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Exosomes in malignant pleural effusion from lung cancer patients impaired the cytotoxicity of double-negative T cells

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A B S T R A C T

$CD3^+CD4^-CD8^-$ double-negative T (DNT) cells are new weapons in cancer immunotherapy. Here, we explored DNT cells in malignant pleural effusions (MPEs) from lung cancer patients. DNT cells, especially $TCR\alpha\beta^+CD56^-$ DNT cells, were increased in MPE from lung cancer patients. DNT cells highly expressed PD-1, TRAIL, NKG2D and DNAM-1. In contrast, FasL was barely detected in DNT cells. Compared with non-MPE cells, MPE-derived DNT cells expressed much higher levels of PD-1 and TRAIL. DNT cells from healthy peripheral blood donors potentially killed lung cancers, which was decreased by MPE supernatant. Exosomes from MPE supernatant expressed PD-1 and CEACAM1 and impaired the cytotoxicity of DNT cells. Blocking PD-1 and TIM3 rescued the cytotoxicity of DNT cells treated with MPE-derived exosomes. Overall, we demonstrated that the frequency of DNT cells in MPE from lung cancer patients was increased and that MPE-derived exosomes impaired the cytotoxicity of DNT cells via the PD-1/PD-L1 and CEACAM1/TIM3 pathways.

Introduction

Lung cancer ranks as the top cause of cancer incidence and mortality [1]. Malignant pleural effusion (MPE) is a common complication in patients with advanced lung cancers. Clinicians observe the predominant subset of T cells in the pleural fluid as a diagnostic indicator of the underlying cause [2]. Among $CD3^+$ cells in pleural effusion, a point of interest is a small subset defined as the expression of CD3 in the absence of CD4 and CD8, which is known as double-negative T (DNT) cells, including $TCR\alpha\beta^+CD56^-$ DNT cells, $TCR\alpha\beta^+CD56^+$ NKT cells and $TCR\gamma\delta^+$ DNT cells [3]. Specifically, DNT cells are defined as T cells expressing αβTCR but lacking coreceptor CD4/CD8 and NK-cell markers, i.e., CD56; these are $TCR\alpha\beta^+CD56^-$ DNT cells [4]. Although few in number, DNT cells are distributed in multiple organs, including the lung and peripheral blood. Recently, DNT cells have been successfully applied in lung cancer immunotherapy [5]. Chimeric antigen receptor DNT (CAR-DNT) cells are effective in infiltration into tumor lung cancers [6]. However, DNT cells in lung cancer MPE remain unexplored.

Exosomes are phospholipid bilayer structure vesicles with a diameter of 40–100 nm. Increasing evidence confirms that MPE-derived exosomes promote the migration, invasion and metastasis of tumor cells by repressing immune effector cells [7]. Proteomic analysis indicates that MPE-derived exosomes express several immune checkpoint molecules [8]. The roles of exosomes on DNT cells have not yet been explored. We hypothesized that lung cancer MPE-derived exosomes suppress the cytotoxicity of DNT cells.

Here, for the first time, we demonstrated that the frequency of DNT cells increased in lung cancer MPE. Moreover, compared with non-MPE DNT cells, lung cancer MPE DNT cells expressed higher levels of PD-1 and TRAIL. Furthermore, we observed that exosomes derived from lung cancer MPE inhibited the antitumor activity of DNT cells. Blocking PD-1 and T-cell immunoglobulin mucin domain-containing protein 3 (TIM3) partially rescued the inhibitory effects of exosomes against DNT cells. Taken together, these data indicated that lung cancer MPE contained potentially malfunctioning DNT cells and that MPE impaired the cytotoxicity of DNT cells through PD-L1- and CEACAM1-enriched

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exosomes. Our work helps close the knowledge gap in DNT immunotherapy against lung cancers.

Materials and methods

Patients

All subjects provided informed consent to participate in the study, which was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University (No. 2020-SR-205). Pleural effusions were collected from 40 patients. According to pleural biopsy and exfoliated cytological pathological examination, subjects were divided into a malignant pleural effusion group (MPE, $n = 24$) and a nonmalignant pleural effusion group (N-MPE, $n = 16$). In the MPE group, 17 cases were adenocarcinoma, 3 cases were squamous cell carcinoma, and 4 cases were small cell lung cancer. Details of the clinical data are shown in Supplementary Table 1. Before the collection of pleural effusion, none of the patients had received antitumor therapy, nor had they used corticosteroids or nonsteroidal anti-inflammatory drugs.

Sample collection and processing

The pleural effusion sample was collected from each patient in a tube pretreated with heparin using standard thoracentesis. The pleural effusion sample was centrifuged at 1200 × g for 5 min. The cell-free supernatant from malignant pleural effusion was immediately frozen and stored at -80 °C after centrifugation for later treatment of DNT cells from peripheral blood of healthy donors. The cell pellet of the pleural effusion was resuspended in PBS, and the mononuclear cells were isolated by density gradient centrifugation to determine the T-cell subsets within 1 h.

Flow cytometry

Flow cytometry was used to determine the T-cell subsets in the pleural effusion. The cell pellet of the pleural effusion was stained with anti-CD3, anti-CD4, and anti-CD8a to analyze T-cell subsets. Three subpopulations of double-negative T cells were analyzed with anti-α/β TCR, anti-γ/δ TCR, and anti-CD56 (NCAM). Molecules related to cytotoxicity function on the membrane of DNT cells were analyzed with anti-CD279 (PD-1), anti-CD178 (FasL), anti-CD314 (NKG2D) and anti-CD226 (DNAM-1). Details of the antibodies used in this study are shown in Supplementary Table 2.

Isolation of double-negative T cells

DNT cells were isolated from the peripheral blood of healthy donors using a double negative T-cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec, cat# 130-092-614). In the first step, $CD4^+$, $CD8^+$, and $CD56^+$ T cells were labeled with a biotin antibody cocktail and antibiotin microspheres. The labeled cells were depleted with an LD column on the autoMACS Separator. In the second step, the flow-through fraction was incubated with anti-TCR α/β-PE and anti-PE microbeads, and then TCR $\alpha/\beta^+CD3^+CD4^-CD8^-$ T cells were enriched with an MS column by positive selection.

