



Bovine colostrum exosomes mediate anti-inflammatory responses in macrophages via the miR-30a-5p/SOX4 axis

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ABSTRACT

Bovine colostrum is essential for neonatal immunity and development, with its exosomal cargo of microRNAs (miRNA) playing a key role in intercellular communication. However, the specific immunomodulatory functions of these exosomes and their temporal variation remain unclear. This study aimed to isolate and characterize exosomes from bovine colostrum at 2 h (2h-Exo) and 24 h (24h-Exo) postpartum, evaluate their effects on cell proliferation and inflammation, and identify the underlying miRNA-mediated mechanisms. Exosomes were successfully isolated using an optimized method combining rennet enzyme digestion, filtration, and differential ultracentrifugation. Small RNA sequencing revealed 69 differentially expressed miRNAs between 2h-Exo and 24h-Exo (22 upregulated, 47 downregulated). Functionally, in an *in vitro* LPS-induced inflammatory model using macrophages, 2h-Exo demonstrated superior anti-inflammatory activity. It significantly reduced the expression of key proinflammatory cytokines, namely TNF- α , IL-1 β , and IL-6, as well as IL-10, compared with 24h-Exo. Bioinformatic and functional analyses identified bta-miR-30a-5p as a critical, temporally enriched miRNA in 2h-Exo. Mechanistically, dual-luciferase reporter assays confirmed that bta-miR-30a-5p directly targets the 3'-untranslated region of the transcription factor *SOX4*. Furthermore, miR-30a-5p mimic transfection recapitulated the anti-inflammatory effect by downregulating *SOX4* and attenuating the expression of TNF- α and IL-6. This study demonstrates that bovine colostrum exosomes, particularly those harvested immediately postpartum, possess potent immunomodulatory capacity. The anti-inflammatory effect is mediated, at least in

part, by the exosomal delivery of bta-miR-30a-5p and its subsequent targeting of *SOX4* mRNA. These findings highlight early colostrum as a valuable source of therapeutic exosomes and elucidate a specific miRNA-mRNA axis for regulating inflammation.

Key words: bovine colostrum, exosome, miR-30a-5p, inflammatory response

INTRODUCTION

Neonatal calves are exceptionally vulnerable to enteric infections due to their underdeveloped immune systems, making the establishment of effective early-life defenses critical. Bovine colostrum, commonly defined as the mammary secretion produced within the first 3 d postpartum (Han et al., 2023), serves as a cornerstone of passive immunity. It delivers not only essential nutrients but also a rich array of immunologically active molecules, including IgG, lactoferrin, and antimicrobial peptides (McGrath et al., 2016; Ghosh and Iacucci, 2021). However, the concentration of these bioactive components declines precipitously after birth. The IgG levels measurably decrease within hours, with colostrum collected at 6 h postpartum already showing significantly lower concentrations than that collected at 2 h postpartum (Larson et al., 1980; Pritchett et al., 1994; Moore et al., 2005). Consequently, collecting high-quality colostrum within the first 2 h is a gold standard to maximize passive immune transfer.

Beyond classical immune factors, colostrum contains extracellular vesicles, particularly exosomes, which are now recognized as pivotal mediators of intercellular communication and regulators of nutritional and immune functions (Santoro et al., 2023; Yang et al., 2024). Exosomes are nanosized (30–150 nm) vesicles that carry a diverse cargo of proteins, lipids, and nucleic acids, including regulatory microRNAs (miRNA; Chavda et al., 2023). Milk is an exceptionally rich source of exosomes,

Received September 24, 2025.

Accepted January 30, 2026.

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-26. Nonstandard abbreviations are available in the Notes.

and they have been isolated from the colostrum and milk of multiple species, including humans, bovines, and pigs (Zempleni et al., 2019; Feng et al., 2021). Studies have reported on the composition and content of miRNAs in milk-derived exosomes from various domestic animals, including Holstein cows (Wang et al., 2022b), buffaloes (Rani et al., 2020), cattle (Ren et al., 2022), pigs (Chen et al., 2014), and donkeys (Chai et al., 2022). Crucially, the bioactivity of milk-derived exosomes exhibits significant functional plasticity, heavily influenced by the lactation stage (Hussain et al., 2025). For instance, exosomes from human colostrum demonstrate superior protective effects against LPS-induced intestinal injury compared with those from mature milk (Wang et al., 2022a). Exosomes derived from different periods of human breast milk production were able to protect intestine organoids against epithelial injury induced by LPS, with colostrum exosomes showing the best protective effects (Gao et al., 2019b). Furthermore, colostrum exosomes are enriched with immune-related miRNAs capable of modulating gene regulation in the newborn (Sun et al., 2013; Ma et al., 2022). These findings support a compelling hypothesis: exosomes harvested during the critical first 2 h postpartum (the same window defined for optimal IgG transfer) may possess unique immunomodulatory potency. Yet, the specific anti-inflammatory functions and mechanisms of these early-collected bovine colostrum exosomes remain unknown.

Inflammation is a fundamental immune response coordinated by innate immune cells such as macrophages. These cells are central orchestrators of innate immunity and can polarize into proinflammatory (M1) or anti-inflammatory (M2) phenotypes in response to stimuli (Taciak et al., 2018). In neonatal calves, many pathogens target the digestive system, making the regulation of local and systemic inflammation critical. To study inflammatory pathways in vitro, the RAW 264.7 macrophage cell line stimulated with LPS serves as a well-established model (Facchin et al., 2022; Mathew et al., 2024). A low dose of LPS is sufficient to activate these immune cells and establish a robust inflammatory model (Zhang et al., 2024c). Furthermore, RAW 264.7 cells, derived from a monocyte-macrophage lineage, exhibit the diversity and plasticity characteristic of primary macrophages. As previously revealed, milk-derived exosomes can modulate macrophage immunity (Pieters et al., 2015), we used this model to investigate the specific anti-inflammatory effects of bovine colostrum exosomes. We examined their effect on the expression of key inflammatory mediators in LPS-stimulated RAW 264.7 cells. The LPS triggers a robust M1 response, driving the production of cytokines such as TNF- α , IL-1 β , and IL-6, and nitric oxide (NO; Serreli et al., 2019; Jhamat et al., 2020), while also inducing a delayed anti-inflammatory feedback

involving IL-10 (Libby, 2007). The TNF- α , IL-6, and IL-1 β are proinflammatory factors, whereas IL-10 is an anti-inflammatory factor. These key inflammatory factors play a crucial role in regulating the inflammatory response and maintaining immune homeostasis. The IL-10 is pivotal in suppressing inflammation and innate immune activation. However, the molecular mechanisms by which bovine colostrum exosomes and their miRNA cargo might modulate this balance are unexplored.

Therefore, to address the significant knowledge gap regarding the functional role of exosomes from the most critical postpartum period, this study aimed to (1) investigate the anti-inflammatory potential of bovine colostrum exosomes collected within 2 h of parturition against LPS-induced macrophage activation and (2) explore the underlying molecular mechanisms, with a focus on the contribution of exosome-derived miRNAs. Our findings provide novel insights into how colostrum exosomes regulate immune responses, offering a theoretical basis for understanding their role in neonatal immune programming and strengthening the rationale for ultra-early colostrum administration.

MATERIALS AND METHODS

Animals and Colostrum Sample Collection

Raw colostrum was obtained from 6 healthy Holstein cows (5–6 yr old, free of clinical mastitis) at the Dairy Cattle Breeding Center of Xinjiang Western Animal Husbandry Co. Ltd. (Shihezi, China) in March, 2023. All animals were housed under identical feeding and management conditions, and calving occurred within a period of 1 wk. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Shihezi University, Protocol Number A2022-420.

Colostrum samples (500 mL per cow) were collected aseptically at 2 h and 24 h after calving. Samples were immediately transported to the laboratory on ice. For each time point, colostrum from each cow was pooled to create a composite sample. The pooled sample was then divided into 50 mL centrifuge tubes and subjected to 2 rounds of centrifugation ($5,000 \times g$, 30 min, 4°C) to remove the upper fat layer and the lower pellet of cellular debris and casein micelles. The clarified, defatted middle layer (skimmed colostrum) was carefully collected, aliquoted, and stored at -80°C until exosome isolation.

