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# PD-L1 deficient exosomes derived from lung cancer cell line enhances NK cell-mediated anti-tumor immunity against lung cancer

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**Abstract:**

As innate immune cells, natural killer (NK) cells play a vital role in combating tumors and infections, making them a promising tool for cancer immunotherapy. Although NK cell has achieved impressive results in treating of hematologic malignancies, the therapeutic efficiency of NK cells on solid tumors remains unsatisfactory. By enhancing the sensitivity of NK cells to specific target cells, it is possible to boost their cytotoxicity and therapeutic effect against solid tumors. Tumor derived exosomes (TEXs) have immunostimulatory effects on NK cells, however, the TEXs carried immune checkpoints ligand also impair NK cells activity. In the present study, PD-L1 was knocked out from lung cancer cell line A549, and the exosomes derived from wild-type A549 cells (WT-EXO) and PD-L1 knocked-out A549 cell (KO-EXO) were used to pre-immunize NK cells. Results showed that, PD-L1 knocking out can reduce the inhibitory effect of WT-EXO on NK cells viability. Meanwhile, A549 cells derived exosomes (WT-EXO and KO-EXO) both boost the cytotoxicity of NK cells, with KO-EXO exhibited better stimulated effect than the other one. The enhanced killing rates of NK cells can be partially attributed to increased IFN-γ secretion following exosomes pre-immunization. Western blot results indicated that the signaling proteins pSTAT1, along with their associated pathways, play important roles in exosomes-induced enhancement of NK cell cytotoxicity. Simultaneously, it was observed that exosomes-preimmunized NK cells exhibited an upregulation of immunological memory markers CD25 and CD159c. Moreover, KO-EXO pre-immunized NK cells showed better infiltration and anti-tumor effects compared to the untreated NK cells in A549 cell bearing mice model. These results highlight the therapeutic potential of engineered TEXs in boosting NK cell based anti-lung cancer immunotherapy.

**Key words:**

lung cancer, exosomes, natural killer (NK) cell, PD-L1, immunotherapy

## Introduction

Natural killer (NK) cell is a type of large granule lymphocyte with the defined phenotype of $CD56^{+}CD3^{-}$, which is able to rapidly attack tumor cell and infected cell independently of pre-immunization. NK cells born from bone marrow, with maturity, they migrated into blood, and settled down in peripheral tissues [1]. On the basis of the balance between activating and inhibitory signals, NK cell kill target cell or keep silent on normal cell. After be stimulated, NK cell directly lyse target cell or induce them apoptosis by secreting IFN-γ, perforin and granzyme. Meanwhile, NK cell can produce cytokines, growth factors, and chemokines to regulate adaptive immune responses through interacting with other immune cells [2]. Because of its cytotoxicity on abnormal cells, immunomodulatory function and extensively distribution, NK cell plays a very important role in many pathological states including infection [3], autoimmune disorder [4], chronic inflammation [5], especially cancer [6], as well as maintaining body health. Numerous studies proved that the infiltration of NK cells in tumor correlated to better prognostic in many types of cancers [7], which highlights the critical role of NK cells in anti-tumor immunity. Adoptive NK cell therapy has been applied in treating various hematological tumors and got satisfactory effect [8]. However, the therapeutic effect of NK cell against solid tumors, especially lung cancer, is still very limited [9]. Many strategies have been developed to enhance the cytotoxicity of NK cells on solid tumor, for instance, build the chimeric antigen receptor (CAR)-NK to increase their reorganization on tumor cells [10]; block inhibitory receptors to promote NK cells activating [11]; and add the cytokine cocktail or immune checkpoint antibodies in NK cell therapy procedure [12].

NK cell has long been classified as a canonical component of the innate immune system owing to lack of antigen specificity and memory function. However, the immunological memory of NK cell has been revealed gradually. Studies have proved that under certain conditions, NK cells exhibit antigen-specific memory capabilities similar to T and B cells. Upon secondary exposure to the same antigen, these memory-like NK cells exhibit more robust and rapid immune response [13]. Based on these, people realized that NK cell can be preimmunized to enhance its cytotoxicity like adaptive immune cell. Tumor antigens have been widely harnessed to prime T cells and DC to enhance their anti-tumor functions [14-16], inspired by this, whether tumor

antigens can activate the memory-like NK cell mediated anti-tumor response deserve further study.

Tumor-specific peptide antigens, tumor lysates, tumor cell membranes, and tumor-associated exosomes, etc. have been used as tumor antigens in the existing literature [17]. Recently, tumor derived exosomes (TEXs) have emerged as a frequently utilized source of tumor antigens in studies [18]. Exosomes, also known as small extracellular vesicle, is the nano-scale biomembrane vesicle secreted by almost all types of cells. Under electron microscope, exosomes exhibits a saucer like double-layer membrane structure with the diameter of 30-150 nm [19]. Exosome is a very important vehicle carrying mRNAs, miRNAs, and proteins derived from parental cells and plays a key role in intercellular communication. The exosomal cargoes reflect the characteristics and status of parental cells and have been often identified as disease markers [20, 21]. As carrying tumor antigens, TEXs have been applied as the vaccine to preimmunize NK cells in tumor immunotherapy [22-24]. In addition, TEXs also carry the molecules that can block the anti-tumor immune response and help tumor escaping from immune surveillance. PD-L1, known as the ligand of important immune checkpoints PD-1, is expressed by lung cancer cells and carried by lung cancer derived exosomes [25]. These PD-L1 carrying exosomes impairs the activity of immunocytes (particular are cytotoxic T lymphocyte (CTL) and NK cell) in tumor microenvironment and promote the progression and metastasis of tumors [26]. By eliminating PD-L1 to block immunosuppression of PD-1/PD-L1 pathway [27] while retaining tumor antigens on TEXs, it is possible to enhance immune cell pre-sensitization and thereby boost the efficacy of anti-tumor immunotherapy.

Consequently, in the present study, through knocking-out the PD-L1 expression on lung cancer cell line A549, the exosomes derived from PD-L1 knocked-out A549 cell (KO-EXO) were prepared. Then, *ex vivo* cultured human primary NK cells were pre-treated by KO-EXO and exosomes derived from wild type A549 cell (WT-EXO) to explore the immunostimulatory activity of lung cancer derived exosomes on NK cells, as well as if the PD-L1 deficient KO-EXO is able to boost the peculiar cytotoxicity of NK cells against lung cancer both *in vitro* and *in vivo*.

## *Materials and methods*

### Ethics statement

All blood donors (n = 16) were recruited from the students and faculties of School of Life Sciences, Northwestern Polytechnical University (7 males and 9 females; age range: 21- 59 years, mean: 43.2 years). All donors were confirmed to be in good health and self-reported no history of major surgical procedures or significant underlying diseases. Blood collecting and animal experiment protocols were approved by the Ethic Committees of Northwestern Polytechnical University, Ethics approval number: 202402061. Every donor signed the informed consent form for blood haemospasia. The ethics form and informed consent file were shown in the supplementary materials.



### Cell lines

The human lung cancer cell line A549 (wild type), human leukemia cell line K562 and 293T cell line were purchased form Cell Bank of Chinese Academy of Sciences (BioVector NTCC, Shanghai, China). They were normal cultured in DMEM or RPMI-1640 cell culture medium (Gibco, No. 11965-084 and 12633012, USA), supplemented with 100 units/mL penicillin, 0.1 mg/mL streptomycin and 10 % (v/v) FBS (Gibco, No. 10082139, USA) and cultured in a humidified 5 % $CO_2$ atmosphere at 37 °C.

### Lentivirus (LV) packaging and infection

The PD-L1 knocked-out A549 cell was established by lentivirus (LVs) infection. The short hairpin (sh) RNA LV (PDS278 __ PL-U6-shRNA-GFP-ccdB-puro (puromycin)), Lentiviral envelope plasmids, packaging plasmids and empty plasmid control (shControl) were obtained from Tsingke Biotechnology Co., Ltd.. The shRNA-PD-L1 sequence is: 5' GGATCCAGTCACCTCTGAACA 3'. Recovered 293T cell was seeded into 6-well plate at 60 % density. The LV plasmids, packaging and envelope plasmids were transfected into 293T cells at the ratio of 2:1:2 using Lipofectamine® 2000 (Invitrogen; ThermoFisher Scientific, Inc.) at

37 °C. After 8 h transfection, discard the culture medium contain transfection mixture and added fresh medium for another 48 h cultivation. Then, the medium was harvested and filtered using 0.45-μm cellulose acetate filters. After concentration by PEG-8000+NaCl buffer, the infectious titer of LVs was determined by a hole-by-dilution titer assay. Following 48 h transfection, the 293T cells were observed under inverted fluorescence microscope to calculate the virus titer. Per-experiment indicated the optimum multiplicity of infection (MOI) of A549 is 10. A549 cells were seeded into 6-well plate and transfected by LVs at the optimum MOI (10, $1\times10^7$ TU mL). After 24 h cultivation, the culture media contain LVs was replaced by normal medium and continue to culture for another 48 h. The stable transfected cells were selected under puromycin (5 μg/mL). The monoclonal of stable transfected cells was selected and the PD-L1 knocked-out efficiency was evaluated by reverse transcription-quantitative PCR (RT-qPCR) and western blot methods.



