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Histodynamics of Natural Killer Cells from a Healthy Donor Exposed to Exosomes from the Blood of Hepatocellular Carcinoma Patients

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Histodynamics of Natural Killer Cells from a Healthy Donor Exposed to Exosomes from the Blood of Hepatocellular Carcinoma Patients

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Abstract

Background: Hepatocellular carcinoma (HCC) is the leading form of liver cancer and the second leading cause of cancer-related deaths globally. Exosomes in the HCC microenvironment can induce significant changes in natural killer (NK) cells during endocytosis. The present study aimed to distinguish exosomes in the blood of HCC patients, analyze changes in NK cell phenotype, and evaluate peroxidase and toluidine blue staining as alternative methods for observing the changes.

Methods: NK cells were collected from healthy donors, and exosomes were extracted from the blood of HCC patients. The exosomes were characterized in accordance with MISEV 2018 guidelines, and NK cells were incubated with HCC-derived exosomes. NK cell phenotype changes were assessed using immunofluorescence, toluidine blue staining, and peroxidase staining.

Results: The identified exosomes measured 34.7 nm, had a charge of −4.33 mV, and were positive for CD81+. Changes in NK cell receptor expression following exposure to HCC exosomes were not significant ($p > 0.05$). Immunofluorescence confirmed exosome endocytosis by NK cells, toluidine blue staining revealed negative metachromasia and peroxidase staining indicated morphological NK cell changes.

Conclusions: This study demonstrates that peroxidase and toluidine blue staining are effective for observing exosome endocytosis in NK cells, enhancing our understanding of HCC exosome-NK cell interactions and beneficial in developing future therapeutic strategies targeting the HCC microenvironment.

Keywords: endocytosis, exosomes, hepatocellular carcinoma, natural killer cells, staining technique

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of liver cancer and the second leading cause of cancer-related deaths worldwide. Exosomes in the HCC microenvironment can induce significant changes in natural killer (NK) cells during endocytosis. Exosomes possess immunogenic properties and can modulate immune responses that induce NK cell activation.[1–4]

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Exosomes released by hepatocellular carcinoma cells are 30–150 nm in size and contain elevated myeloperoxidase (MPO). Increased MPO can trigger the reactions observed in peroxidase staining when NK cells are exposed to exosomes. Additionally, toluidine blue staining revealed changes in the azurophilic granules of NK cells, wherein metachromasia was evident before exposure to exosomes and disappeared afterward, indicating an endocytic effect of the exosomes.[4,5]

This study aimed to evaluate exosomes from the blood of HCC patients and changes in NK cell phenotype, including the expression of activation and inhibition receptors, using flow cytometry and histological staining. This method is a simple alternative for detecting indications of HCC through changes that occur in NK cells following their interactions with exosomes.

METHODS

Whole blood donation from UTD PMI

This study was approved by the ethical committee of Fakultas Kedokteran Universitas Indonesia, with approval number KET-367/UN2.F1/ETIK/PPM.00.02/2023 for obtaining of whole blood from the blood transfusion unit of the Indonesian Red Cross Blood Bank in Jakarta. Healthy-donor blood samples, that is, 3 bags of 350 cc whole blood (triplication), were collected from UTD PMI. The inclusion criteria were individuals who qualify as healthy blood donors according to the UTD PMI guidelines, those aged 18–65 years, and those who have undergone screening to confirm they are negative for infections transmitted through blood transfusion, including HIV, hepatitis B, hepatitis C, and TORCH. Moreover, donors should provide written consent for the use of their blood in this research. Conversely, the exclusion criteria were individuals with a history of infectious or chronic diseases that may affect blood quality, those undergoing specific treatments such as immunosuppressive therapy, pregnant or breastfeeding women, and individuals who had donated blood within 3 months prior to sample collection for this study. These criteria ensure that only blood from healthy donors who meet specific standards is utilized in the research, thereby increasing the reliability of the results.

Isolation of peripheral blood mononuclear cells (PBMC)

PBMC isolation refers to the 2019 PiNK Copyright. Whole blood was poured into a falcon tube containing PBS, with a volume/volume ratio = 1:1. Furthermore, 15 mL of Ficoll-Paque was put into two 50 mL centrifugation tubes (Thermo Fisher Scientific, Singapore). The diluted blood was placed into a 50 mL centrifugation tube containing Ficoll-Paque at a 1:1 ratio. Then, the tube (Eppendorf, Indonesia) was centrifuged with 400xg speedat 4 °C (GEA) (no brake) for 20 minutes. Centrifugation results were collected using a micropipette (Thermo Fisher Scientific, Singapore) in the form of a layer of mononuclear cells (buffy coat). The buffy coat liquid was poured into a 15 mL Falcon tube, and PBS was added to equalize the volume and then centrifuged at 650xg speed at 4 °C (low brake: accelerate, 5 and decelerate, 5) for 10 minutes. This process was performed to wash the mononuclear cell fraction from Ficoll-Paque.

Centrifugation formed pellets and supernatants. The supernatant was discarded. Pellets formed from centrifugation were resuspended in 1 mL PBS, and 10 µL was taken to count the number of cells. Then, cell suspension was mixed with 10 µL trypan blue (Gibco). Cells were counted using Improved Neubauer (Marienfeld, Germany) and were observed under a microscope (Nikon) with 10X magnification in four large squares.