Isolation and Characterization of Bovine Colostrum Exosomes

Bovine colostrum exosomes were isolated and characterized using methods adapted from previously described protocols (Gao et al., 2019a). Briefly, rennet enzymes (1.5

g/L; calf abomasum chymosin, Beijing Doit Biotechnology Co. Ltd.) were activated in a 1% NaCl solution at 37°C for 30 min. This activated rennet enzyme solution was then added to skimmed colostrum and incubated at 37°C for 30 min to remove casein. The whey was collected by low-speed refrigerated centrifugation (Thermo Fisher Scientific, Waltham) at $12,000 \times g$, 4°C for 60 min, and then filtered through a 0.45 μm filter. The filtered supernatant was subjected to high-speed refrigerated centrifugation (CR22N, Hitachi, Chiyoda) at $30,000 \times g$, 4°C for 60 min, followed by filtration through a 0.22 μm filter. The resulting filtrate was then further processed using ultra-fast refrigerated centrifugation (Optima XPN-100, Beckman Coulter Instruments, Fullerton) at $120,000 \times g$, 4°C for 2 h. The supernatant was discarded, and the exosome pellet was resuspended in $1 \times \text{PBS}$ buffer. This resuspended exosome solution was centrifuged again at $120,000 \times g$, 4°C for 2 h, respectively. Finally, the final exosome pellet was collected, resuspended, and stored at -80°C until further use. The concentration of exosome protein was determined using a bicinchoninic acid (BCA) protein concentration assay kit (Beijing Cool Lab Technology Co. Ltd.).

Exosomes were verified by scanning electron microscopy. For this, exosomes dissolved in PBS were deposited onto a 400-mesh copper grid and allowed to stand for 2 min. Excess solution was removed with filter paper, and the grid was stained with 2% phosphotungstic acid for 2 min. After removing the excess staining solution with filter paper, the grid was washed with PBS and dried at room temperature. The morphology and size of the exosomes were observed using a scanning electron microscope (SU8010, Hitachi) at 100 kV.

Finally, classical markers of exosomes, tumor susceptibility gene 101 (TSG101), and ALG-2-interacting protein X (Alix; Lee et al., 2024) were performed for positive expression by western blot.

Cell Culture

Primary Culture of Fetal Lamb Abomasum Smooth Muscle Cells. Primary cultures of fetal lamb abomasum smooth muscle cells were established from tissue aseptically harvested at ~60 d of gestation. The tissue was rinsed 3 times with PBS containing 1% penicillin-streptomycin to remove residual blood. The seromuscular layer was then meticulously dissected, minced, and digested with trypsin. Digestion was terminated by adding complete medium supplemented with 10% fetal bovine serum (catalog no. E600001, Sangon Biotech). The resulting cell suspension was centrifuged, resuspended in fresh medium, transferred to a culture dish, and placed undisturbed in a humidified incubator (37°C, 5% CO_2). The initial medium change was performed after 2 to 3

d, followed by routine medium replacement every 48 h. Once a substantial number of cells had migrated from the explants, the residual tissue fragments were carefully removed. Cells were further expanded, and smooth muscle cell identity was confirmed by morphological assessment and detection of specific marker genes α -smooth muscle actin (α -SAM) and Transgelin (TAGLN; Supplemental Figure S1, see Notes), and the primer sequences are listed in Supplemental Table S1, see Notes.

Macrophage Culture. The RAW 264.7 cells were obtained from iCell (Bioscience Inc.). The cells were cultured in Dulbecco's Modified Eagle Medium (catalog no. BL304A, Biosharp) supplemented with 10% fetal bovine serum (catalog no. E600001, Sangon Biotech) and 100 $\mu\text{g}/\text{mL}$ penicillin-streptomycin solution (catalog no. E607011, Sangon Biotech) at 37°C with 5% CO_2 atmosphere. The medium was changed every 2 d. Cells were passaged upon reaching ~80% confluence.

Coculture with Bovine Colostrum-Derived Exosomes. For uptake and treatment studies, the third-passage abomasal smooth muscle cells and RAW 264.7 cells were seeded into 12-well plates at a density of 5×10^4 cells/mL. After 8 h of incubation, the medium was replaced with fresh complete medium containing colostrum exosomes (at 2 h postpartum [2h-Exo] or 24 h postpartum [24h-Exo]) at a final protein concentration of 200 $\text{ng}/\mu\text{L}$ for 24 h. Control cells were incubated with exosome-free complete medium for the same duration. Each condition was performed with 3 biological replicates, and the entire experiment was independently repeated 3 times.

Proliferation Assay. Cell counting kit-8 (CCK8; Biosharp, catalog no. BL1055B) assays were used to detect cell viability. Cells were seeded in 96-well plates at a density of 5×10^4 cells/mL. After adherent culture, the culture medium was removed, and cells were treated with 2h-Exo or 24h-Exo (200 $\text{ng}/\mu\text{L}$) for 24 h. Following treatment, the medium was removed, and 100 μL of complete medium containing 10% CCK-8 reagent was added to each well. The plate was then incubated for 2 h, and the absorbance was measured at 450 nm. Each experiment was performed with technical triplicates and repeated 3 times independently.

To assess the effect of colostrum exosomes on proliferation, the expression of the proliferation marker genes proliferating cell nuclear antigen (PCNA) and cyclin D1 (CCND1) in treated abomasal smooth muscle cells was analyzed by real-time quantitative PCR (qPCR). All primer sequences are listed in Supplemental Table S1.

Experimental Design for Exosome Treatment in an LPS-Induced Inflammation Model

To investigate the effect of bovine colostrum-derived exosomes (2h-Exo and 24h-Exo) on LPS-induced

inflammation, we implemented 2 distinct treatment protocols in macrophage cultures: (1) therapeutic treatment of established inflammation and (2) prophylactic prevention of inflammation onset. All experiments included the control groups described below and were performed in triplicate. Control group: Cells were maintained in complete medium (treated with PBS as vehicle). The LPS-induced inflammation model: RAW264.7 cells were treated with 1 $\mu\text{g/mL}$ LPS for 24 h to establish the inflammatory baseline, as previously described (Facchin et al., 2022).

Protocol #1: Therapeutic Treatment Model. To assess therapeutic potential, inflammation was first established in RAW 264.7 cells by stimulation with 1 $\mu\text{g/mL}$ LPS for 24 h. Subsequently, the culture medium was replaced with fresh complete medium containing either 2h-Exo or 24h-Exo (200 ng/ μL), and the cells were incubated for a further 24 h.

Protocol #2: Prophylactic Pretreatment Model. To assess prophylactic potential, RAW 264.7 macrophages and primary fetal lamb abomasal smooth muscle cells were first pretreated with complete medium containing either 2h-Exo or 24h-Exo (200 ng/ μL) for 24 h. Following this pretreatment, 1 $\mu\text{g/mL}$ LPS was added directly to the culture medium, and the co-incubation continued for another 24 h.

Measurement of NO Release

The cell culture medium was collected and centrifuged at $\sim 300 \times g$ at room temperature (RT) for 15 min to obtain the supernatant. Following the instructions of the NO detection kit (catalog no. BL1204A, Biosharp), the supernatant was added to a 96-well plate and incubated at 37°C for 30 min. The optical density was measured at wavelength of 540 nm, and the NO concentration was determined by referencing a standard curve.

Colostrum Exosome-Derived miRNA Library Construction and Sequencing

Total RNA was extracted from bovine colostrum exosomes using TRIzol reagent (catalog no. ET111-01-V2, TransGen Biotech). The RNA samples were then prepared for miRNA sequencing. After small RNA library preparation, all libraries were sequenced using the DN-BSEQ-T7 platform (MGI Tech Co. Ltd.). The procedure involved the following steps: total RNA samples were fractionated to isolate small RNA ranging from 16 to 30 nucleotides; the isolated small RNA were gel-purified and ligated with both 3'- and 5'-adaptors; the ligation products were gel-purified, reverse transcribed, and amplified; PCR products within the range of 110 to 130 bp were separated by PAGE electrophoresis; and small RNA sequencing was performed. The valid sequencing data were screened against various databases, including

the miRBase (available at <https://www.mirbase.org/>) and Rfam database (available at <http://rfam.xfam.org>).

Differential expression analysis between 2 groups was performed using the edgeR algorithm, with criteria set to $|\log_2(\text{fold change})| \geq 1$ and a P -value ≤ 0.05 . Target genes were predicted using edgeR (v4.2.0), TargetScan (https://www.targetscan.org/vert_80/), ENCORI (<https://rnasysu.com/encori/>), and miRWalk (<http://mirwalk.umm.uni-heidelberg.de/>). Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted using DAVID.

