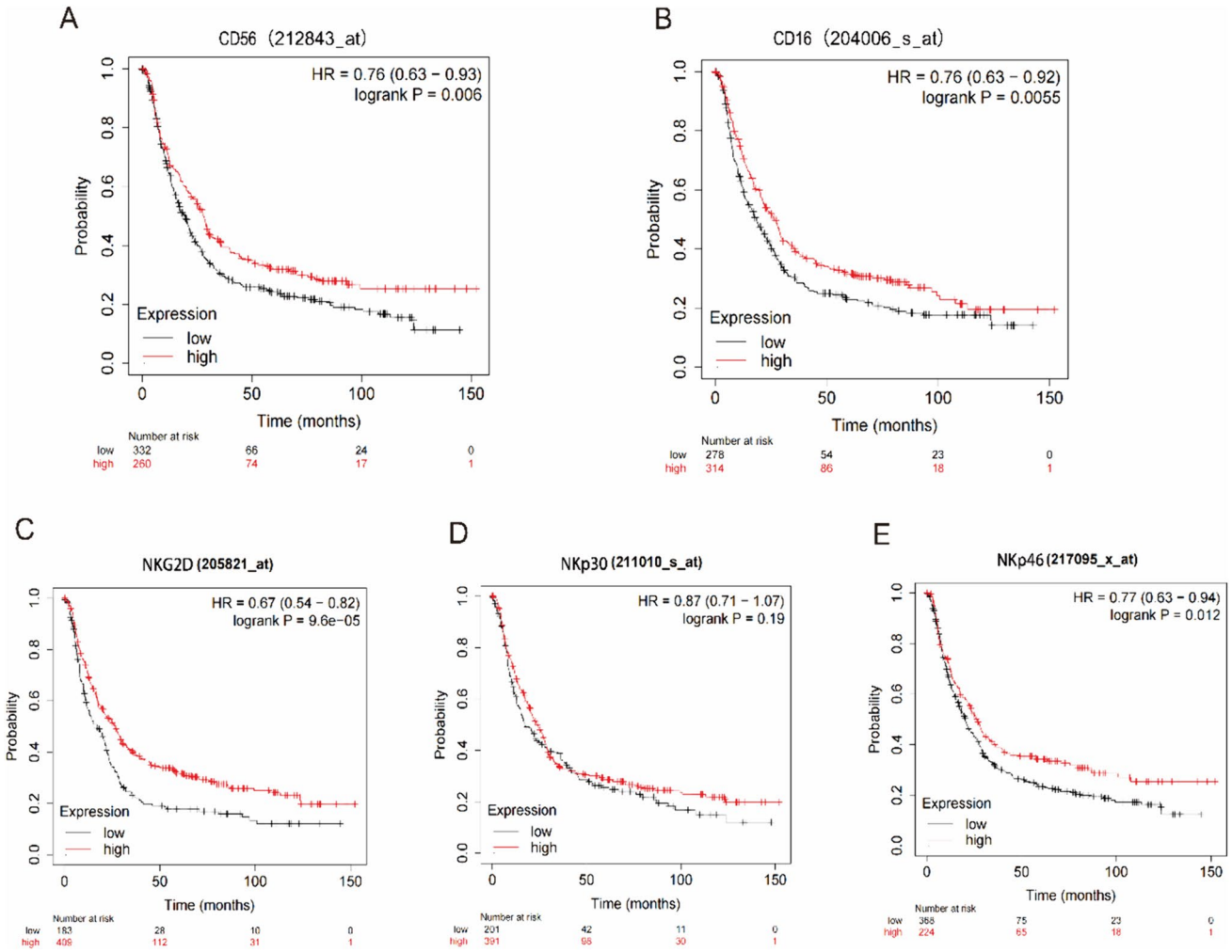

Fig. 3 Correlation of NK cell phenotype and activation receptor gene expression with prognosis in gastric cancer. Kaplan–Meier survival analysis revealed the following associations with poor prognosis in gastric cancer patients: low CD56 expression (**A**), low CD16 expression (**B**), low NKG2D expression (**C**), and low NKp46 expression (**E**). However, no correlation between NKp30 expression and prognosis was observed (**D**)

Induction of NK Cell Dysfunction by Exosomal miR-552-5p

To investigate whether exosome-mediated miR-552-5p induction is responsible for NK cell dysfunction in GC cells, we upregulated miR-552-5p in SGC-7901 and AGS cells using a lentiviral vector, along with a comparable negative control (Lv-NC). qRT-PCR analysis verified significantly high expression of miR-552-5p expression in AGS and SGC-7901 cells (Fig. 6A). Moreover, exosomal miR-552-5p inhibited the expression of NKG2D, NKp30, and NKp46 (Fig. 6B and C). Furthermore, high levels of exosomal miR-552-5p in GC cells markedly suppressed the release of perforin, granzyme B, and IFN-γ by NK-92 cells (Fig. 6E–G). We also assessed the cytotoxicity of NK cells using a CCK8 assay and observed that exosomal miR-552-5p significantly inhibited the cytotoxicity of NK-92 cells compared with that in the control group (Fig. 6D). These findings demonstrate that the GC cell-derived exosomal miR-552-5p inhibits NK-92 cell function.