NK cell isolation from PBMCs

PBMC and NK cell medium were poured into a 15 mL centrifugation tube. Then, the tube was placed into the centrifugation machine for 10 minutes at a speed of 650xg at 4 °C. Centrifuge the cell suspension from PBMC at 300 g at 4 °C for 10 minutes. The supernatant was disposed in a disposal area, and the pellet was resuspended in PBS. Add 10 µL of NK Cell Biotin-Antibody Cocktail (Miltenyi Biotec) per 10^7 total cells. Then, the solution was incubated for 5 minutes in the refrigerator (temperature: 2 °C−8 °C), and PBS and 20 µL of NK Cell MicroBead Cocktail (Thermo Fisher Scientific) per 10^7 total cells were added into the pellet suspension and homogenized and incubated for 10 minutes in the refrigerator at 2 °C–8 °C. After incubation, the pellet was resuspended in 500 µL PBS. The MS column (Miltenyi Biotec) was placed in a magnetic field with the appropriate MACS separator. The cell suspension was transferred to the column slowly, and the formed droplets (NK cells) were placed in a 15 mL centrifugation tube. Afterward, the column was washed with 500 µL buffer, and the centrifugation tube containing the NK cells was labeled. Tubes with non-NK cell labels were arranged in a column, which was then released from the separator, and 1 mL of PBS was added. The plunger column was then pushed into a 15 mL centrifugation tube. NK cells were counted and characterized by flow cytometry (BD FACSCanto II).[6]

HCC peripheral venous blood sampling

This study was approved by the ethical committee of the Faculty of Medicine, Universitas Indonesia, with approval number ND-795/UN2.F1/ETIK/PPM.00.02/2022, dated December 5, 2022, for the collection of blood from hepatocellular carcinoma patients. The inclusion criteria for this study were patients diagnosed with hepatocellular carcinoma based on a comprehensive assessment, including medical history, physical examination, and supporting diagnostic tests, without any age and sex restrictions. Further, patients with a confirmed diagnosis through surgical results examined by the Department of Anatomical Pathology of RSCM were included. Additionally, only those patients who have completed the consent form and were willing to participate in the study were considered for inclusion. Conversely, the exclusion criteria specified that patients with a history of other liver diseases, such as cirrhosis and active hepatitis, would be excluded owing to potential effects on study outcomes. Moreover, individuals undergoing treatments that could influence their health status or laboratory results, including immunosuppressive therapy or chemotherapy, were excluded, as well as pregnant or breastfeeding women to mitigate potential risks. Furthermore, patients with serious medical conditions that could compromise blood quality or research results, such as coagulation disorders or active infections, were excluded. Finally, individuals who were unable to provide written consent to participate in the study were not included. A 10 mL peripheral venous blood sample was extracted from each

eligible patient using a disposable syringe (Terumo, Indonesia). Each sample was placed in an EDTA blood collection tube for transport to SCTE IMERI.

Serum collection from a peripheral venous blood sample

Exosomes were isolated using Ficoll with a density gradient centrifugation method (exosome isolation protocol complies with the granted copyright of PINK protocol). Then, the blood was diluted to 10 mL with PBS, and 10 mL of Ficoll-Paque was placed inside two 50 mL centrifugation tubes. The diluted blood was transferred into a 50 mL centrifugation tube containing Ficoll-Paque and centrifuged at 400 g at 4 °C (no brake) for 20 minutes. Centrifugation formed a layer of serum, which was collected using a micropipette.

Isolation of exosomes from the blood serum of hepatocellular carcinoma patients by ultracentrifugation

Serum obtained from the patient's blood by Ficoll density gradient centrifugation was centrifuged for 15 minutes at 750 g at 20 °C. The supernatant was obtained and centrifuged for 15 minutes at 2000 g. The supernatant from centrifugation was collected using a micropipette. Centrifugation of the supernatant was continued for 45 minutes at 10,000 g. The supernatant was filtered with a 0.2 µm syringe filter. The filtrate was ultracentrifuged for 90 minutes at 100,000 g at 4 °C. This process was conducted at IMERI's molecular biology and proteomic core facilities. Then, the pellet containing the EVs was placed in a 15 mL Falcon. Resuspend the EVs pellet by adding cold D-PBS until the volume reaches 5 mL. The aliquots of EVs were placed inside the cryovial 1 mL each and stored in the cryo box in the refrigerator at −20 °C (Thermo Fisher Scientific) at SCTE IMERI.

Characterization of exosomes from the blood of hepatocellular carcinoma patients

The investigation was conducted at ILRC UI Depok using a particle-size analyzer (PSA) to determine the size and electrical charge of the exosome particles. The ILRC UI laboratory has a Horiba SZ 100z PSA, which can characterize the physical properties of nanosized particles and measure the zeta potential and molecular weight of suspension samples.

Stimulation of healthy donor NK cells with HCC patient exosomes

In addition, 100 µL of the isolated healthy donor NK cells was obtained and transferred to a well on a 96-well plate. Furthermore, 100 µL of coded HCC exosomes was added into the well and was labeled as NK+ exosomes. As a negative control, NK cell culture was added with 100 µL of medium and was not exposed to exosomes. All stimulation stages were repeated in three sets. Then, the wells were incubated for 24 hours in the incubator.

NK cell flow cytometry for receptor activation and inhibition

Further, 10 µL of NK cell suspension, namely, NK cells + exosomes, was placed in each flow cytometry tube that was labeled as unstained, panel 1, panel 2, panel 3, and panel 4. Then, 100 µL PBS was added. Moreover, 200 µL of unstained sample was added and loaded into a flow cytometry tool as gating control. The gating (blank limit) was set to 0.2%.

The antibodies were added according to the panel description below. The NK cell suspensions were divided into four flow cytometry panels (tubes) based on the flow cytometry antibodies for NK cell receptors:

Panel 1 = Double-stain $CD56^{+}$-PE
Panel 2 = Double-stain NKP30-PE and AntiKIR2D/CD158A-FITC
Panel 3 = Double-stain NKP44-PE and NKG2A-FITC
Panel 4 = Double-stain NKG2D-PE

The tubes were incubated in the dark for 30 minutes, added with PBS 200 µL, and washed once by centrifugation. The tubes were analyzed using flow cytometry BD FACS Aria II at SCTE IMERI.

