Changes in gene expression profiles and cytokine secretions in peripheral monocytes by treatment with small extracellular vesicles derived from a canine lymphoma cell line

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ABSTRACT. Interactions between tumor and immune cells within the tumor microenvironment play an important role in tumor progression, and small extracellular vesicles (EVs) derived from these tumor cells have been shown to exert immunomodulatory effects on various immune cells, including macrophages and lymphocytes. Although the immunomodulatory effects of small EVs derived from human cancer cells have been intensively investigated, few studies have investigated the effects of lymphoma-derived small EVs on macrophages in both human and veterinary medicine. Here, we evaluated the effects of canine lymphoma-derived small EVs on canine primary monocytes, which are the major source of macrophages in neoplastic tissues. Comprehensive gene expression analysis of these treated monocytes revealed their distinct activation via the Toll-like receptor (TLR) and NF-κβ signaling pathways. In addition, treatment with lymphoma small EVs increased the secretion of MCP-1, which induces the infiltration and migration of monocytes and lymphocytes in neoplastic and cancer tissues. The results of this study indicate that canine lymphoma small EVs activate monocytes, possibly through the activation of TLR and NF-κβ signaling pathways, and induce monocytes to secrete MCP-1, which might contribute to immune cell infiltration within the tumor microenvironment.

KEYWORDS: diffuse large B-cell lymphoma, dog, monocyte chemotactic protein-1, nuclear factor-kappa β signaling, toll-like receptor signaling

The tumor microenvironment (TME) plays an important role in the initiation and progression of various tumors [28, 29]. TME is composed of stromal cells, fibroblasts, endothelial cells, and innate and adaptive immune cells. Innate immune cells in the TME include monocytes/macrophages, dendritic cells, neutrophils, myeloid-derived suppressor cells, natural killer cells, and innate lymphoid cells, all of which may initiate immune responses that guide tumor progression [13]. The role of TME in the pathophysiology of human lymphomas is well characterized [24]. In diffuse large B cell lymphoma (DLBCL), which is the most common subtype of human B-cell lymphomas, the number of infiltrative T lymphocytes and macrophages has been reported to be associated with the prognosis of patients [19, 24]. These observations indicate that the interactions between tumor and immune cells are important in the pathophysiology of lymphoma.

Intercellular communication is mediated by membrane proteins, cytokines, hormones, and extracellular vesicles (EVs). Small EVs are defined as EVs less than 200 nm in diameter and released from various cell types which may carry RNA, DNA, or proteins [9, 26]. Small EVs transmit signals to target cells via ligand-receptor interactions, phagocytosis, and endocytosis [9]. Small EVs derived from tumor cells have been shown to modify the functions of immune cells to promote tumor progression via the delivery of various biological components [30]. There have been a few reports describing the roles of small EVs in human DLBCL. These include reports describing their interaction with macrophages to induce tumor-promoting effects within TME [17]. A previous study revealed that small EVs derived from human DLBCL cell lines could be incorporated into THP-1 cells, a human
acute monocytic leukemia cell line, activating NF-κβ signaling in these cells [31]. However, there have been no studies, to date, investigating the effects of lymphoma-derived small EVs on primary monocytes from the peripheral blood, which are the main source of macrophages within neoplastic tissues.

In addition, in veterinary medicine, there are very few studies discussing the effects of small EVs on neoplastic tissues. Recently, small EVs derived from pro-inflammatory canine macrophages were reported to exhibit apoptotic effects on canine cancer cell lines [15]. Meanwhile, the effects of cancer cell-derived small EVs on immune cells have not been investigated in veterinary medicine.

We previously analyzed the microRNA and protein profiles of canine lymphoma and leukemia-derived small EVs, which were defined in terms of their size and specific protein markers [1]. However, the biological functions of these small EVs remain unclear. Given this, we designed this study to evaluate the immunomodulatory effects of canine lymphoma-derived small EVs on canine primary monocytes and we evaluated the changes in gene expression profile and cytokine secretion in canine primary monocytes treated with canine DLBCL-derived small EVs.

