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Anticancer effect of LPS and IFN-γ pretreated canine macrophage-derived extracellular vesicles through apoptosis in canine melanoma and osteosarcoma cell lines

개 흑색종 및 골육종 세포주에서 LPS와 IFN-γ 전처리한 개 대식세포 유래 세포외 소포의 세포자멸사를 통한 항암 효과

2020년 8월

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이 논문을 수의학 석사 학위논문으로 제출함

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Abstract

Anticancer effect of LPS and IFN- γ pretreated canine macrophage-derived extracellular vesicles through apoptosis in canine melanoma and osteosarcoma cell lines

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Extracellular vesicles (EVs) are particles naturally released from cells and represent an endogenous mechanism for intercellular communication involving the transport of functionally active biological molecules. In addition to their physiological role, EVs have recently attracted interest as vehicles for delivering therapeutic molecules. The purpose of this study was to evaluate the effect of EV derived from canine M1-polarized macrophages (M1EVs) on canine tumor cells, such as D17 (osteosarcoma cells) and LMeC (melanoma cells), and to determine their effects on immune cells around the tumor. By pretreatment with LPS and IFN-γ, macrophages were polarized to M1 type. In the case of M1EV, the contents of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β was increased, and nitrate/nitrite contents 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. These results suggest that use of 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.

Keywords: Extracellular vesicle, macrophage, anticancer agent, melanoma, osteosarcoma, apoptosis

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Abbreviations

EV, extracellular vesicle; M1EV, EV derived from canine M1-polarized macrophages; TME, tumor microenvironment; TAM, tumor-associated macrophage; M1, type 1 macrophage; M2, type 2 macrophage; P/S, 1% penicillin-streptomycin; cPBMC, canine peripheral blood mononuclear cell; BCA, bicinchoninic acid; CCK-8, Cell Counting Kit-8; M-EV, canine macrophage-derived extracellular vesicle; RT-qPCR, real-time quantitative PCR; NO, nitric oxide; iNOS, inducible nitric oxide synthase

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1. Introduction

Cancer is a leading cause of death in companion animals, with mortality rates of approximately 15~30% in dogs (Yeates and Main, 2009). Cancers can develop in various organs in dogs, including osteosarcoma and melanoma (Baioni et al., 2017). 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 (Mueller et al., 2007). 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 (Chun and de Lorimier, 2003; Coomer et al., 2009; Wycislo and Fan, 2015). Similarly, melanoma is one of the most malignant tumors in dogs, which usually develops in the oral cavity and acral sites (Bastian et al., 2014). 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 (Almela and Ansón, 2019).

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 (Gun et al., 2019; Sica et al., 2006; Zhang et al., 2016). Among these immune cells, tumor-associated macrophages (TAMs) represent the major component. These macrophages mainly have two polarization types: (1) the classically activated type 1 macrophage (M1), which upregulates the production of proinflammatory cytokines and enhances phagocytosis, and (2) the alternatively activated type 2 macrophage (M2), which facilitates the resolution of inflammation and promotes tissue repair and angiogenesis (De Palma and Lewis, 2013). Many studies have confirmed that TAMs are mainly polarized in the TME toward an M2-like phenotype and that this provides a basis for their ability to encourage the growth and vascularization of tumors, resulting in tumor migration and metastasis (Mantovani et al., 2002; Ramanathan and Jagannathan, 2014). In addition, some evidence suggests that tumors with abundant TAMs respond comparatively poorly to cytotoxic therapies (De Palma and Lewis, 2011; Samadi et al., 2016). In other words, the TME can enhance chemoresistance and increase metastatic spread (Mitchem et al., 2013; Schreiber et al., 2011).

Accordingly, various anticancer immunotherapies targeting the TME have been attracting attention as novel therapeutic options (Gun et al., 2019). Previous studies have demonstrated the anticancer effects of extracellular vesicles (EVs) derived from M1 macrophages (Choo et al., 2018; Wang et al., 2019). EVs are nanosized membrane-bound vesicles released by cells into the extracellular space, and these 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 (Andaloussi et al., 2013; Raposo and Stoorvogel,

2013). According to Choo et al., EVs derived from M1 polarized RAW264.7 cells significantly repolarize M2 TAMs to M1 macrophages *in vitro* and *in vivo*. Furthermore, intravenous injection of EVs into tumor-bearing mice suppressed tumor growth, thus indicating that EVs derived from M1 macrophages potentiate antitumor efficacy. However, research on their application in clinical veterinary medicine is lacking, and no studies have investigated their application in canine melanoma and osteosarcoma.