Immunofluorescence endocytosis of $CD81^{+}$ exosomes into NK cells

This examination used previously isolated NK cells that had been fixed with absolute methanol. Remove The fixative solution was removed from the chamber slide, and 100 ul PBS was added. The removed solution was placed inside the Eppendorf tube and centrifuged at 650 g 4 °C for 10 minutes. The pellets were rinsed with PBS and centrifuged again. The exosomes used were taken out from the freezer at −20 °C, and the HCC exosomes were thawed into a beaker glass filled with water at 37 °C. The 300 µL exosomes were transferred to an Eppendorf tube, and 15 µl of CD81-PE antibody was added; then, it was pipetted up and down and incubated in the dark for 60 minutes at 2 °C–8 °C. NKG2A-FITC Ab concentrations were prepared in a 1.5 ml Eppendorf tube for concentrations 1:50 and 1:100 prepared with the PBS mixture. Then, the prepared NKG2A-FITC Ab was added to NK cells and incubated for 30 minutes at 2 °C–8 °C. After exosome incubation with CD81-PE and NK cell incubation with Ab NKG2A-FITC, 100 uL of exosome-CD81 PE was placed inside a tube containing NK cells and incubated for 1 hour. It was then washed with PBST centrifugation, and the cell nucleus was stained with DAPI for 5 minutes at 200 µL at room temperature in a dark room or wrapped in aluminum foil. Then, the DAPI was removed and centrifuged with PBS. The transport box was prepared, and the well chamber slide was observed under a fluorescence microscope starting from negative control.

Peroxidase staining
Slides of NK cells and exosome NK+ cells were fixed, after which they were poured with hydrogen peroxide, horseradish peroxidase (HRP) conjugate, and DAB chromogen [Abcam]. Then, a counterstain was performed with hematoxylin. After staining, the slide can be observed under a light microscope.

Toluidine blue staining
Slides of NK cells and NK+ exosomes were made by dropping the sample onto a glass slide and drying it with a hotplate until all the liquid has evaporated. It was rinsed with water and fixed in absolute methanol for 60 seconds. Toluidine blue (Sigma-Aldrich) was poured until the entire surface of the slide was covered. It was then left to rest for 1–2 minutes and was rinsed with running water and dried. The smear was covered with a cover glass and sealed with entelan. The slides were observed under the microscope as soon as they were ready.

Statistical analysis
The GraphPad Prism 9 software application was used for analysis. First, the variable measurement scale was determined. The average value of the examination results were evaluated. Furthermore, normality and statistical tests were performed to assess its significance. This test is used to show significant or insignificant average differences between more than two groups.

RESULTS

Characterization of the size and electrical mutants of exosomes from the blood of hepatocellular carcinoma patients
Exosome measurements obtained the largest size of 34.8 nm and the smallest size of 33.9 nm. Exosome size examination showed a mean of 34.37 ± 0.45 nm. The typical baseline size of exosomes in healthy individuals was 30–150 nanometers (nm). These results are supported by the polydispersity index value, which averages 0.48 ± 0.45.

The largest electric charge of the exosome was −2.6 mV, and the smallest electric charge was −5.9 mV. The mean electric charge of the exosomes was −4.33 ± 1.65 mV. The baseline for the zeta potential of exosomes generally ranges from −10 mV to −40 mV. In the present study, the mean value of electrophoretic mobility was −0.000003367 ± 0.000001305.

The characterization of exosomes from the blood of cellular carcinoma patients according to the characteristics of exosomes are 30–150 nm in size and have a negative electric charge. The cancer-derived exosomes tended to be smaller, approximately 70 nm, and may exhibit variations in their electric charge due to the influence of the tumor microenvironment. These differences are crucial in intercellular communication and for potential applications in cancer diagnostics.

Characterization of exosomes from the blood of hepatocellular carcinoma patients using flow cytometry
Tetraspanin proteins such as $CD63^+$ and $CD81^+$ are rich in exosome membranes and are used as exosome markers. This characterization used $CD81^+$-PE antibodies with a parent yield of 80.1% and a mean fluorescence intensity (MFI) of 468.

Expression analysis of NK cell phenotype markers ($CD56^+$) isolated results, NK + medium and NK after exposure to exosomes from the blood of hepatocellular carcinoma patients
$CD56^+$ marker (Figure 1a) expression in the NK cell group before exposure to exosomes had a mean parental percentage of 39.6 ± 17.52% and an MFI of 176.7 ± 34.93. $CD56^+$ marker expression in the NK cell group that had been exposed to exosomes had a mean parental percentage of 33.93 ± 16.59% and a mean fluorescence intensity (MFI) of 327 ± 302.7% (Figure 1b).

The mean flow cytometry $CD56^+$ phenotype marker expression in the percent of parents was normally distributed, whereas the mean MFI was not normally distributed. The follow-up statistical test used to evaluate the $CD56^+$ marker expression from the percent parent values was the T-test, and the follow-up statistical test to assess the $CD56^+$ marker expression on the MFI value was the Mann-Whitney test to determine the differences between the two groups. Statistical results from the $CD56^+$ antibody expression test showed no significant difference between the mean $CD56^+$ expression in each group ($p < 0.05$; $p = 0.70$ and $p = 0.99$). Thus, the hypothesis was rejected.

Analysis of activation receptor expression (NKG2D, NKP30, and NKP44) of NK cells before and after exposure to exosomes from the blood of hepatocellular carcinoma patients
Figure 2 shows that NKG2D, NKP30, and NKP44 expressions in the NK cell group before exposure to exosomes had a mean parent percentage of 53.80 ± 6.32%, 60.37 ± 3.27%, and 68.33 ± 10.02%, respectively, and an MFI of 206.7 ± 33.23, 68.33 ± 10.02%, and 490 ± 209.5, respectively. Figure 3 reveals that the NKG2D, NKP30, and NKP44 expressions in the NK cell group that had been exposed to exosomes had a mean percent parent of 50.27 ± 17.60%, 61.97 ± 7.75%, and 54.30 ± 14.27%, respectively, and an MFI of 199.7±3.78, 300.3 ± 159.2, and 451 ± 47.29, respectively. The statistical results from the NKG2D, NKP30, and NKP44 antibody expression tests were $p < 0.05$, $p = 0.75$ and $p = 0.73$; $p < 0.05$, $p = 0.75$ and $p = 0.79$; and $p < 0.05$, $p = 0.23$ and $p = 0.76$. No significant difference was found between the average

activation receptor expression in each group. Hence, the hypothesis was rejected.