Cell lines and cell culture

The lung cancer cell lines A549 and H1975 were obtained from ATCC. SPCA-1 was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. All lung cancer cell lines were maintained in DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (FBS, HyClone) in a humidified incubator with 5% CO_2 at 37 °C.

DNT cells treated with MPE and cytotoxic assay

DNT cells from the peripheral blood of healthy donors were adjusted to a cell density of 1×10^6 cells/ml in a 24-well plate, treated with MPE (volume ratio 20%) for 24 h, washed with phosphate buffered saline (PBS) and resuspended in RPMI-1640 medium supplemented with 10% FBS for cytotoxicity assays and flow cytometry. The cytotoxic activity of DNT cells against lung cancer cells was measured with a cell counting kit-8 (CCK-8) (Yeasen, cat# 40203ES60) according to the manufacturer's instructions. Briefly, cytotoxicity assays of the DNT cells were set to four groups, each group with 3 replicates. One hundred microliters each of lung cancer cell culture medium and DNT cell culture medium were used as the blank group. Lung cancer cells alone and DNT cells alone were used as target cells and effector cells, respectively. Coculture of DNT cells and lung cancer cells was used as the experimental group. In detail, lung cancer cells were placed in a 96-well plate at 5×10^3 cells/well, and then DNT cells were added to the 96-well plate at a specific effector: target (1:1) in a final volume of 200 μl and cocultured for 24 h. In some assays, DNT cells were cocultured with anti-human PD-1 antibody (0.1, 1 μg/ml, R&D Systems, cat# AF1086) for 24 h before seeding into 96-well plates. Then, 20 μl of CCK-8 reagent was added to each well and incubated for 2 h at 37 °C. A_{450} absorbance was detected with a microplate spectrophotometer. The killing rate was calculated according to the following formula: killing rate $(100\%) = \left(1 - \frac{A450(\text{experimentgroup}) - A450(\text{effectorgroup})}{A450(\text{targetgroup}) - A450(\text{blankgroup})}\right) \times 100\%$.

Extraction of exosomes from MPE

Exosomes were isolated from the MPE (400 ml) of patients with lung cancer. The sample was centrifuged at 2000 × g for 10 min and 12,000 × g for 20 min to remove the cells and pellets and then centrifuged at 130,000 × g for 70 min (Ultracentrifuge: 102 (L-100XP)). The supernatant was discarded, the pellet was resuspended in 70 ml PBS, and the resuspension was passed through a 0.22 μm sterile filter and then centrifuged at 130,000 × g for 70 min. The pellet was resuspended in 200 μl PBS and stored at -80 °C.

Transmission electron microscope observation of exosomes

The isolated exosomes were placed on a 200-mesh copper mesh coated with silflurane, negatively stained with uranyl acetate, and observed using a transmission electron microscope (JEOL JEM-1400Flash).

Western blotting analysis

The protein concentration of exosomes was determined using a BCA kit (Beyotime Biotech, cat# P0012S) according to the manufacturer's instructions. Approximately 30 μg of exosome protein was lysed using lysis buffer containing RIPA lysis buffer (Thermo Fisher Scientific, cat# 87788) and protease inhibitor cocktail (Thermo Fisher Scientific, cat# 78430). The lysed protein was electrophoresed on a 12% sodium lauryl sulfate-polyacrylamide gel and then electrotransferred onto a 0.45 μm PVDF membrane. The membrane was blocked with 5% skim milk and then incubated with anti-CD63 (Abcam, cat# ab134045), anti-CD81 (Abcam, cat# ab109201), anti-CEACAM1 (Abcam, cat# ab108397), anti-PD-L1 (Abcam, cat# ab213524) and anti-flotillin-1 (Abcam, cat# 78178) antibodies. The membrane was subsequently incubated with secondary antibody, visualized using a Syngene G: BOX Imaging System, and finally analyzed with ImageJ.

DNT cells cocultured with MPE-derived exosomes

DNT cells were purified from the peripheral blood of healthy donors

using a double negative T-cell isolation kit (2×10^5 cells/well in a 96-well plate) and then incubated with MPE-derived exosomes (0, 5, 15, 25, 50 μg/ml) for 24 h. The treated DNT cells were collected, washed with PBS and cytotoxicity was detected using a CCK-8 assay. For the blocking assay, DNT cells were first preincubated for 24 h with anti-PD-1 (R&D Systems, cat# AF1086) or anti-TIM3 (Genscript, cat# A01834) at a final concentration of 1 μg/ml in the corresponding groups. Then, PD-L1- and CEACAM1-enriched MPE-derived exosomes were added to all the groups to obtain a final concentration of 50 μg/ml. After culturing for 24 h, the cytotoxicity of DNT cells in each group was examined using the CCK-8 method mentioned above.

Statistical analysis

SPSS 25.0 and GraphPad Prism 8 were used for statistical analysis. Comparisons between two groups were assessed by unpaired or paired t tests as indicated in the experiments. Statistical significance was defined as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Results

Increased DNT cells in MPE from patients with lung cancer

Multichannel flow cytometry was used to explore T lymphocyte subsets in MPE and non-MPE (N-MPE) samples. Representative flow cytometry plots showing different T-cell subsets based on their respective markers in MPE and N-MPE are presented in Fig. 1A. The average percentage of $CD3^+CD4^+$ T cells in MPE (58.83 ± 2.97%) was significantly lower than that in N-MPE (68.86 ± 3.71%) (Fig. 1B). In contrast, the proportion of $CD3^+CD8^+$ T cells in MPE (23.86 ± 2.74%) was comparable to that in N-MPE (25.44 ± 3.12%) (Fig. 1C). Correspondingly, the CD4/CD8 ratio in MPE was significantly decreased (Fig. 1D), reflecting that the cellular immune function of patients with MPE was in a suppressed state. In addition, we found that the percentage of $CD3^+CD4^-CD8^-$ DNTs was significantly increased in MPE (5.63 ± 0.67%) relative to the counterpart in N-MPE (3.01 ± 0.36%) (Fig. 1E). To explore the role of DNTs in the differential diagnosis of benign and malignant pleural effusions, we analyzed the correlation between the percentage of DNTs and LDH in MPE (Supplementary Table 1). Notably, the results of correlation analysis showed that the percentage of DNTs in MPE was positively correlated with LDH levels (Fig. 1F). Collectively, DNT cells in MPE from lung cancer patients were increased, which may be potentially involved in lung cancer progression.