Therefore, this study aimed to evaluate the antitumor effect on EVs derived from macrophages polarized with LPS and IFN-γ. The immunomodulatory effect of EVs on immune cells surrounded by tumor cells were discovered, which may induce more sensitive environment for anticancer therapy (Waldmann, 2006). These novel findings contribute to the understanding of the interaction between EVs and their tumor microenvironment.

2. Materials and Methods

2.1. Cell culture

The canine macrophage cell line DH82 was purchased from ATCC. 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 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.

2.2. 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 instruction 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. The blood samples were blended 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.

2.3. Stimulation of M1 polarization with LPS and IFN-γ

For stimulating 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 a 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).

2.4. Isolation and characterization of canine macrophage-derived extracellular vesicles (M-EVs)

DH82 cells were seeded in 6-well plates at a density of 1×10^5 cells/ml and stimulated with LPS, IFN-γ, and LPS and IFN-γ, as described previously. After 24 h, the supernatant was discarded and new media were replenished using DMEM supplemented with 10% exosome-depleted FBS and 1% P/S. After 2 days, the supernatant from the culture medium 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 2000 × *g* for 30 min to remove cellular debris.

Thereafter, the supernatant was mixed with ExoQuick-CG Exosome Precipitation Solution (System Biosciences, Mountain View, California) according to the manufacturer's instruction. After isolation, a bicinchoninic acid (BCA) protein assay was performed for quantifying the M-EVs. To identify whether the isolated protein was an EV, a dynamic light scattering analysis was conducted for measuring the size of the EVs. The shapes of the EVs were determined using transmission electron microscopy. The protein expressions of CD63, CD81, and β-actin were assessed using western blot analysis.

2.5. Cell viability assay

To confirm that the concentrations of LPS and IFN-γ were not cytotoxic for the DH82 cells, a cell viability assay was performed 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 the control which didn't contain LPS and IFN- γ, 10 ng/ml LPS, 50 ng/ml IFN-γ, and 10 ng/ml LPS and 50 ng/ml IFN-γ. I 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.

All the experiments were divided into 5 groups in each tumor cell line: (1) D17 cell line: D17 without M-EVs, D17 with naïve M-EVs, D17 with M-EVs treated with LPS, D17 with M-EVs treated with IFN-γ, and D17 with M-EVs treated with both LPS and IFN-γ; and (2) LMeC cell line: LMeC without M-EVs, LMeC with naïve M-EVs, LMeC with M-EVs treated with LPS, LMeC with M-EVs treated with IFN-γ, and LMeC with M-EVs treated with both LPS and IFN-γ.

2.6. 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 absorbance. 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, Korea). The expression levels of each gene were normalized to that of glyceraldehyde 3-phosphate dehydrogenase. The primer sequences used in this study are listed in Table 1.

2.7. Coculture of cPBMCs with D17 and LMeC cell lines

Twenty-four-well transwell plates (SPL Life Sciences, 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 treated 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.

2.8. Griess assay

The levels of nitric oxide (NO) in M-EVs were assessed indirectly by measuring nitrite and nitrate concentrations (Sun et al., 2003). Quantification of nitrate and nitrite was performed using the Nitric Oxide (NO2/NO3) Detection kit (Enzo Life Sciences) according to the manufacturer's instruction. 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.

2.9. 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, California) 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).

2.10. 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).

2.11. Statistical analysis

Student's t-test and one-way analysis of variance were 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.

3. Results

3.1. LPS and IFN-γ promote M1 macrophage polarization

To evaluate whether the concentrations of LPS and IFN-γ are cytotoxic, we conducted the CCK-8 assay, and found no significant difference between the control and experimental groups (Fig 1A). After stimulation with LPS and IFN-γ, the morphology of the DH82 cells became more roundish than that of the control group (Fig 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 treated with both LPS and IFN-γ. In contrast, the levels of arginase 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 (Fig 1C).

We conducted flow cytometry analysis to identify the phenotype of the DH82 cells. We found that the expression of CD11c was higher when 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 treated with both LPS and IFN-γ (Fig 1D). After stimulating the 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 (Fig 1E).

3.2. 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 groups (Fig 2).

3.3. 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 (Fig 3).

3.4. M-EVs have a cytotoxic effect on the 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 difference was observed among all groups of LMeC cells. However, after 48 h, cell viability was significantly lower in the group cocultured with M-EVs treated 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 (Fig 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 treated with both LPS and IFN-γ (Fig 4B).

3.5. 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 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 treated 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 after treatment with M-EVs treated with both LPS and IFN-γ than after treatment with naïve M-EVs (Fig 5A, Fig 5B).