Cell Transfection

The bta-miR-30a-5p mimic, its inhibitor, and corresponding negative controls (mimic control and inhibitor control) were synthesized by Sangon Biotech. Their nucleotide sequences are provided in Supplemental Table S1. Transfection was performed according to the instructions of TransIntro EL Transfection Reagent (TransGen Biotech, cat. FT201-01). Briefly, RAW 264.7 macrophages were seeded and allowed to reach $\sim 80\%$ confluence. The cells were then transfected with 50 nM of either the bta-miR-30a-5p mimic, inhibitor, or their respective controls. Following transfection, cells were maintained in a standard culture incubator (37°C, 5% CO_2) for 24 h before subsequent analyses. All transfections were performed in triplicate.

Dual-Luciferase Reporter Assay

Wild-type (WT) and mutant (MUT) sequences of the TRAM 3'-untranslated region (UTR), containing the bta-miR-30a-5p binding site, were cloned into the pGL4.23 vector downstream of the luciferase gene. Either the bta-miR-30a-5p mimic or a negative control (NC; Supplemental Table S2, see Notes), along with the luciferase reporter plasmid, was transfected into 293T cells using the Trans-Detect Double-Luciferase Reporter Assay Kit (catalog no. FR201-01, TransGen Biotech). Cells were cocultured for 48 h before lysis. Subsequently, firefly and Renilla luciferase activities were measured according to the Dual-Luciferase Reporter Assay System protocol. Differences in luciferase expression levels among the groups were compared.

Real-Time qPCR

For total RNA isolation from cells, TRIzol reagent (catalog no. ET111-01-V2, TransGen Biotech) was used. The RNA was then converted to cDNA using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (catalog no. AT311-02, TransGen Biotech). The cDNA was then mixed with PerfectStart Green qPCR SuperMix (catalog no. AQ601-01-V2, TransGen Biotech) and corresponding primer pairs to create a 20 μL reaction mixture.

Additionally, β -actin was used as an endogenous house-keeping gene control. The amplification conditions were as follows: initial denaturation at 94°C for 30 s, followed by 45 cycles of 94°C for 5 s and 60°C for 30 s. The relative fold changes were calculated using the $2^{-\Delta\Delta CT}$ method. The sequences of primers used for qPCR in this study are shown in Supplemental Tables S1 and S3, see Notes.

For miRNAs expression analysis, RNA extracted from colostrum exosomes was reverse transcribed to cDNA using miRcute Plus miRNA First-Strand cDNA Kit (KR211, Tiangen). Real-time PCR was conducted using miRcute Plus miRNA qPCR Detection Kit (SYBR Green; FP411, Tiangen), with U6 serving as an endogenous control. The amplification conditions were as follows: an initial denaturation step at 95°C for 15 min, followed by 45 cycles of 94°C for 20 s and 60°C for 34 s. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative fold changes. Primer sequences used for qPCR are listed in Supplemental Table S4, see Notes.

Western Blot Analysis

Exosomes and RAW 264.7 cells were lysed using ice-cold radioimmunoprecipitation assay buffer (catalog no. C500035, Sangon Biotech), containing 1 mM phenylmethylsulfonyl fluoride and 1% (vol/vol) protease and phosphatase inhibitor cocktail (catalog no. IP0280, Solarbio), for 30 min. The lysates were then centrifuged at $13,000 \times g$ for 15 min at 4°C, and the supernatant was collected. Protein concentrations were quantified using a BCA protein assay kit.

Proteins were separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes (0.45 μ m Immobilon-P, catalog no. YA1701, Solarbio). The membranes were blocked with 5% skim milk for 12 h at RT and then incubated overnight at 4°C with primary antibodies. After washing, the membrane was incubated with secondary antibody (Sangon Biotech) for 2 h at RT. Immunoreactive bands were visualized using Pierce ECL Western Blot Substrate (catalog no. BL523B, Biosharp), as previously described (Han et al., 2021). Band densities from exosome-supplementation treatments were normalized to β -actin. Antibodies against ALIX, syntenin-1, and β -actin were purchased from Sangon Biotech (catalog no. D262028, D223488, D110001). Primary antibodies against TNF- α and IL-6 were purchased from Youpin Biotech (catalog no. YP-Ab-15972 and YP-Ab-16022).

Statistical Analysis

Statistical analyses were conducted using SPSS 20.0 software and GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA). All quantitative data were verified in triplicate and presented as the mean $\pm$ SD. Differences between 2 groups were assessed using an unpaired Student's

t-test. For comparisons among multiple groups, ANOVA was used, followed by Tukey's post hoc test. Significance is indicated by asterisks (* $P \leq 0.05$; ** $P \leq 0.01$).

RESULTS

Characterization of Bovine Colostrum Exosomes

Exosomes were successfully isolated from bovine colostrum collected at 2 postpartum time points: 2h-Exo and 24h-Exo. To confirm their identity and purity, we performed structural and biochemical analyses. Scanning electron microscopy revealed that vesicles from both time points exhibited the expected cup-shaped or spherical morphology, with diameters within the characteristic exosomal range of 30 to 150 nm (Figure 1A). Western blot analysis for canonical exosomal markers further confirmed the successful isolation. Both 2h-Exo and 24h-Exo were positive for the markers ALIX and Syntenin-1 (Figure 1B). Notably, the protein levels of both ALIX and Syntenin-1 were markedly higher in 2h-Exo compared with 24h-Exo (Figure 1B), suggesting a temporal change in exosomal composition or yield in early colostrum.

Effect of Colostrum Exosomes on Abomasal Smooth Muscle Cell Gene Expression

We investigated the effect of 2h-Exo and 24h-Exo (200 ng/ μ L) on gene expression in primary fetal sheep abomasal smooth muscle cells (Figure 2A) under normal culture conditions and following an inflammatory challenge with LPS. Under basal conditions, coculture with either 2h-Exo or 24h-Exo significantly upregulated the expression of genes associated with cell proliferation and cycle progression, specifically *PCNA* and *CCND1* (Figure 2B). Concurrently, exosome treatment also increased the mRNA levels of key proinflammatory mediators, including the Toll-like receptor 4 (*TLR4*), transforming growth factor- β (*TGF- β*), and the transcription factor nuclear factor kappa-B (*NF- κ B*; Figure 2C).

In contrast, during LPS-induced inflammation, exosome treatment exerted a distinct anti-inflammatory effect. Compared with cells treated with LPS alone, the addition of either 2h-Exo or 24h-Exo significantly downregulated the expression of the major inflammatory cytokines *IL-6* and *TGF- β* (Figure 2D). The expression of *TNF- α* was downregulated in the 2h-Exo group (Figure 2D).

Bioactivity of Bovine Colostrum Exosomes on Macrophage Inflammation

To establish an optimal inflammatory challenge, RAW 264.7 macrophages were treated with a range of

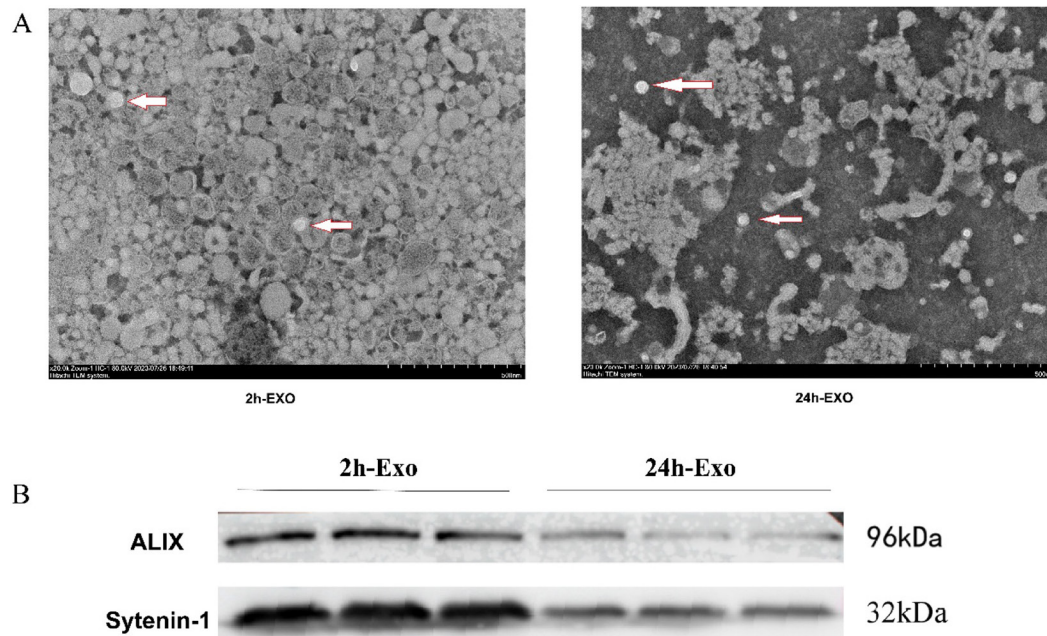


Figure 1. Characterization of bovine colostrum-derived exosomes. (A) Representative scanning electron microscope images of exosomes isolated from colostrum collected at 2 h (left) and 24 h (right) postpartum. Scale bar: 500 nm. White arrows indicate representative exosomes. (B) Western blot analysis demonstrating the presence of the exosomal markers ALIX and Syntenin-1 in isolates from both time points.