Distributions of DNT cell subsets in MPE

DNT cells include three subsets: $TCR\alpha\beta^+CD56^-$ DNT cells, $TCR\alpha\beta^+CD56^+$ NKT cells and $TCR\gamma\delta^+$ DNT cells [3]. Only

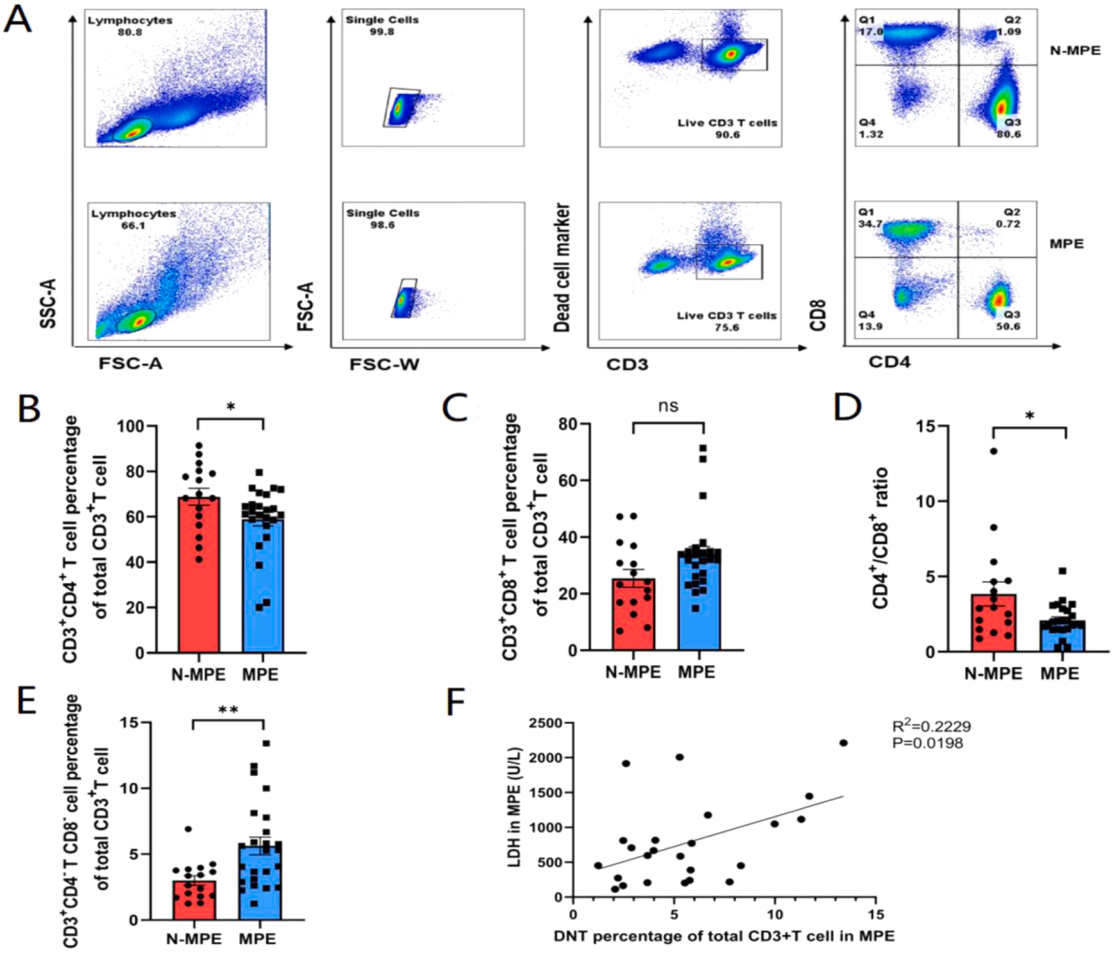

Fig. 1. Increased percentage of DNT cells in MPE from patients with lung cancer. Flow cytometric analysis of T cells from monocytes in N-MPE ($n = 16$) and MPE ($n = 24$). Each point represents data from an individual patient. (A) Representative plots of pleural effusion-derived T-cell subsets by gating on $CD3^+$ cells. (B) The percentage of $CD3^+CD4^+$ T cells in MPE was significantly lower than that in N-MPE. (C) We observed no significant differences in the percentage of CD3+CD8+ T cells in MPE and N-MPE. (D) The $CD3^+CD4^+/CD3^+CD8^+$ ratio in MPE was significantly lower than that in N-MPE. (E) The percentage of $CD3^+CD4^-CD8^-$ T cells in MPE was significantly higher than that in N-MPE (**$P < .01$). (F) The percentage of DNT cells in MPE was positively correlated with LDH levels. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

TCRαβ$^+$CD56$^-$ DNT cells are "true" DNT cells. In contrast, TCRαβ$^+$CD56$^+$ NKT cells are a population of NKT cells expressing αβTCR and CD56 (NK-cell marker) but defective in CD4/CD8. TCRγδ$^+$ DNT cells are a population of γδT cells lacking CD4/CD8. To gain a better understanding of the distribution of DNT cells in MPE, we divided DNT cells into TCRαβ$^+$CD56$^-$ DNT cells, TCRαβ$^+$CD56$^+$ NKT cells and TCRγδ$^+$ DNT cells. Representative flow cytometric dot plots showing the percentages of three DNT cell subsets in MPE and N-MPE are presented in Fig. 2A. As shown in Fig. 2B, C, TCRαβ$^+$CD56$^-$ DNT cells (1.05 ± 0.15%, 2.69 ± 0.49%, P = 0.0062) and TCRαβ$^+$CD56$^+$ NKT cells (0.12 ± 0.023%, 0.39 ± 0.09%, P = 0.0087) in MPE were significantly higher than those in N-MPE. TCRγδ$^+$ DNT cells in MPE were higher than those in N-MPE (1.85 ± 0.29%, 2.67 ± 0.42%, P = 0.1320) (Fig. 2D). The distribution analysis revealed that TCRγδ$^+$ DNT cells predominated in non-MPE; in contrast, TCRαβ$^+$CD56$^-$ DNT cells were major subsets of DNT cells in MPE (Fig. 2E). In short, compared with N-MPE, MPE contained more DNT cells, especially TCRαβ$^+$CD56$^-$ DNT cells.