3.6. *M-EVs increase apoptosis in the osteosarcoma and melanoma cell lines*

We conducted flow cytometry analysis to examine the rate of apoptosis in the 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 treated with both LPS and IFN-γ than in the group treated with naïve M-EVs (Fig 6A, Fig 6B).

3.7. *M-EVs modulate CTLA-4, CCR4, and Foxp3 expressions in cPBMCs cocultured with tumor cell lines*

We cultured cPBMCs and D17 or LMeC cells in transwell plates to imitate the TME. We found that the mRNA expressions 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 the tendency was further decreased by the addition of M-EVs treated 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 treated 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 found in the group treated with M-EVs treated 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 treated 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 treated 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 (Fig 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 a increase when cocultured with cPBMCs and LMeC cells than when cultured with cPBMCs alone. A significant decrease in *CCR4*, *Foxp3*, and *CTLA-4* expressions 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 treated with both LPS and IFN-γ, the mRNA levels of *CCR4*, *Foxp3*, and *CTLA-4* tended to decrease with the addition of M-EVs treated with both LPS and IFN-γ than with the addition of naïve M-EVs (Fig 7B).

4. 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 controlling well-defined cells and in long-term cell storage, while also being expensive with respect to the maintenance of acceptable cell quality (Andre et al., 2004). Because of these drawbacks, the use of EVs is drawing attention as a solution to the technical challenges related to cell-based immunotherapy (Pitt et al., 2016). 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 do living cells (Pitt et al., 2016; Viaud et al., 2008).

We polarized DH82 cells, a canine macrophage cell line, to the M1 type by treating them with LPS and IFN-γ (Martinez et al., 2008) and the morphology of the DH82 cells became more roundish which is a characteristic feature of M1 macrophages (Heinrich et al., 2017). LPS is a major component of the outer wall of Gram-negative bacteria, which triggers the activation of macrophages (Fujihara et al., 2003; Meng and Lowell, 1997). However, LPS potentially acts as a pathogen and can be cytotoxic when used at high doses or for extended periods of time (Doe and Henson, 1978). 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 (Fujiwara et al., 2016; Tabatabaei-Zavareh et al., 2017). Similarly, IFN-γ is one of the most important cytokines in both innate and adaptive immunities, and it can promote macrophage activation and upregulate proinflammatory cytokines (Wu et al., 2014). 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 (Delneste et al., 2003; Lee and Sullivan, 2001; Tabatabaei-Zavareh et al., 2017).

As a result of polarization towards the M1 type in this study, we could confirm that the mRNA expressions of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, were increased in DH82 cells as well as in M-EVs. Furthermore, we performed the Griess assay to indirectly detect NO in M-EVs and verified that M-EVs treated with both LPS and IFN-γ showed significantly higher nitrate and nitrite concentrations. Several studies demonstrated that proinflammatory cytokines played an important role in apoptosis, cell survival, inflammation, and delayed tumor growth (Hess and Neri, 2014; Sawa-Wejksza and Kandefer-Szerszeń, 2018; van Horssen et al., 2006). Furthermore, the formation of NO, the cytotoxic free radical, is regarded as essential for macrophage-mediated defense by inhibiting mitochondrial respiration and DNA synthesis, as well as by inducing tumoricidal effects (Hibbs Jr et al., 1988; Stuehr and Nathan, 1989). Therefore, M-EVs derived from M1-polarized DH82 cells may be responsible for the cytotoxic effect of D17 and LMeC cells.

We proved that caspase-3 and caspase-7 protein expressions in D17 and

LMeC cells were activated when these cells were treated with M-EVs. 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 (Boudreau et al., 2019; Lamkanfi and Kanneganti, 2010; Porter and Jänicke, 1999). In addition, a study demonstrated that caspase activity produces active proinflammatory cytokines and promotes innate immune responses (McIlwain et al., 2013). Furthermore, in this study, we demonstrated that apoptosis is increased in tumor cells by using annexin V and propidium iodide staining. Several reports have proven that apoptosis increases when M1-polarized macrophages are applied as antitumor agents (Cui et al., 1994; Fujiwara and Kobayashi, 2005). Consistent with these reports, the current experimental results demonstrated that apoptosis increased after the application of M-EVs on tumor cells.

