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Influence of Canine Macrophage-derived Extracellular Vesicles on Apoptosis in Canine Melanoma and Osteosarcoma Cell Lines

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Abstract. *Background/Aim: The purpose of this study was to evaluate the effect of extracellular vesicles derived from canine M1-polarized macrophages (M1EVs) on canine tumor cells, such as D17 (osteosarcoma cells) and LMeC (melanoma cells). Materials and Methods: Protein expression was determined by western blot analysis. Gene expression was determined by RT-qPCR. In addition, cell apoptosis was analyzed by Annexin V/PI staining. Results: In the case of M1EV, the levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β were increased, and nitrate/nitrite levels were also increased. M1EV induced apoptosis of tumor cells by increasing caspase-3 and caspase-7 activation. In addition, M1EVs decreased expression of CCR4, Foxp3 and CTLA-4 in canine peripheral mononuclear cells cocultured with tumor cells. Conclusion: M1EV could be an effective anti-cancer therapeutic approach in melanoma and osteosarcoma and M1EVs can be used as immunomodulators in the tumor microenvironment for cancer treatment.*

Cancer is a leading cause of death in companion animals, with mortality rates of approximately 15~30% in dogs (1). Cancers can develop in various dog organs, including the bone (osteosarcoma) and skin/epithelial tissues (melanoma) (2). Osteosarcoma is one of the most malignant tumors affecting the bones in dogs. Appendicular osteosarcoma is the most common form of osteosarcoma in dogs, and accounts for up to 75~85% of all osteosarcoma lesions. This type of cancer is highly aggressive and metastatic, and pulmonary metastasis is common (3). Various modalities have been used to treat osteosarcoma in dogs, including surgical removal, chemotherapy, and radiation therapy, and the median survival time is approximately 103~175 days with surgery and 235~366 days when surgery is combined with chemotherapy (4). Similarly, melanoma is one of the most malignant tumors in dogs, which usually develops in the oral cavity and acral sites (5). Clinical management of melanoma includes surgical resection, radiation therapy, and chemotherapy; however, because of the highly aggressive and metastatic biological behavior of this tumor, controlling the disease using these therapies remains a challenge (6).

The tumor microenvironment (TME) is a complex and heterogenous collection of tissues that comprises not only neoplastic cells, but also other diverse immune cells, such as lymphocytes, macrophages, and neutrophils, and this tumor-immune cell cross-talk results in a rich milieu of cytokines and growth factors that play an important role in tumor progression, advancement of inflammation, and angiogenesis (7). Among these immune cells, tumor-associated macrophages (TAMs) represent the major component. These macrophages are mainly classified in two polarization types: 1) the classically activated type 1 macrophage (M1), which produces high levels of proinflammatory cytokines and shows enhanced phagocytosis, and 2) the alternatively activated type 2 macrophage (M2), which facilitates the resolution of inflammation and promotes tissue repair and angiogenesis (8). Many studies have confirmed that TAMs are mainly polarized in the TME toward an M2-like phenotype and that this provides the basis for their ability to promote the growth and vascularization of tumors, resulting in tumor migration and metastasis (9). In addition, some evidence suggests that tumors

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with abundant TAMs respond comparatively poorly to cytotoxic therapies (10). In other words, the TME can enhance chemoresistance and increase metastatic spread (11).

Accordingly, various anticancer immunotherapies targeting the TME have attracted attention as novel therapeutic options (9). Previous studies have demonstrated the anticancer effects of extracellular vesicles (EVs) derived from M1 macrophages (12, 13). EVs are nanosized membrane vesicles released by cells into the extracellular space; they contain various functional molecules such as lipids, nucleic acids, and proteins. They play a role in cell-to-cell communication, cell maintenance, and immune response stimulation (14, 15).

Therefore, this study aimed to evaluate the antitumor effect on EVs derived from macrophages polarized with LPS and IFN-γ. We also examined the immunomodulatory effect of EVs on immune cells surrounded by tumor cells, which may induce an environment supportive of anticancer therapy (16). These novel findings contribute to the understanding of the interaction between EVs and their tumor microenvironment.

Materials and Methods

Cell culture.The canine macrophage cell line DH82 was purchased from ATCC (ATCC number: CRL-10389, Manassas, VA, USA). DH82 cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM, PAN Biotech, Aidenbach, Germany) supplemented with 15% FBS (PAN Biotech) and 1% penicillin-streptomycin (P/S) at 37˚C in a humidified atmosphere with 5% CO_2. The canine osteosarcoma cell line D17 (ATCC, ATCC number: CCL-183) was cultured in DMEM supplemented with 10% FBS and 1% P/S at 37˚C in a 5% CO_2 incubator. The canine melanoma cell line LMeC was incubated with Roswell Park Memorial Institute-1640 medium (PAN Biotech) supplemented with 10% FBS and 1% P/S. The media were replaced every 3 days. When the cells reached about 80~90% confluence, subculture was conducted.

Isolation of canine peripheral blood mononuclear cells (cPBMCs). cPBMCs were collected using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Uppsala, Sweden) according to the manufacturer's instructions and the protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-191017-7). Briefly, whole blood samples were collected from healthy beagle dogs kept at the Veterinary Medical Teaching Hospital of Seoul National University. The blood samples were mixed with an equal volume of PBS and placed on Ficoll-Paque PLUS (GE Healthcare Life Sciences). The cloudy cell layer was collected after centrifugation at 750 × *g* for 30 min. RBC lysis buffer was added to reduce residual RBCs, which were then washed with PBS and resuspended in DMEM supplemented with 15% FBS and 1% P/S.

Stimulation of M1 polarization with LPS and IFN-γ. To stimulate DH82 cells with LPS (Sigma-Aldrich, St. Louis, MO, USA) and IFN-γ, the cells were plated at a density of 1×10^5 cells/ml in 6-well plates and incubated for 24 h. The supernatant was discarded and 10 ng/ml LPS, 50 ng/ml IFN-γ, and the combination of 10 ng/ml LPS and 50 ng/ml IFN-γ were added. The cells were cultured another 24 h for stimulation. To examine cellular morphology, images of randomly selected cells at 200× magnification were acquired using a microscope (Olympus CK2 Inverted Microscope, Olympus, Center Valley, PA, USA).