Induction of NK Cell Dysfunction via the PD-1/PD-L1 Axis by Exosomal miR-552-5p

NK cells can enhance the expression of PD-L1 in the PD-1/PD-L1 pathway in tumor cells, thereby regulating tumor progression [16]. Furthermore, PD-L1 is expressed not only on tumor cells but also on certain immune cells, such as NK cells and macrophages, in the tumor microenvironment. Some tumor cells with low PD-L1, such as the K562 cell line or AML patient-derived tumor cells,

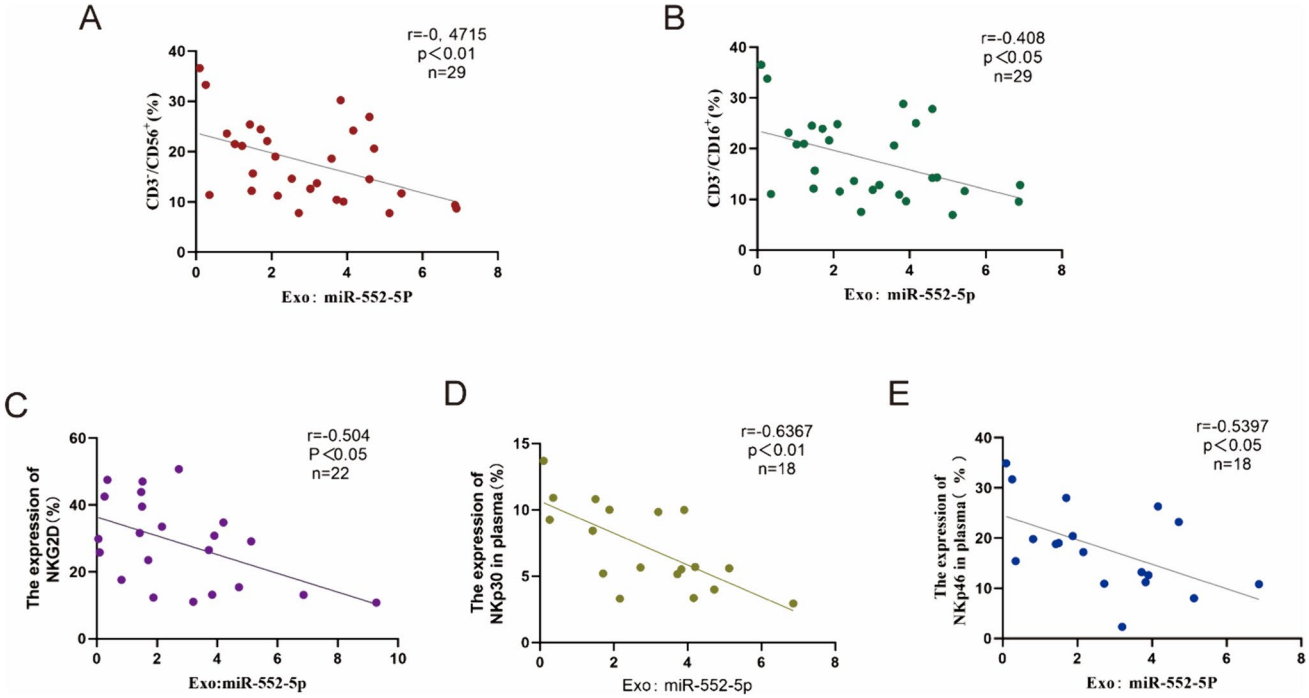

Fig. 4 Correlation of plasma exosomal miR-552-5p with NK cell phenotypes and activated receptors in gastric cancer. **A** and **B** Negative correlation between plasma exosomal miR-552-5p expression and the distribution of CD3−/CD56+ and CD3−/CD16+ subpopulations in peripheral blood NK cells of gastric cancer patients. **C–E** Negative correlation between plasma exosomal miR-552-5p expression and the expression of peripheral blood NK cell-activating receptors NKG2D, NKp30, and NKp46 in gastric cancer

can stimulate NK cells in the tumor microenvironment to upregulate PD-L1 expression. Consequently, these NK cells demonstrate enhanced tumor-killing capabilities [17]. To verify the altered expression of PD-L1 in NK-92 cells, we co-cultured GC cells transfected with Lv-miR-552-5p and Lv-NC with NK-92 cells and detected PD-L1 expression in NK-92 cells using flow cytometry. We observed downregulation of PD-L1 expression in NK cells co-cultured with Lv-miR-552-5p compared with that in the control group (Fig. 7A–C). To further investigate the effect of miR-552-5p on NK cell function via the PD-1/PD-L1 axis, we stimulated NK-92 cells with or without the anti-PD-L1 antibody durvalumab. The experiment included three groups: Lv-NC, Lv-miR-552-5p, and Lv-miR-552-5p + Anti-PD-L. Flow cytometry and ELISA were used to assess the expression of PD-L1 + NK-92 cells, granzyme B, perforin, and IFN-γ. We observed an increase in PD-L1 expression in NK-92 cells stimulated with PD-L1 antibody compared with that in the group stimulated without the anti-PD-L1 antibody (Fig. 7D–F). Furthermore, the expression levels of GZMS-B, perforin, and IFN-γ were restored in NK-92 cells (Fig. 7G–I). These findings indicate that GC cell-derived exosomal miR-552-5p can induce NK cell dysfunction via the PD-1/PD-L1 axis (Fig. 8).

Discussion

NK cells play a crucial role in immunological homeostasis and tumor surveillance by directly increasing cytotoxicity via mechanisms including activation of surface receptors, suppression of receptor dynamic expression, and release of perforin and granzyme B. They also produce antitumor-active chemicals, immunomodulatory cytokines, such as IFN-γ and TNF-α, and chemokines to induce cytotoxicity in tumors and limit their spread, thereby activating early cellular defenses. Therefore, NK cell depletion or dysfunction could promote tumor growth [18, 19]. In this study, we observed a significant reduction in the ratio of peripheral blood NK cell-activated receptors NKG2D and NKp30 in GC patients, along with the downregulation of plasma cytokines IFN-γ and TNF-α. Furthermore, the expression of NK cell-related genes CD56, CD16, NKG2D, and NKP46 was reduced in GC tissues. The survival prognosis of GC patients was found to be poor. These findings suggest that dysfunction or depletion of NK cells is associated with the development and progression of GC.