MATERIALS AND METHODS

Cell line validation and culture conditions

We used a canine B-cell lymphoma cell line, CLBL-1, established and validated in a previous study [22] for our experiments. Mycoplasma contamination was excluded using a Takara PCR Mycoplasma Detection Set (Takara Bio Inc., Kusatsu, Japan). The cell line was cultured in RPMI-1640 medium at 37°C, with 10% fetal bovine serum (Biowest, Nuaille, France) and penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) in a humidified atmosphere containing 5% CO2.

Small EV isolation from the culture supernatant of CLBL-1

Small EVs were isolated from the culture medium of CLBL-1 as previously reported [1]. Briefly, 3 × 10^7 CLBL-1 cells were cultured in growth medium without fetal bovine serum for 24 hr, and small EVs were isolated from cell culture media using a Total Exosome Isolation (from cell culture media) kit (Thermo Fisher Scientific). In the small EV isolation procedure, the culture supernatant was removed after the last centrifugation step, which precipitates the small EVs, according to the manufacturer’s instructions. We previously demonstrated that the average size of these small EVs was 100–150 nm and was concordant with the size definition [1, 26]. Isolated small EVs were resuspended in phosphate-buffered saline (PBS) and lysed by RIPA buffer followed by 10 times dilution with distilled water as previously described [25]. The concentration of the obtained protein was measured using a Micro BCA Protein Assay (Thermo Fisher Scientific) following the manufacturer’s instructions.

Isolation of primary monocytes

Peripheral blood was collected from healthy beagle dogs kept for experimental purposes in our laboratory, and peripheral blood mononuclear cells (PBMCs) were isolated via gradient centrifugation with Ficoll-Paque (1.077 g/ml; GE Healthcare, Chicago, IL, USA). CD14^+ monocytes were isolated from freshly prepared PBMCs using the MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) with a selection column as previously described, with some modifications [6]. Briefly, PBMCs were incubated with the anti-CD14 mouse monoclonal antibody, TUK4 (Thermo Fisher Scientific), and secondary goat anti-mouse microbeads (Miltenyi Biotec). Thereafter, the incubated cells were positively selected using a selection column (MACS column MS, Miltenyi Biotec) and then washed. This method isolated CD14^+ monocytes with a purity of more than 95%, as determined via flow cytometry (Supplementary Fig. 1). This animal experiment was approved by the Animal Care Committee of The University of Tokyo (approval no. P19-120).

Small EV treatments for canine monocytes

Isolated monocytes were seeded in 24-well plates at a concentration of 2 × 10^5 cells/ml with or without small EVs. The monocytes were cultured in RPMI-1640 medium at 37°C, with 10% exosome-depleted fetal bovine serum (Thermo Fisher Scientific) and penicillin-streptomycin in a humidified atmosphere containing 5% CO2. Small EVs were added to the monocytes at a final concentration of 80 μg/ml based on the previous reports [2, 3], and the same volume of PBS without small EVs was added to the control groups. After 4 hr of incubation, non-attached cells were discarded [5], and total RNA was extracted from the attached cells using an RNasey mini kit (Qiagen, Milan, Italy).

RNA sequencing

RNA integrity was assessed using a BioAnalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA), and samples with an RNA integrity number (RIN) of >7.5 were used for RNA-seq. RNA was extracted from monocytes isolated from a healthy dog to minimize variance in gene expression levels between different donors. One microgram of total RNA was amplified using the SMARTer Ultra Low RNA Kit for sequencing (Clontech Laboratories, Mountain View, CA, USA) following the manufacturer’s instructions. Next, 0.2 ng of cDNA was subjected to 11 cycles of PCR. The quality of the sequencing library was assessed using a BioAnalyzer 2100, and three cDNA libraries from small EV-treated monocytes and three cDNA libraries from control monocytes were constructed. These libraries were sequenced on the Illumina NovaSeq 6000 platform using a 150-base pair paired-end strategy. Base calling was performed using the Real Time Analysis software (v3.4.4, Illumina, San Diego, CA, USA) and data were demultiplexed and converted to the FASTQ format using bcl2fastq software (v2.20, Illumina). Mapping of the reads was conducted using STAR (2.6.0c) and the gene expression levels were calculated and annotated using Genedata Profiler Genome.
Identifying DEGs in small EV-treated monocytes