Isolation and characterization of canine macrophage-derived extracellular vesicles (M-EVs). DH82 cells were seeded in 6-well plates at a density of 1×105 cells/ml and stimulated with LPS, IFN-γ, and LPS and IFN-γ, as described previously. After 24 h, the supernatant was discarded, and cells were refed with DMEM supplemented with 10% exosome depleted FBS and 1% P/S. After 2 days, the cell culture supernatant was collected and centrifuged at 300 × *g* for 10 min to remove the cells. After centrifugation, the supernatant was transferred to another tube and centrifuged at 2,000 × *g* for 30 min to remove cellular debris. Thereafter, the supernatant was mixed with ExoQuick-CG Exosome Precipitation Solution (System Biosciences, Mountain View, CA, USA) according to the manufacturer's instructions. After isolation, a bicinchoninic acid (BCA) protein assay was performed for quantifying M-EVs. To determine whether the isolated vesicles were EV, a dynamic light scattering analysis was conducted for measuring the size of EVs. The shapes of the EVs were determined using transmission electron microscopy. The protein expression of CD63, CD81, and β-actin was assessed using western blot analysis.

Cell viability assay. To confirm that the concentrations of LPS and IFN-γ were not cytotoxic to DH82 cells, cell viability was examined using the Cell Counting Kit-8 (CCK-8) assay (Donginbio, Seoul, Korea). The cells were seeded at a density of 1×10^4 cells/well in a 96-well plate. After 24 h, the medium was replaced with control medium, which didn't contain LPS and IFN-γ (10 ng/ml LPS, 50 ng/ml IFN-γ). We performed the CCK-8 assay after stimulating the cells for 24 h according to the manufacturer's instruction. Moreover, to examine the antitumor effect of the M-EVs derived from the DH82, D17, and LMeC cell lines, the M-EVs were plated at a density of 1×10^4 cells/well in a 96-well plate. After 24 h, the medium was replaced with new medium containing 100 μg/ml M-EVs isolated from the naïve DH82 cell line (naïve M-EV) or M-EVs isolated from DH82 cell lines treated with LPS and IFN-γ. The CCK-8 assay was conducted after stimulating the cells for 24, 48, and 72 h.

Each tumor cell line received three different treatments: 1) D17 cell line: D17 treated without M-EVs, D17 treated with naïve M-EVs, D17 treated with M-EVs stimulated with LPS, D17 treated with M-EVs treated with IFN-γ, and D17 treated with M-EVs stimulated with both LPS and IFN-γ; and 2) LMeC cell line: LMeC treated without M-EVs, LMeC treated with naïve M-EVs, LMeC treated with M-EVs stimulated with LPS, LMeC treated with M-EVs stimulated with IFN-γ, and LMeC treated with M-EVs stimulated with both LPS and IFN-γ.

RNA extraction, cDNA synthesis, and real-time quantitative PCR (RT-qPCR). The Easy-BLUE Total RNA Extraction kit (Intron Biotechnology, Seongnam, Korea) was used to isolate RNA according to the manufacturer's instructions. For each sample, total RNA concentration was measured at 260-nm. cDNA was synthesized using CellScript All-in-One 5× 1st cDNA Strand Synthesis Master Mix (CellSafe, Seoul, Korea), and the samples were detected using AMPIGENE qPCR Green Mix Hi-ROX with SYBR Green dye (Enzo Life Sciences, Farmingdale, NY, USA) and forward and reverse primers (Cosmo Genetech, Seoul, Republic of Korea). The expression levels of each gene were normalized to that

Table I. *Sequences of PCR primers*.

Target gene	Primer	Sequence	Reference
Canine iNOS	Forward	GAG ATC AAT GTC GCT GTA CTC C	(48)
	Reverse	TGA TGG TCA CAT TTT GCT TCT G	
Canine arginase	Forward	CAG AAG AAT GGA AGA GTC AG	(48)
	Reverse	CAG ATA TGC AGG GAG TCA CC	
Canine TNF-α	Forward	TCA TCT TCT CGA ACC CCA AG	(48)
	Reverse	ACC CAT CTG ACG GCA CTA TC	
Canine IL-6	Forward	ATG ATC CAC TTC AAA TAG TCT ACC	(48)
	Reverse	AGA TGT AGG TTA TTT TCT GCC AGT G	
Canine IL-1β	Forward	AGT TGC AAG TCT CCC ACC AG	(48)
	Reverse	TAT CCG CAT CTG TTT TGC AG	
CCR4	Forward	CGA GCG CAA CCA TAC CTA CT	(49)
	Reverse	CGG CAA AGA CCA TCC TCA CT	
Foxp3	Forward	AAA CAG CAC ATT CCC AGA GTT C	(48)
	Reverse	AGG ATG GCC CAG CGG ATC AG	
CTLA-4	Forward	TTC TCC AAA GGG ATG CAT GT	(50)
	Reverse	TCA CAT TCT GGC TCA GTT GG	
Canine GAPDH	Forward	AGT ATG TCG TGG AGT CTA CTG GTG T	(48)
	Reverse	AGT GAG TTG TCA TAT TTC TCG TGG T	

of glyceraldehyde 3-phosphate dehydrogenase. The primer sequences used in this study are listed in Table I.

Coculture of cPBMCs with D17 and LMeC cell lines. Twenty-four-well transwell plates (SPL Life Sciences, Pocheon-Si, Republic of Korea) were used to mimic the TME in vitro. The tumor cell lines, D17 or LMeC, were plated onto a 0.4-μm-pore size insert at a density of 1×10^5 cells/ml, and 2×10^7 cPBMCs were seeded at the bottom. The total number of wells were divided into 4 groups: cPBMCs at the bottom and no insert, cPBMCs and tumor cells in the upper chamber, cPBMCs containing 100 μg/ml naïve M-EVs and tumor cells in the upper chamber, and cPBMCs containing 100 μg/ml M-EVs stimulated with both LPS and IFN-γ and tumor cells in the upper chamber. All plates were incubated for 48 h, and the cPBMCs were harvested for further study.

Griess assay. The levels of nitric oxide (NO) in M-EVs were assessed indirectly by measuring nitrite and nitrate concentrations (17). Quantification of nitrate and nitrite was performed using the Nitric Oxide (NO_2/NO_3) Detection kit (Enzo Life Sciences) according to the manufacturer's instructions. Briefly, M-EVs from each group were quantified using the BCA protein assay, and 100 μg of M-EVs were treated with the Griess reagent for 10 min at room temperature. The concentrations of nitrite and nitrite were measured at 540-nm absorbance.