**q-PCR**

The RNA of A549 cells was extracted using TRIzol™ Reagent (Invirtogen, Carlsbad, CA) following the manufacturer's instructions. The concentration and purity of the RNA were determined by NanoDrop-1000. cDNA was synthesized by HiScript® II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) as follows procedure: 4×gDNA wiper Mix, template RNA and RNase free dd$H_2O$ were mixed proportionally and incubated at 42 °C for 2 minutes. Then the 5×HiScript II qRT SuperMix IIwas added in to the mixture and incubated at 50 °C for 15 min and 85 °C for 5 s for cDNA synthesis. After that, TransStart Top Green qPCR SuperMix kit (TransGen Biotech, Beijing, China) was used for quantitative PCR as follows: SYBR qPCR Master Mix, forward and reverse primers, dd$H_2O$ and cDNA were mixed proportionally. Then, the reaction mixtures were incubated for 30 min at 48 °C, followed by 40 cycles of PCR at 94 °C for 5 s, 55 °C for 15 s, and 72 °C for 10 s. At the end of the 40 cycles, the melting curve analysis was performed to confirm the presence of only a single amplified product of the expected size. Fold change was calculated as $2^{-\Delta\Delta CT}$ method [28]. The primers used for PCR were shown in Table 1.

**NK cells *ex vivo* expansion**

According to our previous work, human primary NK cells were expanded from peripheral blood mononuclear cells (PBMCs) [29]. Briefly, ten milliliters peripheral venous blood was collected from healthy donors (n = 16). The PBMCs were separated using lymphocyte separation liquid (Haoyang TBD, Tianjin, China). After counting, PBMCs were co-cultured with genetically modified stimulating cell line which was prepared according to our previous work at the ratio of PBMCs : stimulating cell = 30 : 1 [29]. The mixture was cultured in RPMI-1640 cell culture medium supplemented with 100 units/mL penicillin, 0.1 mg/mL streptomycin, 10 % (v/v) FBS and 100 units/mL IL-2. Every two days, half-volume medium was changed. Undergo 14 days culture, the proportion of NK cell ($CD56^+CD3^-$) in PBMC was determined by flow cytometry (BD FACS Calibur, San Jose, CA, USA) through labeled with CD56-PE (BD, Cat No. 561904, CA, USA) and CD3-FITC monoclonal antibodies (mAbs) (BD, Cat No. 561806, CA, USA).

**Exosomes collection and identification**

According to the initial ultracentrifugation isolation method [19] with slight modification, the exosomes were isolated from culture supernatant of wild type A549 cells (WT-EXO) and PD-L1 knocked out A549 cells (KO-EXO). Briefly, cell culture supernatant was collected and centrifuged (1×10 min, 300×g; 1×30 min, 2000×g) to remove debris. After preliminarily centrifugation, the supernatant containing exosomes was collected and filtered by 0.22-μm filter. Filtrate was ultracentrifuged (1×60 min, 10,0000×g) using a fixed-angled rotor (Ti-70, Beckman Coulter, Inc., Brea, CA, USA) at 4 °C. Then, the filtrates were washed by PBS, and ultracentrifuged (1×70 min, 10,0000×g) at 4 °C. The sediment was stored in -80 °C for the subsequent detection.

The sediments were suspended in 2 % paraformaldehyde aqueous solution and mixed gently. Exosomes suspension (3-5 μL) was dripping onto cleaned mica chips. After critical point drying and gold sputtering, mica chips were imaged by scanning electron microscope (SEM, VEGA 3 SBH, Tescan, Czech Republic) to exam the morphology of exosomes. The particle size of

isolated exosomes was detected by the Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Then, the expression of exosomal markers CD9, CD63, CD81, as well as negative marker Calnexin on isolated exosomes were detected by western blot. The information of antibodies was shown in Table 2. The concentrations of exosomes were evaluated by BCA assay (BCA Protein Assay Kit, Beyotime, No. P0012-1).

**Viability and cytotoxicity of NK cell**

The NK cells were treated by WT-EXO and KO-EXO ($2\times10^6$ NK cells with 10 μg exosomes in each group) for 24 h-7 d. After washed by PBS, NK cells in each group were resuspended in 1 mL RPMI-1640 media (IL-2 free). Two hundred microliters of NK cell suspension were added into a well of 96-well plate and this operation was repeated five times. Twenty microliters of CCK-8 (Cell Counting Kit-8, Dojindo, Japan) were added to each well of NK cell and then the plate was incubated in a 5 % $CO_2$ incubator at 37 °C for 2 h. The optical density (OD) value of each well was recorded at 450 nm in a microplate reader (BioTek synergy H1, USA) to examine the viability of the NK cells.



The NK cells cytotoxicity was tested by calculating the rate of NK cell kill target cell according to our publication [30]. Briefly, after 24 h exosomes treatment and washed by PBS, NK cells ($2\times10^6$) were re-suspended in RPMI-1640 (IL-2 free) and added into the well of a 96-well plate at 100 μL. Target cell (K562 or A549) ($4\times10^5$) was re-suspended in 100 μL medium and added to the NK cell well (mix well) to set the effector-to-target ratio (E:T) at 5:1. The wells contain only NK cell (effector (e) well) and K562/A549 cell (target (t) well) respectively, were set simultaneously with the final volume to 200 μL in each. After 4 h incubation at normal culture condition, 20 μL of CCK-8 reagent was added to each well, and the plate was incubated for another 2 h at 37 °C environment. The OD values were recorded at 450 nm. The killing rate was calculated by following equation:

$$\text{Killing rate (\%)} = [1- (OD_{mix}-OD_e)/OD_t] \times 100\ \%.$$

**Apoptosis and functional receptor expression of NK cell**

The apoptosis and functional receptors expression of NK cell after 24 h exosomes treatment were assessed as well. Annexin-V/PI double dye method was employed to test the apoptosis of NK cell. They were washed by PBS and collected after exosomes treatment. Annexin V-FITC and PI (AnnexinV-FITC Apoptosis Detection Kit, Cat No. C1062M. Beyotime Institute of Biotechnolog, China) were used to label apoptotic NK cells. Following 10 min staining, NK cells in each group were tested by flow cytometry (BD Calibur, California, USA). The fluorescence intensities of FL1 and FL2 channels were analyzed by Cellquest (BD) software.

Fluorescent antibodies (NKG2A, NKG2D, NKp30, NKp44, NKp46, and CD226) were used to test the expression of NK cell functional receptors. Following 10 min staining, each sample was tested by flow cytometry, respectively, and analyzed by Cellquest (BD) software. The information of all antibodies was shown in Table 2.



**Cytokines assay using CBA technique**

The BD™ Cytometric Bead Array (CBA) testing kits (BD$^{TM}$ CAB, Human IFN-γ Flex Set, Cat.No.:558269; BD$^{TM}$ CAB, Human Granzyme B Flex Set, Cat.No.:560304) were used to assess the secretion level of cytokines (IFN-γ and granzyme B) by NK cells. After exosomes treatment, the culture supernatant of NK cells in each group were collected. The detection beads were mixed with standard sample and testing samples, respectively, and stained for 1 h. Then the detection antibodies were added into mixture and stained for an additional 2 h. After washed by PBS, all testing samples were analyzed by flow cytometry. The door of FSC/SSC chart was enclosed based on the position of beads. Then the fluorescence intensity of labeled standard samples were determined by flow cytometry. The standard curve was calculated according to the concentration and fluorescence intensity of standard. The fluorescence intensity of testing samples was detected, and the concentration of cytokines in each sample was calculated according to the standard curve.

**Western blot**

The NK cells after exosomes treatment were lysed for Western blot (WB) detection. Anti-IFN-γ (5 μg/mL) was used to validate the regulatory role of IFN-γ in exosomes stimulated cytotoxicity of NK cells. The information of used primary antibodies of immune checkpoints (TIGIT, PD-1, CTLA-4, and TIM3), signaling pathway proteins (PI3K, p-PI3K, STAT1, p-STAT1, JAK2, p-JAK2, and SOCS3) and anti-IFN-γ is detailed in Table 2. ImageJ software was employed to perform quantitative analysis on WB bands.