To understand the heterogeneity of immune cells, clarifying their distribution and phenotypic characteristics in functional immune status during tumor progression is necessary [20]. The number of infiltrating NK cells, expression of surface receptors, and release of immune activators are

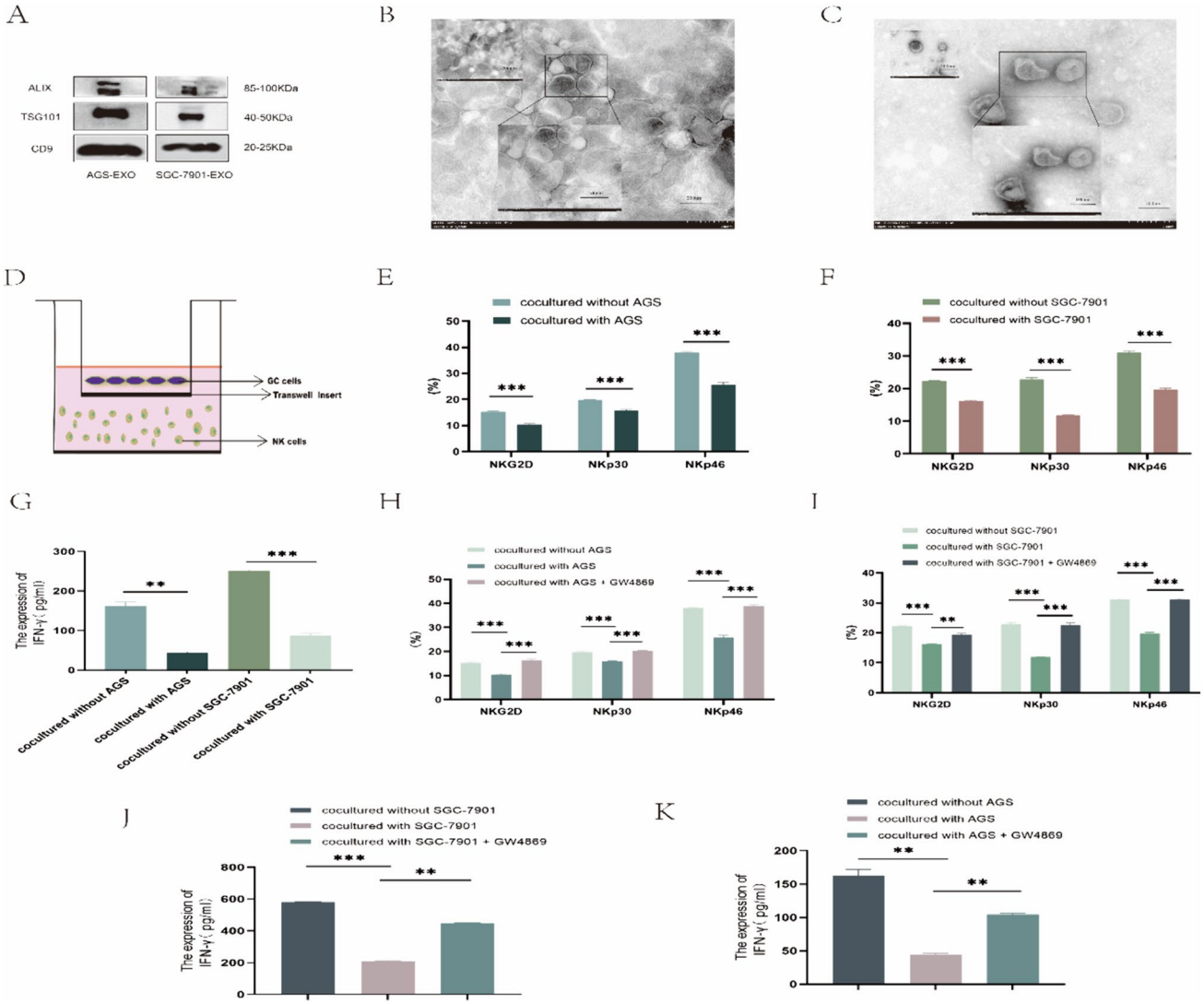

Fig. 5 Induction of NK cell dysfunction by gastric cell-derived exosomes. **A** Western blotting analysis of exosome marker (ALIX, TSG101, and CD9) expression. **B** and **C** Representative electron micrographs of exosomes isolated from SGC-7901 and AGS cell culture medium. Scale bars: 200 and 100 nm. **D** Schematic diagram of the co-cultivation model **E**–**F** Inhibition of the expression of NK cell-activation receptors NKG2D, NKp30, and NKp46 by gastric cancer cells. **G** Inhibition of the secretion of the NK cell cytokine IFN-γ by gastric cells. **H** and **I** GW4869 inhibits gastric cancer cell-induced downregulation of NK cell-activated receptors. **J** and **K** GW4869 reverses the inhibitory effect of gastric cancer cells on IFN-γ secretion by NK cells. *P < 0.05; **P < 0.01; ***P < 0.001

associated with the development and prognosis of multiple human tumors [21, 22]. In our study, we found no significant change in the distribution of CD3−/CD56+ and CD3−/CD16+ subpopulations of NK cells in the peripheral blood of GC patients and healthy volunteers, which is consistent with the findings of Han et al. [10]. NK cells are divided into distinct subpopulations and mature bodies based on different stages of differentiation, and the expression of representative subpopulations of NK cells varies at different stages of maturation, maintaining a dynamic equilibrium [23]. However, further characterization revealed a significant decrease in the percentage of NK cell activation receptors NKG2D and NKp30 expression in the peripheral blood of GC patients. The downregulation of NKG2D promotes immune escape in several malignancies, including cervical cancer, pancreatic cancer, and melanoma [24]. Decreased expression levels of NK cell activation receptors often result in insufficient activation of NK cells, impairing their tumor-fighting effects [25]. We found that the plasma levels of IFN-γ and TNF-α in GC patients were significantly lower than those in healthy volunteers. The reduced ability of NK cells to secrete their killing effectors is the primary factor contributing to the dysfunction of NK cells [26]. Bioinformatics analysis combined with our findings revealed the

