

Immunomodulatory Effects of Human Breast Milk-Derived Exosomes on Myeloid Cells and Chondrocytes

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Abstract

Human breast milk (HBM) is an ideal nutritional source for the growth and development of infants. In addition, HBM contains hormones, growth factors, microRNAs and exosomes that perform various physiological functions. This study investigates the immunomodulatory effects of HBM-derived exosomes on myeloid cells and chondrocytes, and implications for juvenile idiopathic arthritis. HBM-derived exosomes were isolated and characterized using nanoparticle track analyzer and Western blotting. The HBM-derived exosomes treatment decreased the expression of inflammatory mediators and proinflammatory cytokines in mouse peritoneal macrophages upon lipopolysaccharide stimulation. Flow cytometry analysis of bone marrow-derived macrophages indicated that exosomes promoted M2 polarization, as evidenced by a decrease in cells expressing CD80 (M1 marker) and a concurrent increase in cells expressing M2 marker CD206. In addition, exosome treatment attenuated the mitogen-activated protein kinase signaling pathway by reducing the phosphorylation of extracellular signal-regulated kinase, c-Jun N-terminal kinase, p38 mitogen-activated protein kinase, and I κ B- α , thereby reducing the expression of inducible nitric oxide synthase, cyclooxygenase-2, metalloprotease (MMP)-1, MMP-3, and MMP-13 in SW1353 chondrocytes following IL-1 β stimulation. These findings suggest that HBM-derived exosomes promote macrophage polarization toward an anti-inflammatory M2 phenotype and exert significant immunomodulatory effects.

Key Words: Human breast milk, Exosome, Macrophage, Chondrocytes, Inflammation

INTRODUCTION

Human breast milk (HBM) is an ideal and essential source of nutrition for the healthy development of infants (Celik *et al.*, 2024), and accordingly, the World Health Organization (Aryeetey and Dykes, 2018), the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (Nutrition *et al.*, 2009), and the American Academy of Pediatrics recommend exclusive breastfeeding for the six months of life (Meek and Noble, 2022). In addition, HBM contains diverse biological compounds such as immune-regulating factors, which contribute to the maintenance and enhancement of the neonatal immune system (Andreas *et al.*, 2015).

Exosomes are membrane-bound extracellular vesicles (EVs) ranging in size between 30 nm and 150 nm (Colombo *et al.*, 2014). They carry complex molecular cargos such as proteins, lipids, mRNAs, and microRNAs (miRNAs), which reflect the physiological state of the maternal cell, modulate cellular responses, and serve as important mediators of intercellular

communication (Ballard and Morrow, 2013; Zonneveld *et al.*, 2014). Exosomes are present in various body fluids including HBM, and HBM-derived exosomes have been shown to resist digestive degradation and enter systemic circulation to influence gene expression or immune signaling in recipient cells (Celik *et al.*, 2024). Specifically, Kim *et al.* have reported that HBM-derived exosomes modulate macrophage and T cell responses, promoting regulatory T cell (Treg) differentiation while attenuating pro-inflammatory Th1 and Th17 activity (Kim *et al.*, 2025).

Juvenile idiopathic arthritis (JIA) is the most common chronic rheumatic disease in children, characterized by persistent joint inflammation with onset before the age of 16 (Sudhakar and Kumar, 2024). JIA represents a clinically heterogeneous group of disorders and includes multiple subtypes, such as systemic, oligoarticular, and polyarticular forms. The pathogenesis of JIA is believed to involve both innate and adaptive immune dysfunction (Long and Marston, 2023). Systemic JIA, in particular, is more autoinflammatory in nature, character-

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ized by excessive activation of monocytes, macrophages, and neutrophils leading to cytokine storms driven by IL-1, IL-6, and IL-18. Other forms of JIA, however, are more autoimmune and driven by autoreactive T cells and autoantibodies (Prakken *et al.*, 2011). Although the etiology of JIA is multifactorial, involving genetic predisposition and environmental triggers, increasing evidence suggests that early-life immune modulation plays a significant role in disease susceptibility (Bansal *et al.*, 2023).

Epidemiological studies have reported an inverse association between breastfeeding and the incidence of JIA (Kindgren *et al.*, 2017). A case-control study in a prospective birth cohort in Sweden showed that breastfeeding for more than four months was associated with a reduced risk of JIA (Kindgren *et al.*, 2017). Similar findings were reported in cohort studies conducted in UK (Hyrich *et al.*, 2016), indicating that early-life exposure to breast milk may modulate immune responses in a way that reduces autoimmune susceptibility (Kindgren *et al.*, 2017). However, the mechanisms by which breastfeeding confers such protection are not well understood. Specifically, the role of HBM-derived exosomes in modulating inflammatory responses relevant to JIA has not been systematically investigated. Given the immunoregulatory potential of breast milk exosomes, particularly their capacity to influence T cell differentiation and inflammatory signaling, it is plausible that they contribute to the observed protective effects.

In this study, we aim to isolate and characterize exosomes from HBM, and investigate their immunomodulatory effects in the context of JIA. By elucidating how these exosomes regulate immune pathways involved in JIA pathogenesis, we seek to uncover novel insights into the early-life origins of autoimmunity and the therapeutic potential of natural exosomal cargo.