The TME consists of diverse cells comprising not only tumor cells but also stromal cells, immune cells such as macrophages, and lymphoid cells (Kim and Bae, 2016). Immune cells play an important role in the 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 (Li et al., 2007). To indirectly confirm the effect of M-EVs derived from the immune cells constituting the TME, we created an experimental setup similar to the TME by using a transwell plate. We found that the mRNA expressions 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. Cancer can prevent the immune system from destroying cancer cells via the excessive activity of regulatory T cells. *CCR4* is a chemokine receptor found mainly in the regulatory T cells, and it plays an important role in regulatory T cell function and immunosuppression (Ishida and Ueda, 2006). *CTLA-4* is a T cell surface receptors 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 (Keilholz, 2008). *Foxp3* is found in regulatory T cells and is critical for the maintenance of immune homeostasis (Li et al., 2015). One study demonstrated that *Foxp3* expression plays a crucial role in the tumor escape mechanism (Hinz et al., 2007). In the current study, we discovered that M-EV treatment significantly reduced *CCR4*, *CTLA-4*, and *Foxp3* mRNA expressions 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 cells in the TME.

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 disadvantages, 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. This study could serve as a basis for future *in vivo* studies on the antitumor effects and clinical applications of canine M-EVs.

5. Conclusion

We demonstrated that M-EVs derived from M1-polarized DH82 cells showed apoptosis effects in D17 and LMeC cells, and M-EVs had inhibitory potential of tumor immune-escape. This novel study could play a role as a basis for clinical applications of anticancer therapy.

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Table 1. Sequences of PCR primers

Target gene	Primer	Sequence	Reference
Canine iNOS	Forward	GAG ATC AAT GTC GCT GTA CTC C	(An et al., 2020)
	Reverse	TGA TGG TCA CAT TTT GCT TCT G	
Canine arginase	Forward	CAG AAG AAT GGA AGA GTC AG	(An et al., 2020)
	Reverse	CAG ATA TGC AGG GAG TCA CC	
Canine TNF-α	Forward	TCA TCT TCT CGA ACC CCA AG	(An et al., 2020)
	Reverse	ACC CAT CTG ACG GCA CTA TC	
Canine IL-6	Forward	ATG ATC CAC TTC AAA TAG TCT ACC	(An et al., 2020)
	Reverse	AGA TGT AGG TTA TTT TCT GCC AGT G	
Canine IL-1β	Forward	AGT TGC AAG TCT CCC ACC AG	(An et al., 2020)
	Reverse	TAT CCG CAT CTG TTT TGC AG	
CCR4	Forward	CGA GCG CAA CCA TAC CTA CT	(Park et al., 2013)
	Reverse	CGG CAA AGA CCA TCC TCA CT	
Foxp3	Forward	AAA CAG CAC ATT CCC AGA GTT C	(An et al., 2020)
	Reverse	AGG ATG GCC CAG CGG ATC AG	
CTLA-4	Forward	TTC TCC AAA GGG ATG CAT GT	(Tagawa et al., 2017)
	Reverse	TCA CAT TCT GGC TCA GTT GG	
Canine GAPDH	Forward	AGT ATG TCG TGG AGT CTA CTG GTG T	(An et al., 2020)
	Reverse	AGT GAG TTG TCA TAT TTC TCG TGG T	