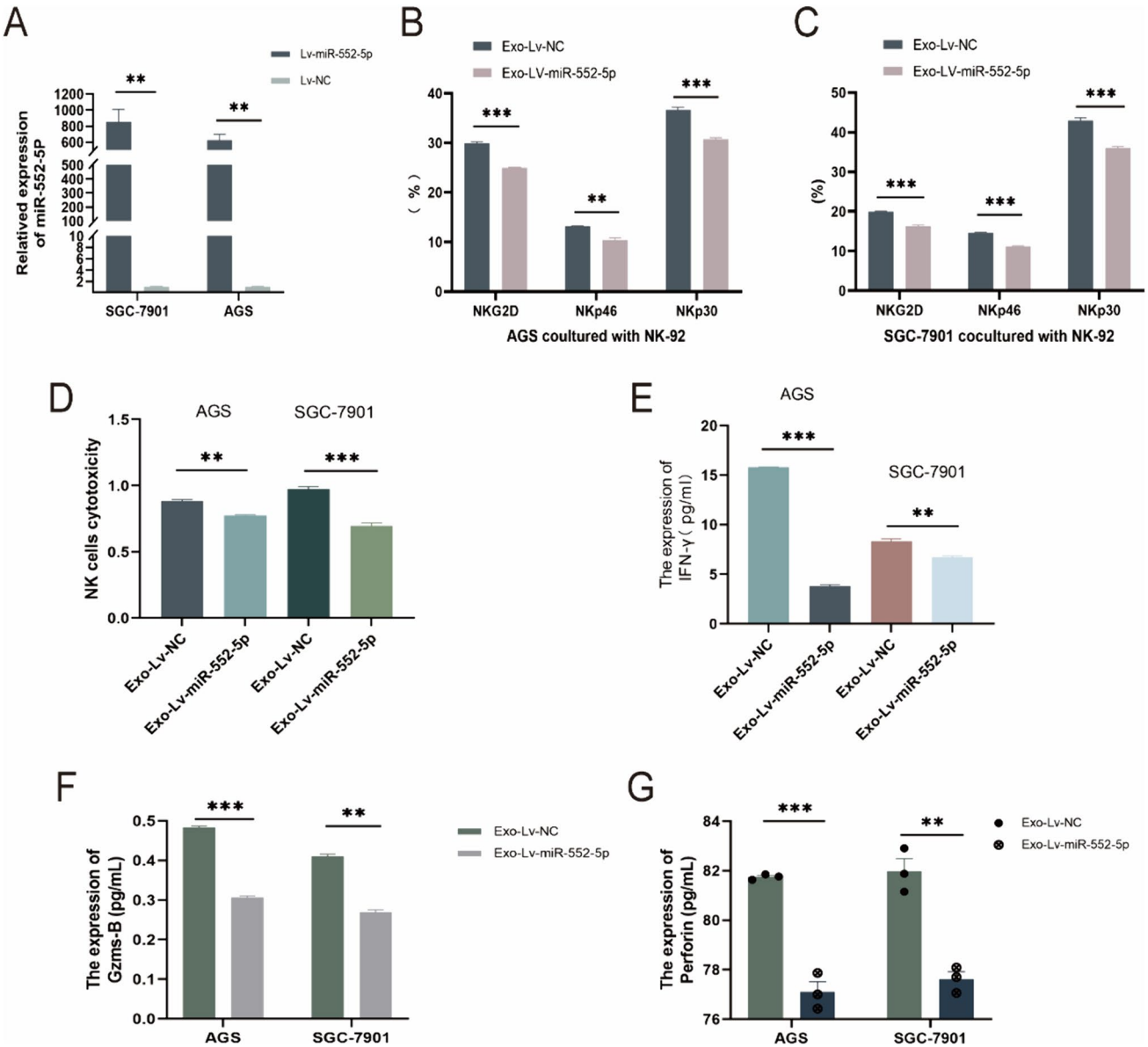

Fig. 6 Induction of NK cell dysfunction by gastric cancer cell-derived exosomal miR-552-5p. **A** qRT-PCR analysis of miR-552-5p levels in lentiviral vector-transfected cells with upregulated miR-552-5p (Lv-miR-552-5p) and corresponding negative control (Lv-NC). **B** and **C** Flow cytometry analysis examining the effect of gastric cancer cell-derived exosomal miR-552-5p on the expression of NKG2D, NKp30, and NKp46 receptors. **D** CCK-8 cytotoxicity assay evaluating the impact of gastric cancer cell-derived exosomal miR-552-5p on NK cell cytotoxicity. **E–G** ELISA assessing the effect of gastric cancer cell-derived exosomal miR-552-5p on IFN-γ, granzyme B, and perforin production by NK cells. *P < 0.05; **P < 0.01; ***P < 0.001

downregulation of NK cell-related genes CD56 and NKG2D in GC tissues, and the expression levels of CD56, CD16, NKG2D, and NKp46 were correlated with the survival prognosis of GC patients. Therefore, it is reasonable to assume that NK cell function is systematically downregulated in GC, leading to a suppressed state of NK cells. In melanoma, downregulation of NKG2D, NKp46, and DNAM-1 receptors is associated with NK cell dysfunction and serves as a key marker of disease progression and poor prognosis [27]. Earlier studies have reported a decrease in NK cell function in both GC tissue and peripheral blood [28]. However, owing to the limited number of GC patients included in our cohort, elucidating the correlation between expression levels of peripheral blood NK cell-activating receptors and the disease stage and prognosis of GC patients in this study was not possible. Further studies with expanded patient cohorts are necessary to provide comprehensive insights into this association.