LPS concentrations (0–4 $\mu\text{g/mL}$). The LPS induced a concentration-dependent increase in NO release (Figure 3A). Concurrently, LPS dose-dependently upregulated the gene expression of the inflammatory cytokines *IL-6* and *TNF- α* (Figure 3B). Based on these results, 1 $\mu\text{g/mL}$ LPS was selected for all subsequent experiments.

Before immunomodulation studies, we assessed the effect of exosomes on macrophage viability. The CCK-8 assays confirmed that neither 2h-Exo nor 24h-Exo (200 ng/ μL) exhibited cytotoxic effects on RAW 264.7 cells, both 2h-Exo and 24h-Exo could significantly promote RAW 264.7 cell growth compared with control (Figure 3C). We next evaluated the ability of colostrum exosomes to modulate the inflammatory response in coculture experiment. Gene expression analysis showed significantly higher levels of inflammatory cytokines *IL-6*, *IL-1 β* , and *TNF- α* (Figure 3D) in the 2h-Exo group compared with the control group. Indicating that the colostrum-derived exosomes effectively upregulated these cytokines.

Potential of Bovine Colostrum Exosomes to Attenuate LPS-Induced Inflammation in Macrophages

To evaluate the preventive (pretreatment) effect of bovine colostrum exosomes on inflammation, RAW 264.7 macrophages were pretreated with 2h-Exo or 24h-Exo (200 ng/ μL) for 2 h before stimulation with LPS (1 $\mu\text{g/mL}$) for 24 h.

First, we confirmed that LPS successfully induced classical M1 macrophage polarization, characterized by a proinflammatory phenotype (Figure 4A). A key functional readout of M1 activation is the production of NO. Analysis of culture supernatants using the Griess reaction showed that LPS robustly induced NO release. However, pretreatment with colostrum exosomes significantly attenuated this response. Specifically, 24h-Exo pretreatment significantly reduced NO levels compared with the LPS-only control group (Figure 4B). Notably, the inhibitory effect of 2h-Exo was even more potent, resulting in significantly lower NO release than that observed with 24h-Exo pretreatment (Figure 4B).

We next analyzed the expression of key inflammatory mediators at the mRNA level. Quantitative PCR analysis revealed that exosome pretreatment effectively suppressed the LPS-induced upregulation of multiple cytokines. Pretreatment with 2h-Exo significantly reduced the mRNA expression of proinflammatory *TNF- α* , *IL-6*, and *IL-1 β* genes, compared with the LPS-only control (Figure 4C). Pretreatment with 24h-Exo also produced a significant anti-inflammatory effect, significantly inhibiting the expression of *IL-10* (Figure 4C). This was confirmed at the protein level by western blot analysis. Consistent with the gene expression data, pretreatment with either 2h-Exo or 24h-Exo led to a significant decrease in the protein levels of *TNF- α* and *IL-6* compared with the LPS-induced control group (Figure 4D). Furthermore,

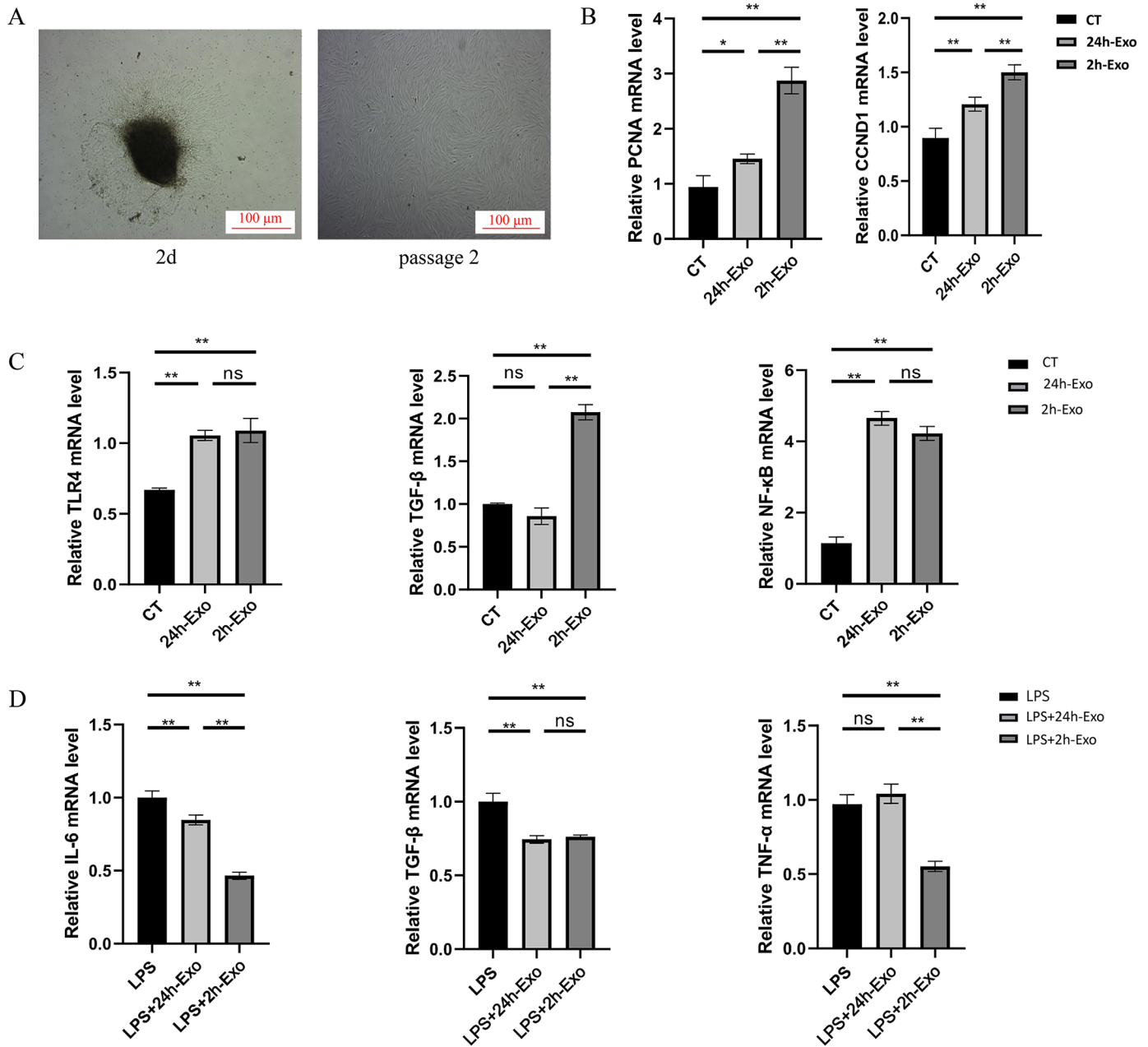


Figure 2. Effects of colostrum exosomes on abomasal smooth muscle cell gene expression. (A) Representative phase-contrast images of primary fetal sheep abomasal smooth muscle cells, showing typical spindle-shaped morphology at d 2 of culture and at passage 2. (B) Exosomes upregulate proliferation-related genes under basal conditions. (C) Exosomes enhance the expression of inflammatory mediators under basal conditions. (D) Exosomes attenuate inflammatory cytokine expression during LPS challenge. Data are expressed as mean $\pm$ SEM ($n = 3$ biological replicates). Significance versus the appropriate control (untreated for B, C; LPS-only for D) was determined as follows: ns (not significant), $P > 0.05$; * $P < 0.05$; ** $P < 0.01$. Both 2h-Exo and 24h-Exo were used at 200 ng/ μ L. CT = control.

2h-Exo was consistently more effective than 24h-Exo in reducing the protein abundance of both cytokines under LPS challenge conditions (Figure 4D). In summary, pretreatment with bovine colostrum exosomes, particularly 2h-Exo, effectively alleviates LPS-induced inflammation in macrophages by reducing the production of NO and the expression of key proinflammatory cytokines.