The RNA-seq analysis generated at least 61 million raw reads and had a mapping ratio of >86% for each sample (Supplementary Table 2). Differential expression analysis of the small EV-treated and control monocytes identified 446 DEGs, which were defined as genes with log2 fold change >1 and false discovery rate <0.1 (Supplementary Table 3). Of these, 320 were upregulated and 126 were downregulated in response to small EV treatment (Fig. 1A). When we evaluated the annotated genes, we saw that the top three downregulated genes included integrin beta 5 (ITGB5), eosinophil peroxidase (EPX), and rap guanine nucleotide exchange factor 4 (RAPGEF4), whereas the top three upregulated genes included Ets homologous factor (EHF), transmembrane serine protease 6 (TMPRSS6), and C-C motif chemokine ligand 13 (CCL13). Hierarchical clustering analysis using these DEGs showed that small EV-treated and control monocytes clustered separately (Fig. 1B).

Validation of the RNA-seq results using RT-qPCR

We went on to evaluate the expression levels of six DEGs, Interleukin (IL)-1β, IL6, tumor necrosis factor α (TNFa), ITGB5, C-X-C motif chemokine ligand 12 (CXCL12), and OTU deubiquitinase 1 (OTUD1) in small EV-treated and control monocytes using RT-qPCR to validate the RNA-seq results. IL-1β, IL6, and TNFa were selected from the upregulated DEGs in small EV-treated monocytes in RNA-seq according to the results of a previous report [4], and other 3 genes were randomly selected from downregulated DEGs in small EV-treated monocytes in RNA-seq. The results demonstrated that the transcription of IL-1β, IL6, and TNFa were significantly upregulated in small EV-treated monocytes when compared to the control, whereas ITGB5, CXCL12, and OTUD1 transcription was significantly downregulated in the small EV-treated monocytes compared to the control (P<0.05) (Fig. 2), confirming the RNA-seq results.

GO terms, KEGG pathway enrichment, and IPA for DEGs

GO biological processes, GO cellular component, and GO molecular function analysis using extracted DEGs showed that 30, 7, and 13 terms were significantly enriched, respectively (-log10 (P)>1.3) (Fig. 3A–C). Among the GO biological process terms,
immune response and inflammatory response were the most significantly enriched, whereas in the GO cellular component terms, the extracellular space was the most significantly enriched. Cytokine activity was the most significantly enriched in the GO molecular function terms. DAVID-mediated KEGG pathway analysis identified 20 significantly enriched pathways (-log10 (P) > 1.3) including the TNF, Jak-STAT, and NF-κβ signaling pathways (Fig. 3D).

Canonical pathway analysis via IPA revealed 14 significantly activated and 2 significantly inhibited pathways, with P < 0.05 and z-score > |2| (Fig. 4). Significantly activated pathways included the toll-like receptor (TLR) and IL-6 signaling pathways, whereas the significantly inhibited pathways were identified as liver X receptor/retinoid X receptor (LXR/RXR) activation and peroxisome proliferator-activated receptor signaling. Upstream regulator analysis via IPA identified 242 significantly induced and 124 significantly inhibited regulators with P < 0.05 and z-score > |2| (Supplementary Table 4). The top 10 most significantly induced and inhibited regulators, except for the chemical pathways, are shown in Table 1. The significantly upregulated upstream regulators included TLR4, TLR2, TLR9, TLR3, and NF-κβ.

Quantification of cytokine concentrations via cytokine array analysis

The concentrations of 10 cytokines, IL-2, IL-6, IL-8, IL-10, GM-CSF, MCP-1 (CCL2), RAGE, SCF, TNF-α, and VEGF-A, in the culture supernatant of control and small EV-treated monocytes were evaluated using a cytokine array. Representative figures for each group are shown in Supplementary Fig. 2. Of the 10 cytokines evaluated in this study, only MCP-1 demonstrated significant difference between the two groups, with the average concentration of MCP-1 increasing in the culture supernatant of small EV-treated monocytes (4,021 ± 527 pg/ml) when compared to the control (1,304 ± 894 pg/ml) (P = 0.021, Fig. 5). When we examined the expressions of MCP-1 mRNA by RT-qPCR, it was shown that the expression levels of MCP-1 mRNA expression were significantly higher in small EV-treated monocytes than control ones (P = 0.031, Supplementary Fig. 3).
Fig. 3.
DISCUSSION