MATERIALS AND METHODS

Collection of human breast milk samples

HBM was obtained from consenting lactating mothers at the Chung-Ang University Breastfeeding Research Institute in Seoul, South Korea, under IRB approval (IRB NO: 1041078-20251016-BR-375). Freshly collected milk was aliquoted into 15 mL tubes and stored at -80°C until use.

Isolation of breast milk-derived exosomes

To minimize donor variability, HBM from at least 10 individuals was pooled and thawed. Exosome isolation involved sequential centrifugation. First, samples were centrifuged at $2,000\times g$ for 10 min and $12,000\times g$ for 60 min at 4°C to obtain the whey. The resulting supernatants were transferred to Optiseal tubes (Beckman Coulter, Brea, CA, USA) and ultracentrifuged at $30,000\times g$ and $70,000\times g$ for 60 min each at 4°C (Beckman Coulter Optima XE-100 with Type 50.2 Ti Fixed angle ultracentrifuge rotor). The final supernatants were filtered through 0.8, 0.45, and 0.22 μm syringe filters (Sartorius AG, Göttingen, Germany), then centrifuged at $100,000\times g$ for 120 min at 4°C . The resulting pellets were resuspended in PBS and loaded onto a 35 nm qEV column (Izon Science Ltd., Christchurch, Canterbury, New Zealand), and fractions 7-9 were used for experiments.

Nanoparticle tracking analysis (NTA)

Exosome size and concentration were measured using a

NanoSight NS300 (Malvern Panalytical Ltd., Malvern, UK) with NTA 3.4 software. Samples were analyzed in flow mode at a syringe pump speed of 30; each result was captured ten times for 30 s using a 488 nm laser.

Isolation of peritoneal macrophages

Peritoneal macrophages were isolated from male C57BL/6 mice (≥ 5 weeks; Raonbio, Seoul, Korea) by injecting 10 mL PBS into the peritoneal cavity, performing a gentle massage, and collecting the suspension. After centrifugation, the cells were resuspended in RPMI 1640 (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS; Welgene), and 1% (v/v) penicillin/streptomycin (P/S; Gibco, Grand Island, NY, USA). The cells were incubated for at least 4 h at 37°C , and adherent cells were subsequently used for experiments.

In vitro differentiation of bone marrow-derived macrophages (BMM)

Bone marrow was extracted from the tibia and femur of euthanized male mice (C57BL/6) by flushing with ACK lysing buffer, and then centrifuged. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Welgene) supplemented with 10% FBS, 1% P/S, and 20 ng/mL M-CSF (Biolegend, San Diego, CA, USA). On day 5, adherent cells were seeded onto plates and treated with HBM-derived exosomes (250 or 500 $\mu\text{g/mL}$). After 24 h, M1 differentiation was induced with IFN- γ (Biolegend) and lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA), and M2 differentiation was induced with IL-4 (Biolegend) and IL-13 (Biolegend) for an additional 24 h.

SW1353 cell culture

SW1353 cells, the human chondrosarcoma cell line, were maintained in DMEM containing 10% (v/v) FBS and 1% (v/v) P/S at 37°C . When cells reached appropriate confluency, they were detached and passaged using a 1X working solution of Trypsin-EDTA (Welgene; 0.5% trypsin and 5.3 mM EDTA in 0.9% NaCl) prepared from a 10X stock. The culture medium was replaced two to three times per week.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to quantify the levels of TNF- α , IL-1 β , and IL-6 secreted by BMMs and peritoneal macrophages. Cells were seeded in 12-well plates and pretreated with exosomes (250 or 500 $\mu\text{g/mL}$) for 24 h, followed by stimulation with lipopolysaccharide (LPS, 1 $\mu\text{g/mL}$; Sigma-Aldrich) for 18 h. After stimulation, culture supernatants were collected and clarified by centrifugation to remove cell debris. Cytokine concentrations were determined by sandwich ELISA using specific antibody pairs according to standard protocols. Information on the antibody pairs used is as follows. Primary antibodies: hTNF- α (Mab1, Biolegend), hIL-6 (MQ2-13A5, eBioscience, San Diego, CA, USA), hIL-1 β (CRM56, eBioscience), mTNF- α (TN3-19.12, eBioscience), mIL-6 (MP5-20F3, BD Biosciences, Franklin Lakes, NJ, USA), mIL-1 β (B122). Secondary antibodies: hTNF- α (Mab11, Biolegend), hIL-6 (MQ2-39C3, eBioscience), hIL-1 β (CRM57, eBioscience), mTNF- α (13-7341-85, eBioscience), mIL-6 (MP5-32C11, BD Biosciences), mIL-1 β (13-7112-85, eBioscience).

To generate a standard curve, recombinant human TNF- α , IL-6, and IL-1 β cytokines (PeproTech®, Rocky Hill, NJ, USA) were used and streptavidin-conjugated alkaline phosphatase (Code:016-050-084, Jackson ImmunoResearch, West Grove,

Table 1. Primer sequences used in this study

GENE	Primer	Sequence
iNOS	Forward	5'-AGG GAC AAG CCT ACC CCT C-3'
	Reverse	5'-CTC ATC TCC CGT CAG TTG GT-3'
COX-2	Forward	5'-CTG GCG CTC AGC CAT ACA G-3'
	Reverse	5'-CGC ACT TAT ACT GGT CAA ATC CC-3'
MMP-1	Forward	5'-ATG AAG CAG CCC AGA TGT GGA G-3'
	Reverse	5'-TGG TCC ACA TCT GCT CTT GGC A-3'
MMP-3	Forward	5'-CAC TCA CAG ACC TGA CTC GGT T-3'
	Reverse	5'-AAG CAG GAT CAC AGT TGG CTG G-3'
MMP-13	Forward	5'-CCA ACC CTA AAC ATC CAA AAA C-3'
	Reverse	5'-AAA AAC AGC TCC GCA TCA AC-3'

iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; MMP-1, metalloprotease (MMP)-1.