Flow cytometry analysis. The DH82 cells were stimulated with LPS and IFN-γ as described above, and then labeled with mouse CD11c antibody (eBioscience, San Diego, CA, USA) to evaluate the polarization of M1 macrophages by using the FACS Aria II system (BD Biosciences, Franklin Lakes, NJ, USA). In addition, after treating the tumor cell lines with M-EVs, the D17 or LMeC cells were stained with FITC-conjugated annexin-V (Enzo Life Sciences) and propidium iodide (Enzo Life Sciences) according to the manufacturer's recommendations. Flow cytometry analysis was conducted for detecting apoptosis. All data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Western blot analysis. Total proteins were extracted from the DH82, D17, and LMeC cells by utilizing PRO-PREP Protein Extraction Solution (Intron Biotechnology) according to the manufacturer's recommendations. The proteins were quantified using Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) and the samples were separated using SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked and exposed to primary antibodies against TNF-α (Santa Cruz Biotechnology, Dallas, TX, USA), IL-6 (Santa Cruz Biotechnology), IL-1β (Santa Cruz Biotechnology), CD63 (Santa Cruz Biotechnology), Caspase-3 (Santa Cruz Biotechnology), Caspase-7 (Santa Cruz Biotechnology), and CD81 (Santa Cruz Biotechnology) overnight at 4˚C. After incubation, the membranes were washed and incubated with suitable secondary antibodies at room temperature for 1 h. The immunoreactive bands were detected via chemiluminescence (Advansta, Menlo Park, CA, USA) and normalized to β-actin levels (Santa Cruz Biotechnology).

Statistical analysis. Each experiment was performed at least three times. One-way analysis of variance was conducted, followed by the Bonferroni multiple comparison test, using GraphPad Prism v.6.01 software (GraphPad Software, La Jolla, CA, USA), and the data were expressed as mean±standard deviation. p-Values <0.05 were considered statistically significant.

Results

LPS and IFN-γ promote M1 macrophage polarization. To evaluate whether the concentrations of LPS (10 ng/ml) and IFN-γ (50 ng/ml) were cytotoxic, we conducted the CCK-8 assay, and found no significant difference between the

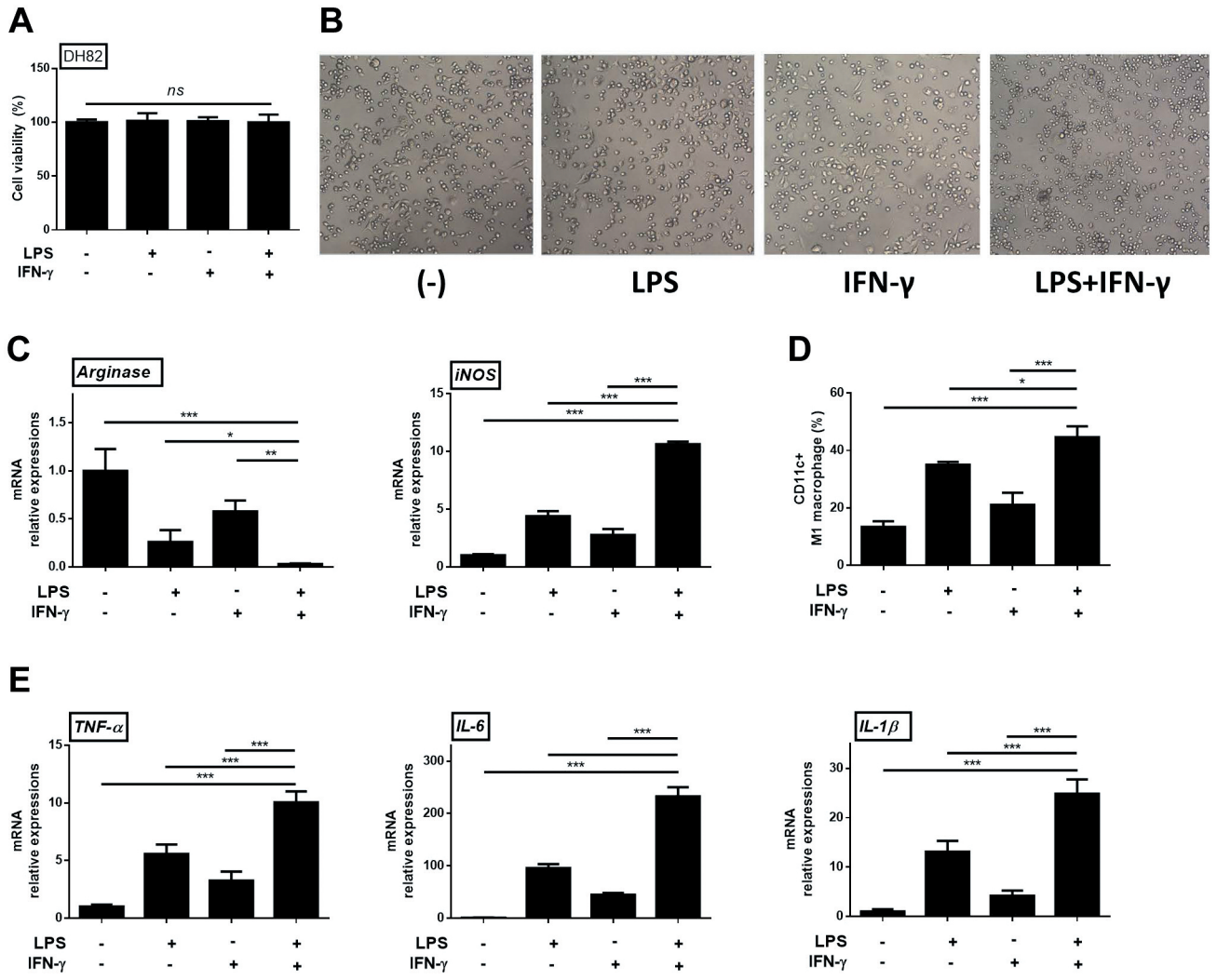

Figure 1. *LPS and IFN-γ promote type 1 macrophage (M1) polarization in canine macrophages. A. Cell viability assay using CCK-8 assay. There were no significant differences between the different groups B. Morphology of macrophage stimulated with LPS and IFN-γ. When stimulated with LPS and IFN-γ, DH82 cells showed more roundish morphology. C. mRNA expression levels of M1 (iNOS) and M2 (Arginase) markers. There was significant increase in iNOS expression levels and significant decrease in mRNA expression levels of Arginase. D. Percentage of CD11c+ cells in LPS/ IFN-γ stimulated DH82 cells. There was significant increase of polarization to M1 of cells stimulated with LPS, IFN-γ or both LPS and IFN-γ compared to control group. E. mRNA expression levels of proinflammatory cytokines in canine macrophages. There was significant increase of TNF-α, IL-6 and IL-1β levels in the presence of LPS, IFN-γ or both LPS and IFN-γ compared to control group. Results are shown as mean±standard deviation of triplicate samples of three independent experiments. *p<0.05, **p<0.01, ***p<0.001. ns: Not significant, as determined by one-way ANOVA.*

control and experimental groups (Figure 1A). After stimulation with LPS and IFN-γ, the morphology of DH82 cells became more roundish than that of the control group (Figure 1B). The polarization of the DH82 cells was evaluated using RT-qPCR and flow cytometry. The levels of inducible nitric oxide synthase (iNOS) were higher in the experimental groups than in the control group. Among the treated cells, the LPS group showed higher levels of iNOS than did the IFN-γ group. Moreover, iNOS levels showed the greatest increase when the cells were treated with both LPS and IFN-γ. In contrast, the levels of arginase were decreased to a greater extent in the cells treated with LPS and IFN-γ than in the control group. The levels of arginase were more highly reduced in the LPS group than in the IFN-γ group. Moreover, the reduction was greater when LPS and IFN-γ were used together (Figure 1C).