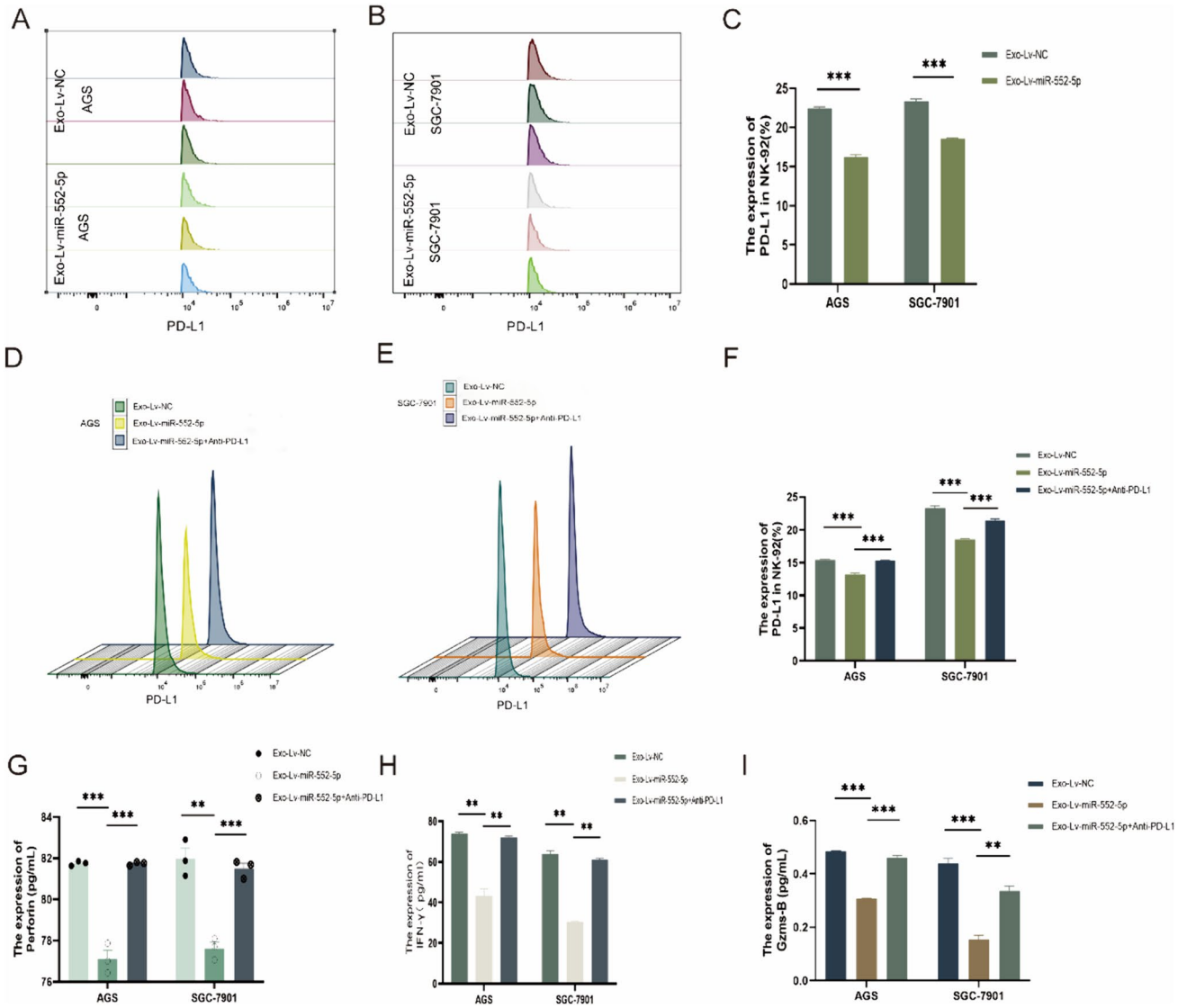

Fig. 7 Induction of NK cell dysfunction by gastric cancer cell-derived exosomal miR-552-5p via the PD-1/PD-L1 axis. **A**–**C** Flow cytometry demonstrating the expression level of PD-L1 on the surface of NK cells under the influence of gastric cancer cell-derived exosomal miR-552-5p, where **C** is a quantitative plot of the flow assay results. **D**–**F** Blockage of the downregulation of PD-L1 in NK cells induced by gastric cancer cell-derived exosomal miR-552-5p, where **F** is a quantitative plot of the flow assay findings. **G**–**I** Reversal of the inhibitory effect of gastric cancer cell-derived exosomal miR-552-5p on IFN-γ, granzyme B, and perforin secretion from NK cells through anti-PD-L1 antibody. *P < 0.05; **P < 0.01; ***P < 0.001

Exosomes are primarily produced by human body fluids and encapsulate nucleic acids, proteins, cholesterol, and lipids. They not only reflect the malignant phenotype of donor cells but also transmit oncogenic signals to recipient cells, thereby accelerating tumor development [29]. Exosomes can influence NK cells by inhibiting the expression of NK cell-associated receptors or cytokines, affecting the functional state of NK cells against tumor cells, and inducing immunosuppression [30]. Exosomal miRNAs serve as important carriers of information for reprogramming immune response factors and immune target cells such as dendritic cells (DCs), NK cells, and T lymphocytes. Through the regulation of NK cell immune function, exosomal miRNAs contribute to immune tolerance in tumor cells. This process is complex, involving multiple links, targets, and factors. The interaction between exosomes and NK cells often leads to changes in the expression levels of NK cell antitumor factors, such as IL-4, IFN-γ, activating receptors, granzyme B, and perforin, thereby promoting the immunosuppressive effects of NK cells [31, 32]. In a previous study by our research group, we found higher levels of plasma exosomal miR-552-5p in GC patients than in healthy donors. The expression of exosomal miR-552-5p was correlated with tumor infiltration depth, lymph node metastasis,