PA, USA) served as the detection reagent. For substrate development, substrate tablets (Sigma-Aldrich, N2765, 20 mg) were freshly dissolved in substrate buffer at a final concentration of 1 mg/mL. Absorbance was measured at 405 nm using a microplate reader (Emax microplate reader, Molecular Devices, Sunnyvale, CA, USA).

Quantitative analysis of mRNA expression

Total RNA was isolated from 3×10^5 cells using RNAiso Plus (Takara Bio Inc., Kusatsu, Japan), followed by chloroform (Sigma-Aldrich) extraction and isopropanol (2-Propanol, 99.5%; Samchun Pure Chemical Co., Korea) precipitation. After washing the RNA pellet with 75% ethanol, samples were air-dried and dissolved in RNase-free water. The RNA was incubated at 55–60°C to enhance solubilization. The extracted RNA was then reverse-transcribed into cDNA using a commercially available reverse transcription kit. Quantitative PCR was conducted using the TOPreal™ SYBR Green RT-qPCR Kit (Enzynomics, Daejeon, Korea) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Gene expression was analyzed in macrophages and SW1353 cells. The primer sequences used in this study are listed in Table 1.

Western blotting

Western blotting was performed to verify exosome identification and assess the activation of inflammatory signaling pathways.

To verify successful exosome isolation, whey and exosomes were separately collected into 1.5 mL tubes and stored at –20°C before use. Protein concentration of both samples were quantified using Pierce™ BCA Protein Assay Kit (Thermo-Fisher Scientific, Rockford, IL, USA), and exosome-specific markers Flotillin-1 (#18634, Cell signaling Technology, Danvers, MA, USA), HSP70 (#4876, Cell signaling Technology), CD81 (#56039, Cell signaling Technology), CD9 (#13174, Cell signaling Technology), Annexin V (#8555, Cell signaling Technology) were analyzed by western blotting. The detailed western blotting procedure was identical to the method described below.

SW1353 cells were seeded in 12-well plates at a density of 3×10^5 cells/mL under four experimental conditions: negative control (NC), positive control (PC), and treatment groups with 250 µg/mL and 500 µg/mL exosomes, respectively. The day after seeding, cells in the treatment groups were treated with

exosomes at 250 or 500 µg/mL. After 24 h, all wells except the NC group were stimulated with Human recombinant IL-1β (Cat. #200-01B, PeproTech®) and incubated for 30 min to activate inflammatory signaling pathways.

Cells were lysed using Pierce™ RIPA Buffer (Thermo-Fisher Scientific), and total protein was quantified using the Pierce™ BCA Protein Assay Kit (Thermo-Fisher Scientific). Equal amounts of denatured protein were separated on 12% SDS-PAGE gels, and transferred to polyvinylidene fluoride (PVDF) membranes (Amersham, Piscataway, NJ, USA) using a wet transfer system at 4°C.

Membranes were blocked with 5% skim milk in TBS-T for 1 h, then incubated overnight at 4°C with primary antibodies targeting phospho-p38 mitogen-activated protein kinase (MAPK) (Thr180/Tyr182, #4631S, Cell signaling Technology), total p38 mitogen-activated protein kinase (p38 MAPK) (clone: Poly6224, BioLegend), phospho-ERK1/2 (Thr202/Tyr204, #4370T, Cell signaling Technology), total ERK1/2 (sc-514302, Santa Cruz Biotechnology, Dallas, TX, USA), phospho-JNK (Thr183/Tyr185, #4668S, Cell signaling Technology), total c-Jun N-terminal kinase (JNK) (#9252, Cell signaling Technology), phospho-IκBα (Ser32/36, #9246S, Cell signaling Technology), total IκBα (#4812S, Cell signaling Technology), and GAPDH (#2118S, Cell signaling Technology). Following incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG-HRP, anti-mouse IgG-HRP), for 1 h, protein bands were visualized using the West-saves Femto™ enhanced chemiluminescence substrate (Ab- Frontier, Seoul, Korea) on a Fusion Solo X imaging system (Vilber, Paris, France). Band intensities were quantified using Fusion software, and phosphorylated proteins were normalized to their respective total protein levels or GAPDH.