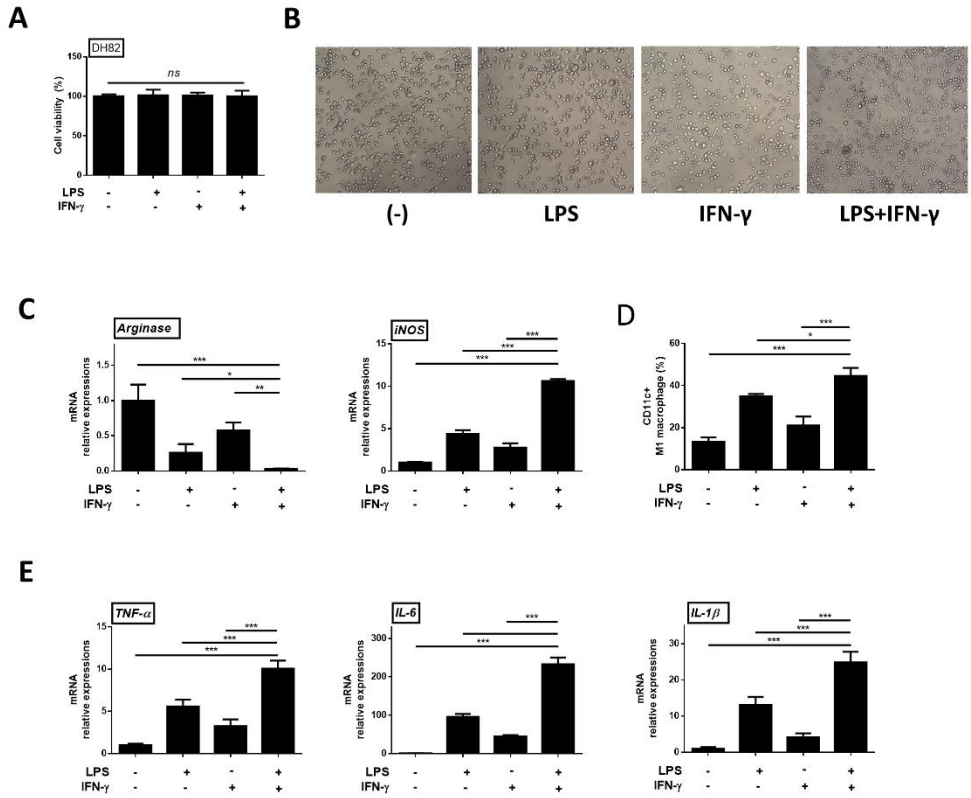


Figure 1. LPS and IFN-γ promote type 1 macrophage (M1) polarization in canine macrophages. **A**. cell viability assay using CCK-8 assay. There were no significant differences between all groups **B**. Morphology of macrophage stimulated with LPS and IFN-γ. When stimulated with LPS and IFN- γ, the morphology of DH82 cells showed more roundish **C**. mRNA expression levels of M1(iNOS) and M2 (Arginase) marker. There was significant increase in iNOS expression levels and significant decrease in mRNA expression level of Arginase. **D**. Percentage of CD11c+ cells in LPS/ IFN-γ stimulated DH82 cells. There was significant increase of polarization to M1 treated 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 presence LPS, IFN- γ or both LPS and IFN- γ compared to control group. Results are shown as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not significant.

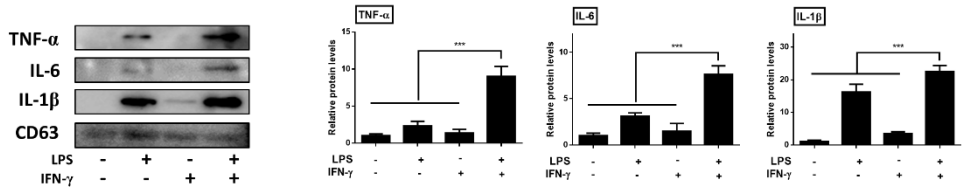


Figure 2. Expression of proinflammatory cytokines in macrophage derived extracellular vesicles (M-EV). There was significant increase of TNF-α, IL-6 and IL-1β in group treated both LPS and IFN- γ than the other groups. Results are shown as mean ± standard deviation. ***P < 0.001.

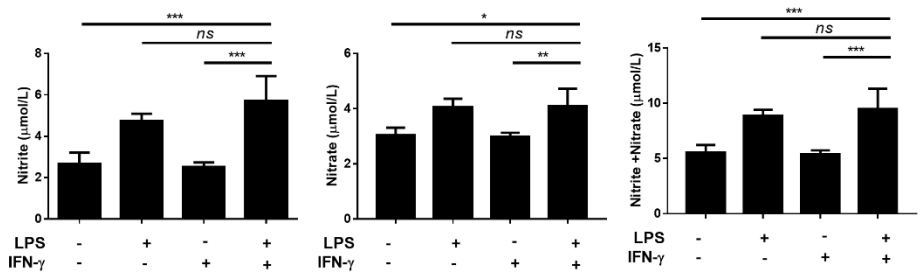


Figure 3. Nitrate and nitrite concentration in M-EV . The concentration of nitrate and nitrite was measured in M-EV. The nitrate and nitrite levels were significantly increased in M-EV treated with both LPS and IFN- γ compared with the other groups. Results are shown as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not significant.