DNT cells in lung cancer MPE expressed higher levels of PD-1 and mTRAIL

It has been reported that DNTs can utilize different effector molecules to target cancer cells by ligand–receptor binding, such as natural killer group 2 member D (NKG2D), DNAX accessory molecule-1 (DNAM-1), membrane-bound tumor necrosis factor-related apoptosis inducing ligand (mTRAIL), and factor-related apoptosis ligand (FasL) [9]. To explore the cytotoxic activity of DNTs in MPE, we quantified the expression of these molecules. In the MPE group, the frequencies of PD1$^+$ DNTs (26.55 ± 2.67%, 39.42 ± 3.96%, P = 0.0133) and TRAIL$^+$ DNTs (28.67 ± 6.63%, 48.29 ± 4.34%, P = 0.0207) were significantly higher than those in the N-MPE group (Fig. 3A, B). Meanwhile, the frequencies of FasL$^+$ (0.74 ± 0.31%, 3.83 ± 1.57%, P = 0.0659), NKG2D$^+$ (72.89 ± 4.00%, 71.39 ± 6.01%, P = 0.8371) and DNAM-1$^+$ (38.45 ± 3.97%, 42.67 ± 4.76, P = 0.5020) DNT cells (Fig. 2 C–E) were comparable in both the MPE and N-MPE groups. Overall, these results indicated that, with concurrent higher expression of the coinhibitory molecule PD-1 and the costimulatory molecule mTRAIL, lung cancer MPE-derived DNT cells may function aberrantly.

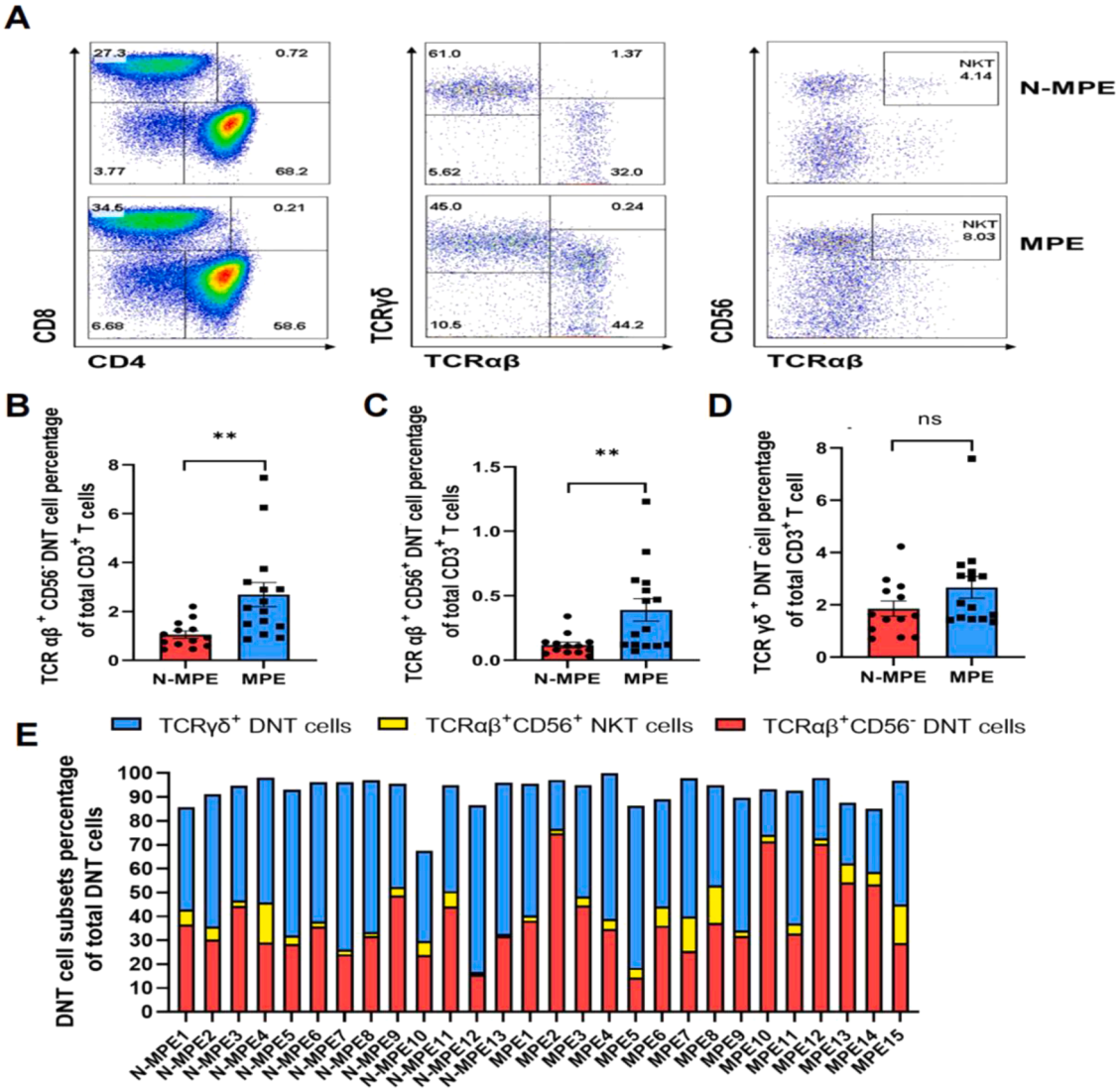

Fig. 2. Increased percentage of each DNT cell subset in MPE from patients with lung cancer. Flow cytometric analysis of DNT cell subsets in N-MPE (n = 13) and MPE (n = 15). Each point represents data from an individual patient. (A) Representative plots of pleural effusion-derived DNT cell subsets by gating on CD3$^+$CD4$^-$CD8$^-$ cells. (B, C) The percentage of TCRαβ$^+$CD56$^-$ DNT cells or TCRαβ$^+$CD56$^+$ NKT cells in MPE was significantly higher than that in N-MPE. (D) We observed no significant differences in the percentage of TCRγδ$^+$ DNT cells in MPE and N-MPE. (E) The distribution of DNT cell subsets in pleural effusions from each patient is presented with a stacked histogram. ** $P < 0.01$; ns, not significant.