Flow cytometry

M1/M2 differentiation of BMM was analyzed by flow cytometry. Following cytokine treatment, cells were harvested, washed, and incubated with Fc block (0.1 µL per sample) to minimize nonspecific binding. Subsequently, cells were stained with fluorochrome-conjugated antibodies against F4/80 (BioLegend, 123107), CD11b (BioLegend, 101212), CD80 (BioLegend, 104733), and CD206 (BioLegend, 162503) (0.2 µL per sample, with PE-conjugated antibodies at 0.1 µL). Samples were incubated for 30 min at 4°C in the dark, washed, and then subjected to fixation and permeabilization (Fix:Perm=1:3, 200 µL per sample) for 30 min. Intracellular

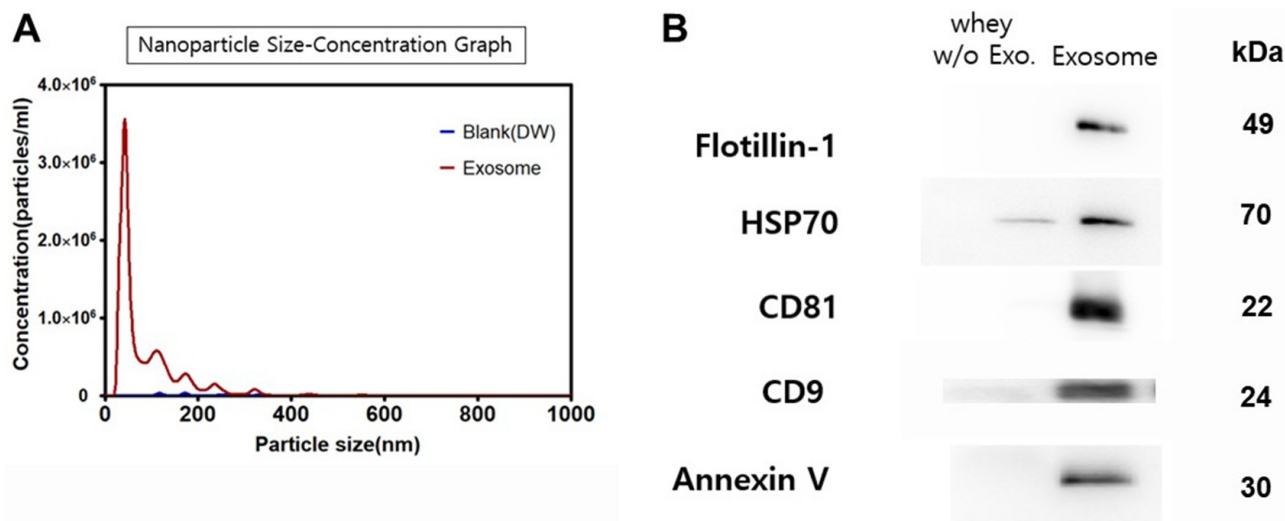


Fig. 1. Characterization of HBM-derived exosomes. (A) Particle size distribution of HBM-derived exosomes as determined by NTA. (B) Western blot analysis confirming the expression of standard exosomal markers.

staining was carried out using the same antibody concentrations prepared in permeabilization buffer.

Data were acquired on a FACS Calibur flow cytometer (BD Biosciences), and analysis was conducted using CellQuest Pro software. Macrophage populations were gated on CD11b⁺F4/80⁺ cells, and their polarization status was determined by identifying CD80⁺CD206⁻ cells as M1 macrophages and CD80⁻CD206⁺ cells as M2 macrophages.

Statistical analysis

All experiments were performed in at least three replicates, and the results from each replicate are presented as the mean $\pm$ SEM using GraphPad Prism v. 5 software (GraphPad, San Diego, CA, USA). Statistical analysis was conducted using one-way ANOVA, and differences were considered significant at $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$.

RESULTS

Characterization of HBM-derived exosomes

HBM-derived exosomes were collected using ultracentrifugation. Their size was analyzed through NTA, which indicated a mean size of approximately 88.1 nm, and a most frequently detected particle size of 42.5 nm (Fig. 1A). Additionally, the expression of exosomal markers such as Flotillin-1, HSP70, CD81, CD9, and Annexin V was confirmed via Western blot (Fig. 1B).

Anti-inflammatory effects of HBM-derived exosomes on peritoneal macrophages

To evaluate the anti-inflammatory effects of HBM-derived exosomes on peritoneal macrophages, ELISA, qPCR, and Western blot analysis were conducted. Exosome treatment decreased the secretion of pro-inflammatory cytokines including TNF- α , IL-6, and IL-1 β , upon LPS stimulation (Fig. 2A). Additionally, the expression of inflammatory factors such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2

(COX-2) was decreased at both mRNA (Fig. 2B) and protein (Fig. 2C) levels.

Effects of HBM-derived exosomes on BMM differentiation

To investigate the effects of HBM-derived exosomes on M1/M2 differentiation of BMMs, FACS and ELISA analysis were performed. FACS results demonstrated that HBM-derived exosomes decreased the expression of the M1 marker CD80 while increasing the expression of the M2 marker CD206 dose-dependently (Fig. 3A, 3B). Furthermore, ELISA analysis of the supernatant revealed a concentration-dependent decrease in the secretion of pro-inflammatory cytokines, including TNF- α , IL-6, and IL-1 β , in BMM upon LPS stimulation (Fig. 3C).

Anti-inflammatory effects of HBM-derived exosomes in SW1353 cells

Building on the previously observed anti-inflammatory effects of exosomes on macrophages, we addressed their effects on chondrocytes. SW1353 chondrocytes were stimulated with IL-1 β to induce inflammation, and the expression of MMP-1, MMP-3, MMP-13, iNOS, and COX-2 was assessed by qPCR. MMPs, as zinc-dependent endopeptidases, are crucial for the degradation of extracellular matrix components and play significant roles in various processes, including tissue remodeling, inflammation, and wound healing. Measuring MMP expression levels allowed us to evaluate the potential of HBM-derived exosomes to reduce IL-1 β -induced inflammation and cartilage degradation. The data showed that exosomes reduced the expression of MMP-1, MMP-3 and MMP-13, as well as the inflammatory mediators, iNOS and COX-2 (Fig. 4).