We conducted flow cytometry analysis to identify the phenotype of the DH82 cells. We found that the expression of

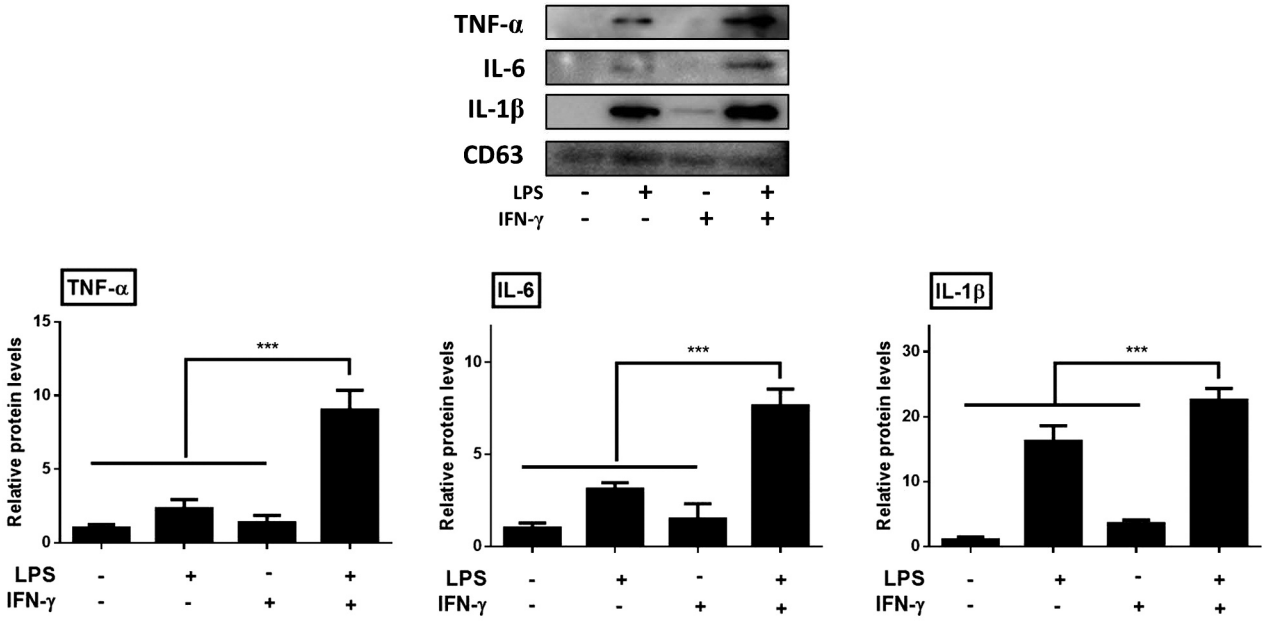

Figure 2. *Expression of proinflammatory cytokines in macrophage derived extracellular vesicles (M-EVs). There was a significant increase of TNF-α, IL-6 and IL-1β in the group stimulated with both LPS and IFN-γ than the other groups. Results are shown as mean±standard deviation of triplicate samples of three independent experiments. ***p<0.001, as determined by one-way ANOVA.*

CD11c was higher when cells were treated with LPS and IFN-γ, and that the levels of CD11c were much higher in the LPS group than in the IFN-γ group. The levels of CD11c were the highest when the cells were treated with both LPS and IFN-γ (Figure 1D). After stimulating DH82 cells with LPS and IFN-γ, we performed RT-qPCR to identify the levels of proinflammatory cytokines, such as *TNF-α*, *IL-6*, and *IL-1β*, and found that their levels were significantly higher in the treated groups than in the control group. Stimulation with both LPS and IFN-γ showed the highest levels of mRNA expression of inflammatory cytokines (Figure 1E).

EVs derived from macrophages stimulated with LPS and IFN-γ increase the expression of inflammatory cytokines
After isolating EVs, we performed western blot analysis to measure the levels of inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, in the EVs. Although no significant difference was observed among the control group, the group treated with LPS, and the group treated with IFN-γ, significantly higher protein levels were observed in the group treated with both LPS and IFN-γ than in any other group (Figure 2).

Concentrations of nitrite and nitrate increase in M-EVs derived from M1-polarized macrophages. Compared with the other M-EVs, those treated with both LPS and IFN-γ showed significantly higher nitrate, nitrite, as well as both nitrate and nitrite levels (Figure 3).

M-EVs have a cytotoxic effect on osteosarcoma and melanoma cell lines. To determine whether the M-EVs derived from the DH82 cell line had anticancer effects, we performed the CCK-8 assay at 24, 48, and 72 h after treating the D17 and LMeC cells with 100 μg/ml of M-EVs. At 24 h after treatment, no significant differences were observed among all groups of LMeC cells. However, after 48 h, cell viability was significantly lower in the group cocultured with M-EVs stimulated with both LPS and IFN-γ than in the other groups. After 72 h, although no significant difference was observed between the control group and the group cocultured with naïve M-EVs, a significant decrease in cell viability was observed in the other groups (Figure 4A). At 24 h after treatment, the D17 cell lines showed no significant difference between the control group and the group cocultured with naïve M-EVs, but the other groups showed a significant decrease in cell viability. Similar results were observed at 48 and 72 h, but at 72 h, cell viability tended to decrease more when the cells were cocultured with M-EVs stimulated with both LPS and IFN-γ (Figure 4B).