**Lung cancer bearing mice model**

Balb/c nude mice (5 weeks old) were purchased from Animal Experiment Center of Xi'an Jiaotong University (license key SYXK-2020005). After one-week conventional raise, mice were randomly divided into four groups with seven mice in each (n = 7). WT-A549 cells ($2 \times 10^6$ each mouse) were implanted subcutaneously into the flanks of each mouse in a volume of 100 μL DMEM/Matrigel (1:1 v/v, BD Biosciences). NK cells were pre-treated by WT-EXO (exosomes derived from wild-type A549 cell) or KO-EXO (exosomes derived from PD-L1 knocked out A549 cells), respectively for 24 h ($2\times10^6$ NK cells with 10 μg exosomes). One week following tumor inoculation, each group of NK cells (untreated, WT-EXO preimmunized, and KO-EXO preimmunized) in 100 μL normal saline were subcutaneously injected around the tumor ($1\times10^6$ NK cells per mouse). All mice were set as four group, which are Group 1 (injected KO-EXO preimmunized NK cells), Group 2 (injected WT-EXO preimmunized NK cells), Group 3 (injected untreated NK cells), and Control (injected normal saline). NK cell injection procedure was performed every week, with a total of three repetitions. The mouse weight and size of tumors were measured every three days. Tumor size was calculated according to the following formula [31]:

$V = 1/2 \times ab^2$ [maximum (a) and minimum (b) length of the tumor]

All mice were sacrificed in the fifth week; tumors were separated and weighed. Four tumors in each group (4/7) were digested into single-cell suspension using digestive solution (Matrixtru Tissue Digestion Solution, Cat. EXCM2703, CTRUEBLUE) and labeled by CD56 and CD3 anti-bodies, respectively. All cell suspension samples were analyzed using flow cytometry (BD Celesta, California, USA), and data was analyzed using BD FASDiva software to test the NK cells ($CD56^+CD3^-$) infiltration in tumor tissues. The remaining three tumors of each group (3/7) were collected and fixed for immunohistochemistry staining. Tumors in each group were cut into slices, after dewaxing and hydration, the slices were blocked by 3 % hydrogen peroxide and then blocked by 3 % BSA. After sequential incubation with primary and secondary antibodies, the slices were stained by freshly prepared DAB, followed by hematoxylin solution counterstain. After dehydration and mounting, all slices were checked under a bright-field microscope. The antibodies information was shown in Table 2.



**Data analysis**

Graphpad Prism8 software was employed to analyze the data. All data were presented as the mean ± SEM. Comparisons between two group were performed using analysis of student' *t*, one way *ANOVA* was performed to compare the means among multiple groups. Statistical significance was defined as $p < 0.05$.

**Results**

***PD-L1 knock out efficiency of A549 cells***

By using fluorescence microscope, the packaging efficiency of LVs in 293T cells were examined, which is about 85 % and the LVs titer is $1\times10^8$ TU/mL (data not show). Pre-experiment established an optimal MOI of 10 ($1\times10^7$ TU/mL) for A549 cells. Fluorescence imaging demonstrated that the PD-L1-targeting shRNA was successfully transfected into A549 cells, with a transduction efficiency is approximately 75 % (Supplementary Figure S1C). The q-PCR results

indicated that the knock out efficiency of PD-L1 is approximately 70 % in gene level (data not show). Since IFN-γ has been reported to induce PD-L1 expression in tumor cells [32], the expression levels of PD-L1 in A549 cell in the presence and absence of IFN-γ were examined. Western blot results demonstrated that in the presence of IFN-γ, WT A549 cells exhibited PD-L1 expression, and there was almost no PD-L1 expressed in PD-L1 knocked out A549 cells regardless of the presence or absence of IFN-γ (Supplementary Figure S1D). These data demonstrated the PD-L1 expression was efficiently blocked in A549 cells.

***The exosomes identification***

The exosomes were isolated from culture supernatant of wild-type A549 and PD-L1 knocked out A549 cells. After the multi-step centrifugation, the collected vesicles were morphological analyzed by SEM. It was shown that, these vesicles presented typical sphere and saucer-like structure with the diameter between 30-200 nm (Figure 1A). The mean diameter of vesicles is 146.8 ± 74.4 nm (figure 1B). Western blot experiment confirmed the presence of exosomal bio-markers of CD63, CD81, and CD9, on these vesicles (Figure 1C). Meanwhile, compared to the cell lysate, these vesicles lacked detectable levels of the negative markers Calnexin (Figure 1C). Furthermore, PD-L1 was detected on WT-EXO but not on KO-EXO (Figure 1D). These results indicated that the collected vesicles fit the characteristics of exosomes. Meanwhile, knockout of PD-L1 in A549 cells produced PD-L1-deficient exosomes. Moreover, the secretion of exosomes was quantified before and after PD-L1 knock out on A549 cells. It was found that there is no significant difference between the size, protein concentration and micro-morphology of WT-EXO and KO-EXO (Supplementary Figure S2), which indicated that PD-L1 knock out will not affect the secretion of exosomes by A549 cells.

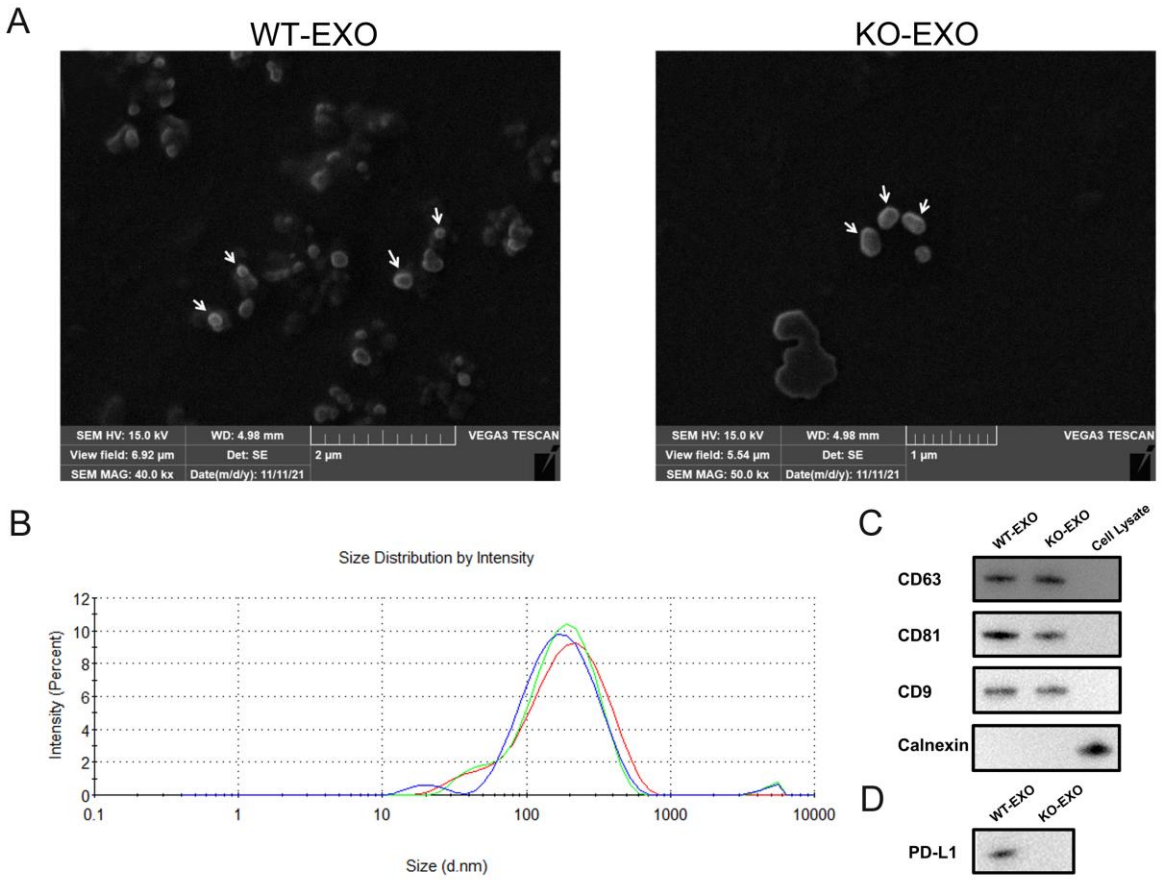


**Figure 1 Identification of exosomes**

A: Exosomes morphology under SEM; B: Particle size distribution of exosomes (exosomes were diluted by 1 × PBS, and measured by a Zetasizer Nano) (n = 3); C: Western blot analysis of the exosomes markers (CD63, CD81, CD9) and negative markers (Calnexin). D: the exosomal expression of PD-L1.

***The viability and apoptosis of NK cells following exosomes treatment***

The purity of NK cells and their growth process were shown in supplementary materials (Supplementary Figure S1 A, C). The results indicated that NK cells cultured *in vitro* grew well with a purity of over 90 % (supplementary figure S1A, data derived from the blood sample No. 12). Since PD-L1 expression in A549 cells upon IFN-γ stimulation has been reported [32] and was also observed by ourselves (data not show), we collected the exosomes of two types of A549 cells in the presence or absence of IFN-γ. There was no observable change of NK cell $OD_{450}$

following 24 h treatment except the WT-EXO+ treated group ("+" and "-" represent the WT/KO A549 cell pre-stimulated by IFN-γ or not) which demonstrated WT-EXO collected under IFN-γ pre-stimulated is able to impair the viability of NK cells (Figure 2A). However, NK cells apoptosis didn't show significant change in each group (Figure 2B and C), which indicated WT-EXO can affect NK cells viability but not by inducing them apoptosis.