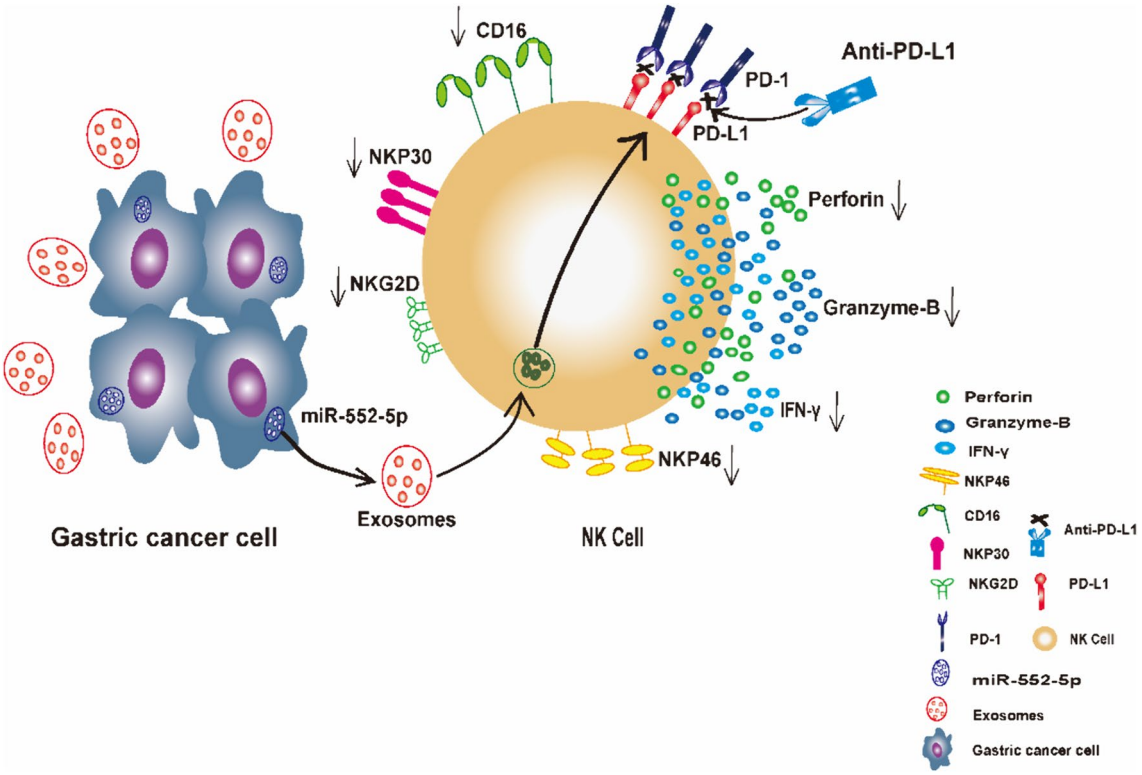

Fig. 8 Schematic representation of how GC cell-derived exosomal miR-552-5p promotes NK cell dysfunction. Exo-miR-552-5p acts on NK cells, leading to dysregulation of NK cell-activating receptor NKG2D, NKp30, and NKp46 expression and reduced secretion of IFN-γ, perforin, and granzyme B. This outcome may be regulated through the PD-1/PD-L1 axis

and TNM stage [15]. Hence, we examined the expression levels of plasma exosomal miR-552-5p in GC patients to explore its association with NK cells. We observed a negative correlation between the expression of plasma exosomal miR-552-5p and the ratio of peripheral blood NK cell subpopulations CD3−/CD16+ and CD3−/CD56+ as well as the NK cell-activating receptors NKG2D, NKp30, and NKp46. Exosomal miRNAs are closely associated with NK cell activity and function in various cancers, including neuroblastoma, breast cancer, and ovarian cancer [33, 34]. Moreover, a negative correlation exists between circUHRF1 expression in plasma exosomes and the proportion of NK cells in the blood of hepatocellular carcinoma patients [35].

Exosomes have been extensively studied in tumor-related fields. Compared with normal cells, tumor cells tend to release a higher quantity of exosomes, which exhibit strong immunomodulatory capabilities in the tumor microenvironment [36]. In renal clear cell carcinoma, tumor cell-derived exosomes induce NK cell dysfunction by regulating the TGF-β/SMAD axis, thereby facilitating immune evasion by the tumor [37]. In pancreatic ductal adenocarcinoma, tumor cell-derived exosomes influence NK cells, resulting in the downregulation of NKG2D, TNF-α, and INF-γ and consequently inducing NK cell dysfunction [38]. In hepatitis B patients, exosomes carry HBV to uninfected hepatocellular carcinoma cells, leading to NK cell dysfunction by inhibiting RIG-I expression and downstream signaling pathways [39]. These findings underscore the crucial role of exosomes in NK cell immunomodulation. Based on these observations, we hypothesized that the GC cell-derived exosomal miR-552-5p may also affect NK cells and contribute to their dysfunction. To validate this hypothesis, we conducted in vitro experiments in which we exposed NK cells to GC cell-derived exosomal miR-552-5p. Our results demonstrated that exposure to exosomal miR-552-5p induced functional impairment in NK cells, including the downregulation of NKG2D, NKp30, and NKp46 receptors, reduced expression levels of IFN-γ, decreased secretion of granzyme B and perforin, and decreased toxicity of NK cells. These findings suggest that exosomal miR-552-5p plays a key role in inducing NK cell dysfunction in GC.