Expression analysis of inhibitory receptors (KIR2D and NKG2A) of NK cells before and after exposure to exosomes from the blood of hepatocellular carcinoma patients

Figure 4 presents that the expression of the KIR2D marker in the NK cell group before exposure to exosomes had a mean parental percentage of 68.33 ± 10.02% and an MFI of 490 ± 209.5. Moreover, the KIR2D marker expression in the group of NK cells that had been exposed to exosomes had a mean parental percentage of 54.30 ± 14.27% and an MFI of 451 ± 47.29. Additionally, the NKG2A marker expression in the NK cell group before exposure to exosomes had a mean parent percentage of 44.33 ± 11.14% and an MFI of 216 ± 142. The expressions of KIR2D and NKG2Ain the NK cell group that had been exposed to exosomes had a mean parental percentage of 26.43 ± 5.44% and an MFI of 404 ± 303.3. The KIR2D and NKG2A antibody expression tests showed significant differences in the average expression of NK cell inhibitory receptors in each group ($p < 0.05$; $p = 0.44$; $p = 0.61$ and $p < 0.05$; $p = 0.06$; $p = 0.38$). Therefore, the hypothesis was rejected.

Immunofluorescence of exosome endocytosis to NK cells

Figure 3a shows the NK cell nucleus with a dark blue round shape. Figure 3b reveals NK cells stained with DAPI and exosomes stained with $CD81^+$-PE. The red distribution indicates the location of the exosome to NK cells, which is outside the nucleus or cytoplasm of NK cells and outside the NK cells. NKG2A-FITC was used to detect NKG2A transmembrane inhibitory receptor expression in NK cells (in green). Figure 3c presents that the NK cell nucleus is slightly oval in the center of the cell and is surrounded by the cell membrane. Figure 4d indicates the presence of exosome endocytosis in the NK cell cytoplasm.

Toluidine blue and peroxidase staining of exosome endocytosis to NK cells

Simple histological staining (toluidine blue and peroxidase) was performed on NK cells. Figure 5a shows that NK cells appear round and 4 µm in size. Toluidine blue staining showed NK cells with darker nuclei and a purplish cytoplasm. In Figure 5b, NK cells from a group of NK cells exposed to hepatocellular carcinoma exosomes appear round and 5 µm in size, with large purple nuclei. The NK cell nucleus is surrounded by a clear blue cytoplasm. Toluidine blue staining in NK cells without exosomes demonstrated metachromasia of all cells, that is, approximately 100%. Observation of NK cells exposed to exosomes revealed that 50% of NK cells had clear blue cytoplasm and 50% of NK cells had positive metachromasia. In Figure 5c, the NK cells are spherical and 6 µm in size. The large NK cell nucleus almost fills the entire cell and is red, and the cytoplasm has a paler red color, indicating the absence of a peroxidase enzyme reaction in NK cells. In Figure 5d, NK cells exposed to hepatocellular carcinoma exosomes are spherical and 5 µm in size. An NK cell nucleus and cytoplasm with brown granules were noted. Peroxidase staining in NK cells revealed a negative peroxidase reaction in all cells (100% peroxidase negative). Moreover, observation of NK cells exposed to exosomes found NK cells with a positive peroxidase reaction of 33.3% and NK cells with a negative peroxidase reaction of 66.6%.

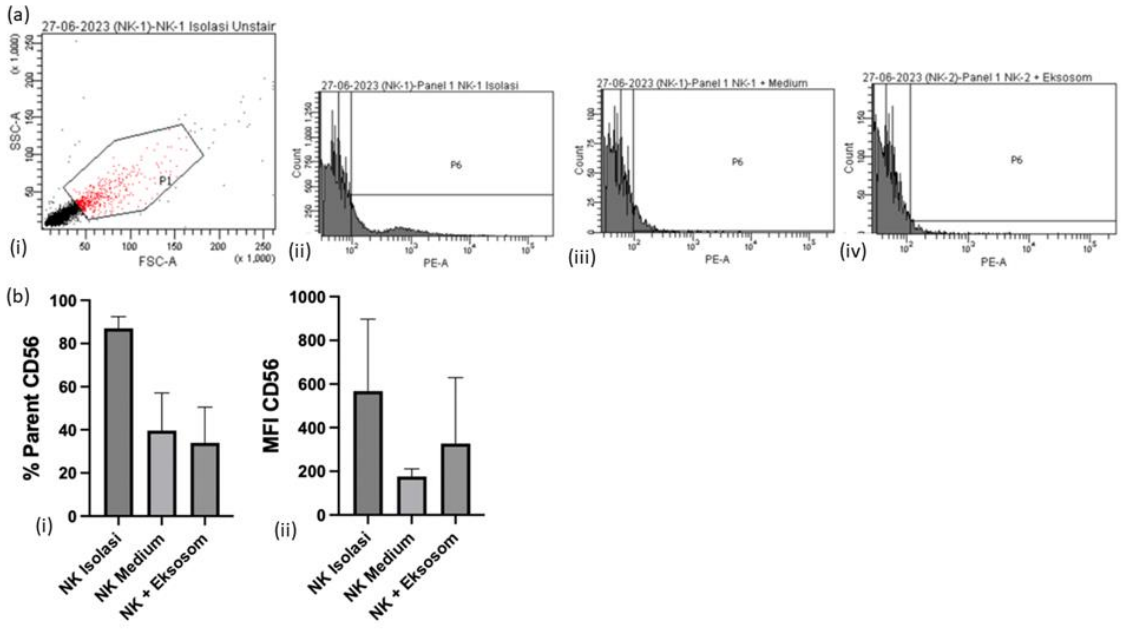


FIGURE 1. (a) Flow cytometry of $CD56^+$ antibody expression: (i) gating strategy, (ii) $CD56^+$ antibody expression in NK cells isolated from MNC (NK isolasi), (iii) $CD56^+$ antibody expression on NK cells + medium (NK+medium), and (iv) $CD56^+$ NK cell

antibody expression + exosomes from the blood of hepatocellular carcinoma patients (NK+eksosom); (b) graph of CD56+ expression of NK cells isolated from MNC (NK isolasi), NK cells + medium (NK+medium), and NK cells + exosomes from the blood of hepatocellular carcinoma patients (NK+eksosom) analyzed by flow cytometry: (i) comparison of the average percentage of CD56+ expression parents and (ii) comparison CD56+ expression MFI mean