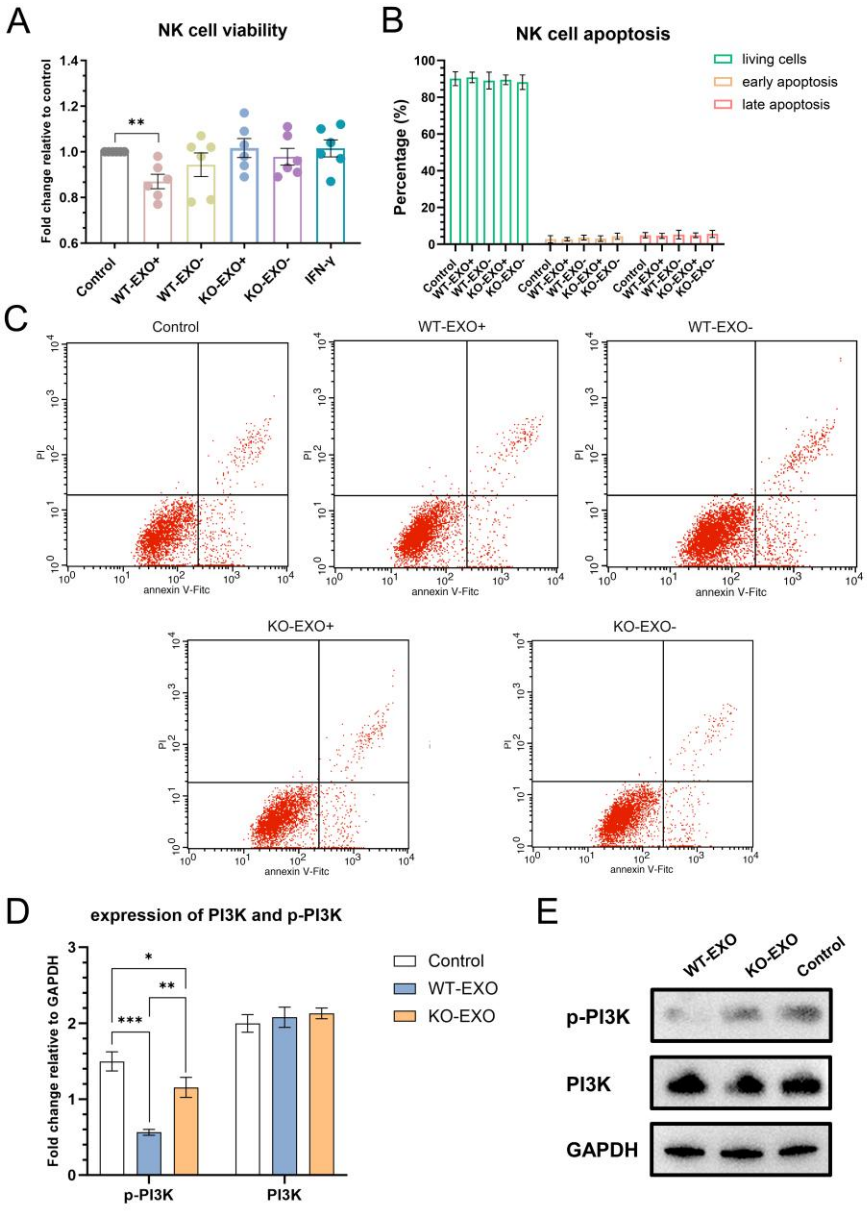


**Figure 2 The viability and apoptosis of NK cells following exosomes treatment**

A: The viability of NK cells following A549 cell exosomes treatment (n = 6). B: The statistical result of NK cells apoptosis (n = 4); C: The representative flow cytometry data of NK cells apoptosis. "Control" represented

NK cells untreated with exosomes, "WT-EXO+" represented NK cells treated with WT-EXO in presence of IFN-γ; "WT-EXO-" represented NK cells treated with WT-EXO in the absence of IFN-γ; "KO-EXO+" represented NK cells treated with KO-EXO in presence of IFN-γ; "KO-EXO-" represented NK cells treated with KO-EXO in the absence of IFN-γ; IFN-γ represented NK cells cultured only in presence of IFN-γ. E: The statistical result of PI3K and p-PI3K expression in NK cells; F: Western blot data of PI3K and p-PI3K expression. One way *ANOVA*, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

To explore the underlying causes, we examined the molecular pathways associated with NK cell viability. Western blot results demonstrated that the WT-EXO (collected in presence of IFN-γ) treatment attenuated the phosphorylation of PI3K, which was triggered by PD-1/PD-L1 pathway [33]. Then, the attenuated PI3K signal further suppressed the activity of NK cells [34, 35]. Taken together, these results indicate that in the presence of IFN-γ, exosomes derived from A549 cells may modulate PI3K related signaling pathways, via their carried PD-L1, thereby influencing NK cell viability.



To further investigate the time dependent effects of A549 exosomes on NK cells, we treated the cells with WT-EXO or KO-EXO (collected after IFN-γ stimulation) over a sustained period. In an experiment with a duration of 7 days, WT-EXO exhibited a certain inhibitory effect on NK cells viability in 1- and 2-days treatment. And then from the third day, this effect becomes less noticeable (Supplementary Figure S3). It can be inferred that NK cells have a certain degree of self-recovery ability after being inhibited by exosomes. However, the fact that KO-EXO did not significantly impair NK cell viability demonstrates their safety (Supplementary Figure S3).

***The killing rate and memory markers expression of NK cells following exosomes treatment***

Sixteen primary NK cell samples (isolated from peripheral blood of healthy donors and cultured *ex vivo*) were used to examine the killing rates of NK cells against A549 and K562 cell lines following A549 exosomes treatment. Results indicated that exosomes derived A549 cells exhibit a stimulated effect on NK cells cytotoxicity. Furthermore, the KO-EXO showed better stimulated effect on NK cells cytotoxicity than WT-EXO against A549 cells (Figure 3A). The killing rate of

NK cells against K562 cell was also detected to further explore the NK cell activation. Results showed that, KO-EXO can enhance the cytotoxicity of NK cells against K562 cells as well. However, the enhancement of killing rate was less pronounced compared to that against A549 cells (Figure 3B).

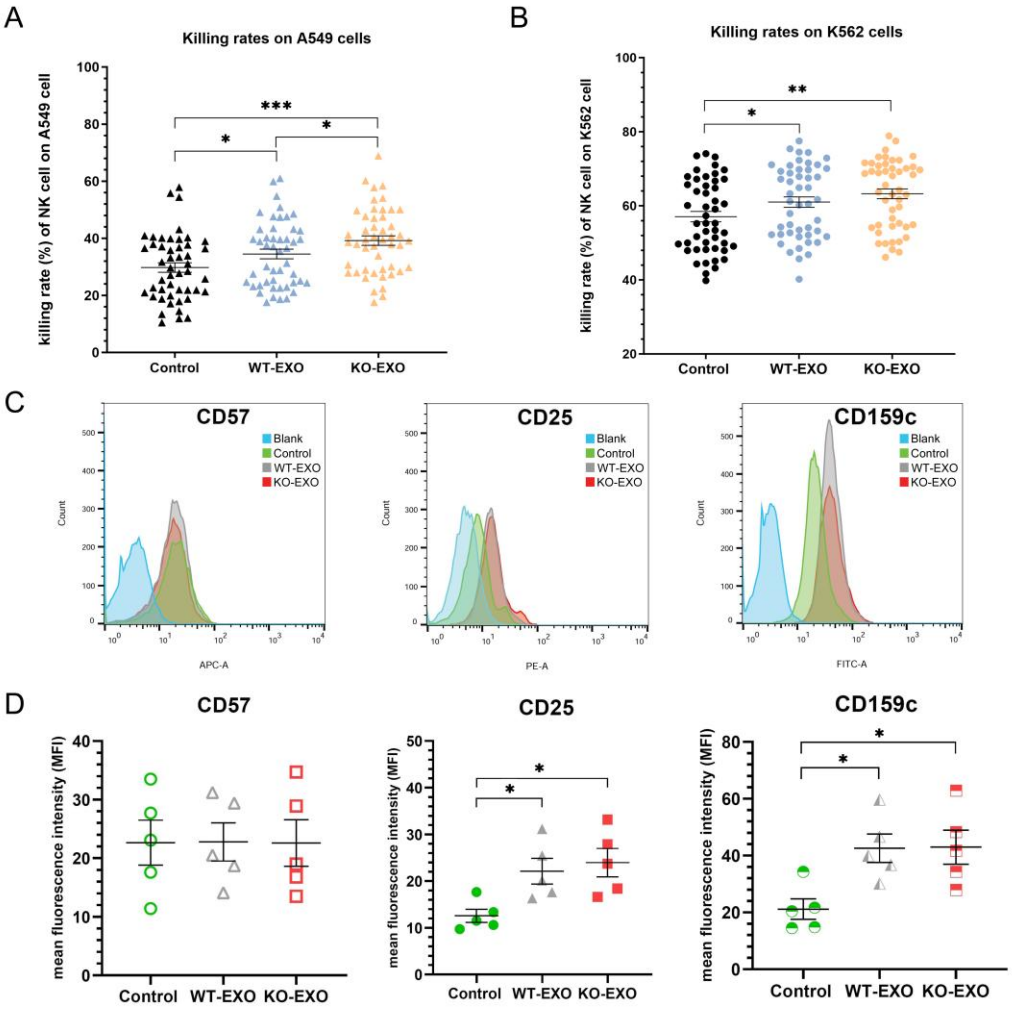


**Figure 3 The killing rate and memory markers expression of NK cells following exosomes treatment**

A: Killing rate of NK cells on A549 cells (n = 16); B: Killing rate of NK cells on K562 cells (n = 16); C: The representative flow cytometry data of NK cells memory markers expression. D: The statistical results of NK cells memory biomarkers expression (n = 5). One way *ANOVA*, *: $p < 0.05$; **: $p < 0.01$. ***: $p < 0.001$