M-EVs upregulate the protein levels of caspase-3 and caspase-7 in the osteosarcoma and melanoma cell lines. We demonstrated that M-EVs had a cytotoxic effect on D17 and LMeC cells by using the CCK-8 assay. Additionally, we conducted western blot analysis to evaluate whether M-EVs induced apoptosis in D17 and LMeC cells by detecting

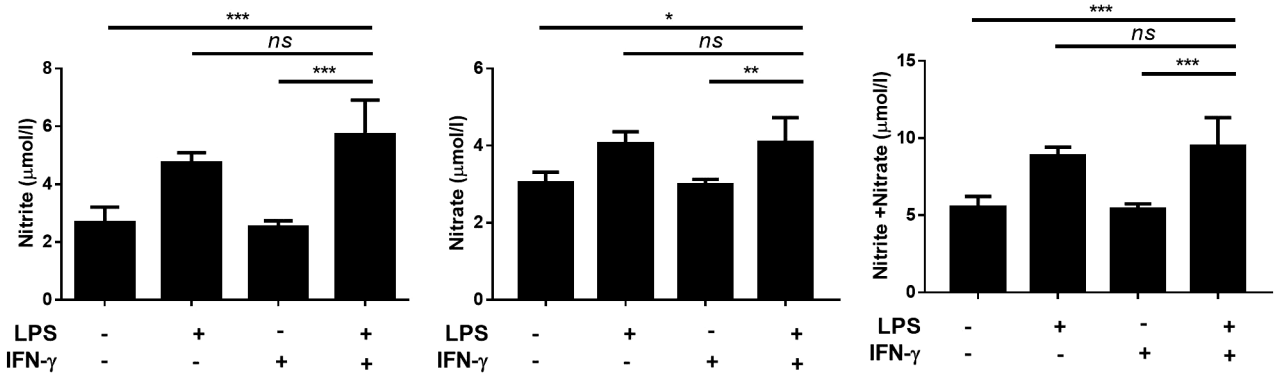

Figure 3. *Nitrate and nitrite concentration in M-EVs. The concentration of nitrate and nitrite was measured in M-EVs. The nitrate and nitrite levels were significantly increased in M-EVs treated with both LPS and IFN-γ compared with the other groups. Results are shown as mean±standard deviation of triplicate samples of three independent experiments. *p<0.05, **p<0.01, ***p<0.001. ns: Not significant, as determined by one-way ANOVA.*

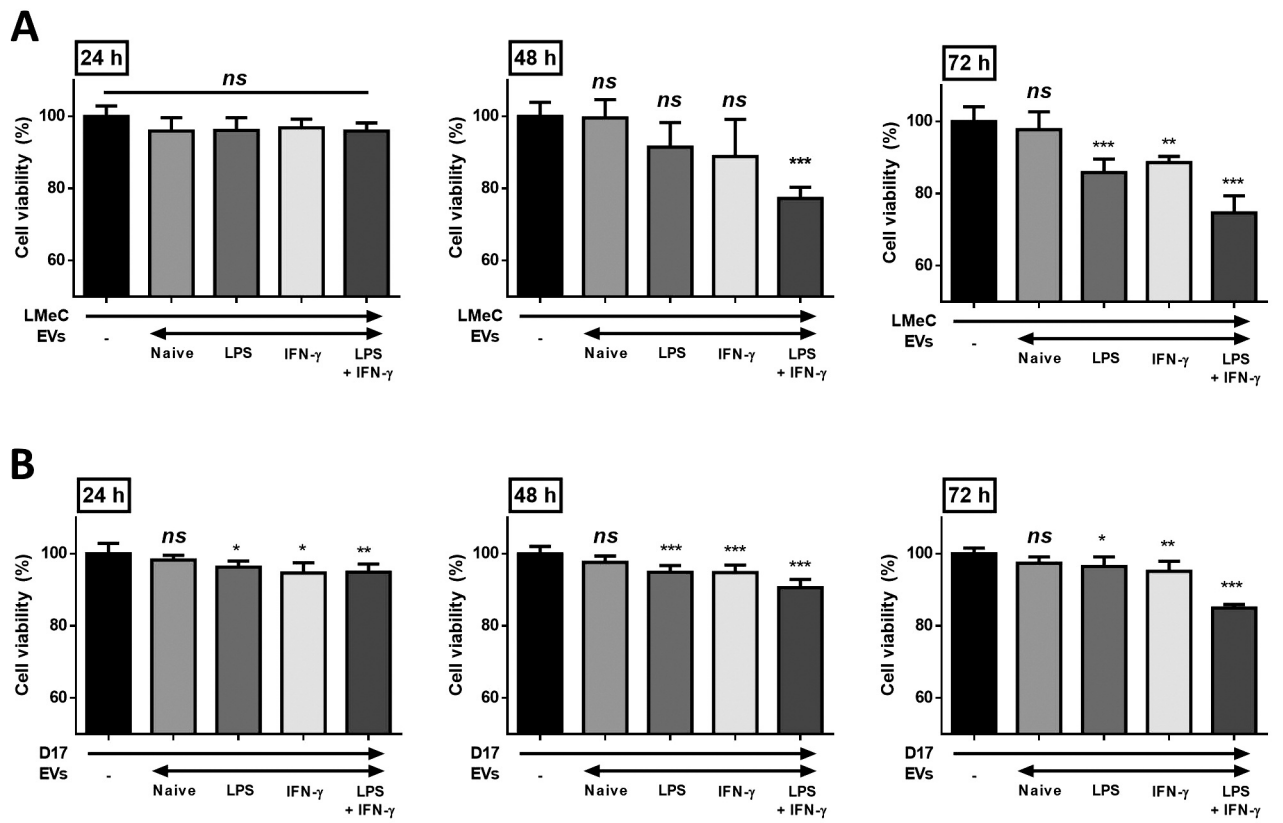

Figure 4. *Cell viability of D17 cells and melanoma LMeC cells treated M-EVs for 24 h, 48 h, and 72 h. A. In LMeC cells, there was no difference of cell viability between the different groups at 24 h. The rate of cell viability was significantly decreased following treatment with M-EVs stimulated with both LPS and IFN-γ at 48 h. At 72 h, there was no difference between the control group and the group treated with naïve M-EVs, but cell viability was significantly decreased in the other groups. B. In D17 cells, there was no difference between the control group and the group treated with naïve M-EV but there was a significant decrease in the other groups at 24 h. Similarly, there was no significant change in cell viability between the control group and the group treated with naïve M-EVs, but the cell viability was significantly declined in the other groups at 48 h and 72 h. Results are shown as mean±standard deviation of triplicate samples of three independent experiments. *p<0.05, **p<0.01, ***p<0.001. ns: Not significant, as determined by one-way ANOVA.*