Furthermore, Western blot analysis demonstrated that HBM-derived exosomes attenuated the phosphorylation of extracellular signal-regulated kinase (ERK), JNK, p38, and I κ B α in a dose-dependent manner (Fig. 5A). ERK, JNK, and p38 are major components of the MAPK pathway, while I κ B α is a key regulator of NF- κ B activation. Quantification based on the phospho/total protein ratio also confirmed that higher concentrations of exosomes consistently led to greater suppression

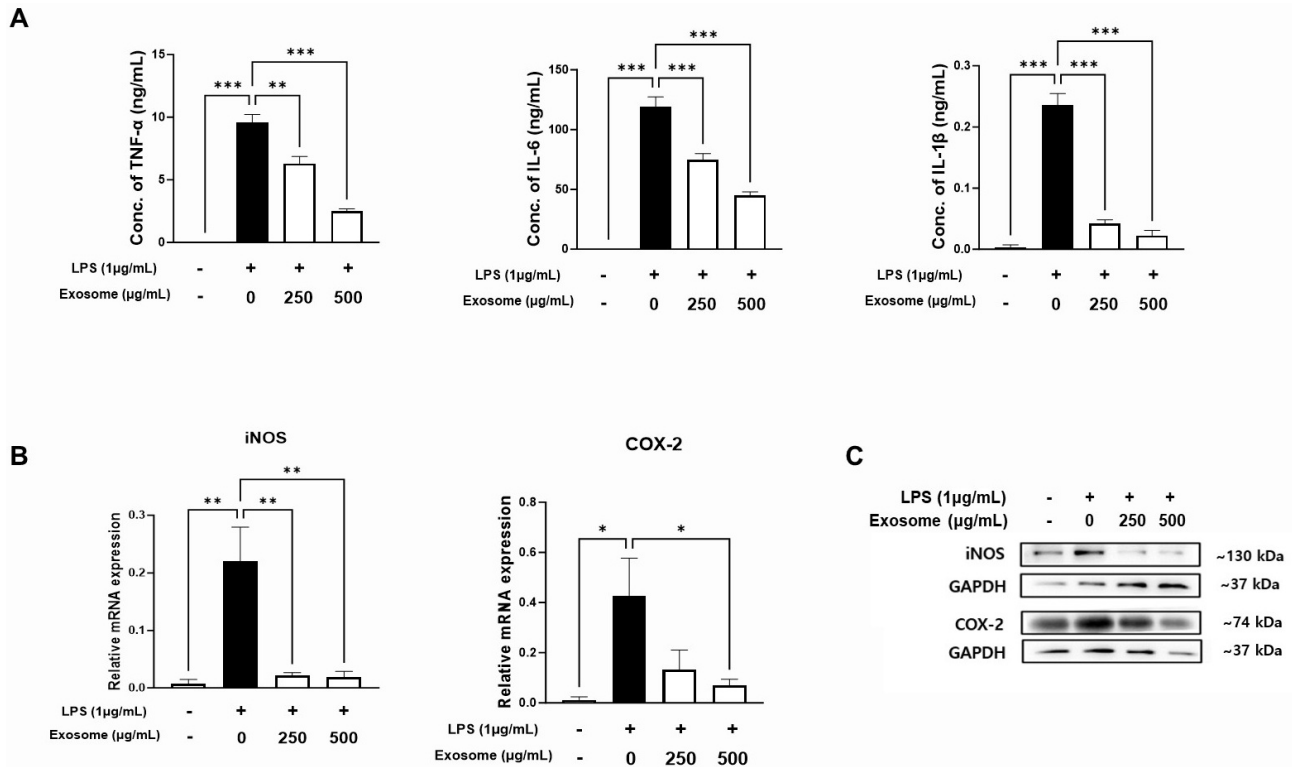


Fig. 2. Anti-inflammatory effects of HBM-derived exosomes on peritoneal macrophages. (A) Macrophages were treated with HBM-derived exosomes and then stimulated with LPS. The supernatant was subsequently analyzed by ELISA to measure the secretion of TNF- α , IL-6, and IL-1 β . (B) qPCR and (C) Western blot analysis were performed to measure the expression of iNOS and COX-2 at mRNA and protein levels, respectively. Data shown are representative of three independent experiments with similar results. Statistical significance was denoted as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

of these signaling molecules (Fig. 5B).

DISCUSSION

HBM is rich in both macronutrients and micronutrients essential for the health of infants (Kim and Yi, 2020). Beyond its nutritional value, HBM contains numerous bioactive components which crucially support the infant's growth and development. Exosomes specifically extracted from HBM were first studied in 2007, and marked an important milestone in understanding the role of maternal bioactive compounds (Admyre *et al.*, 2007). These exosomes transfer proteins, lipids, nucleic acids, and other cellular materials to recipient cells, thereby influencing various biological processes, including inflammation, stress defense, stem cell growth, etc. (Chutipongtanate *et al.*, 2022). Breastfeeding, therefore, offers notable health benefits to both mother and infant (Dieterich *et al.*, 2013), and according to the Developmental Origins of Health and Disease hypothesis, early exposure to environmental factors in HBM may influence long-term health outcomes and disease risk (Holme and Sitras, 2020).