This study was designed to examine the effects of canine DLBCL cell line-derived small EVs on canine primary monocytes. Our results indicated that treatment with small EVs upregulated the expression of various immune response genes including IL6 and TNFα and activated several intracellular signaling pathways including TLR and NF-κβ signaling in primary monocytes. In addition, small EV-treated monocytes secreted significantly higher concentrations of MCP-1 compared to control monocytes. Gene ontology and pathway analysis revealed that small EV treatment modulated various pathways, especially those related to inflammatory responses in primary monocytes. Among the modulated pathways, NF-κβ signaling pathway was the most significantly activated in small EV-treated monocytes. These results are consistent with those of a previous study that reported the activation of NF-κβ signaling pathway in THP-1 cells following their treatment with small EVs derived from human DLBCL cell lines [31]. NF-κβ is an important transcription factor that regulates macrophage activation in response to diverse factors [14], thus its activation under these conditions suggest that small EV treatment might activate canine monocytes through this signaling pathway.

Table 1. Top 10 activated and inhibited upstream regulator in small extracellular vesicle-treated monocytes compared with control monocytes

| Upstream Regulator | Activation z-score | P-value of overlap | Activated signaling | Inhibited signaling |
|-------------------|-------------------|--------------------|--------------------|--------------------|
| IL1B              | 4.144             | 1.34E-08           |                    |                    |
| EGF               | 4.142             | 0.00283            |                    |                    |
| TLR4              | 3.798             | 0.00000134         |                    |                    |
| JUN               | 3.619             | 0.000141           |                    |                    |
| TLR2              | 3.516             | 0.0000128          |                    |                    |
| TLR9              | 3.513             | 0.0000211          |                    |                    |
| TLR3              | 3.505             | 0.00000554         |                    |                    |
| NFkB              | 3.488             | 0.000731           |                    |                    |
| F2                | 3.373             | 0.00135            |                    |                    |
| IL2               | 3.349             | 0.0138             |                    |                    |
| NR1H2             | −2.58             | 0.000246           |                    |                    |
| IL37              | −2.593            | 6.75E-08           |                    |                    |
| COL18A1           | −2.621            | 0.000554           |                    |                    |
| HBB               | −2.63             | 0.00000334         |                    |                    |
| CISH              | −2.63             | 0.000149           |                    |                    |
| HMOX1             | −2.73             | 0.000997           |                    |                    |
| RPSA              | −2.771            | 1.66E-08           |                    |                    |
| ZFP36             | −2.798            | 0.000104           |                    |                    |
| miR-155-5p        | −2.935            | 0.00136            |                    |                    |
| Nr1h              | −3.326            | 0.0000705          |                    |                    |

Fig. 4. Histogram showing the enriched pathways in the canonical pathway analysis by Ingenuity Pathway Analysis (IPA) and the differentially expressed genes in small extracellular vesicle (EV)-treated monocytes. Red bar indicates a z-score of >2. Blue bar indicates a z-score of <−2.

Fig. 3. Histograms showing results of gene ontology analyses and pathway analysis using differentially expressed genes extracted from the comparisons of small extracellular vesicle (EV)-treated and control monocytes. (A) Histogram showing the enriched gene ontology biological process terms using differentially expressed genes in small EV-treated monocytes. (B) Histogram showing the enriched gene ontology cellular component terms using differentially expressed genes in small EV-treated monocytes. (C) Histogram showing the enriched gene ontology molecular function terms using differentially expressed genes in small EV-treated monocytes. (D) Histogram showing enriched pathways identified by KEGG pathway enrichment analysis of the differentially expressed genes in small EV-treated monocytes.
Fig. 5. Comparisons of the concentrations of 10 cytokines in the culture supernatant of control and small extracellular vesicle (EV)-treated monocytes. N=3 for all tests. * indicates P<0.05. Data are shown as the mean + SD.

In conclusion, canine DLBCL cell line-derived small EVs activate canine peripheral monocytes, possibly through the activation of the TLR and NF-κβ signaling pathways. These activations might result in an increase in the secretion of MCP-1, a cytokine that induces immune cell infiltration of the TME.

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