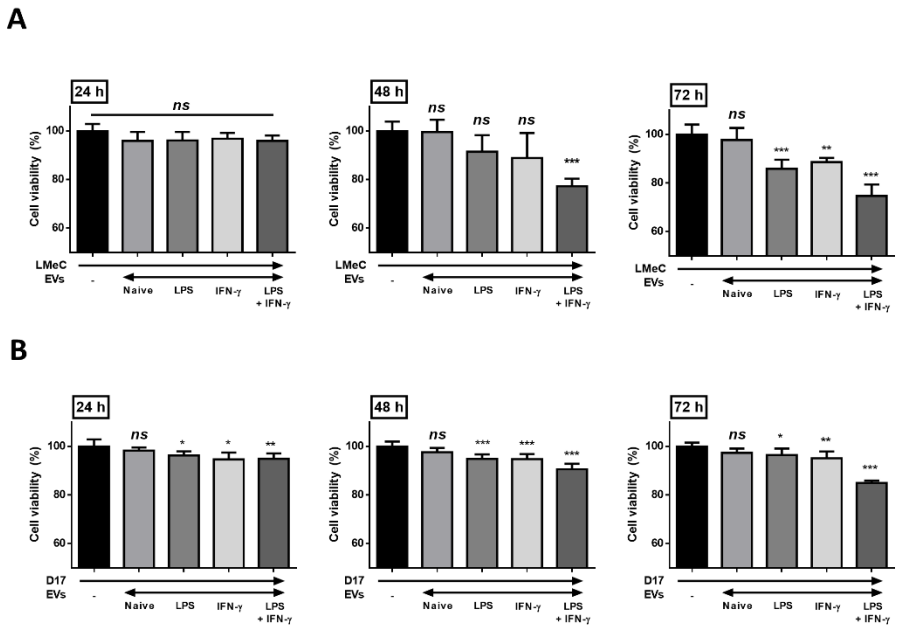


Figure 4. Cell viability of D17 cells and melanoma LMeC cells under M-EV after 24 h, 48 h, and 72 h. A. In LMeC cells, there was no difference of cell viability between all groups at 24 h. The rate of cell viability was significantly decreased with M-EV treated both LPS and IFN-γ at 48 h. At 72 h, there was no difference between control group and the group treated with naïve M-EV, but the cell viability was significantly decreased in other groups. **B**. In D17 cells, there was no difference between control group and the group treated with naïve M-EV but there was significant decrease in other groups at 24 h. Similarly, there was no significant change in the cell viability between control group and the group treated with naïve M-EV, but the cell viability was significantly declined in other groups at 48 h and 72 h. Results are shown as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not significant.

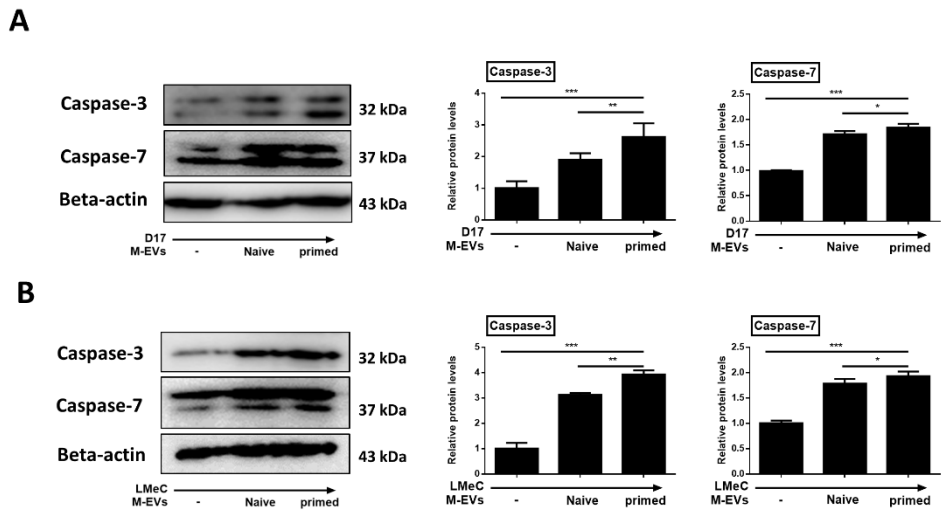


Figure 5. Effect of naïve M-EV and M-EV treated with both LPS and IFN-γ on caspase-3/ 7 protein expression in D17 and LMeC cells. A. In D17 cells, there were significant increases of caspase-3 and caspase-7 protein expression when cultured with M-EV. Also, cultured with M-EV treated both LPS and IFN-γ 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 significantly increased when incubated with navie or M-EV treated both LPS and IFN-γ. Cultured with M-EV treated both LPS and IFN-γ showed much more significantly increased levels of caspase-3 and caspase-7. Results are shown as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001.