Given the established importance of HBM-derived exosomes, we aimed to evaluate their anti-inflammatory properties through *in vitro* experiments involving peritoneal macrophages, BMM, and the SW1353 chondrocyte cell line. In peritoneal macrophages, where inflammation was induced

by LPS, HBM-derived exosome treatment led to a dose-dependent reduction in pro-inflammatory cytokine secretion, and iNOS and COX-2 expression (Fig. 2). To further characterize this anti-inflammatory activity, we evaluated the impact of HBM-derived exosomes on macrophage polarization in BMM. Macrophages are central players in inflammation and can adopt a spectrum of polarization states, from pro-inflammatory M1 to anti-inflammatory M2 phenotypes (La Bella *et al.*, 2023; Locati *et al.*, 2020; Luo *et al.*, 2024; Schmidt *et al.*, 2020). M1 macrophages promote tissue damage by secreting TNF- α and IL-1 β , while M2 macrophages aid in tissue repair and immune resolution (Chen *et al.*, 2023; Locati *et al.*, 2020; Luo *et al.*, 2024). Macrophages have emerged as central players in synovial inflammation, where dysregulated innate immune responses contribute to the progression of various inflammatory joint conditions such as rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) (Fall *et al.*, 2007; Udalovala *et al.*, 2016; Inoue and Schulert, 2023). Our results demonstrated that HBM-derived exosomes modulate macrophage polarization toward an anti-inflammatory M2 phenotype (Fig. 3), implying the potential importance of HBM-derived exosomes in modulating inflammatory joint diseases.

Furthermore, to investigate the potential relevance of these findings to arthritis, we used SW1353 chondrocyte cell line. Inflammation was induced by IL-1 β , and the effect of HBM-derived exosomes on the expression of iNOS, COX-2, MMP-1, MMP-3, and MMP-13 was assessed. Exosome treatment

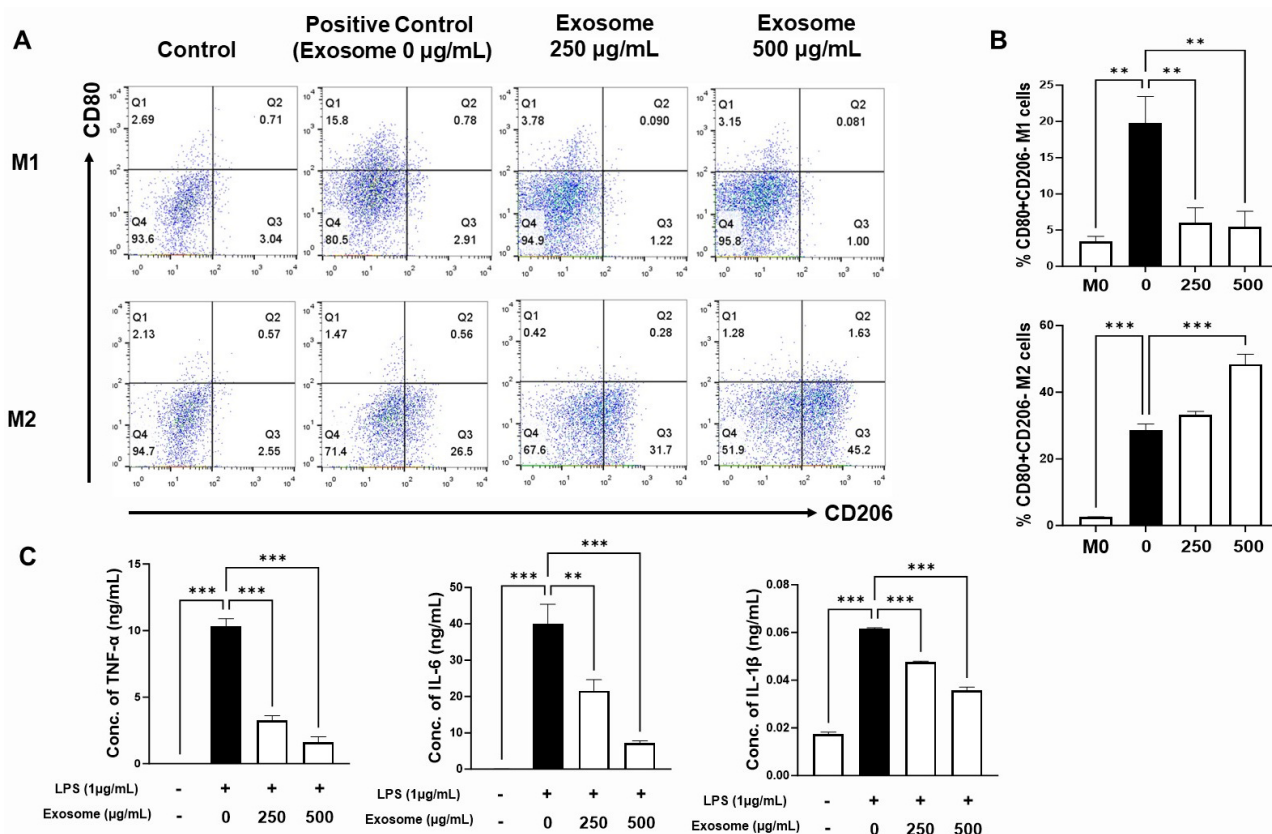


Fig. 3. The effects of HBM-derived exosomes on BMM differentiation. (A) Flow cytometry analysis of BMM differentiation. CD11b⁺ F4/80⁺ cells were gated to assess the effects of exosomes on the differentiation of M1 (CD80⁺CD206⁺) and M2 (CD80⁺CD206⁺) macrophages. (B) Quantification of M1 and M2 macrophage differentiation. (C) ELISA analysis of proinflammatory cytokine secretion (TNF-α, IL-6, and IL-1β) from LPS-stimulated macrophages. Data are presented as mean ± SEM and are representative of three independent experiments with similar results. Statistical significance was denoted as follows: ***p* < 0.01; ****p* < 0.001.