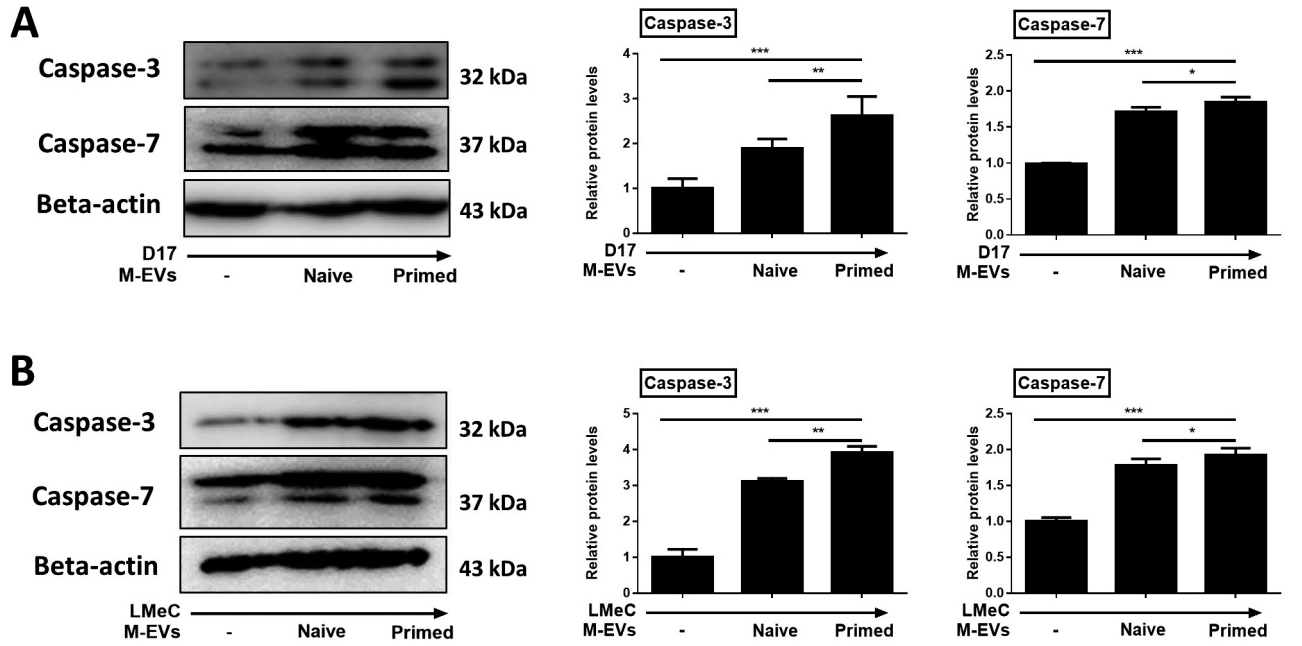

Figure 5. *Effect of naïve M-EVs and M-EVs stimulated with both LPS and IFN-γ on caspase-3/7 protein expression in D17 and LMeC cells. A. In D17 cells, there were significant increases in caspase-3 and caspase-7 protein expression when cultured with M-EVs. Also, incubation with M-EVs stimulated with both LPS and IFN-γ resulted in even more significant increase of caspase-3 and caspase-7 expression. B. In LMeC cells, the protein levels of caspase-3 and caspase-7 were significantly increased when incubated with naive or M-EVs stimulated with both LPS and IFN-γ. Incubation with M-EVs stimulated with both LPS and IFN-γ resulted in significantly higher levels of caspase-3 and caspase-7. Results are shown as mean±standard deviation of triplicate samples of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, as determined by one-way ANOVA.*

the protein levels of caspase-3 and caspase-7. Western blot analysis was performed 48 h after treatment with naïve M-EVs or M-EVs stimulated with both LPS and IFN-γ in each tumor cell line. Eventually, the protein levels of caspase-3 and caspase-7 were significantly higher in the treatment groups than in the control group. Among the treatment groups, we observed significantly higher levels of apoptosis proteins after treatment with M-EVs stimulated with both LPS and IFN-γ than after treatment with naïve M-EVs (Figure 5).

M-EVs increase apoptosis in the osteosarcoma and melanoma cell lines. We conducted flow cytometry analysis to examine the rate of apoptosis in D17 and LMeC cell lines after treatment with naïve M-EVs or M-EVs treated with both LPS and IFN-γ. We found a more significant increase in the rate of apoptosis in the treatment groups than in the control group. Moreover, the rate of apoptosis was much higher in the group treated with M-EVs stimulated with both LPS and IFN-γ than in the group treated with naïve M-EVs (Figure 6).

M-EVs modulate CTLA-4, CCR4, and Foxp3 expressions in cPBMCs cocultured with tumor cell lines. We cultured cPBMCs with D17 or LMeC cells in transwell plates to imitate the TME. We found that the mRNA expression of *CCR4* and *Foxp3* were significantly higher in cPBMCs cocultured with tumor cell lines than in cPBMCs cultured alone. No significant differences were observed in *CTLA-4* mRNA expression levels between the group cultured with only cPBMCs and the group cocultured with cPBMCs and D17 cells, but a tendency towards an increase in *CTLA-4* mRNA expression levels was observed in the cPBMCs cocultured with D17 cells. *CCR4* mRNA expression levels seemed to decrease to a greater extent in the group treated with naïve M-EVs than in the group cocultured with cPBMCs and D17 cells, and there was a tendency for further decrease by the addition of M-EVs stimulated with both LPS and IFN-γ. In addition, mRNA expression was more significantly reduced after the addition of M-EVs treated with both LPS and IFN-γ than after treatment with the group cocultured with cPBMCs and D17 cells.

Similar to *CCR4* expression, the expression of *Foxp3* tended to decrease with the addition of naïve M-EVs or M-EVs stimulated with both LPS and IFN-γ. Although no significant difference was found between the group cocultured with cPBMCs and D17 cells and the group treated with naïve M-EVs, a significant decrease in the expression of *Foxp3* was