Therapeutic Effect of Bovine Colostrum Exosomes on LPS-Induced Inflammation in Macrophages

To evaluate the therapeutic potential of bovine colostrum exosomes, RAW 264.7 macrophages were treated with 2h-Exo or 24h-Exo (200 ng/ μ L) after establishing inflammation via a 24-h LPS (1 μ g/mL) challenge

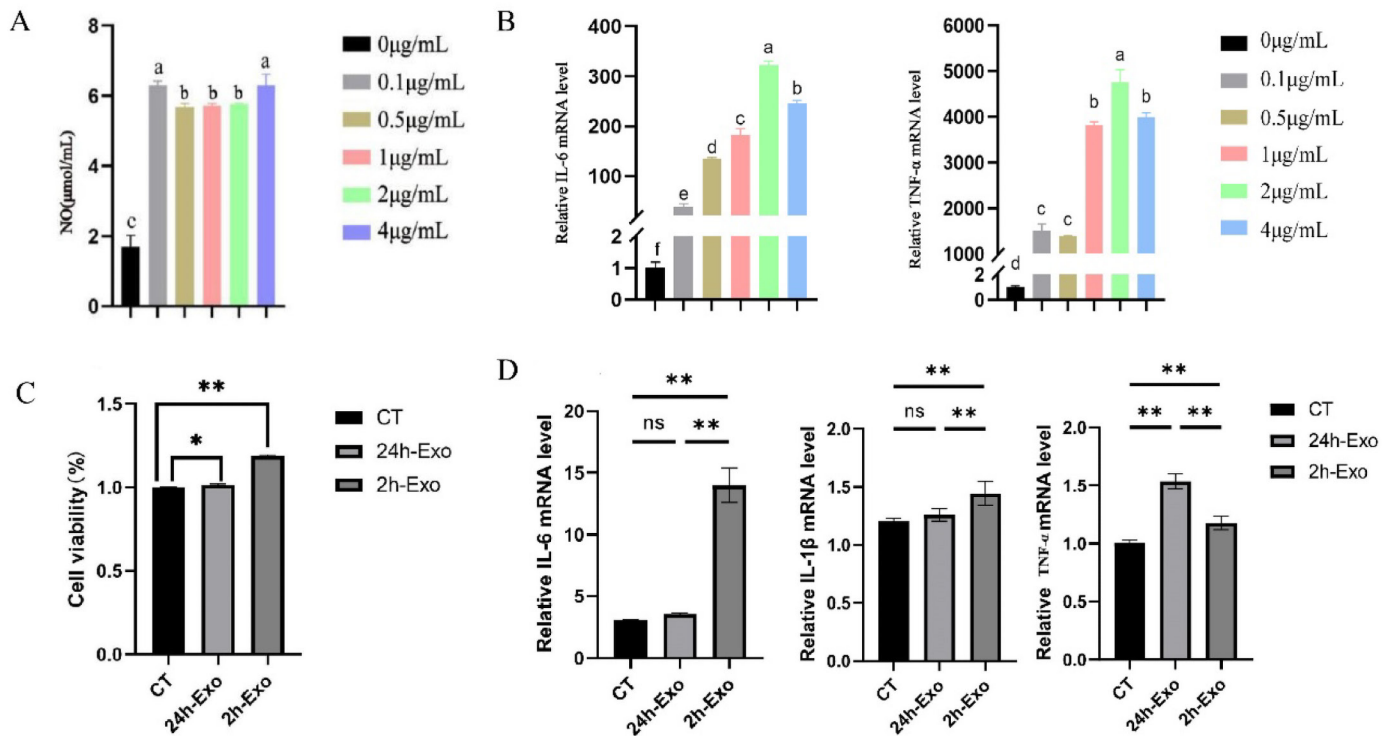


Figure 3. Effects of colostrum exosomes on macrophage proliferation and inflammatory factor gene expression. (A) Nitric oxide (NO) release and (B) inflammatory gene expression in RAW 264.7 cells treated with increasing concentrations of LPS (0–4 μg/mL) for 24 h. (C) Cell viability assessed by CCK-8 assay shows that treatment with 2h-Exo or 24h-Exo (200 ng/μL) is not cytotoxic. (D) Effect of colostrum exosomes on the gene expression of inflammatory factors in macrophages. Data are presented as the mean ± SEM (n = 3 per group). Different lowercase letters in the figure represent significant differences ($P < 0.05$), and significance was determined as follows: ns (not significant), $P > 0.05$; * $P < 0.05$; ** $P < 0.01$. Both 2h-Exo and 24h-Exo were used at a concentration of 200 ng/μL in cell culture. CT = control.

(treatment model). First, the production of NO, a key inflammatory mediator, was measured. Analysis of culture supernatants revealed that treatment with either exosome type significantly inhibited NO release compared with the LPS-only control group (Figure 5A). Notably, the inhibitory effect of 2h-Exo was significantly greater than that of 24h-Exo (Figure 5A).

The qPCR analysis showed that exosome treatment effectively suppressed the expression of multiple LPS-induced cytokines. Treatment with 2h-Exo significantly reduced the mRNA levels of *TNF-α*, *IL-6*, *IL-1β*, and *IL-10* compared with the LPS control (Figure 5B). Similarly, 24h-Exo treatment significantly reduced the expression of *TNF-α* and *IL-1β* (Figure 5B). Consistent with the NO data, 2h-Exo was more effective than 24h-Exo at lowering *TNF-α* and *IL-6* mRNA levels. This anti-inflammatory effect was confirmed at the protein level by western blot analysis. Treatment with 2h-Exo or 24h-Exo led to a significant decrease in the protein abundance of TNF-α and IL-6 compared with cells treated with LPS alone (Figure 5C). Furthermore, 2h-Exo demonstrated superior efficacy, reducing the

protein levels of both cytokines more effectively than 24h-Exo (Figure 5C).

In summary, therapeutic administration of bovine colostrum exosomes, particularly 2h-Exo, significantly mitigates established LPS-induced inflammation in macrophages by reducing the production of NO and suppressing the expression of key proinflammatory cytokines at both the mRNA and protein levels.

Differential miRNA Expression Profiles in 2h-Exo and 24h-Exo

To investigate the molecular basis for the differential bioactivity of 2h-Exo and 24h-Exo, we performed small RNA sequencing (DNBSEQ-T7) on exosomes isolated from bovine colostrum at 2 and 24 h postpartum. Sequencing generated over 85 million clean reads (Supplemental Table S5, see Notes), with sequenced miRNAs averaging 22.6 nt in length. Comparative analysis revealed a distinct miRNA expression profile between the 2 exosome types. Compared with 24h-Exo, 22 miRNAs were upregulated and 47 were downregulated in 2h-Exo