In the tumor microenvironment, PD-L1 is commonly expressed on tumor cells, while T cells often express its receptor PD-1. The binding of PD-L1 from tumor cells to the PD-1 receptor on T cells transmits inhibitory signals, eventually leading to T cell failure [40]. However, PD-L1 is reportedly expressed not only on tumor cells but also on NK cells, macrophages, and T cells. High expression of PD-L1 can

enhance the immune response of NK cells [41–43]. Therefore, we further investigated the expression of PD-L1 in NK cells in GC. Our findings revealed a decrease in PD-L1 levels in NK cells in GC, and the use of an anti-PD-L1 antibody restored the ability of NK cells to secrete perforin, granzyme B, and IFN-γ. These results are consistent with those of a recent study demonstrating that compared with PD-L1-negative NK cells, PD-L1-positive NK cells in acute leukemia patients exhibited larger size, higher secretion of the effector molecules CD107a and IFN-γ, and increased tumor-killing potency. Moreover, patients who achieved complete remission after two weeks of standard chemotherapy had significantly higher levels of PD-L1 positive NK cells than those who did not achieve complete remission [17]. Collectively, our findings suggest that cancer cell-derived exosomal miR-552-5p exerts immunosuppressive effects on NK cells in GC, and the small molecule inhibitor of PD-L1, durvalumab, can reverse miR-552-5p-induced dysfunction of NK cell. Additionally, the PD-1/PD-L1 axis is associated with NK cell immunosuppression in GC.

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Author's contribution CT contributed to the design, documentation of clinical information, experimental work and analysis, and wrote the manuscript. JY contributed to the analysis of the study and writing of the manuscript. JQ and HC contributed to experimental work and analysis. HW contributed to the design. CT and JY contributed equally to this work. AL and LG contributed to experimental design and provided funding. All authors contributed to the article and approved the submitted version.

Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

References

1. Shifrin N, Raulet DH, Ardolino M. NK cell self tolerance, responsiveness and missing self recognition. *Semin Immunol* 2014;26:138–144. https://doi.org/10.1016/j.smim.2014.02.007.
2. O'Brien KL, Finlay DK. Immunometabolism and natural killer cell responses. *Nat Rev Immunol* 2019;19:282–290. https://doi.org/10.1038/s41577-019-0139-2.
3. Abel AM, Yang C, Thakar MS et al. Natural killer cells: development, maturation, and clinical utilization. *Front Immunol* 2018;9:1869. https://doi.org/10.3389/fimmu.2018.01869.
4. Wensveen FM, Jelenčić V, Polić B. NKG2D: a master regulator of immune cell responsiveness. *Front Immunol* 2018;9:441. https://doi.org/10.3389/fimmu.2018.00441.
5. Wu S-Y, Fu T, Jiang Y-Z et al. Natural killer cells in cancer biology and therapy. *Mol Cancer* 2020;19:120. https://doi.org/10.1186/s12943-020-01238-x.
6. Sung H, Ferlay J, Siegel RL et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021;71:209–249. https://doi.org/10.3322/caac.21660.
7. Nakauchi M, Vos E, Tang LH et al. Outcomes of neoadjuvant chemotherapy for clinical stages 2 and 3 gastric cancer patients: analysis of timing and site of recurrence. *Ann Surg Oncol* 2021;28:4829–4838. https://doi.org/10.1245/s10434-021-09624-5.
8. Zhao L, Liu Y, Zhang S et al. Impacts and mechanisms of metabolic reprogramming of tumor microenvironment for immunotherapy in gastric cancer. *Cell Death Dis* 2022;13:378. https://doi.org/10.1038/s41419-022-04821-w.
9. Du Y, Wei Y. Therapeutic potential of natural killer cells in gastric cancer. *Front Immunol* 2018;9:3095. https://doi.org/10.3389/fimmu.2018.03095.
10. Han B, Mao F, Zhao Y et al. Altered NKp30, NKp46, NKG2D, and DNAM-1 expression on circulating NK cells is associated with tumor progression in human gastric cancer. *Journal of Immunology Research* 2018;2018:1–9. https://doi.org/10.1155/2018/6248590.
11. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science* 2020;367:eaau6977. https://doi.org/10.1126/science.aau6977.
12. Zou Y, Zhao X, Li Y et al. miR-552: an important post-transcriptional regulator that affects human cancer. *J Cancer* 2020;11:6226–6233. https://doi.org/10.7150/jca.46613.
13. Han T, Zhang Y, Yang X et al. miR-552 regulates liver tumor-initiating cell expansion and sorafenib resistance. *Mol Ther Nucleic Acids* 2020;19:1073–1085. https://doi.org/10.1016/j.omtn.2019.12.043.
14. Feng X, Zhu M, Liao B et al. Upregulation of miR-552 predicts unfavorable prognosis of gastric cancer and promotes the proliferation, migration, and invasion of gastric cancer cells. *Oncol Res Treat* 2020;43:103–111. https://doi.org/10.1159/000505377.
15. Zhu L, Zhang S, Chen S et al. Exosomal miR-552-5p promotes tumorigenesis and disease progression via the PTEN/TOB1 axis in gastric cancer. *J Cancer* 2022;13:890–905. https://doi.org/10.7150/jca.66903.
16. Bellucci R, Martin A, Bommarito D et al. Interferon-γ-induced activation of JAK1 and JAK2 suppresses tumor cell susceptibility to NK cells through upregulation of PD-L1 expression. *Oncoimmunology* 2015;4:e1008824. https://doi.org/10.1080/2162402X.2015.1008824.
17. Dong W, Wu X, Ma S et al. The mechanism of anti-PD-L1 antibody efficacy against PD-L1-negative tumors identifies NK cells expressing PD-L1 as a cytolytic effector. *Cancer*