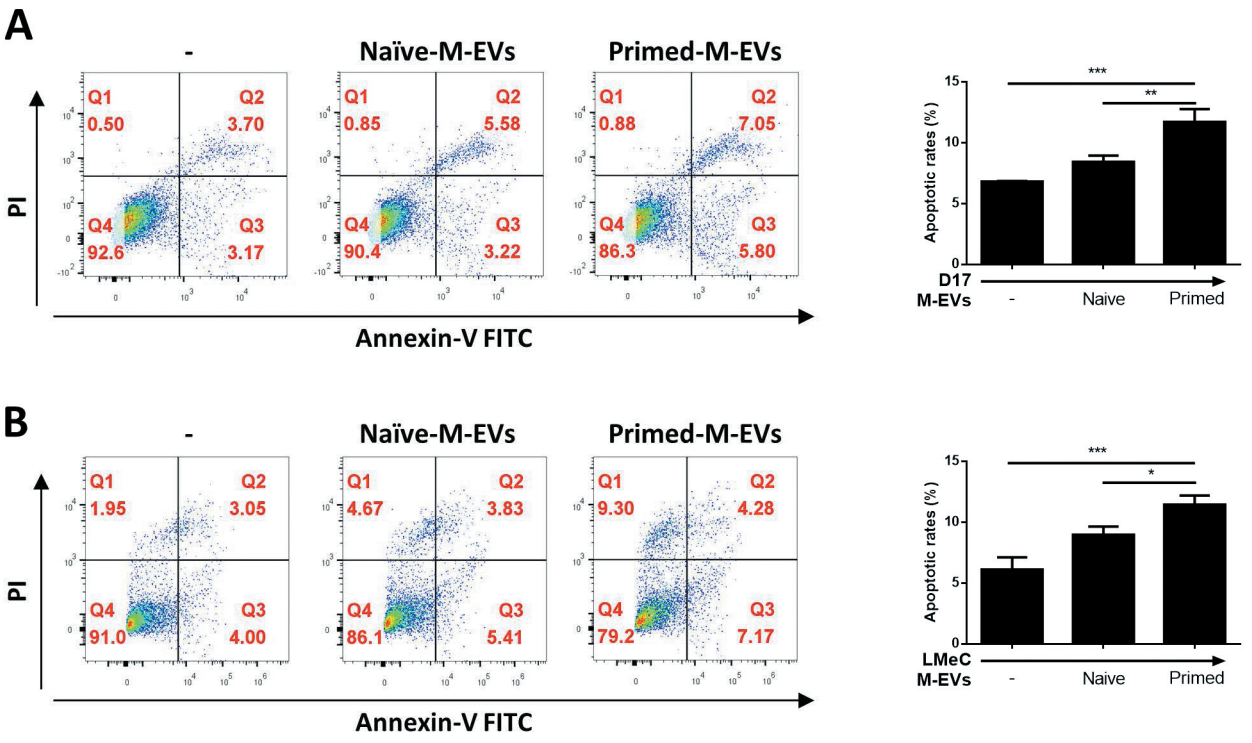

Figure 6. *Annexin-V/PI staining of D17 and LMeC cells. A. Incubation with M-EVs resulted in significant increase in apoptosis of D17 cells, especially in the group treated with M-EVs stimulated with both LPS and IFN-γ. B. In LMeC cells, there was significantly higher apoptosis when incubated with M-EVs compared to control group. There was higher apoptosis in the group cultured with M-EVs stimulated with both LPS and IFN-γ. Results are shown as mean±standard deviation of triplicate samples of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, as determined by one-way ANOVA.*

found in the group treated with M-EVs stimulated with both LPS and IFN-γ than in the group cocultured with cPBMCs and D17 cells. A significant decrease was also observed in *Foxp3* levels in the group treated with M-EVs stimulated with both LPS and IFN-γ than in the group treated with naïve M-EVs. Likewise, the expression of *CTLA-4* decreased significantly with the addition of M-EVs. Although no significant differences were found between the group treated with naïve M-EVs and the group treated with M-EVs stimulated with both LPS and IFN-γ, a significant decrease was observed when these groups were compared to the group cocultured with cPBMCs and D17 cells (Figure 7A). The results were similar to those obtained for the D17 cells when the cPBMCs and LMeC cells were cocultured in transwell plates. The mRNA expression of *CCR4* and *Foxp3* were significantly increased when cocultured with cPBMCs and LMeC cells. Although not significant, the mRNA expression of *CTLA-4* showed a greater tendency towards an increase when cocultured with cPBMCs and LMeC cells than when cultured with cPBMCs alone. A significant decrease in *CCR4*, *Foxp3*, and *CTLA-4* expression was observed in the groups treated with M-EVs than in the group cocultured with cPBMCs and LMeC cells. Although no significant difference was observed between naïve M-EVs and M-EVs stimulated with both LPS and IFN-γ, the mRNA levels of *CCR4*, *Foxp3*, and *CTLA-4* tended to decrease with the addition of M-EVs stimulated with both LPS and IFN-γ than with the addition of naïve M-EVs (Figure 7B).

Discussion

Through this study, we confirmed that EVs derived from canine M1 macrophages have antitumor effects and regulate immune cell polarization. Compared to EV therapy, cell therapy poses difficulties in maintaining the phenotype and health of cells during long-term cell storage, while being also expensive with respect to the maintenance of an acceptable cell quality (18). Because of these drawbacks, the use of EVs is drawing attention as a solution to the technical challenges related to cell-based immunotherapy (19). EVs can play a role in the physiological function of the original cells and can be cryopreserved for at least 6 months owing to the stable properties of their exosomal membranes. In addition, EVs can be strictly regulated and monitored, thereby posing fewer risks than living cells (20).