significantly reduced the expression of these inflammatory mediators and MMPs (Fig. 4). Since MMPs are crucial for the degradation of extracellular matrix components and are deeply implicated in cartilage degradation and chronic inflammation, this finding strongly suggests that HBM-derived exosomes can suppress IL-1β induced-catabolic activity. Moreover, Western blot analysis demonstrated that HBM-derived exosomes attenuated the phosphorylation of ERK, JNK, p38 – major components of the MAPK pathway – and IκBα – a key regulator of NF-κB activation (Fig. 5). Together, these pathways orchestrate the induction of pro-inflammatory cytokines and matrix-degrading enzymes in response to IL-1β stimulation. Our results indicate that HBM-derived exosomes mitigate IL-1β-induced inflammation in SW1353 cells by downregulating MMPs, iNOS, and COX-2 expression through the inhibition of key MAPK and NF-κB signaling cascades.

In a previous study (Kim *et al.*, 2023), we performed comprehensive proteomic and miRNA profiling of milk-derived exosomes and identified approximately 377 proteins and 208 miRNAs. Functional enrichment analyses (GO and KEGG) revealed that these components are significantly involved in modulating various physiological processes, particularly inflammatory signaling pathways. We speculate that specific bioactive molecules within the exosomal cargo, such as miRNAs and proteins associated with signal transduction, work

synergistically to suppress the MAPK and NF-κB signaling cascades, thereby reducing the expression of MMPs, iNOS, and COX-2. While further research is needed to identify the exact contribution of individual component, our data provide a strong mechanistic basis for the anti-inflammatory and anti-catabolic effects of HBM-derived exosomes in chondrocytes.

In addition, while our findings provide valuable insights into the immunomodulatory effects of HBM-derived exosomes, this study has some limitations. Although the IL-1β-stimulated SW1353 system is a useful *in vitro* model for studying inflammatory responses, it does not fully recapitulate the complex, multi-cellular pathophysiology of inflammatory joint diseases. Specifically, this model lacks the intricate interactions between immune cells and synovial tissues. Furthermore, while primary human chondrocytes or synoviocytes would offer higher clinical relevance, we utilized cell lines in this study. Future studies should employ primary human-derived systems or *in vivo* models to further validate these findings in a clinical context.

In conclusion, our findings demonstrate that HBM-derived exosomes modulate macrophage polarization toward the M2 phenotype and exhibit significant anti-inflammatory and anti-catabolic effects by inhibiting the MAPK and NF-κB signaling pathways. These results offer new insights into the potential role of HBM-derived exosomes in managing inflammatory response relevant to joint diseases.