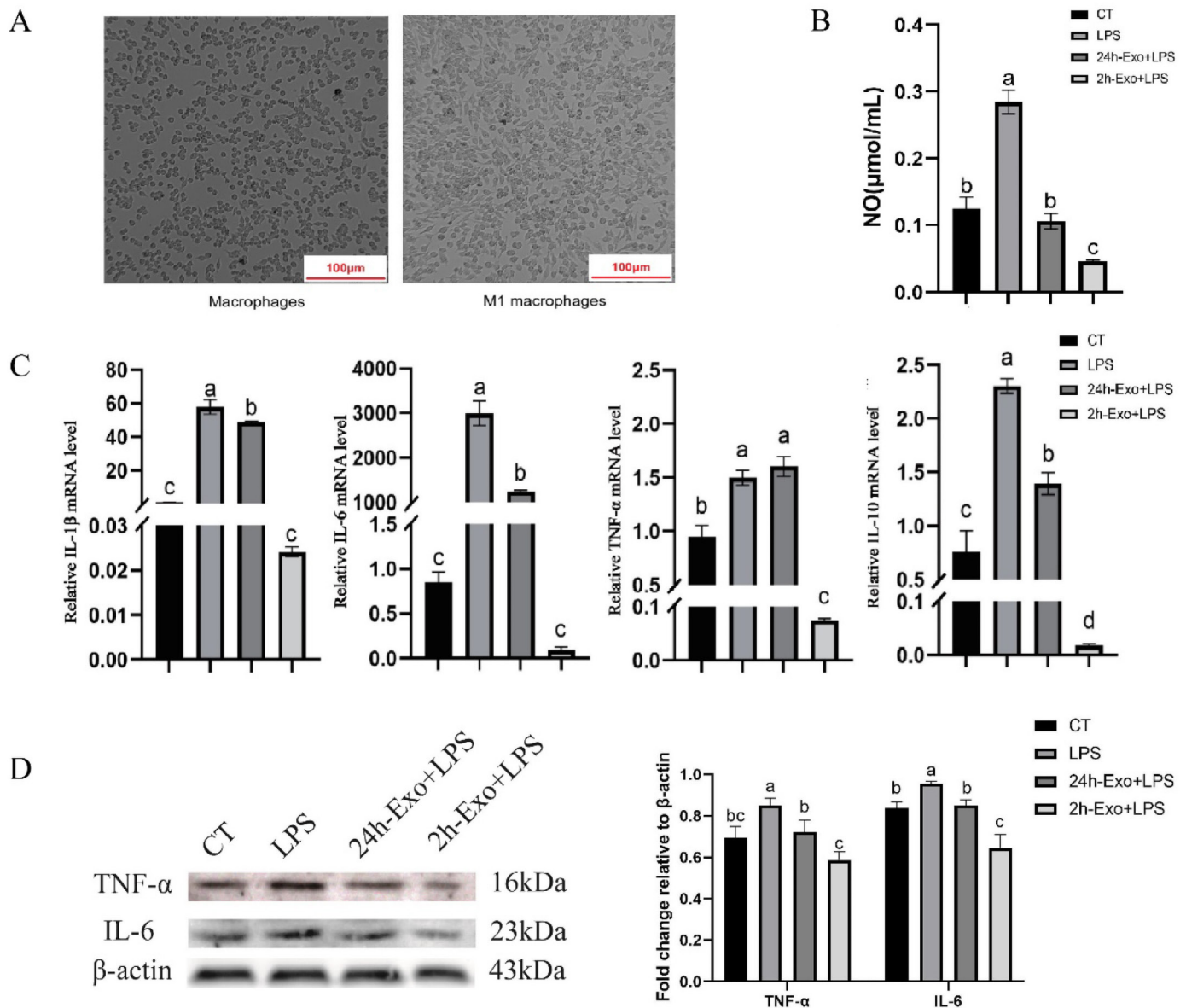


Figure 4. Pretreatment with bovine colostrum exosomes attenuates LPS-induced inflammation in RAW 264.7 macrophages. (A) Representative characterization of M1 macrophage polarization induced by LPS. (B) Nitric oxide (NO) release in culture supernatants, measured by Griess reaction. (C) mRNA expression levels of inflammatory cytokines (*TNF-α*, *IL-6*, *IL-1β*, *IL-10*) determined by qPCR. (D) Left: representative western blots of *TNF-α* and *IL-6* protein; β -actin served as a loading control. Right: densitometric quantification of protein signals. Data are presented as mean $\pm$ SEM ($n = 3$). Different lowercase letters indicate significant differences ($P < 0.05$) between all groups. CT = control.

(Figure 6A). The top 10 differentially expressed miRNAs in 2h-Exo are highlighted (Figure 6B, Supplemental Table S6, see Notes).

To understand the functional implications, we performed KEGG pathway enrichment analysis on the predicted mRNA targets of miRNAs upregulated in 2h-Exo. The top 30 enriched pathways included adherens junction, TGF- β signaling, ubiquitin-mediated proteolysis, Hippo signaling, focal adhesion, and the PI3K-Akt signaling pathway (Figure 6C), strongly implicating

these miRNAs in the regulation of inflammatory and cellular signaling processes.

Among the highly expressed miRNAs, bta-miR-30a-5p was notable for its high abundance and conservation across colostrum and milk in multiple species (Su et al., 2020; Li et al., 2022; Gao et al., 2024). Functional enrichment analysis of bovine colostrum miR-30a-5p targets genes is detailed in Supplemental Table S7, see Notes. Finally, we validated the sequencing data by quantifying selected differentially expressed miRNAs

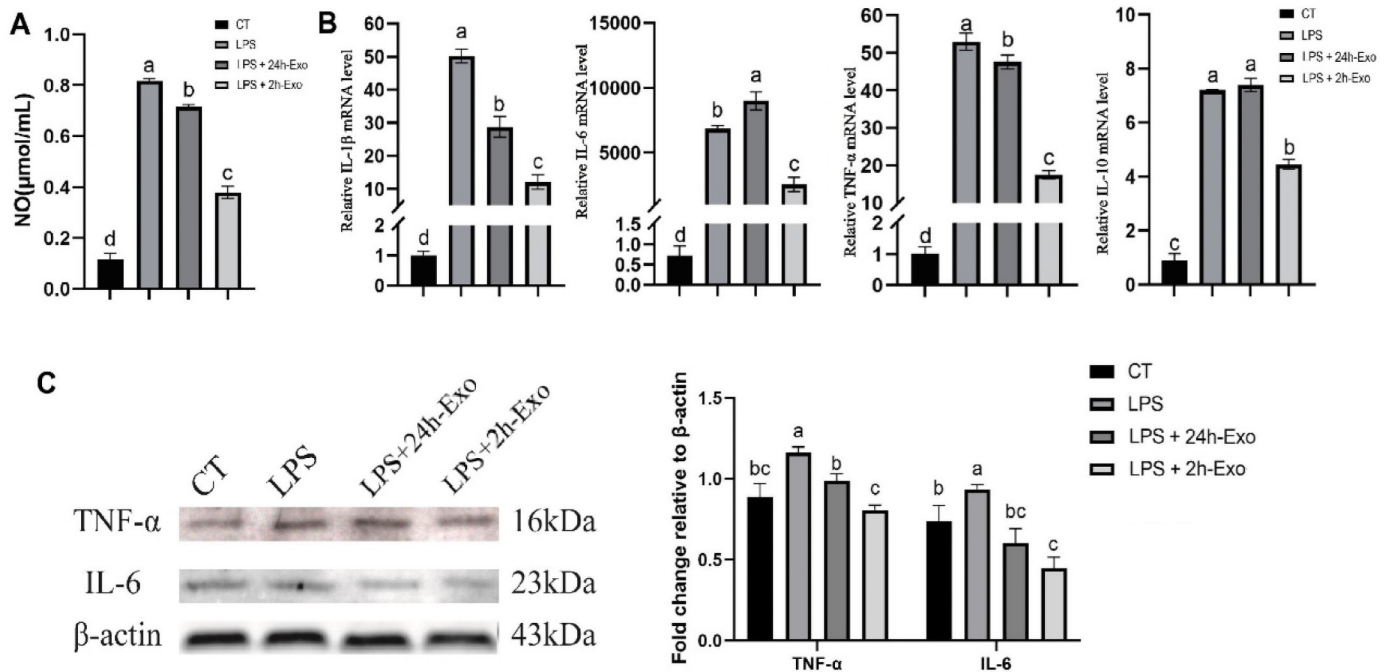


Figure 5. Therapeutic administration of colostrum exosomes alleviates LPS-induced inflammation in RAW 264.7 macrophages. The RAW 264.7 cells were first stimulated with LPS (1 μg/mL) for 24 h to establish inflammation, followed by treatment with 2h-Exo or 24h-Exo (200 ng/μL) for 24 h. (A) Nitric oxide (NO) release in culture supernatants measured by Griess reaction. (B) mRNA expression levels of inflammatory cytokines (*TNF-α*, *IL-6*, *IL-1β*, *IL-10*) determined by qPCR. (C) Left: representative western blots of *TNF-α* and *IL-6* protein; β-actin served as a loading control. Right: densitometric quantification of protein signals. Data are presented as mean ± SEM (n = 3). Different lowercase letters indicate significant differences ($P < 0.05$) between all groups. CT = control.

via qPCR, which confirmed their distinct expression patterns in 2h-Exo versus 24h-Exo (Figures 6D and 6E).

Identification of *SOX4* mRNA as a Direct Target of bta-miR-30a-5p

Bioinformatic analysis predicted that the SRY-box transcription factor 4 (*SOX4*) is a potential target of bta-miR-30a-5p, with a binding site for the miR-30a-5p seed sequence (ACAAUG) in its 3'-UTR (Figure 7A). We validated this interaction using a dual-luciferase reporter assay. Cotransfection of a miR-30a-5p mimic with a reporter plasmid containing the wild-type *SOX4* 3'-UTR (*SOX4*-WT) significantly reduced luciferase activity compared with an NC mimic. In contrast, the miR-30a-5p mimic had no effect on a reporter with a mutated binding site (*SOX4*-MUT; Figure 7B). This confirms *SOX4* mRNA as a direct target of bta-miR-30a-5p.

Functional Role of bta-miR-30a-5p in Macrophage Inflammation

To investigate the functional consequence of this targeting, we modulated bta-miR-30a-5p levels in RAW 264.7 macrophages. Transfection with a bta-miR-30a-5p

mimic successfully increased, although a specific inhibitor decreased, the intracellular levels of mature bta-miR-30a-5p, as confirmed by qPCR (Figures 7C and 7D).