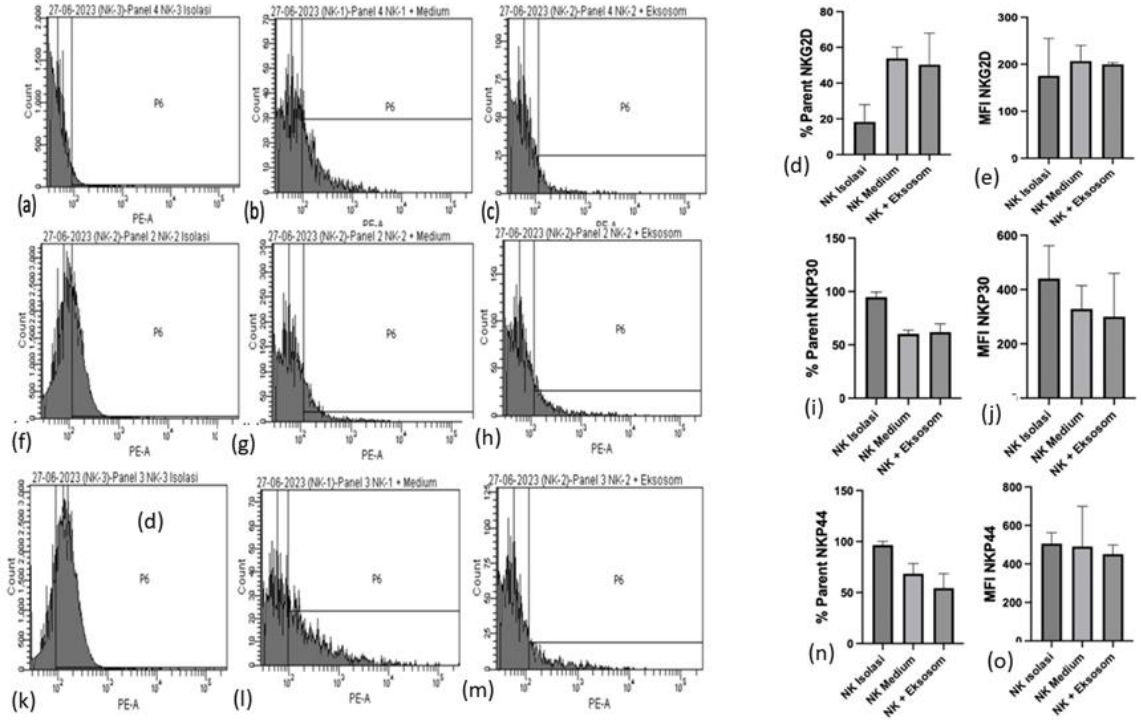


FIGURE 2. Flow cytometry of NK cell activation receptor expression, (a) NKG2D antibody expression in NK cells isolated from MNC (NK isolasi), (b) NKG2D antibody expression in NK cells + medium (NK+medium), (c) NKG2D antibody expression in NK cells + exosomes from the blood of hepatocellular carcinoma patients (NK+eksosom), (e) NKG2D expression mean based on the percent value of the parent, (f) mean NKG2D expression based on MFI values, (f) NKP30 antibody expression in NK cells isolated from MNCs (NK isolasi), (g) NKP30 antibody expression in NK cells + medium (NK+medium), (h) NKP30 antibody expression in NK cells + exosomes from the blood of hepatocellular carcinoma patients (NK+eksosom), (i) NKP30 expression mean based on the parent percent value, (j) NKP30 expression mean based on MFI values; (k) NKP44 antibody expression in NK cells isolated from MNC (NK isolasi), (l) NKP44 antibody expression in NK cells + medium (NK+medium), (m) NKP44 antibody expression in NK cells + exosomes from the blood of hepatocellular carcinoma patients (NK+eksosom), (n) NKP44 expression mean based on the parent percent value, and (o) average NKP44 expression based on MFI values.

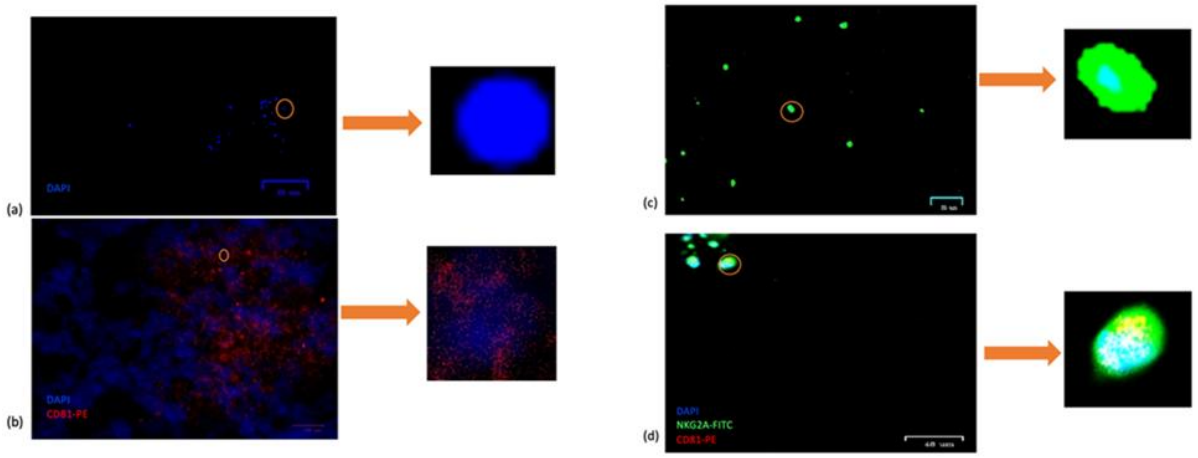


FIGURE 3. Immunofluorescence results of NK cells and exosomes, (a) DAPI-labeled NK cell nuclei, (b) DAPI-stained NK cell nuclei and CD81+-PE-stained exosomes, (c) NK cells with NKG2A-FITC antibody, and (d) NK cells exposed to exosomes