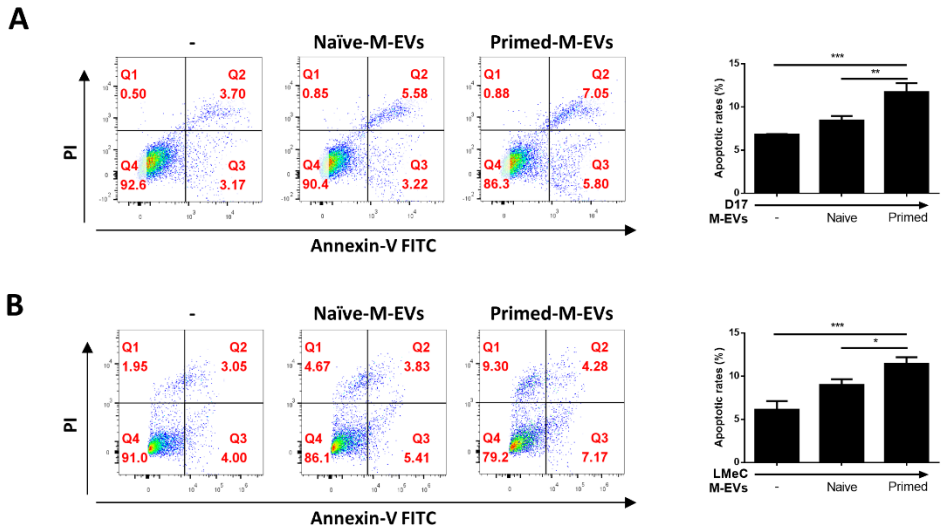


Figure 6. Annexin-V/PI staining of D17 and LMeC cells. A. Culture with M-EV resulted in significant increase in apoptosis of D17 cells, especially in the group treated with M-EV treated both LPS and IFN-γ **B**. In LMeC cells, there was significantly higher apoptosis when added M-EV compared to control group. There was much more apoptosis in the group cultured with M-EV treated both LPS and IFN-γ. Results are shown as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001.

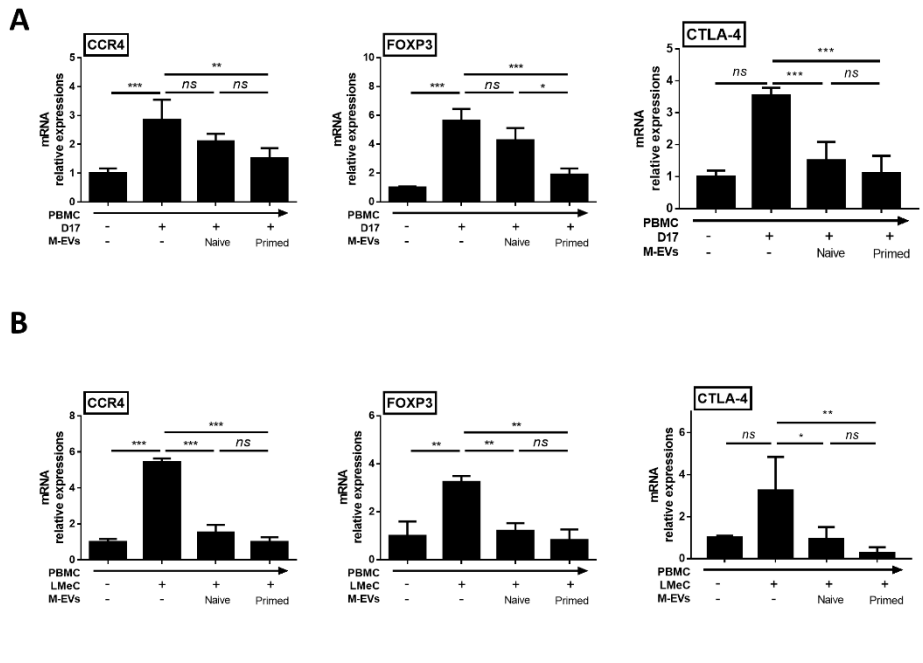
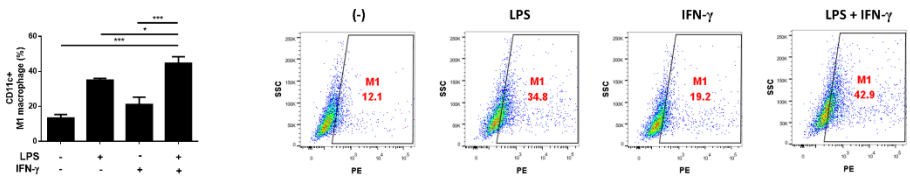
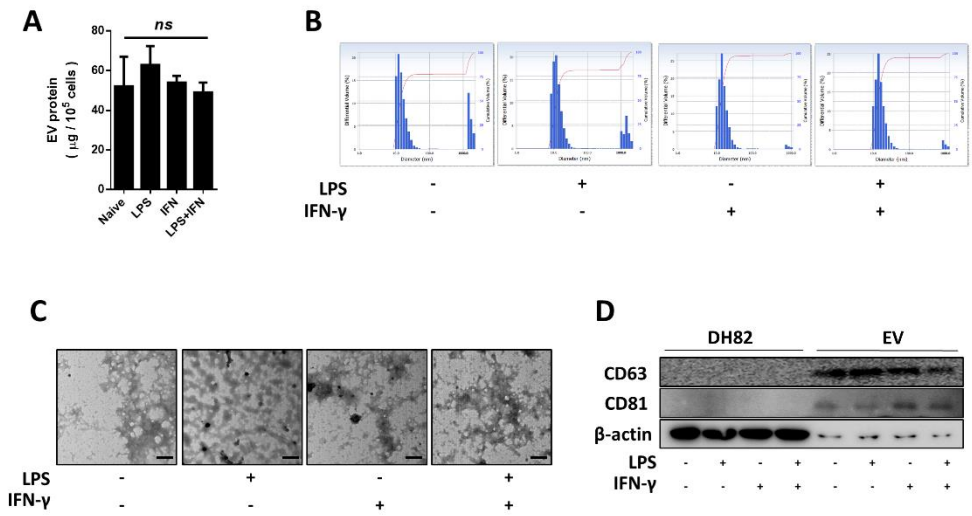