We then assessed the effect on LPS-induced inflammation. In cells challenged with LPS, overexpression of bta-miR-30a-5p (mimic) significantly suppressed the mRNA expression of the proinflammatory cytokines *IL-6*, *IL-1β*, and *TNF-α* and the immunoregulatory cytokine *IL-10* compared with the NC-mimic control (Figure 7E). Conversely, inhibition of endogenous bta-miR-30a-5p (inhibitor) exacerbated the inflammatory response, leading to a significant increase in the expression of all 4 cytokines compared with the NC-inhibitor control (Figure 7F).

DISCUSSION

Although the benefits of bovine colostrum are well-established, and milk exosomes are recognized for their roles in growth and immune regulation (Ren et al., 2022), research specifically addressing their anti-inflammatory potential is limited. In this study, we isolated exosomes from colostrum at 2h-Exo and 24h-Exo postpartum to investigate their immunomodulatory effects. Our findings demonstrate that colostrum exosomes, particularly

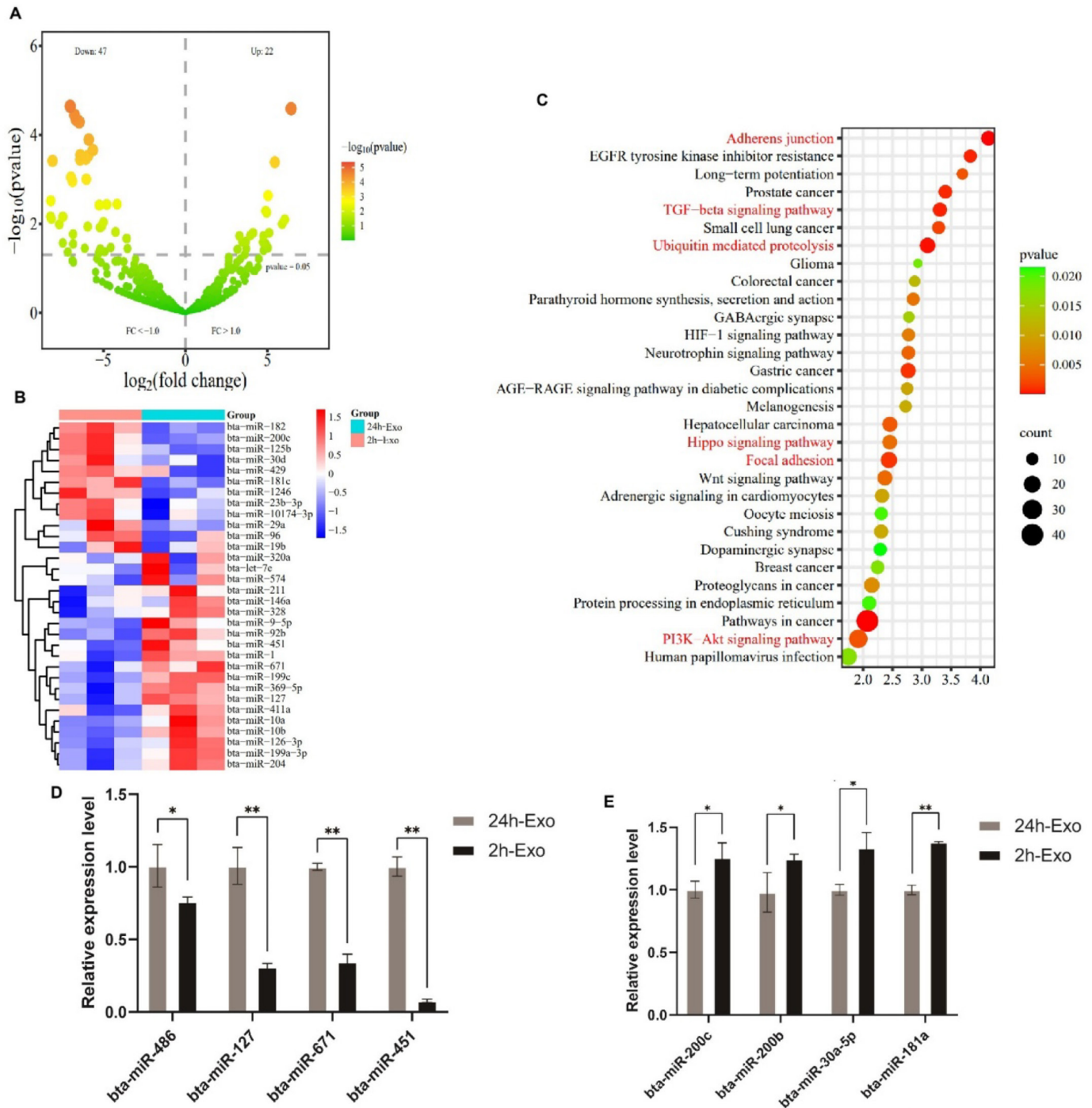


Figure 6. Differential miRNA expression and functional enrichment in 2h-Exo versus 24h-Exo. (A) Volcano plot showing differentially expressed miRNAs. Dots represent individual miRNAs; red indicates significant upregulation (Up), and green indicates significant downregulation (Down) in 2h-Exo compared with 24h-Exo (threshold: $|\text{fold change}| \geq 1.5$, $P < 0.05$). (B) Heatmap of hierarchical clustering for selected differentially expressed miRNAs. Red denotes higher expression; blue denotes lower expression. (C) The KEGG pathway enrichment analysis of predicted targets for miRNAs upregulated in 2h-Exo. The top 30 enriched pathways are shown; pathways related to immune and barrier function are shown in red. (D and E) Validation of selected differentially expressed miRNAs by qPCR. Data confirm the sequencing results, showing distinct expression patterns of specific miRNAs in 2h-Exo and 24h-Exo. * $P < 0.05$; ** $P < 0.01$.

2h-Exo, effectively alleviate cellular inflammation. Comprehensive miRNA profiling revealed a distinct molecular signature in 2h-Exo, from which bta-miR-30a-5p was identified as a key regulator. Furthermore, we established that miR-30a-5p exerts its anti-inflammatory effect, at least in part, by directly targeting the mRNA encoding transcription factor *SOX4*.

The isolation of exosomes from colostrum is complicated by its high casein content, which constitutes ~80% of colostrum protein and can co-pellet with vesicles. The κ -CN, the predominant form, is effectively removed by rennet enzymes, specifically chymosin, which cleaves κ -CN to disrupt micelle stability (Hanieh et al., 2020). To address this, we employed a rennet pretreatment followed by density gradient centrifugation, which proved to be an effective method for obtaining high-purity exosomes from bovine colostrum. Notably, this protocol yielded a greater quantity of exosomes from the 2h-Exo compared with the 24h-Exo, consistent with the known temporal decline in κ -CN and other milk components postpartum (Rzemieniewski et al., 2013). This optimized isolation was crucial for the subsequent functional comparisons.

Macrophages are central orchestrators of inflammation, polarizing toward a proinflammatory (M1) state in response to LPS via NF- κ B-driven cytokine production (TNF- α , IL-1 β , IL-6; Ahmad Mir et al., 2024). Our findings reveal that bovine colostrum exosomes, particularly those from the 2h-Exo, exert a sophisticated, context-dependent regulatory influence on this system. Under basal conditions, 2h-Exo promoted macrophage proliferation and the expression of certain inflammatory pathway genes, suggesting a priming or homeostatic function. Which is consistent with a previous study showing that milk exosomes can stimulate the proliferation of colonic epithelial cells and macrophages and improve the inflammatory response (Han et al., 2022). In stark contrast, during LPS challenge, these exosomes exhibited a potent anti-inflammatory effect. 2h-Exo was consistently more effective than 24h-Exo in suppressing LPS-induced expression of TNF- α , IL-1 β , IL-6, and the immunoregulatory cytokine IL-10, along with nitric oxide production, in both preventive (pretreatment) and therapeutic (post-treatment) models. This coordinated downregulation of both proinflammatory cytokines and the canonical anti-inflammatory cytokine IL-10 is noteworthy. Although IL-10 typically functions to resolve inflammation, its expression is also a hallmark of late-stage, regulatory macrophage responses (Zhang et al., 2025). Our results suggest that 2h-Exo may accelerate the resolution process by broadly dampening the entire activated cytokine cascade, including the IL-10 feedback loop, rather than selectively enhancing an anti-inflammatory profile. This aligns with the exosomes' concurrent inhibition of NO, another key inflammatory mediator. Collectively, this

dual role (enhancing baseline immune readiness while powerfully suppressing an acute inflammatory response) may be physiologically rational for neonatal colostrum. It provides initial immune education and proliferative signals while equipping the newborn with a potent mechanism to prevent damaging hyperinflammation upon first microbial exposure. The superior bioactivity of 2h-Exo correlates with the known temporal decline of bioactive components in colostrum, highlighting the critical importance of early harvest timing.