To investigate the underlying mechanism by which preimmunization enhances the killing rate of NK cells against target cells, we measured the expression levels of NK cell memory markers (CD25, CD57 and CD159c). Data of flow cytometry indicated that the NK cell memory markers CD25 and CD159c exhibited an increased expression following KO-EXO treatment compared with control, however, the expression of CD57 did not exhibit any significant alteration (Figure 3C and D)

***The expression of functional receptors and cytokines of NK cells following exosomes treatment***

NK cells derived from five blood samples were employed to test the expression of functional receptors and cytokines following exosomes treatment. q-PCR and flow cytometry were used to examine expression of these proteins in NK cells. The data demonstrate that among a panel of NK cell functional receptors, the expression of NKG2D exhibited the most pronounced alteration, with a marked down regulation following exosomes treatment. However, in KO-EXO treatment group, NKG2D protein expression did not exhibit a significant decrease compared with the control. Meanwhile, the expression levels of other functional receptors, including NKG2A, NKp30, NKp44, CD226, and ICAM-1 showed no significant alterations. Moreover, IFN-γ exhibit the significant up-regulated expression following both WT-EXO and KO-EXO treatment (Figure 4D, E and F).

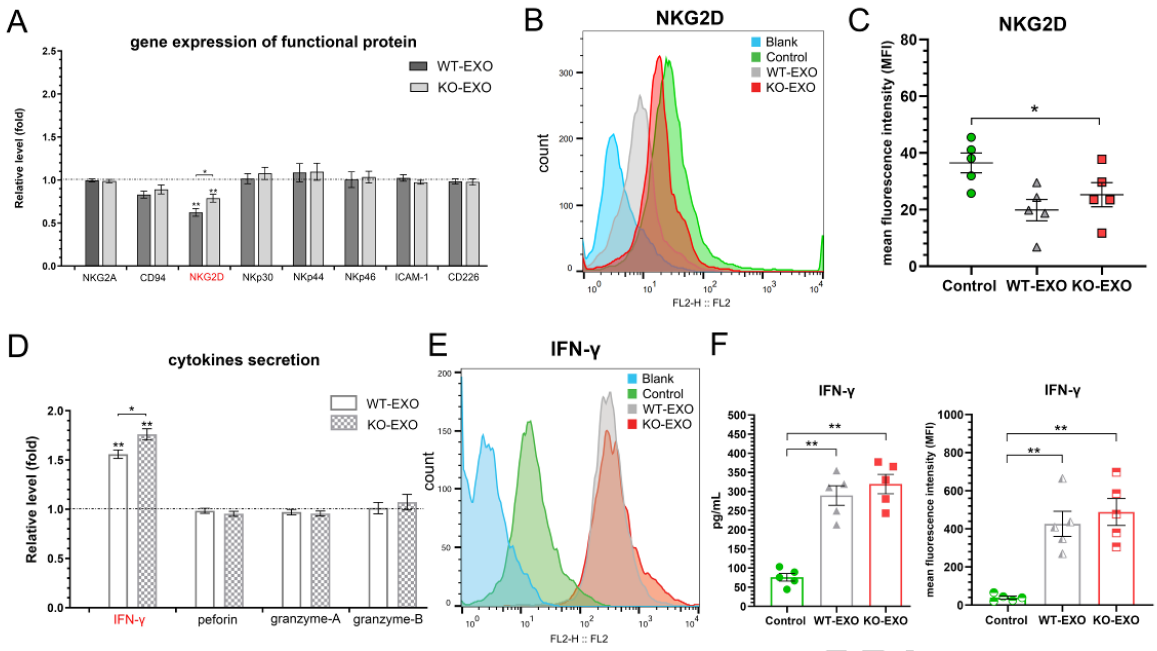


**Figure 4 Expression of functional receptors and cytokines on NK cells following exosomes treatment**

A: The q-PCR data of the NK cells functional receptors expression (NKG2A, CD94, NKG2D, NKp30, NKp44, NKp46, ICAM-1 and CD226) (n = 3); B: The representative flow cytometry data of NKG2D expression; C: The statistical result of NKG2D expression (n = 5). D: The q-PCR data of cytokines secretion in NK cells; E: The representative flow cytometry data of IFN-γ expression; F: The statistical results of IFN-γ expression (n = 5). One way *ANOVA*, *: $p < 0.05$; **: $p < 0.01$.

To further explore the underlying mechanism of enhanced NK cells cytotoxicity after exosomes treatment, we detected the expression of signaling pathway proteins and immune checkpoints in NK cells. Western blot results indicated that KO-EXO treatment enhanced the expression of phosphorylated JAK2 (p-JAK2) and STAT1 (p-STAT1) in NK cells compared to the WT-EXO group and control. Furthermore, SOCS3 was found to be significantly activated by KO-EXO as well. It is worth noting that in the presence of an IFN-γ antibody, the activation of both p-JAK2 and p-STAT1 as well as SOCS3 were suppressed to some extent, indicating that the exosomes can activate NK cell cytotoxicity through an IFN-γ-dependent pathway. Moreover, Exosomes derived from A549 cell can alter the expression level of immune checkpoint proteins. PD-1, the functional receptor for PD-L1, and CTLA-4 are slight down-regulated following exosomes

treatment, but without significance (Figure 5A and B). Moreover, another immune checkpoint protein TIM3 showed significant up-regulated in both WT-EXO and KO-EXO treatment. However, the KO-EXO-treated group showed a lesser increase in TIM3 expression compared to the WT-EXO-treated group (Figure 5A and B).

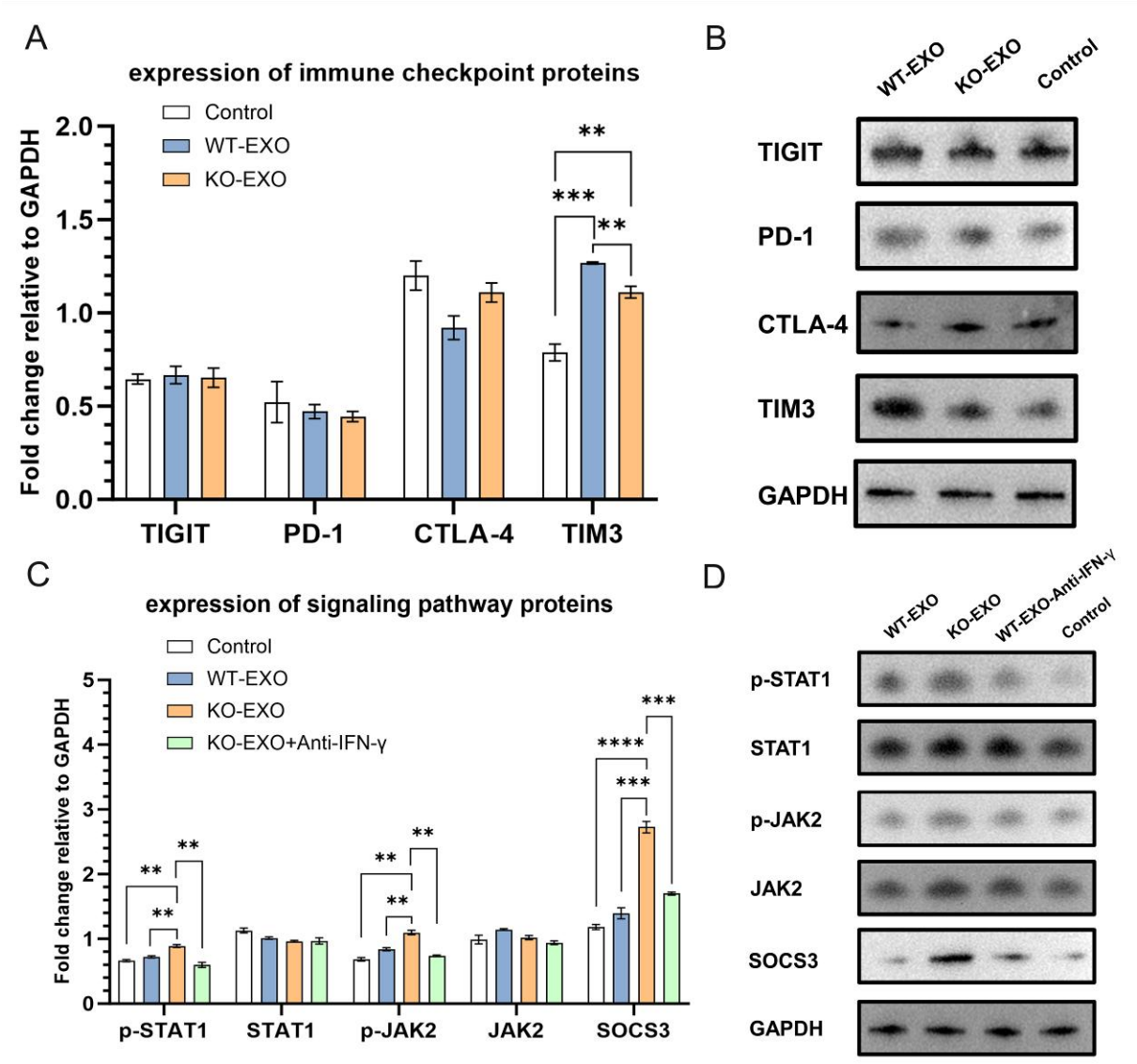


**Figure 5 The expression of signaling pathway proteins and immune checkpoints in NK cells following exosomes treatment**