Figure 7. mRNA expressions of *CCR4, Foxp3* and *CTLA-4* in canine PBMC (cPBMC) A. The levels of *CCR4* and *Foxp3* tended to decrease with naïve M-EV and the levels of *CTLA-4* were significantly downregulated when treated with naïve M-EV. The mRNA expressions of *CCR4*, *Foxp3*, and *CTLA-4* were significantly decreased when cultured M-EV treated both LPS and IFN-γ **B**. When cultured with naïve M-EV or M-EV treated both LPS and IFN-γ, the mRNA expressions of *CCR4, Foxp3* and *CTLA-4* were significantly decreased. Results are shown as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not significant.



Supplementary figure 1. Results of flow cytometry in figure 1D. The polarization toward M1 was examined to detect CD11c+ DH82 cells. Compared to control group, the percentage of CD11c+ cells was significantly increased in the presence of LPS, IFN-γ or LPS and IFN-γ. Results are shown as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001.



Supplementary figure 2. Characterization of pre-conditioning macrophage derived EV **A**. The protein quantifications of M-EV were determined using BCA protein assay. As a result, no significant differences of yields were observed between all groups **B**. The size of M-EV was measured by DLS and its size was approximately 10~100 nm **C**. The morphology of M-EV was taken by TEM **D**. To identify if the proteins were M-EV, western blot analysis was conducted. As a result, M-EV revealed CD63+, CD81+ contrary to DH82 cells which were negative to CD63 and CD81. Results are shown as mean ± standard deviation. ns, not significant.

국문 초록

개 흑색종 및 골육종 세포주에서 LPS와 IFN-γ 전처리한 개 대식세포 유래 세포외 소포의 세포자멸사를 통한 항암 효과

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이 경 미

세포외 소포는 세포로부터 자연적으로 방출된 입자이며 기능적으로 활성화된 생물학적 분자에 대한 수송을 포함한 세포 간 커뮤니케이션을 위한 내인성 메커니즘을 나타낸다. 이러한 생물학적 기능뿐만 아니라, 세포외 소포는 최근 치료 분자를 전달하는 매개체로서 관심을 끌고 있다. 이 연구의 목표는 1형으로 분극된 개 대식세포주 (DH82 cell line)로부터 얻은 세포외 소포(M1EV)의 항암 효과를 개 흑색종 세포주 (LMeC cell line)와 개 골육종 세포주 (D17 cell line)를 통해 평가하고, 주변의 면역 세포에 미치는 영향을 결정하는 것이였다. LPS와 IFN-γ를 전처치하여 DH82 세포를 제1형으로 분극화한 후 세포외 소포를 획득하였다. M1EV 내에 함유된 TNF-α, IL-6 and IL-1β와 같은

전염증성 사이토카인이 증가하였고, nitrate/nitrite 함유량도 또한 증가하였다. 또한 M1EV는 caspase-3와 caspase-7 활성을 증가시킴으로써 종양 세포의 세포자멸사를 유도하였다. 그리고 개 말초 단핵세포와 종양 세포를 공배양하였을 때, M1EV는 개 말초 단핵 세포의 CCR4, Foxp3, 그리고 CTLA-4 발현을 감소시켰다. 이러한 결과는 M1EV가 흑색종 및 골육종에 대한 효과적인 항암 치료 접근법일 수 있고, M1EV가 종양 치료에 있어서 종양 미세환경 내에서의 면역 조절 역할을 할 수 있음을 시사한다.

주요어 : 세포외 소포; 대식구; 항암제; 흑색종; 골육종; 세포자멸사

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