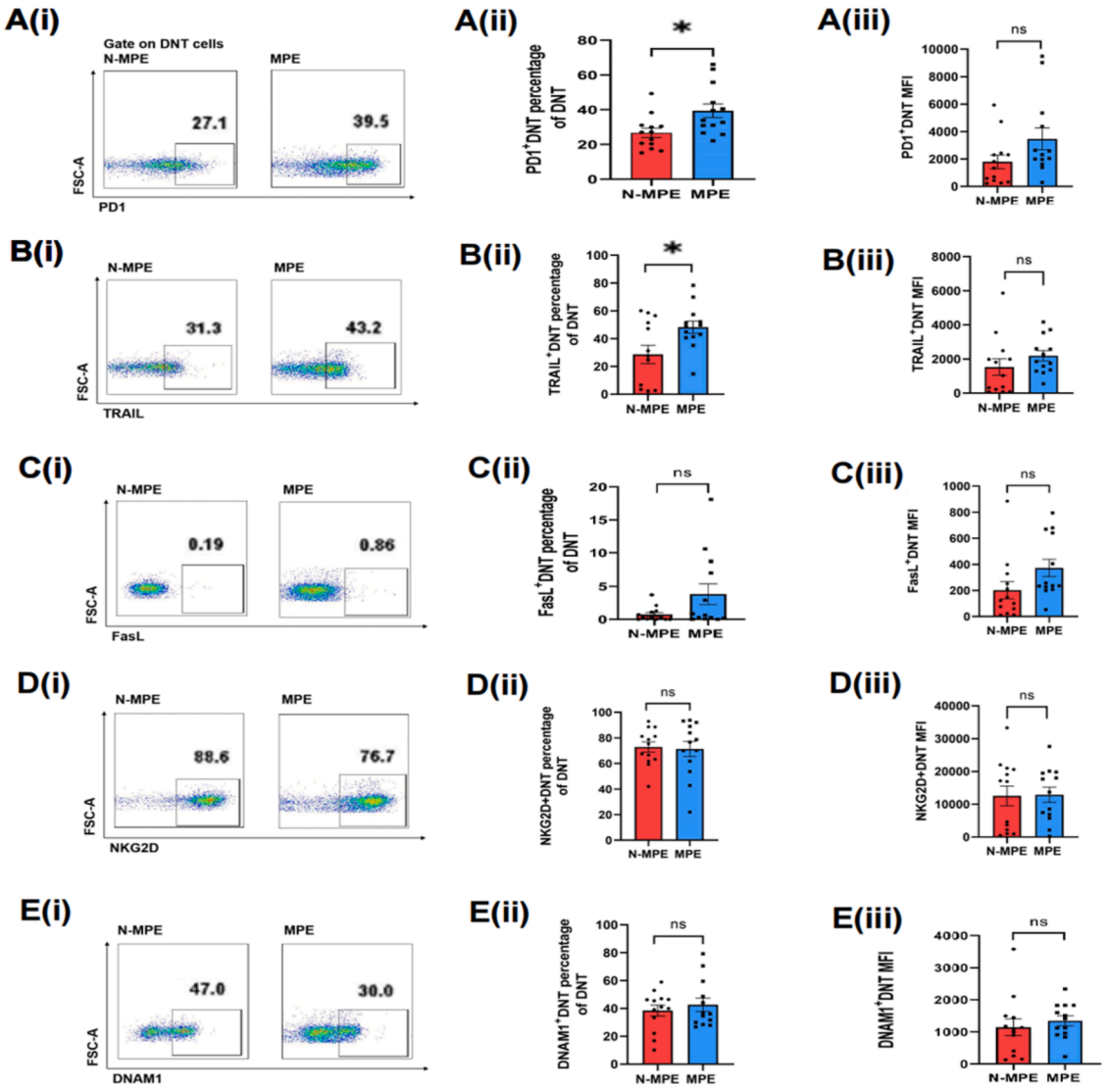

Fig. 3. Functional molecules on DNT cells. Flow cytometric analysis of effector molecules expressed on DNT cells in MPE ($n = 13$) and N-MPE ($n = 13$) by gating on $CD3^+CD4^-CD8^-$ cells. Each point represents data from an individual patient. (A, B) The percentage of PD-1^+DNT cells or $TRAIL^+$DNT cells in MPE was significantly higher than that in N-MPE. (C–E) There was no significant difference in the percentage of $FasL^+$DNT cells, $NKG2D^+$DNT cells or $DNAM1^+$DNT cells or in MPE and N-MPE. No significant difference was observed in the mean fluorescence intensity (MFI) of PD-1, TRAIL, FasL, NKG2D or DNAM1 in DNT cells. *, $P < 0.05$; ns, not significant.

MPE supernatant decreased the cytotoxicity of DNT cells

DNT cells in pleural effusion are very limited in number, which hinders direct exploration of the functions of DNT cells. Previous studies have confirmed that malignant pleural effusion affects the number and function of tumor-infiltrating lymphocytes and plays an important role in tumor immune escape [10]. To investigate the influence of the tumor microenvironment of MPE on the cytotoxicity of DNT cells, DNT cells from peripheral blood (PB) of healthy donors were treated with MPE supernatant (volume ratio 20%) for 24 h. Flow cytometry analysis was used to characterize isolated double-negative T cells (Fig. 4Ai). As shown in Fig. 4Aii, after MPE treatment, the cytotoxic activity of DNT cells from healthy donors was significantly compromised (34.84 ± 1.66%, 23.71 ± 2.22%, $P = 0.009$). Meanwhile, the percentage of PD-1^+ DNTs treated with MPE was notably increased compared with that in the medium alone (7.66 ± 1.23%, 14.50 ± 1.28%, $P = 0.0049$) (Fig. 4B). Furthermore, the cytotoxicity of DNT cells was restored by 1 μg PD-1 neutralization antibody (37.82 ± 5.25%, 55.69 ± 3.10%, $P = 0.0427$) (Fig. 4C). These results suggested that lung cancer MPE supernatant impaired the cytotoxic activity of DNT cells via the PD-L1/PD-1 axis.