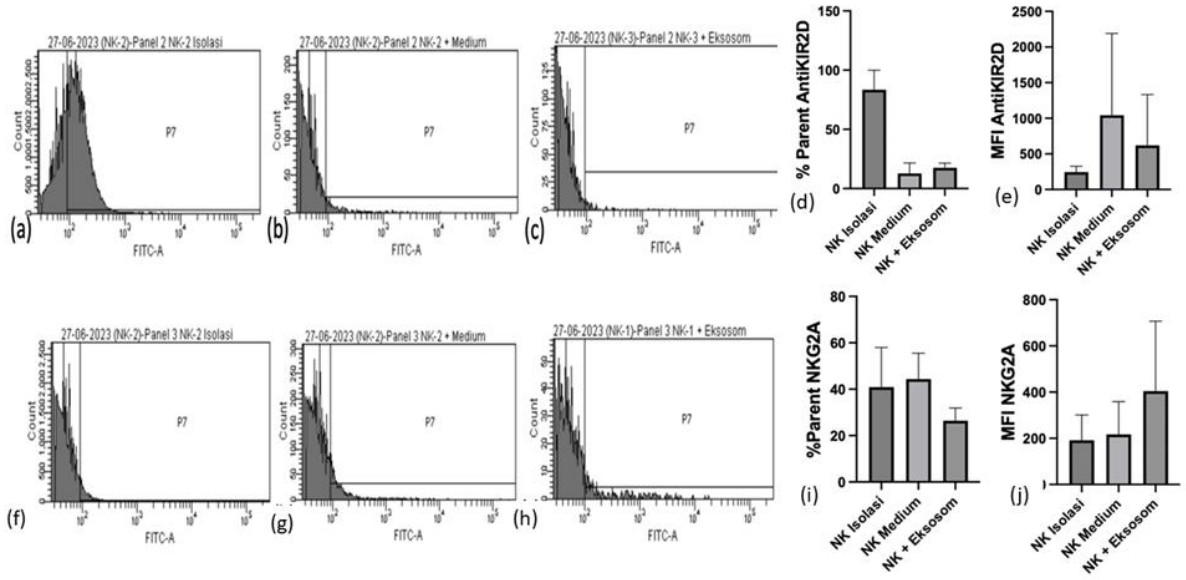
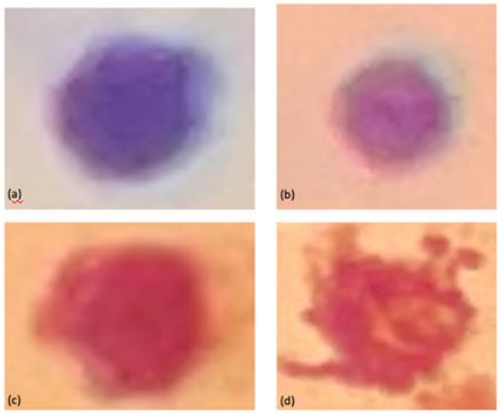


FIGURE 4. Flow cytometry of NK cell inhibitory receptor expression, (a) KIR2D expression in the NK cell group isolated from MNC (NK isolasi), (b) KIR2D expression in the medium + NK cell group (NK+medium), (c) KIR2D expression in the NK cell group + exosomes from the blood of hepatocellular carcinoma patients (NK+eksosom), (d) KIR2D expression mean based on parent percent value, (e) KIR2D expression mean based on MFI values; (f) NKG2A expression in the NK cell group isolated from MNC (NK isolasi), (g) NKG2A expression in the NK + medium cell group (NK+medium), (h) NKG2A expression in the NK cell group + exosomes from the blood of hepatocellular carcinoma patients (NK+eksosom), (i) expression mean based on parent percent value, and (j) average expression based on MFI value



Toluidine Blue Staining	NK Cells only	NK cells + HCC Exosome
Metachromasia (+)	100%	50%
No metachromasia (−)	0%	50%

Peroxidase Staining	NK cells only	NK Cells + HCC exosome
Positive (+)	0%	33.3%
Negative (−)	100%	66.6%

FIGURE 5. Simple coloring results, (a) Toluidine blue staining of NK cells, (b) toluidine blue staining of NK cells + exosomes, (c) NK cell peroxidase staining, and (d) NK cell peroxidase staining + exosomes (objective lens magnification 40x)

DISCUSSION

Characteristics of exosomes from the blood of hepatocellular carcinoma patients

Exosomes isolated from the blood of hepatocellular carcinoma patients were sized according to typical exosomes, negatively charged, and had transmembrane proteins according to the characteristics of exosomes, according to MISEV 2018. The nanoparticles isolated from the blood of hepatocellular carcinoma patients were exosomes. Notably, in exosomes isolated from the blood of patients with hepatocellular carcinoma, examining the electric charge with the zeta potential found a negative electrical charge. These results are similar to those of a study by Yukawa *et al*., who isolated hepatocellular carcinoma exosomes from the supernatant of HepG2 cell culture medium, which was incubated for 4 days.[8] Negative electrically charged exosomes can interact with molecules and target cell surfaces through a receptor-ligand recognition mechanism. This indicates that exosomes may be involved in the transfer of information and signals between cells. The characteristics of the exosome's electrical charge are related to the interaction between the exosome and the environment or target cells.[9–11]

Exosomes isolated from the blood of cancer patients showed CD81$^+$ expression as an exosome marker and an assessor for exosome purity. CD81$^+$ expression in the exosomes isolated from the blood of this carcinoma patient was 80.1%, whereas CD81$^+$ expression in the exosomes isolated from the supernatant of HepG2 cell medium was 99.9%. The difference in the results of this exosome isolation is due to the source and method used to isolate the exosome. Exosomes sourced from the cell medium supernatant HepG2 were isolated using the ExoQuick-TC kit.[8,12]