A: The statistical result of immune checkpoints (TIGIT, PD-1, CTLA-4 and TIM3) expression in NK cells (n = 3); B: Western blot data of immune checkpoints expression; C: The statistical result of signaling pathway proteins expression in NK cells (n = 3); D: Western blot data of signaling pathway proteins expression. One

way *ANOVA*, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. ****: $p < 0.0001$

***Anti-tumor effects of A549 exosomes preimmunized NK cell in vivo***

One week after the inoculation with A549 cells to establish the model, the 28 mice were randomly divided into four groups (seven in each, Figure 6) which are Control (injected normal saline), Group 1 (injected KO-EXO preimmunized NK cells), Group 2 (injected WT-EXO preimmunized NK cells), and Group 3 (injected untreated NK cells). Results showed that, the mice in Group 1 displayed minimum tumor volume and higher body weight (Figure 6B and C). The injection of KO-EXO preimmunized NK cells resulted in a significant inhibition of tumor growth compared to the Control, as well as Group 2 and 3 (Figure 6B). NK cells preimmunized with WT-EXO (Group 2) exhibited modest tumor-inhibitory activity, but the difference was not statistically significant compared to the untreated NK cells (Group 3). Furthermore, these groups exhibited a superior anti-tumor effect relative to the group receiving normal saline (Control). These results suggested that KO-EXO exhibit the most potent anti-tumor effect *in vivo*. Whereas WT-EXO stimulated NK cells showed no significant improvement over unstimulated cells, indicating that WT-EXO has a limited capacity to enhance NK cell function in mice model.



H&E staining results (Figure 6E) showed that tumors in Group 1 displayed numerous cavities with irregular cell morphology, and a high abundance of cells with apoptotic and necrotic features (nuclear pyknosis and intercellular spaces increased). Although groups of 2 and 3 also showed increased apoptotic and necrotic cells compared to the control, these effects were less pronounced than those in Group 1. This result indicated the superior anti-tumor efficacy of KO-EXO preimmunized NK cells *in vivo*. Furthermore, the result of TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) (Figure 6E), revealed that there were the highest number of apoptotic cells (green fluorescence signal) in Group 1, followed by Group 2 and 3 when compared with Control, confirming the enhanced apoptosis-inducing effect of KO-EXO preimmunized NK cells on tumors *in vivo*. The expression level of the nuclear protein Ki67 is a well-established marker of cellular proliferative activity. The Ki67 staining result demonstrated the reduced Ki67 positivity in tumor cells from Group 1 compared to the Control,

suggesting a potent inhibitory effect on tumor growth by KO-EXO preimmunized NK cells (Figure 6E).

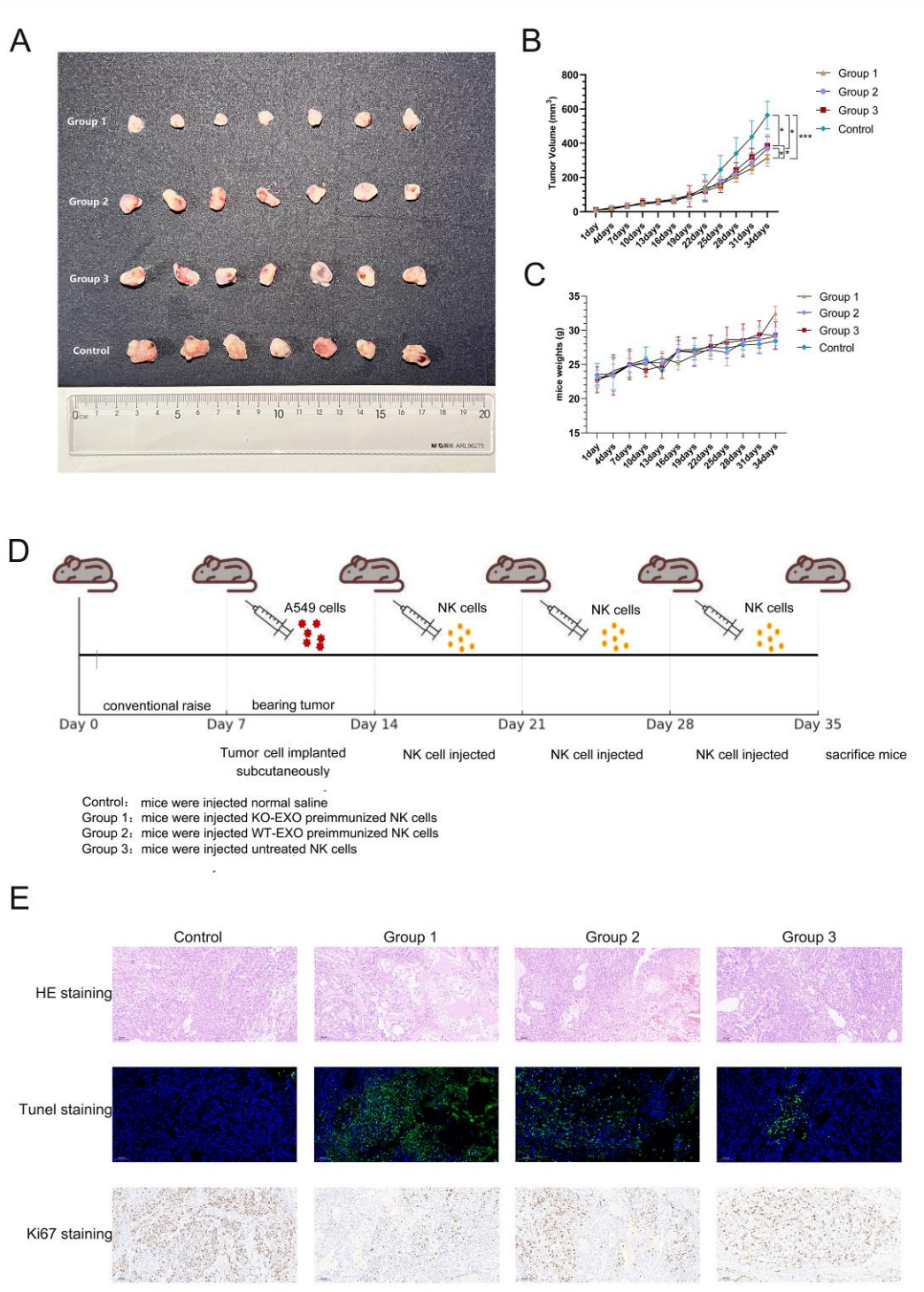


**Figure 6 Anti-tumor effect of A549 exosomes preimmunized NK cell *in vivo***

A: Tumors of A549 cell bearing mice (n = 7); B: Tumor volume during experiment (n = 7); C: Mice weight during experiment (n = 7); D: Schematic diagram of drug administration; E: The representative HE staining of tumor slices (n = 3); The representative TUNEL staining of tumor slices (n = 3); The representative Ki67 staining of tumor slices (n = 3); One way *ANOVA*, *: $p < 0.05$; ***: $p < 0.001$.

To further investigate the NK infiltration across the groups, the tumors were digested into single-cell suspension to quantify the infiltrating NK cells ($CD56^{+}CD3^{-}$) by flow cytometry. As shown in Figure 7A and 7B, the highest level of NK cell infiltration was observed in Group 1, where NK cells accounted for approximately 12 % of the total cells followed by WT-EXO treated and untreated NK cells, respectively. As expected, no NK cells infiltration was detected in the Control as they received saline. Furthermore, the immunohistochemistry results (Figure 7C) validated the flow cytometry data, showing that the tumor in Group 1 exhibited most extensive infiltration of $CD56^{+}$ cells (visualized as brown staining).