MPE-derived exosomes reduced the cytotoxicity of DNT cells

As previously reported, exosomes, which are significantly abundant in MPE supernatant, are potent immunosuppressive factors [10], and we hypothesized that MPE-derived exosomes may potentially impair the cytotoxicity of DNT cells. As expected, we successfully purified exomes from MPE, which were round-shaped membrane vesicles (~100 nm) [11] enriched with CD63, CD81 and Flotillin-1 (Fig. 5A, B). To determine whether MPE-derived exosomes directly contributed to the suppression of the antitumor activity of DNT cells, we treated DNT cells from healthy donors with MPE-derived exosomes. As shown in Fig. 5C–E, MPE-derived exosomes significantly decreased the cytotoxic activity of DNT cells against the lung cancer cell lines A549, H1975 and SPCA-1. In brief, lung cancer MPE-derived exosomes compromised the antitumor activity of DNTs.

MPE-derived exosomes impaired the cytotoxicity of DNT cells via the PD-L1/PD-1 and CEACAM1/TIM3 pathways

Previous studies revealed that MPE-derived exosomes expressed

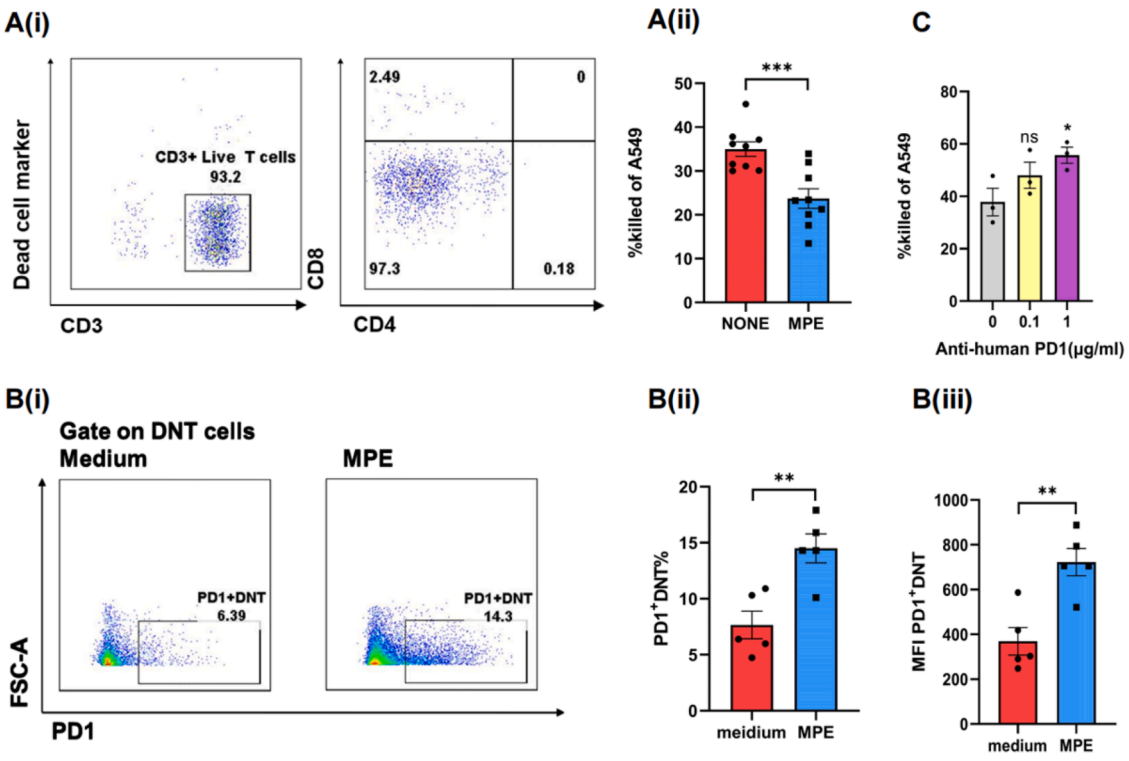

Fig. 4. MPE decreased the cytotoxicity of DNT cells. (A) (i) Flow cytometric analysis of the viability and purity of DNT cells isolated from the peripheral blood of healthy donors using a double negative T-cell isolation kit. (ii) DNT cells were incubated with MPE supernatant (volume ratio 20%) from different patients or medium for 24 h and then cocultured with A549 cells for 24 h at a 1:1 ratio. The cytotoxicity of DNT cells to A549 cells was calculated using the CCK-8 method. The cytotoxicity of DNT cells after incubation with MPE supernatant was significantly decreased ($n = 9$). (B) (i) DNT cells were incubated with MPE supernatant (volume ratio 20%) from different patients or medium for 24 h, and then PD-1 expressed on DNT cells was analyzed by flow cytometry. (ii) The percentage of PD-1$^+$DNT cells after incubation with MPE supernatant was significantly increased ($n = 5$). (iii) The mean fluorescence intensity (MFI) of PD-1$^+$DNT cells after incubation with MPE supernatant was significantly increased ($n = 5$). (C) Anti-human PD-1 antibody (0, 0.1, 1 μg/ml) was administered for 24 h to DNT cells. After treatment, DNT cells were cocultured with A549 cells for 24 h at a 1:1 ratio. The cytotoxicity of DNT cells to A549 cells was calculated using the CCK-8 method. The cytotoxicity of DNT cells was restored by 1 μg/ml anti-human PD1 Ab ($n = 3$). *$P < 05$, **$P < 0.01$***, $P < 0.001$, ns, not significant.

several immune checkpoint molecules, including CEACAM1 [12]. First, we demonstrated that MPE-derived exosomes expressed PD-L1 and CEACAM1 (Fig. 6A). To determine whether PD-L1 and CEACAM1 in MPE-derived exosomes were crucial factors in the inhibition of the cytotoxicity of DNT cells, we used neutralizing antibodies (anti-PD-1 and anti-TIM3) to block the PD-L1 and CEACAM1 signaling pathways. As expected, anti-PD-1 and anti-TIM3 antibodies restored the cytotoxicity of DNT cells stimulated with MPE-derived exosomes (Fig. 6B). Taken together, we found that MPE-derived exosomes impaired the cytotoxicity of DNT cells at least partially through the PD-L1/PD-1 and CEACAM1/TIM3 pathways.