The current study used the same ultracentrifugation rate as that of Zhu *et al*. In this study, the ultracentrifugation time was three times shorter than that of Zhu *et al*. Moreover, this study isolated exosomes from the culture medium supernatant of human hepatocellular carcinoma Huh-7 cells by ultracentrifugation for 5 hours. The results of exosome isolation in this study were similar to the characteristics of hepatocellular carcinoma exosomes isolated using the ExoQuick-TC kit. Therefore, the isolation of carcinoma exosomes in this protocol was more efficient because it can be used to isolate exosomes with shorter times and large-scale samples.[8,13–15]

Expression of NK cell phenotype markers (CD56$^+$) isolated from NK + medium and NK after exposure to exosomes from the blood of hepatocellular carcinoma patients

NK cell receptors in the form of CD56$^+$ were observed. A difference was noted in the receptor expression on NK cells before and after being exposed to exosomes. However, the difference was not significant. CD56$^+$ is a crucial NK cell marker in the immune response against cancer cells. These indicate that the exposed exosomes did not directly affect CD56$^+$ receptor expression as a marker of healthy NK cells. Further, this shows that CD56$^+$ receptor expression on the surface of NK cells remains relatively stable after being exposed to exosomes from the blood of hepatocellular carcinoma patients.[2,3,16,17]

Expression of activating receptors (NKG2D, NKP30, and NKP44) of NK cells before and after exposure to exosomes from the blood of hepatocellular carcinoma patients

This result is different from that of a study by Zhu *et al*., regarding the effect of oral cancer exosomes on NKG2D, NKp30, NKp44, NKp46, and NKG2A NK cell expression, which showed significant results of increasing the expression of NK cell activation receptors. In Zhu *et al*.'s study, the exosomes used were derived from the supernatant of an oral cancer cell culture medium. The source of these exosomes is different from that of this study, which was obtained from the blood of hepatocellular carcinoma patients.[18] In a study, Hong *et al*., revealed decreased expression of NKG2D in healthy NK cells exposed to acute myeloid leukemia (AML) exosomes. In Hong *et al*.'s research, exosomes were derived from the plasma of AML patients. NK cells (1x 10^6) were incubated with exosomes (50 µg) for 48 hours. Lv *et al*., revealed that NK cells exposed to exosomes derived from HepG2 had decreased expression of CD69, NKG2D, and NKp44 receptors. They used exosomal protein at a dose of 5, 10, and 20 g/mL combined with IL-2 and incubated with NK cells for 4 days. The same result was expressed by Li *et al*., who incubated healthy NK cells with tumor exosomes for 48 hours, resulting in reduction of NKG2D and NKP44 receptors. The method differs from that used in the present study in the duration of incubation and exosome dose used.[19–21]

Expression of inhibitory receptors (KIR2D and NKG2A) of NK cells before and after exposure to exosomes from the blood of hepatocellular carcinoma patients

In the current study, flow cytometry showed no significant changes in the expression of the KIR2D and NKG2A phenotype markers in healthy NK cells before and after exposure to exosomes from the blood of hepatocellular carcinoma patients. Based on the results, KIR2D expression has the same pattern as other NK cell receptors. The expression of KIR2D NK cells + medium is the same as the group of NK cells exposed to exosomes from the blood of hepatocellular carcinoma patients and different from early (postisolated) NK. However, in the present study, differences in expression patterns (increasing or decreasing) that are opposite between percentage and MFI were noted. The KIR2D receptor is an NK cell surface receptor that mediates signals for inhibition. The KIR2D receptor is a paired receptor that shares the same ligand, but with a different binding affinity. KIR2D has several subtypes, such as KIR2DS2, which can show increased antitumor activity in NK cells.

KIR2D and NKG2A receptors are inhibitory NK cell receptors that characterize HLA class I downregulation and NK cell inhibitory signaling. This insignificant result indicates that exosomes from the blood of hepatocellular carcinoma patients do not provide sufficient signals to increase or decrease NK cell activity compared with the medium + NK cell group. The difference in percentage and MFI results of KIR2D expression. Studies have not been found regarding the expression of NK cell KIR2D against hepatocellular carcinoma tumor exosomes. Thus, the results of KIR2D expression in the present study are novel.[22,23]

MFI is the amount of fluorescence at each cell "event" or antibody expression level, whereas the percentage of cells showed the entire cell population antibody expression level. The NK cell population had extremely high KIR2D expression in response to hepatocellular carcinoma exosomes that increase MFI, whereas the overall percentage of cells expressing NK cells did not show the same trend. The BD FACSDiva software was used to set up a flow cytometer, collect data, and analyze the resulting data. This software generates numbers by converting the

fluorescence signal generated by the analyzed cells into user-readable numbers. The factors that influence the difference between percentage and MFI include voltage, compensation, antibody dilution, tandem dye degradation, laser fluctuations, sample and reagent handling, instrumentation or maintenance of flow cytometry, and data analysis.[24]

In the present study, NKG2A expression had the same pattern as other NK cell receptors. NKG2A is an inhibitory NK cell receptor. NKG2A also expressed HLA class I downregulation and NK cell inhibitory signaling. The results in this study are different from those of studies by Lv *et al*., and Zhu *et al*., which revealed a decrease in NKG2A expression in tumor-exposed NK cells.[18,21]

Moreover, in this study, the expression of receptor activation and inhibition of healthy NK cells was similar between the NK + medium and NK + exosomes from the blood of hepatocellular carcinoma patients, which had a different trend from the isolated NK cell group. These results indicate that healthy NK cell cultures in vitro have sufficient stimulation to produce a different phenotype from the initial NK cells (MACS isolation results), although not significantly different. The relatively stable phenotype of healthy NK cells, even when incubated with exosomes from the blood of hepatocellular carcinoma patients, may be because of the release of exosomes from healthy NK cells during in vitro culture. Several in vitro studies have shown that exosomes released by healthy NK cells have a higher cytotoxic capacity and antitumor activity. This indicates that the exosomes of hepatocellular carcinoma incubated with NK cell cultures experience resistance by exosomes released by healthy NK cells. The antitumor activity of exosomes released by healthy NK cells will maintain the phenotype of healthy NK cells amidst a micro/in vitro environment containing the exosomes of hepatocellular carcinoma.[25–27]