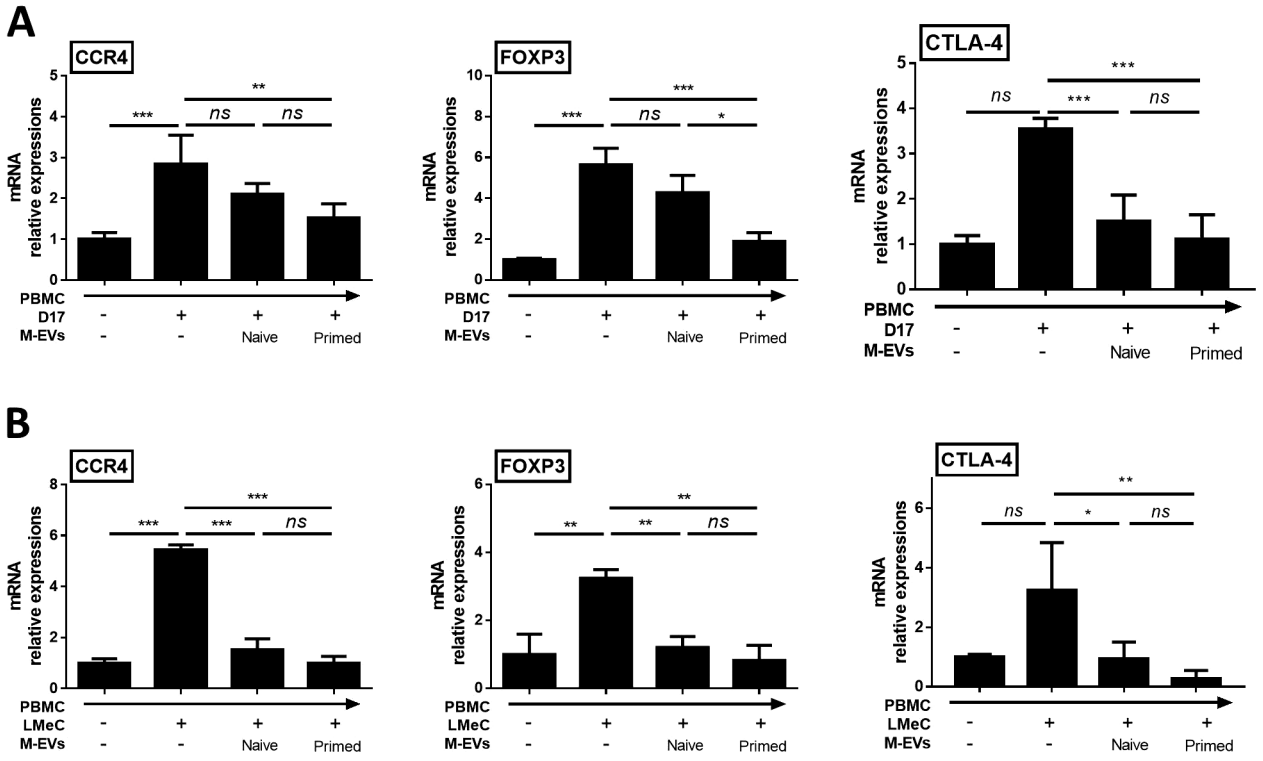

Figure 7. *mRNA expression of CCR4, Foxp3 and CTLA-4 in canine PBMC (cPBMC). A. The levels of CCR4 and Foxp3 tended to decrease following incubation with naïve M-EVs and the levels of CTLA-4 were significantly down-regulated when cells were treated with naïve M-EVs. The mRNA expression of CCR4, Foxp3, and CTLA-4 were significantly decreased when cells were cultured with M-EVs stimulated with both LPS and IFN-γ. B. When cells were cultured with naïve M-EVs or M-EVs stimulated with both LPS and IFN-γ, the mRNA expression of CCR4, Foxp3 and CTLA-4 were significantly decreased. Results are shown as mean±standard deviation of triplicate samples of three independent experiments. *p<0.05, **p<0.01, ***p<0.001. ns: Not significant, as determined by one-way ANOVA.*

We polarized DH82 cells, a canine macrophage cell line, to the M1 type by treating them with LPS and IFN-γ (21) and the morphology of the DH82 cells became more roundish which is a characteristic feature of M1 macrophages (22). LPS is a major component of the outer wall of Gram-negative bacteria, which triggers the activation of macrophages (23, 24). However, LPS potentially acts as a pathogen and can be cytotoxic when used at high doses or for extended periods of time (25). Therefore, based on previous research data, we selected an appropriate concentration of LPS that could activate macrophages without inducing cytotoxicity, and we confirmed that the concentration of LPS used to treat DH82 cells did not exhibit cytotoxicity by using the CCK-8 assay (26, 27). Similarly, IFN-γ is one of the most important cytokines in both innate and adaptive immunity, and it can promote macrophage activation and upregulate proinflammatory cytokines (28). Therefore, IFN-γ was also used for macrophage stimulation, and after selecting suitable concentrations based on various research data, the cytotoxicity of IFN-γ was evaluated using the CCK-8 assay (29, 30).

Several studies have demonstrated that proinflammatory cytokines played an important role in apoptosis, cell survival, inflammation, and delayed tumor growth (31-33). Furthermore, the formation of NO, the cytotoxic free radical, is regarded essential for macrophage-mediated defense by inhibiting mitochondrial respiration and DNA synthesis, as well as by inducing tumoricidal effects (34, 35). Therefore, M-EVs derived from M1-polarized DH82 cells may be responsible for the cytotoxic effect of D17 and LMeC cells.

Caspases are a family of proteins that are crucial mediators of apoptosis, acting as a central pathway for inhibiting and controlling the progression of cancer. Caspases are typically classified into initiator caspases and executioner caspases. Among them, caspase-3 and caspase-7 are executioner caspases that are cleaved and then activated at the end of the caspase cascade, leading to apoptosis (36-38). In addition, a study has demonstrated that caspase activity produces active proinflammatory cytokines and promotes innate immune responses (39). Furthermore, in this study, we demonstrated that apoptosis is increased in

tumor cells by using annexin V and propidium iodide staining. Several reports have shown that apoptosis increases when M1-polarized macrophages are applied as antitumor agents (40, 41). Consistent with these reports, the current experimental results demonstrated that apoptosis increased after the application of M-EVs on tumor cells.

TME consists of diverse cells comprising not only tumor cells but also stromal cells, immune cells such as macrophages, and lymphoid cells (42). Immune cells play an important role in TME by being involved in immunosuppression, invasion, metastasis, and angiogenesis *via* cell-to-cell communication, activation of molecular crosstalk, and production of various growth factors and chemokines (43). To indirectly confirm the effect of M-EVs derived from the immune cells constituting the TME, we generated an experimental setup similar to the TME by using a transwell plate. We found that the mRNA expression of *Foxp3*, *CTLA-4*, and *CCR4* in the immune cells decreased in the presence of M1-EVs. Tumor-infiltrating regulatory T cells contribute to the stabilization of the immunosuppressive TME, thereby hindering the development of effective antitumor immunity. *CCR4* is a chemokine receptor found mainly on regulatory T cells, and it plays an important role in regulatory T cell function and immunosuppression (44). *CTLA-4* is a T cell surface receptor which acts as a major negative regulator of T cell activation and mediates immune-response attenuation. Decreased expression of *CTLA-4* causes the activation of immune cells and T cell expansion (45). *Foxp3* is found in regulatory T cells and is critical for the maintenance of immune homeostasis (46). One study has demonstrated that *Foxp3* expression plays a crucial role in the tumor escape mechanism (47). In the current study, we discovered that M-EV treatment significantly reduced *CCR4*, *CTLA-4*, and *Foxp3* mRNA expression in the immune cells around the tumor. Thus, we confirmed that M-EVs not only exert an anticancer effect by inducing apoptosis in tumor cells, but also affect the surrounding TME cells.

In this study, we discovered the *in vitro* effects of M-EVs, but we could not determine whether similar effects occur *in vivo*. However, in order to compensate for such disadvantage, we created an environment similar to the TME by using a transwell plate and confirmed that M-EVs affected the expression of immune factors such as *Foxp3*, *CCR4*, and *CTLA-4* around tumor cells. Moreover, we could perform the experiments only in melanoma and osteosarcoma cells and not in other tumor cells; therefore, the effects of M-EVs in other tumor cell lines remain unclear. Nevertheless, this is the first study to reveal the mechanism by which M1-EVs increase apoptosis in canine melanoma and osteosarcoma cells, and to show how M1-EVs affect immune cells around the tumor cells.

In conclusion, the present study demonstrated that M-EVs derived from M1-polarized DH82 cells induced apoptosis in D17 and LMeC cells. Moreover, M-EV derived from M1-polarized DH82 cells exhibited immunomodulatory effects on immune cells surrounding the tumor. This study could serve as a basis for future *in vivo* studies on the antitumor effects and clinical applications of canine M-EVs.

Conflicts of Interest

The Authors have no conflicts of interest to report in relation to this study.

Authors' Contributions

KML: Data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, writing-original draft. JHA: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, writing-original draft, writing-review & editing. SJY: data curation. SMP: data curation. JHL: data curation. HKC: data curation. WJS: Conceptualization, data curation, formal analysis, investigation, methodology, supervision, validation, visualization, writing-review & editing. HYY: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, resources, software, supervision, validation, visualization, writing-review & editing.

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