Discussion

Immunotherapy has radically changed the landscape of lung cancer treatments [13]. Adoptive cell transfer, including dendritic cells (DC) [14], natural killer (NK) cells [15], and γδ T cells [16], to lung cancer patients is well tolerated. As NK and γδ T cells, DNT cells are non-MHC-restricted cytotoxic cells that have emerged as new players in lung cancer immunotherapy [5]. However, the efficiency of adoptive cell transfer remains controversial in lung cancer treatments [17]. One plausible explanation is that immune cells, including DNT cells, may play dual roles in the tumor microenvironment. On one hand, DNT cells directly kill tumor cells [18]. On the other hand, DNT cells suppress CD8$^+$ cytotoxic T cells and promote immune tolerance [19], which may be beneficial for tumor survival. Herein, we demonstrate that increased DNT cells in lung cancer MPE expressed higher levels of the costimulatory molecule mTRAIL and the coinhibitory molecule PD1, deliberately reflecting the complicated biological functions of DNT cells. Moreover, MPE-derived exosomes decreased the cytotoxicity of DNT cells, suggesting that the tumor killing ability of DNT cells may be influenced by the immune microenvironment. Blocking the PD-1/PD-L1 and CEACAM1/TIM-3 pathways partially reversed the inhibitory effects of MPE-derived exosomes, implying that adoptive DNT cell immunotherapy should be combined with checkpoint therapy in lung cancer treatments.

Compared with that in the benign pleural effusion, the percentage of DNT cells in the MPE from lung cancer patients was increased. DNT cells comprised three subsets: TCRαβ$^+$CD56$^-$ DNT cells, TCRαβ$^+$CD56$^+$ NKT cells and TCRγδ$^+$ DNT cells [3]. In the present study, all three DNT subsets in lung cancer MPE were increased. These increased DNT cells in MPE were in accordance with the increased frequency of DNT cells in blood from NSCLC patients [20]. In contrast, some investigators reported that the frequency of DNT cells was decreased in lung cancer tissues and in the peripheral blood of NSCLC patients [21]. In addition, we found that the increase in DNT cells in MPE was significantly positively related to LDH, a disease progression marker of lung cancer [22]. The decrease in DNT cells was associated with improvements in melanoma patients [23]. Therefore, we inferred that the increased frequency of DNT cells in MPE may be a sign of tumor exacerbation.

The origin of DNT cells in pleural effusion is largely unknown. Studies have suggested that DNT cells may originate from the thymus by escaping negative selection and then migrating around the periphery to amplify when they experience antigen stimulation [24]. On the other hand, data have shown that DNT cells are produced in the periphery rather than in the thymus [25]. CD3$^+$ CD4$^+$ CD8$^+$ T thymocytes could downregulate the expression of CD4 and CD8 after being stimulated by high-affinity antigen, thereby generating a large number of DNT cells

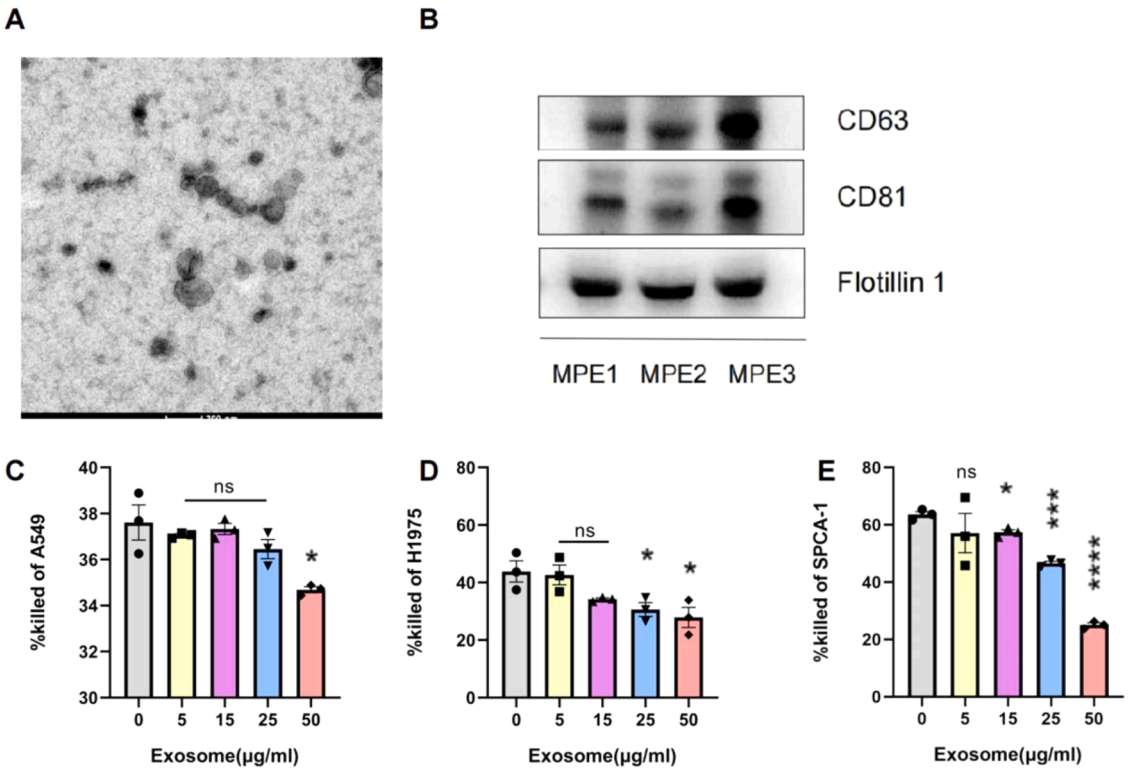

Fig. 5. MPE exosomes decreased the cytotoxicity of DNT cells. (A) MPE-derived exosomes were detected by transmission electron microscopy. (B) The surface markers (CD63 and CD81) expressed on MPE-derived exosomes were confirmed by Western blot. (C–E) MPE-derived exosomes (0, 5, 15, 25, 50 μg/ml) were added to DNT cell cultures. Twenty-four hours post-treatment, DNT cells were used to kill lung cancer cells for 24 h in a 1:1 ratio. The cytotoxicity of DNT cells to lung cancer cells was calculated using the CCK-8 method. The cytotoxicity of DNT cells to A549 cells, H1975 cells or SPCA-1 cells was decreased ($n = 3$). *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