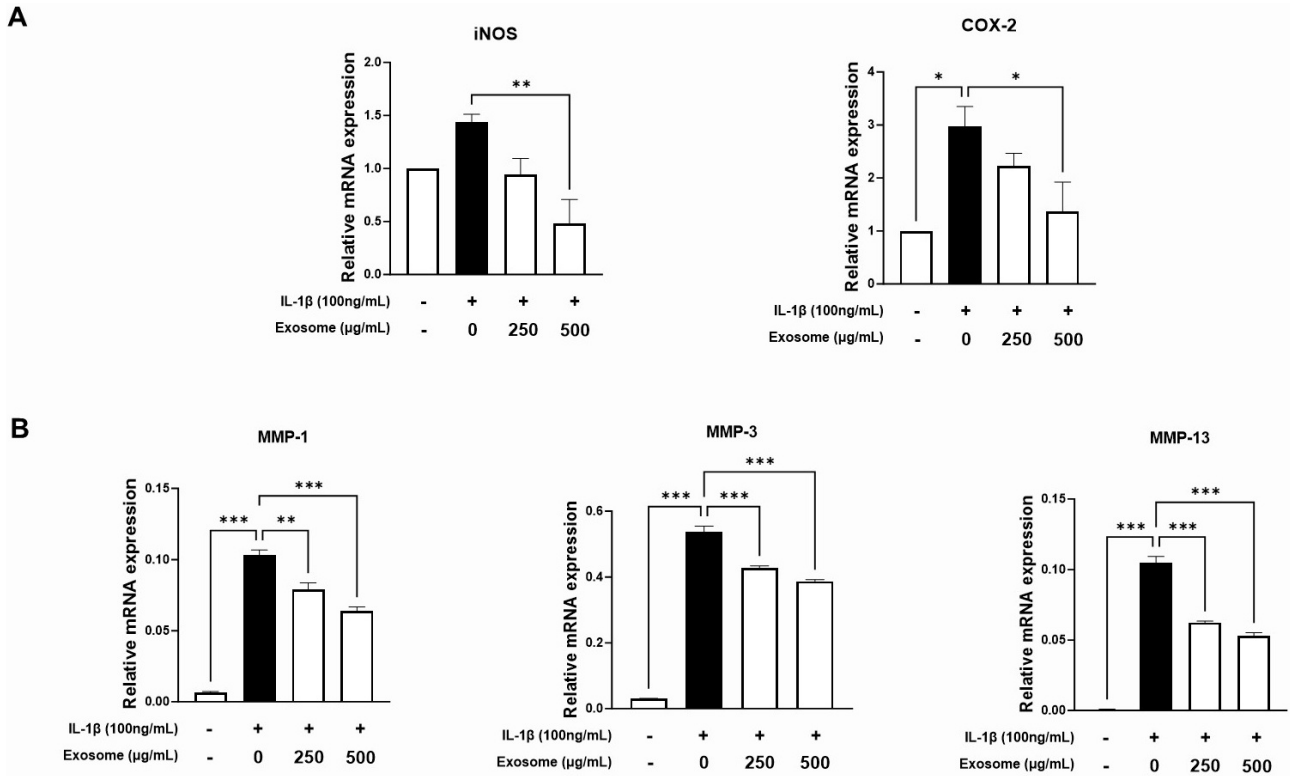


Fig. 4. Suppression of IL-1 β -induced inflammation in SW1353 cells by exosome treatment. SW1353 chondrocytes were stimulated with IL-1 β (100 ng/mL), and the effects of HBM-derived exosomes on the mRNA expression of iNOS and COX-2 (A), and MMP-1, MMP-3, and MMP-13 (B) were measured by qPCR. Data are presented as mean $\pm$ SEM and are representative of three independent experiments with similar results. Statistical significance was denoted as follows: * p <0.05; ** p <0.01; *** p <0.001.

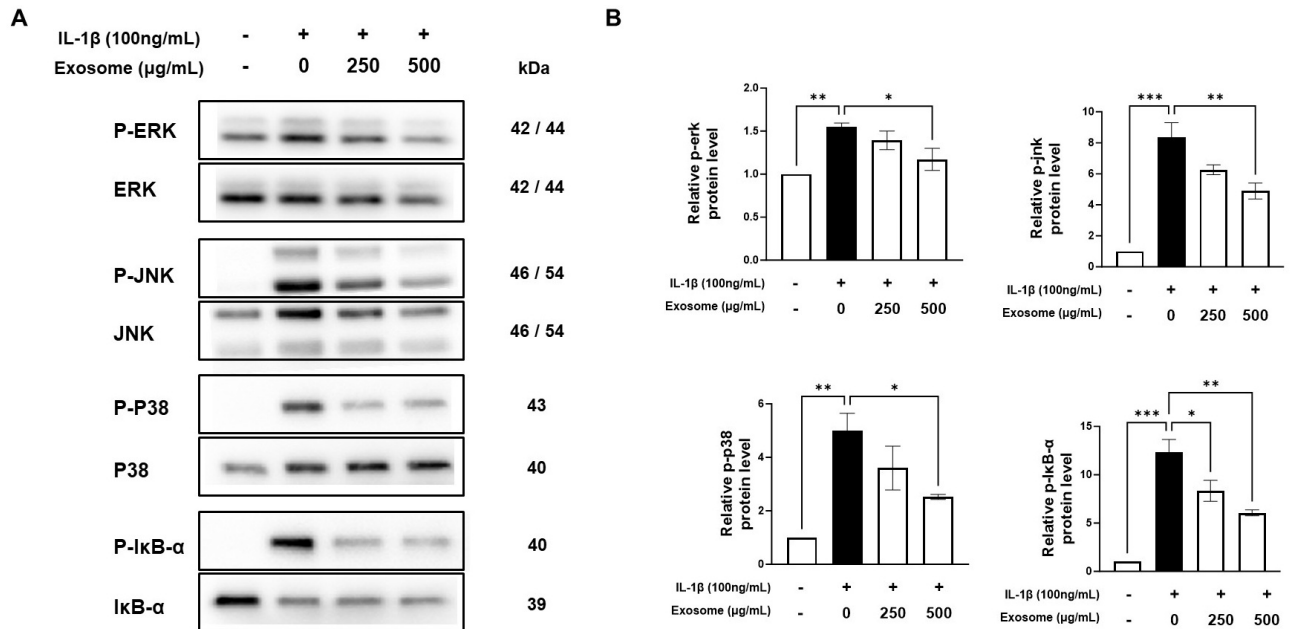


Fig. 5. Molecular mechanism of anti-inflammatory effects of HBM-derived exosomes in SW1353 cells. (A) Western blot analysis of phosphorylated and total ERK, JNK, p38, and I κ B α in SW1353 cells stimulated with IL-1 β (100 ng/mL) with or without exosome treatment (0, 250, or 500 μ g/mL). (B) Quantification of phosphorylation levels normalized to total protein (phospho/total ratio). Data are shown as mean $\pm$ SEM and are representative of three independent experiments with similar results. Statistical significance was denoted as follows: * p <0.05; ** p <0.01; *** p <0.001.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare. All authors have seen and agree with the contents of the manuscript.

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AUTHOR CONTRIBUTIONS

Methodology, Formal analysis, and Writing-original draft: D.K., and J.Y.Y. Methodology: K.-B.D., and K.-U.K. Conceptualization: J.-Y.L. and H.M. Writing-original draft, Writing-review & editing, Project administration and Supervision: H.M. All authors reviewed the manuscript.

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