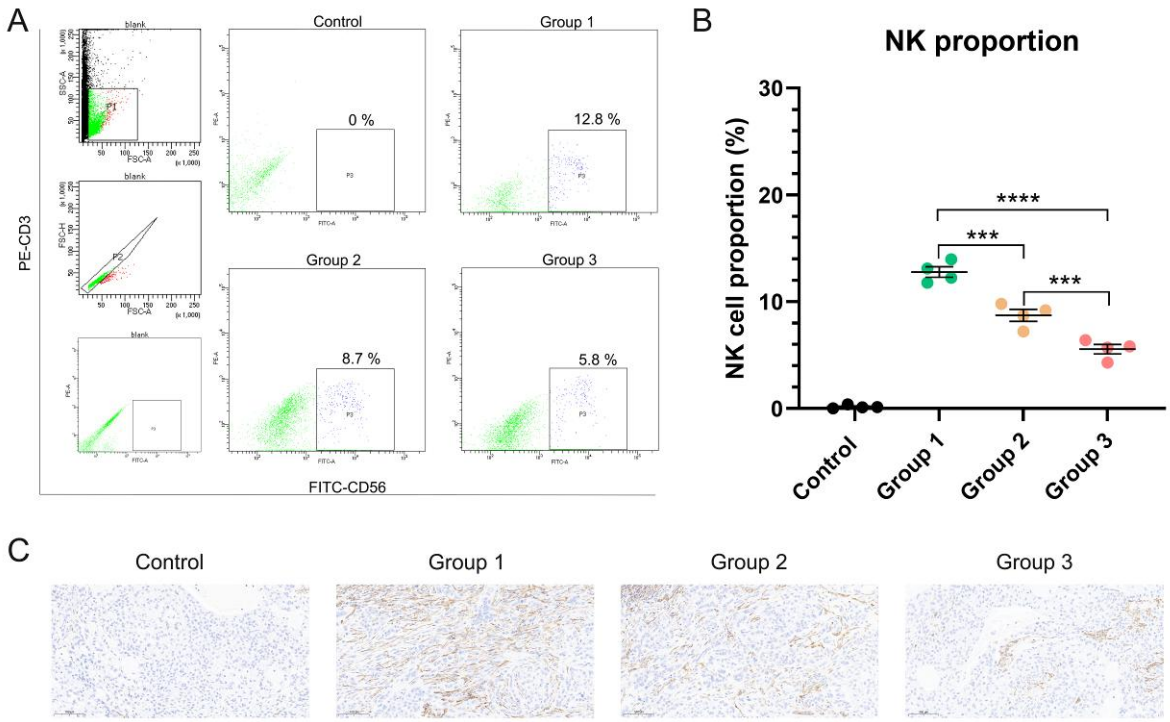


**Figure 7 NK cell infiltration in tumors**

A: The representative flow cytometry data of NK cell infiltration (n = 4). B: The statistical result of NK cell infiltration; C: CD56 staining of tumor slices (n = 3). Control: mice were injected normal saline; Group 1: mice were injected KO-EXO preimmunized NK cells; Group 2: mice were injected WT-EXO preimmunized NK cells; Group 3: mice were injected untreated NK cells. One way *ANOVA*, **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

## Discussion

Tumor-derived exosomes (TEXs) contain diverse biological constituents inherited from their parent cells, including proteins and nucleic acids (e.g., DNA, mRNA, miRNA, lncRNA) [36] which play important roles in promoting tumor growth, angiogenesis, metastasis, and in modulating immune surveillance. Meanwhile, TEX can inhibit tumor progression by activating anti-tumor immune responses as well [37]. Due to a growing understanding of their functions, TEXs are being used for various biomedical applications, including targeted drug delivery, tumor diagnosis, and vaccine preparation [38, 39].

Various stress factors, including starvation, heat shock, certain drugs, and irradiation, can not only increase the secretion of TEXs but also alter their profile, leading to a stronger immune response. For instance, in a study from Zhou *et al*., exosomes derived from immunogenically dying tumor cells were isolated, loaded with CCL22-siRNA and modified by MART-1 peptide. As a therapeutic vaccine, these engineered exosomes indicated significant antitumor efficacy in a model of pancreatic cancer [40]. With regard to NK cell stimulation, Lv *et al*., demonstrated that under stress condition, hepatocellular carcinoma cells can release heat shock proteins (HSP)-bearing exosomes. These exosomes efficiently stimulated NK cell cytotoxicity and granzyme B production, down regulated the expression of inhibitory receptor CD94, and up regulated the expression of activating receptors CD69, NKG2D, and NKp44. These results suggested that stress induced hepatocellular carcinoma derived exosomes have the superior immunogenicity in inducing HSP-specific NK cell responses [41]. Subsequent research revealed that TEXs produced in response to stress carry damage-associated molecular patterns (DAMPs) that activate innate immune cell populations [42]. Particularly, TEXs carried stress-induced ligands for the NKG2D are able to activate NK cells as well [43].

Due to its immunostimulatory properties, TEX has been utilized in the development of various tumor vaccines. Wang *et al.* developed a dendritic cell vaccine by using lung cancer cells derived tumor-associated exosomes. In tumor bearing mice, these exosomes have more potent in promoting DC maturation, enhancing MHC cross presentation, and inducing more robust tumor-specific cytotoxic T lymphocyte (CTL) response than tumor cell lysates (TCLs) [44]. In another study, through loading human neutrophil elastase (ELANE) and Hiltonol (TLR3 agonist) into α-lactalbumin (α-LA)-engineered breast cancer-derived exosomes, Huang *et al*, prepared an *in situ* DC vaccine (HELA-Exos). In mice model and patient-derived tumor organoids, HELA-Exos can effectively induce immunogenic cell death (ICD) in breast cancer cells by activating type one conventional DCs *in situ* and cross-primed tumor-reactive $CD8^+$ T cell responses [45].

The PD-1/PD-L1 blockade strategy has been extensively utilized to promote T cell-dependent anti-tumor immunity [46]. Meanwhile, the PD-1 expression in human NK cells has been widely proved [47]. Therefore, the blockage of PD-1/PD-L1 axis may also improve NK cells antitumor cytotoxicity [48]. It is reasonable to speculate that decreasing exosomal PD-L1 expression may attenuate immunosuppressive effects while preserving the immunogenicity of lung cancer-derived exosomes. Our findings highlight the immunostimulatory effect of exosomes derived from PD-L1-deficient lung cancer on NK cells. Admittedly, when conditions permit, the use of autologous tumor-derived TEXs would theoretically yield superior immunotherapeutic outcomes. However, the cultivation of patient-specific tumors and the collection of autologous TEXs cost time and labor. Therefore, in preclinical research, the use of cell line-derived TEXs offer distinct advantages. Once the technology matures, it will also facilitate future translation to autologous TEXs for priming immune cells, thereby achieving more optimal therapeutic efficacy.

Memory NK cell existed in the host tissue with the memory to infectious agents. When the body is attacked by the previous antigen, memory NK cells are able to quickly recognize the pathogen and make an effective immune response, providing faster and stronger immune protection [49-51]. With the growing recognition of NK cell memory functions, NK cells have emerged as potential targets for vaccine activation [52].

Existing literature suggests that cytokine-induced memory-like (CIML) NK cells exhibit the features include increased expression of CD25 (IL-2Ra), decreased expression of KIRs, and TGF-β receptors, which indicates that CD25 is a potential marker for memory NK cells [53]. The subpopulation of NK cells with CD159c (also known as NKG2C receptor) expression is expanded under human cytomegalovirus infection (HCMV). These $NKG2C^{+}$ cells exhibited enhanced proliferative capacity and cytokine secretion post-HCMV exposure. Correspondingly, CD159c is regarded as another marker of memory NK cells [54, 55]. Our results showed that preimmunized by A549 cell-derived exosomes effectively enhances the cytotoxicity of NK cells against A549 cells. Correspondingly, the killing rates of NK cells on K562 cells were increased to a certain extent as well, but not as same degree as the them on A549 cell. Flow cytometry data demonstrated that following exosomes treatment, the expression of CD25 and CD159c on NK cells is up-regulated, which propose that tumor antigen carried by A549 cells derived exosomes induces memory-like responses in NK cells. And these memory-like NK cells exhibited enhanced cytotoxicity against A549 cells when re-exposed to them. Furthermore, exosomes from A549 cells induce NK cells to secrete IFN-γ. Employing an IFN-γ blocking assay, we demonstrated that the increased IFN-γ secretion is the reason for enhanced cytotoxicity of NK cells against A549 cells themself and other target cells (K562). IFN-γ plays a crucial role in anti-tumor immunity [56]. It binds to a heterodimeric receptor composed of IFNGR1 and IFNGR2, thereby activating the JAK-STAT signaling pathway in T cells [57, 58]. Additionally, IFN-γ promotes the accumulation, activation, and cytotoxic function of NK cells [59]. We also found that IFN-γ can directly induce tumor apoptosis to some extent [see the details in supplementary figure S4].

Additionally, our data demonstrate that WT-EXO attenuates NK cells viability by suppressing the PI3K signaling pathway and it also inhibits the expression of the activating receptor NKG2D. However, KO-EXO did not exhibit a significant inhibitory effect on either NK cell viability or NKG2D protein expression. Meanwhile, the KO-EXO has stronger stimulation effect on NK cells cytotoxicity and IFN-γ secretion than WT-EXO, suggesting its greater potential in activating NK cells. Western blot results suggested that enhanced JAK2 and STAT1 phosphorylation and upregulated SOCS3 expression are the underlying mechanism by which

KO-EXO augments NK cell cytotoxicity against A549 cells. In NK cells, p-STAT1 translocates into the nucleus, to initiate the expression of genes encoding perforin and granzymes, which are key mediators of NK cell cytotoxicity [60, 61]. SOCS3 (Suppressor of Cytokine Signaling 3) belongs to the SOCS protein family and is a key negative-feedback regulator of the JAK/STAT pathway [62]. Therefore, the increase in negative feedback factors also suggests the activation of the JAK/STAT signaling pathway in the KO-EXO treatment group.