Discov 2019;9:1422–1437. https://doi.org/10.1158/2159-8290.CD-18-1259.
18. Perera Molligoda Arachchige AS. Human NK cells: from development to effector functions. *Innate Immun* 2021;27:212–229. https://doi.org/10.1177/17534259211001512.
19. Liu S, Galat V, Galat Y et al. NK cell-based cancer immunotherapy: from basic biology to clinical development. *J Hematol Oncol* 2021;14:7. https://doi.org/10.1186/s13045-020-01014-w.
20. Galon J, Angell HK, Bedognetti D et al. The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. *Immunity* 2013;39:11–26. https://doi.org/10.1016/j.immuni.2013.07.008.
21. Xu H. Expressions of natural cytotoxicity receptor, NKG2D and NKG2D ligands in endometriosis. *J Reprod Immunol* 2019;136:102615. https://doi.org/10.1016/j.jri.2019.102615.
22. Schwinn N, Vokhminova D, Sucker A et al. Interferon-gamma down-regulates NKG2D ligand expression and impairs the NKG2D-mediated cytolysis of MHC class I-deficient melanoma by natural killer cells. *Int J Cancer* 2009;124:1594–1604. https://doi.org/10.1002/ijc.24098.
23. Huntington ND, Cursons J, Rautela J. The cancer-natural killer cell immunity cycle. *Nat Rev Cancer* 2020;20:437–454. https://doi.org/10.1038/s41568-020-0272-z.
24. Duan S, Guo W, Xu Z et al. Natural killer group 2D receptor and its ligands in cancer immune escape. *Mol Cancer* 2019;18:29. https://doi.org/10.1186/s12943-019-0956-8.
25. Lanier LL. Natural killer cells: from no receptors to too many. *Immunity* 1997;6:371–378. https://doi.org/10.1016/s1074-7613(00)80280-0.
26. Vitale M, Cantoni C, Pietra G et al. Effect of tumor cells and tumor microenvironment on NK-cell function. *Eur J Immunol* 2014;44:1582–1592. https://doi.org/10.1002/eji.201344272.
27. Mirjačić Martinović KM, Babović NL, Džodić RR et al. Decreased expression of NKG2D, NKp46, DNAM-1 receptors, and intracellular perforin and STAT-1 effector molecules in NK cells and their dim and bright subsets in metastatic melanoma patients. *Melanoma Res* 2014;24:295–304. https://doi.org/10.1097/CMR.0000000000000072.
28. Chen J, Yang J, Jiang J et al. Function and subsets of dendritic cells and natural killer cells were decreased in gastric cancer. *Int J Clin Exp Pathol* 2014;7:8304–8311.
29. Mashouri L, Yousefi H, Aref AR et al. Exosomes: composition, biogenesis, and mechanisms in cancer metastasis and drug resistance. *Mol Cancer* 2019;18:75. https://doi.org/10.1186/s12943-019-0991-5.
30. Hosseini R, Sarvnaz H, Arabpour M et al. Cancer exosomes and natural killer cells dysfunction: biological roles, clinical significance and implications for immunotherapy. *Mol Cancer* 2022;21:15. https://doi.org/10.1186/s12943-021-01492-7.
31. Sun Z, Shi K, Yang S et al. Effect of exosomal miRNA on cancer biology and clinical applications. *Mol Cancer* 2018;17:147. https://doi.org/10.1186/s12943-018-0897-7.
32. Takahashi R-U, Prieto-Vila M, Hironaka A et al. The role of extracellular vesicle microRNAs in cancer biology. *Clin Chem Lab Med* 2017;55:648–656. https://doi.org/10.1515/cclm-2016-0708.
33. Fanini F, Fabbri M. Cancer-derived exosomic microRNAs shape the immune system within the tumor microenvironment: state of the art. *Semin Cell Dev Biol* 2017;67:23–28. https://doi.org/10.1016/j.semcdb.2016.12.004.
34. Wang B, Wang Q, Wang Z et al. Metastatic consequences of immune escape from NK cell cytotoxicity by human breast cancer stem cells. *Cancer Res* 2014;74:5746–5757. https://doi.org/10.1158/0008-5472.CAN-13-2563.
35. Zhang P-F, Gao C, Huang X-Y et al. Cancer cell-derived exosomal circUHRF1 induces natural killer cell exhaustion and may cause resistance to anti-PD1 therapy in hepatocellular carcinoma. *Mol Cancer* 2020;19:110. https://doi.org/10.1186/s12943-020-01222-5.
36. Paskeh MDA, Entezari M, Mirzaei S et al. Emerging role of exosomes in cancer progression and tumor microenvironment remodeling. *J Hematol Oncol* 2022;15:83. https://doi.org/10.1186/s13045-022-01305-4.
37. Xia Y, Zhang Q, Zhen Q et al. Negative regulation of tumor-infiltrating NK cell in clear cell renal cell carcinoma patients through the exosomal pathway. *Oncotarget* 2017;8:37783–37795. https://doi.org/10.18632/oncotarget.16354.
38. Zhao J, Schlößer HA, Wang Z et al. Tumor-derived extracellular vesicles inhibit natural killer cell function in pancreatic cancer. *Cancers (Basel)* 2019;11:874. https://doi.org/10.3390/cancers11060874.
39. Yang Y, Han Q, Hou Z et al. Exosomes mediate hepatitis B virus (HBV) transmission and NK-cell dysfunction. *Cell Mol Immunol* 2017;14:465–475. https://doi.org/10.1038/cmi.2016.24.
40. Sun C, Mezzadra R, Schumacher TN. Regulation and function of the PD-L1 checkpoint. *Immunity* 2018;48:434–452. https://doi.org/10.1016/j.immuni.2018.03.014.
41. Terme M, Ullrich E, Aymeric L et al. Cancer-induced immunosuppression: IL-18-elicited immunoablative NK cells. *Cancer Res* 2012;72:2757–2767. https://doi.org/10.1158/0008-5472.CAN-11-3379.
42. Hartley GP, Chow L, Ammons DT et al. Programmed cell death ligand 1 (PD-L1) signaling regulates macrophage proliferation and activation. *Cancer Immunol Res* 2018;6:1260–1273. https://doi.org/10.1158/2326-6066.CIR-17-0537.
43. Iraolagoitia XLR, Spallanzani RG, Torres NI et al. NK cells restrain spontaneous antitumor CD8+ T cell priming through PD-1/PD-L1 interactions with dendritic cells. *J Immunol* 2016;197:953–961. https://doi.org/10.4049/jimmunol.1502291.