Immunofluorescence compared with simple histological staining (toluidine blue and peroxidase) to identify exosome endocytosis in NK cells

This study compared the immunofluorescence images of NK cells and NK cells + exosomes to observe exosome endocytosis in NK cells. The combination of NK cell and exosome staining allows for interactions between NK cells and exosomes. This is similar to that in a study by Zhu *et al*., who observed the endocytosis of exosomes to NK cells. This study demonstrated that tumor exosomes reside in the cytoplasm of NK cells. In contrast to this study, Zhu *et al*., used DiO staining for exosomes.[18] This difference in NK cell staining can affect the results of immunofluorescence. NK cell staining appears to be better by staining cell membrane surface receptors than transmembrane such as NKG2A-FITC. These findings demonstrate the successful endocytosis of exosomes from the blood of hepatocellular carcinoma patients to healthy NK cells and the distribution of exosomes in NK cells. The presence of NK cell endocytosis is used as a gold standard to observe the histodynamics of NK cells owing to exposure to exosomes from the blood of hepatocellular carcinoma patients. This is related to the ability of NK cells to absorb hepatocellular carcinoma exosomes as their target and the potential of NK cells to regulate their function.[18,28,29]

Toluidine blue staining revealed metachromasia in all NK cells without exosomes. This staining is used to observe changes in azurophilic granules in the cytoplasm of NK cells due to exosome endocytosis from the blood of hepatocellular carcinoma patients. In the NK + exosome cell group, the cell nucleus is dark blue and the cytoplasm is clear blue, indicating no metachromasia. However, in the NK cell + exosome group, NK cells with metachromasia were found. This indicates that metachromasia can be found in all NK cells that are not exposed to hepatocellular carcinoma exosomes and that NK cells exposed to hepatocellular carcinoma exosomes show negative metachromasia. Metachromasia changes from positive to negative in the NK + exosome cell group. This reveals that NK cells exposed to exosomes have NK cells that experience a reaction to toluidine blue, resulting in metachromasia, and that some NK cells change due to exosomes of hepatocellular carcinoma by not reacting to metachromasia and display clear blue cytoplasm.

The occurrence of metachromasia in this NK cell group is similar to that in a case reported by Agarwal *et al*., who discovered metachromasia in blood smears from acute lymphoblastic leukemia. None of the previous studies revealed the existence of metachromasia in lymphoid blood cell lines. NK cells are lymphoid strains. The presence of metachromasia indicates an ionic interaction in the form of polyanions between toluidine blue dye and polyanion azurophilic granules in the cytoplasm of NK cells. No metachromasia or a clear blue appearance in the exosome-exposed NK cell cytoplasm indicates that changes in the NK cell cytoplasm are caused by exosome hepatocellular carcinoma. The results of this study decreased the percentage of positive metachromasia and increased the percentage of negative metachromasia in NK cells exposed to exosomes. This reveals that the histodynamics of NK cells due to endocytosed hepatocellular carcinoma exosomes can be NK cells. The findings in this study can support the postulate for observing the histodynamics of NK cells by observing the absence of metachromasia in exosome endocytosis in NK cells.[30–33]

In this study, peroxidase staining of NK cells showed that all cells did not experience peroxidase reactions or were negative. In NK cells + exosomes, NK cells with brownish or positive granules were found and NK cells without brownish or negative granules. Based on the visual analog scale, the results of NK + exosome staining, which appeared to be brownish granules, were level 2, which

means they had moderate staining compared to the negative reaction NK cell group. This staining reaction occurs because the peroxidase enzyme, derived from hepatocellular carcinoma exosome myeloperoxidase and the HRP used, catalyzes oxidation reactions using hydrogen peroxide as a substrate and produces products that can be colored by chromogenic reagents. In this staining test, the results of NK cells and NK cells + exosomes differed. NK cells, which are known to have a negative result on peroxidase staining, became positive to form a brown precipitate in the cytoplasm of NK cells after being exposed to exosomes from the blood of hepatocellular carcinoma patients. The results of this study decreased the percentage of negative peroxidase reactions and increased the percentage of positive peroxidase reactions in NK cells exposed to exosomes. This indicates that the histodynamics of NK cells due to endocytosed hepatocellular carcinoma exosomes can be NK cells.[34,35]

The simple staining performed in this study was chosen because of the specific character of NK cells. Toluidine blue staining describes the presence of metachromasia (cell shape), while peroxidase staining describes the presence of azurophilic granules, which are characterized by a peroxidase reaction due to the presence of the myeloperoxidase enzyme from the exosomes of hepatocellular carcinoma in NK cells (cell physiology). The results of this staining can show the histodynamics of NK cells with NK cells exposed to hepatocellular carcinoma exosomes. In this simple staining, something can and cannot be seen for exosome endocytosis to NK cells. Studies on the staining of toluidine blue and peroxidase in NK cells could not be found.

CONCLUSIONS

In this study, histodynamics of NK cells due to endocytosis of hepatocellular exosomes was not only from histology staining. Flow cytometry showed a change in NK cell receptor phenotype (mainly KIR2D). Furthermore, immunofluorescence revealed the presence of exosomes in the cytoplasm of NK cells in the form of CD81 + -PE positive granules in the background of NK cell nuclei stained with DAPI. Additionally, yellow granules in the cytoplasm of NK cells stained with NKG2A-FITC can be analyzed by toluidine blue histology staining for a decrease in the percentage of metachromasia positive cells and an increase in the percentage of metachromasia negative cells and peroxidase staining with an increase in the percentage of peroxidase positive cells.

CONFLICT OF INTEREST

The author(s) declare no competing interest in this study.

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