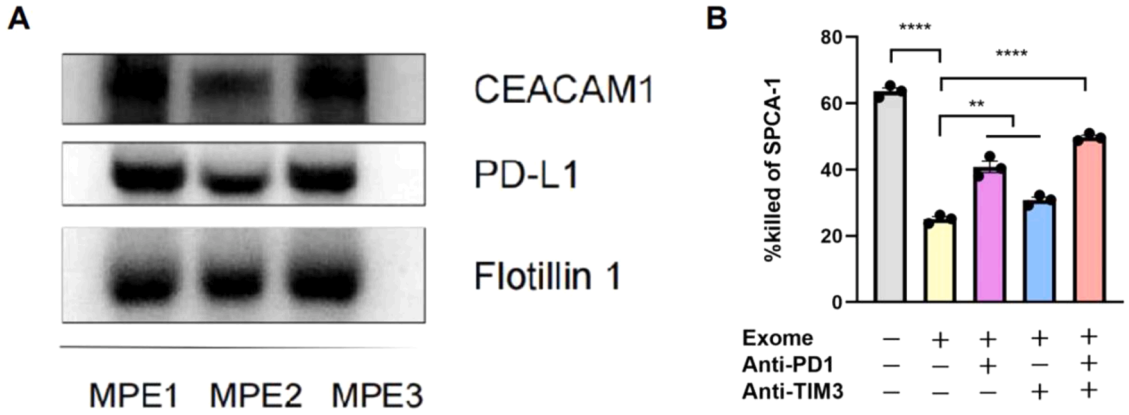

Fig. 6. MPE-derived exosomes impaired the cytotoxicity of DNT cells via the PD-L1/PD-1 and CEACAM1/TIM3 pathways. (A) PD-L1 and CEACAM1 expression on MPE-derived exosomes was confirmed by Western blot. (B) Anti-PD1 or anti-TIM3 was added to the DNT cell culture system, which was supplied with PD-L1- and CEACAM1-enriched MPE-derived exosomes. After culturing for 24 h, the cytotoxicity of DNT cells in each group was examined using the CCK-8 method. The impaired cytotoxicity of DNT cells after incubation with $PDL1^{+}CEACAM1^{+}$ MPE-derived exosomes was restored by anti-human PD1 Ab or anti-human TIM3 Ab ($n = 3$). **$P < 0.01$, ****$P < 0.0001$.

[26]. In addition, some evidence indicates that DNT cells may be directly derived from $CD8^{+}$ T cells due to the DNA methylation of CD8 [27]. Some scholars have put forward different views that DNT cells may be derived from $CD4^{+}$ T cells rather than $CD8^{+}$ T cells [28]. We could not preclude the possibility that increased DNT cells in lung cancer MPE may have different origins.

DNT cells show antitumor activity with various molecules. For example, FasL on DNT cells mediates the apoptosis of pancreatic cancer cells [29]. NKG2D and DNAM-1, expressed by DNT cells, are required for cytotoxicity against acute myeloid leukemia (AML) cells [30]. The combination of NKG2D, DNAM-1 and mTARIL initiates the attack of DNT cells on lung cancer cells [31]. In contrast, PD-1 negatively modulates the killing potential of DNT cells [21]. In the present study, we observed that DNT cells barely expressed FasL. Meanwhile, NKG2D, DNAM-1, mTARIL and the checkpoint molecule PD-1 were highly expressed by DNT cells. Moreover, DNT cells in lung cancer MPE expressed much higher TRAIL. Considering that DNT cells from lung cancer patients or healthy controls were similar in cytotoxicity against lung cancer cells [32], we speculated that TRAIL and PD-1 may balance the killing potentials of DNT cells.

Extracellular vehicles (EVs), including exosomes, microvesicles, and apoptotic bodies, range from 30 nm to 4 μm in size. Either malignant pleural mesothelioma or benign reactive mesothelial proliferations produced pleural effusion, which contained exosomes. The proportions of several angiogenesis proteins (i.e., galectin-1, mesothelin, osteopontin, and VEGF) were higher, whereas angiopoietin-1 was lower in exosomes in malignant samples than in benign samples. These angiogenesis proteins may regulate tumor metastasis [8]. Similar to the present study, pleural elusions from patients with pulmonary tuberculosis (TPE) or with lung cancer (MPE) were analyzed; metabolomics and lipidomics analysis revealed that exosomes from MPE and TPE were different in phenylalanine, leucine and some other metabolites [33]. An increasing number of studies have demonstrated that MPE-derived exosomes can affect the number and functions of immune cells [34]. In particular, MPE-derived exosomes contributed to the maintenance of Treg numbers and suppressive functions [10]. Exosomes from cancer cells also modulate the function of $CD8^+$ T cells [35], NK cells [36], dendritic cells [37] and neutrophils [38]. For the first time, we demonstrated that MPE-derived exosomes decreased the antitumor activity of DNT cells. We further clarified that PD-L1 and CEACAM1 on MPE-derived exosomes were involved in the inhibition of the killing function of DNT cells. PD-1/PD-L1 checkpoint therapy combined with adoptive DNT cell transfer effectively killed lung cancer cells [21]. CEACAM1 is a promising candidate in lung cancer therapy [39]. Indeed, CEACAM1 blocking further enhanced the PD-1 efficiency in lung cancer cells [40]. The combined therapy of CEACAM1/PD-1 blockade and adoptive DNT cell transfer in lung cancers warrants further research. However, DNT cells in blood were decreased in melanoma patients responsive to checkpoint therapy [23], suggesting that the fluctuation of DNT cells should be monitored in the combined therapy of CEACAM1/PD-1 blockade against lung cancer patients.

In summary, we demonstrate that the frequency of DNT cells was increased in MPE lung cancer patients. DNT cells expressed PD-1, TRAIL, NKG2D and DNAM-1 and barely expressed FasL. The elevated expression of PD-1 and TRAIL may balance the cytotoxicity of DNT cells from lung cancer patients with MPE. In addition, exosomes from lung cancer MPE inhibited the antitumor potential of DNT cells, which was dependent on the PD-1/PD-L1 and TIM3/CEACAM1 pathways. Recently, a nanoparticle effective to eradicate oncogenic exosomes was reported [41]. Therapeutically, our findings shed light on the indispensable combination of DNT cell transfer, exosome targeting and checkpoint molecule blockade in lung cancer therapy.

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Declaration of Competing Interest

The authors declare that they have no competing financial interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101564.

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