Nowadays, many strategies have been developed to increase the immunogenicity of TEXs, including 1. Thermal stress, this process can make exosomes carrying more heat shock proteins, tumor antigen peptides and chemokines, which activate the response of immune cells [63]; 2. The costimulatory molecules, interleukins (ILs) or CD40L are engineered onto exosomes to further increase the immune response [64-66]; 3. To stimulate immunocytes by TEXs along with interferon regulatory factor 1 (IRF-1), or inhibitor of PD-L1 simultaneously [67, 68]. A latest study demonstrated that engineered bacterial-derived vesicles carrying IFN-γ and a tumor antigen significantly inhibited tumor growth and metastasis in a breast cancer model. This effect was mediated by increasing the infiltration of tumor-specific effector T cells and reshaping the tumor microenvironment [69]. Collectively, these findings indicate that TEXs hold significant potential for the development of anti-tumor vaccines.



Our study demonstrates that exosomes derived from PD-L1 knockout lung cancer cells can effectively enhance NK cells anti-tumor activity against lung cancer by inducing immunological memory. Consequently, these exosomes hold considerable potentiality in arming the *ex vivo* expanded primary NK cells, thereby improving the efficacy of adoptive NK cell therapy for lung cancer *in vitro* and in mice model. This method presents a safer alternative to *in vivo* PD-1/PD-L1 blockade, as it avoids the direct systemic inhibition of this pathway that may lead to unforeseen immune dysfunction. However, it is important to acknowledge several limitations in our study. First is the relatively small sample size, which might limit the robustness of the findings. Furthermore, as the study relied on cell lines, it may not fully capture the intricate crosstalk within the tumor-immune microenvironment. Additionally, the antigens presented by

the A549 cell line might be insufficient to elicit a robust NK cell cytotoxicity against primary tumor cells.

In future, researchers could explore the use of exosomes derived from TEXs preimmunized dendritic cells (DCs) to activate NK cells to make it more closely mimic the physiological antigen presentation process *in vivo*. Additionally, if specific tumor markers are available, a detailed comparison of tumor antigen levels across different tumor derived extracellular vehicles (EVs) including classic exosomes, microvesicle and apoptotic body, as well as newly discovered blebbisome and ectosomes [70, 71] should be conducted to identify the optimal antigen carrier. Since the repertoire of TEXs is still poorly understood. A study system for the comprehensive assessment of the immunostimulatory effects of TEXs is critically needed. And, it is necessary to make a profound study on the mechanism of NK cell heterogeneity when responding to TEXs stimulation among individuals. For instance, the application of high-throughput sequencing may help to reveal the reasons behind NK cell response heterogeneity which will facilitate to predict the immunotherapeutic effect of NK cell on cancer and develop more individualized and effective NK cell activators.



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## Authors' Contributions

**Qi Li,** Experimental design and article writing, flow cytometry detection; **Juanping Chen,** q-PCR and WB experiments; **Wuli Guo**, Blood samples collection and NK cell culture, PD-L1 knock-out A549 cell line construction; **Qi Shu**, Exosomes extraction and identification. Exosomes extraction and identification; **Yuan Yin,** Mouse feeding; **Yuanyuan Qu**, Mouse feeding; **Yutong Feng**, Mouse feeding; **Zijie Liu,** Mouse feeding; **Wei Zhang**, Blood sample collection and funding support; **Ting Huyan**, Experimental design, establish the mouse model and funding support.

## Data availability

No datasets were generated or analyzed during the current study.

## Declarations

## Ethics approval and consent to participate

We confirmed that all methods involving the human blood, mouse and cells adhered with the relevant institutional, national, and international guidelines and legislation.



## Competing interests

The authors declare no competing interests.

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**Table1**. **Primers sequences used for quantitative real time PCR of NK cell related genes**

| *Gene* | *Primers (5'-3')* |
|---|---|
| **NKG2A (F)** | AATGGCCTCTGTGGTAACGA |
| **NKG2A (R)** | GGCCACAATGACGTGCTTTC |
| **CD94 (F)** | AGCCAGCATTTACTCCAGGAC |
| **CD94 (R)** | AGCTGAAGCAGGCTGGATTT |
| **KLRK1 (F)** | TTTTTCAACACGATGGCAAAAGC |
| **KLRK1 (R)** | GGGCCACAGTAACTTTCGGT |
| **NCR3 (F)** | CCCCTGAGATTCGTACCCTG |
| **NCR3 (R)** | CTCCACTCTGCACACGTAGAT |
| **NCR2 (F)** | CCTCTCGATTCACAATCTGGGA |
| **NCR2 (R)** | AGTTGTCAGAAGGGCCGGTAGA |
| **NCR1 (F)** | CCACCGAGGGACATACCGAT |
| **NCR1 (R)** | GTGCAAGGCTGGTGTTCTCA |
| **ICAM-1 (F)** | ATGCCCAGACATCTGTGTCC |
| **ICAM-1 (R)** | GGGGTCTCTATGCCCAACAA |
| **CD226 (F)** | GATGTTGGCTACTATTCCTGCTC |
| **CD226 (R)** | CTGAACCACCTGTATCACCTTC |
| **IFN-γ (F)** | TCGGTAACTGACTTGAATGTCCA |
| **IFN-γ (R)** | TCGCTTCCCTGTTTTAGCTGC |
| **PRF-1 (F)** | GTGGGACAATAACAACCCCAT |
| **PRF-1 (R)** | TGGCATGATAGCGGAATTTTAGG |
| **GZMA (F)** | TCTCTCTCAGTTGTCGTTTCTCT |
| **GZMA (R)** | GCAGTCAACACCCAGTCTTTTG |
| **GZMB (F)** | TACCATTGAGTTGTGCGTGGG |
| **GZMB (R)** | GCCATTGTTTCGTCCATAGGAGA |
| **TIGIT (F)** | TCTGCATCTATCACACCTACCC |
| **TIGIT (R)** | CCACCACGATGACTGCTCT |

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|---|---|
| **PD-L1 (F)** | CCTACTGGCATTTGCTGAACGCAT |
| **PD-L1 (R)** | ACCATAGCTGATCATGCAGCGGTA |
| **PD-1 (F)** | ACGAGGGACAATAGGAGCCA |
| **PD-1 (R)** | GGCATACTCCGTCTGCTCAG |
| **CTLA-4 (F)** | GCCCTGCACTCTCCTGTTTTT |
| **CTLA-4 (R)** | GGTTGCCGCACAGACTTCA |
| **TIM3 (F)** | TTGGACATCCAGATACTGGCT |
| **TIM3 (R)** | CACTGTCTGCTAGAGTCACATTC |
| **GAPDH (F)** | CTGGGCTACACTGAGCACC |
| **GAPDH (R)** | AAGTGGTCGTTGAGGGCAATG |

## Table 2. Antibodies information

| Antibody | Brand | Catalog Number |
|---|---|---|
| PE-humanNKG2A antibody | R&D | FAB1059P |
| PE-Mouse human CD314 (NKG2D) antibody | BD Pharmingen | 554680 |
| NKp30 antibody | Abcam | ab186425 |
| PE-Mouse human NKp44 (CD336) antibody | BD Pharmingen | 558563 |
| PE-Mouse human NKp46 (CD335) antibody | BD Pharmingen | 557991 |
| FITC-CD56 (NCAM) antibody | eBioscience | 11-0566-42 |
| PE-human CD3 antibody | Peperotech (BioGems) | 05131-60-25 |
| PE-CD226-antibody | Abcam | ab33337 |
| FITC-human CD25 Antibody | BioLegend | 356105 |
| APC-human CD57 Antibody | BioLegend | 359609 |
| PE-human CD159c (NKG2C) Antibody | BioLegend | 375003 |
| PD-1 antibody | Cell Signaling | 86163 |
| CTLA-4 antibody | Cell Signaling | 53560 |
| TIM-3 antibody | Cell Signaling | 45208 |
| TIGIT antibody | Cell Signaling | 99567 |
| CD9 antibody | Cell Signaling | 13403 |
| CD81 antibody | Cell Signaling | 52892 |
| CD63 antibody | Abcam | ab271286 |
| Calnexin antibody | proteintech | 10427-2-AP |
| STAT1 antibody | proteintech | 66545-1-Ig |
| Phospho-STAT1 antibody | proteintech | 82674-10-RR |
| PI3K antibody | Abcam | ab247274 |
| Phospho- PI3K antibody | Abcam | ab278545 |
| JAK2 antibody | Abcam | ab245303 |
| Phospho- JAK2 antibody | Abcam | ab195055 |
| SOCS3 antibody | Abcam | ab16030 |
| IFN-γ antibody | Thermo Fisher | 14-7318-81 |

| | | |
|---|---|---|
| PD-L1 antibody | Abcam | ab243877 |
| CD56 antibody | Servicebio | GB112671 |
| Ki67 antibody | Servicebio | GB121141 |