The functional effects of exosomes are largely attributed to their conserved miRNA cargo, which enables cross-species modulation of cellular processes (Nail et al., 2023). Our sequencing analysis revealed a distinct miRNA signature in 2h-Exo compared with 24h-Exo, with 69 differentially expressed miRNAs. Pathway enrichment indicated that miRNAs upregulated in 2h-Exo target networks central to inflammation and cell signaling, providing a molecular rationale for its enhanced bioactivity. The expression of miRNAs in bovine milk exosomes varies depending on the lactation period, breed, and living environment of the animals (Rani et al., 2017; Colitti et al., 2019; Cendron et al., 2024). Studies have also compared miRNA profiles between colostrum and milk exosomes (Yun et al., 2021; Cendron et al., 2025). Certain miRNAs detected in the current study are commonly found in previous studies of colostrum and milk exosomes (Yun et al., 2021; Cendron et al., 2025).

Milk-derived exosomal miRNAs exhibit diverse functions in immune regulation, with several specific miRNAs emerging as crucial regulators of immune cell function. We focused on bta-miR-30a-5p due to its high abundance in colostrum (Cendron et al., 2025). Recent studies have explored the potential of this miRNA to attenuate LPS-induced intestinal inflammation. Zhang et al. (2024a) discovered that miR-30a-5p, abundantly expressed in colostrum, inhibits the downstream NF- κ B inflammatory pathway by targeting TRAM. Furthermore, bovine colostrum bta-miR-30a-5p antagonizes the decrease in cell viability induced by inflammation in Caco-2 cells, and its mimic inhibits LPS-induced phosphorylation of p65 and expression of proinflammatory mediators, suggesting it forms a regulatory chain with TRAM and NF- κ B to exert anti-inflammatory functions (Chu et al., 2024). In our study, transfection of a bta-miR-30a-5p mimic notably decreased the gene expression of TNF- α , IL-6, IL-1 β , and IL-10 in RAW 264.7 cells, demonstrating its role as a potent regulator in mitigating inflammatory responses. Interestingly, bta-miR-30a-5p has also been implicated as a tumor suppressor in various cancers (e.g., ovarian, lung), often by targeting specific genes such as *SOX4* (Zhou et al., 2015; Quan et al., 2019; Zeng et al., 2022; Du et al., 2023; Peng et al., 2023; Zhang et al., 2024b). Given that inflammatory responses are key for the initia-

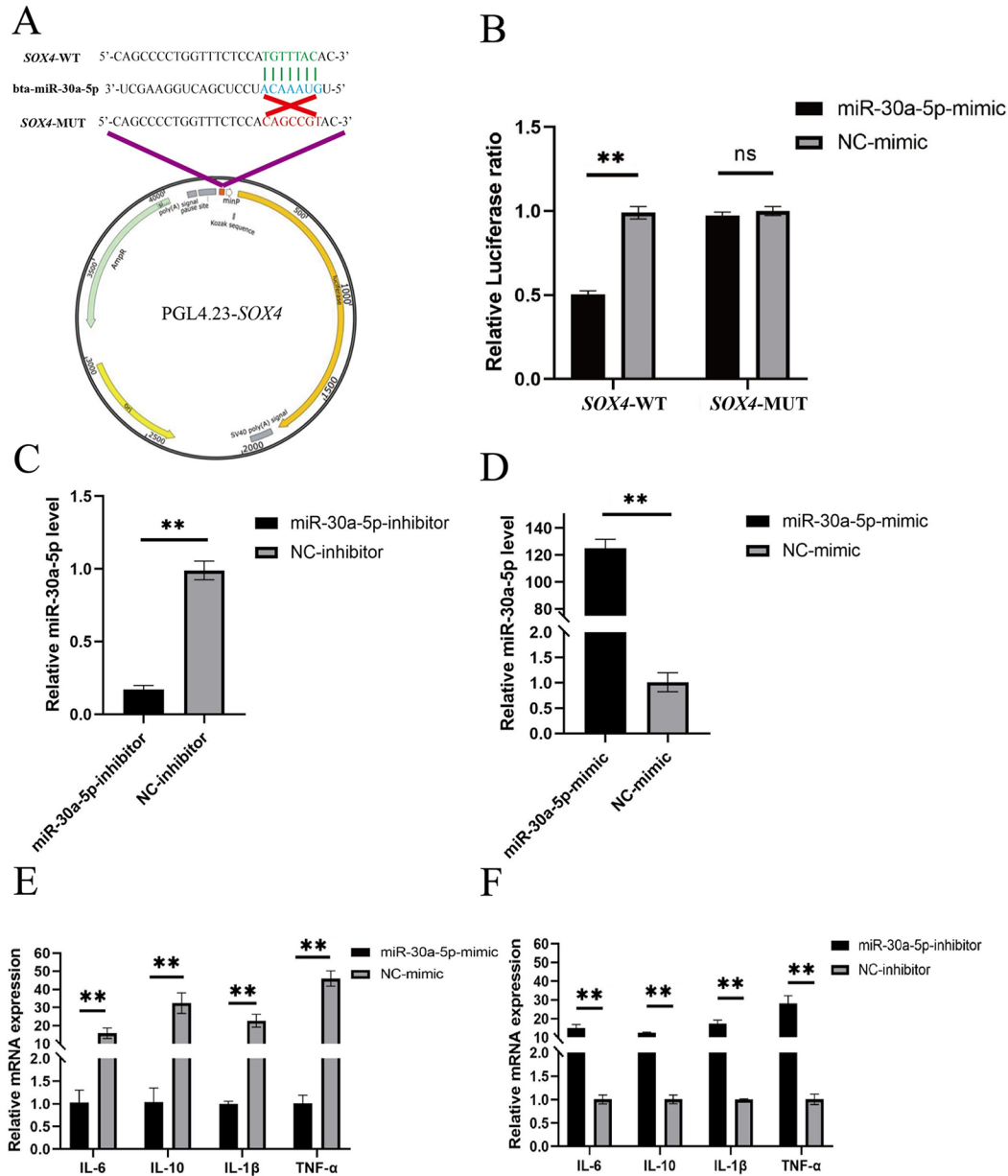


Figure 7. bta-miR-30a-5p targets *SOX4* to regulate inflammatory responses in macrophages. (A) Schematic diagram of the predicted binding site of bta-miR-30a-5p in the wild-type (*SOX4*-WT) and mutant (*SOX4*-MUT) 3'-UTR of *SOX4*. Red crossed lines = mutated nucleotides; purple V-shaped line = complementary base pairing between miRNA and target site. (B) Dual-luciferase reporter assay validating the direct interaction. Luciferase activity is shown for cells cotransfected with the *SOX4*-WT or *SOX4*-MUT reporter and either a bta-miR-30a-5p mimic or a negative control (NC) mimic. (C and D) qPCR validation of bta-miR-30a-5p levels in RAW 264.7 cells after transfection with a mimic (C) or an inhibitor (D). (E and F) Effect of bta-miR-30a-5p modulation on LPS-induced cytokine gene expression. Cells were transfected with mimic/NC (E) or inhibitor/NC (F), then stimulated with LPS (1 µg/mL). The mRNA levels of *IL-6*, *IL-1β*, *TNF-α*, and *IL-10* were measured by qPCR. Data are presented as mean ± SEM (n = 3). Significance: ns (not significant), $P > 0.05$, $**P < 0.01$ versus the respective NC group.

tion of tumor development, a common anti-inflammatory and antioncogenic mechanism is plausible.

SOX4, a crucial developmental transcription factor often elevated in cancers, is also deeply linked to the identity, maintenance, and function of various stem and progenitor cells. Indeed, *SOX4* is often elevated in vari-

ous cancers (Lu et al., 2015; Moreno, 2020). Xie and Xu (2024) found that *SOX4* knockdown reduces oxidative stress and inflammatory responses by diminishing NF-κB pathway activity. Furthermore, *SOX4* is a predicted and validated target of miR-30a-5p in several cancer contexts, suggesting the miR-30a-5p/*SOX4* axis may be important

for disease prevention. Therefore, we hypothesized that miR-30a-5p may function as an anti-inflammatory regulator in immune cells by targeting *SOX4* mRNA. Using bioinformatics and dual-luciferase assays, we confirmed a targeting relationship between miR-30a-5p and *SOX4* mRNA. A significant reduction in fluorescence was observed only in cells co-transfected with the miR-30a-5p mimic and the WT *SOX4* plasmid ($P < 0.05$), indicating that miR-30a-5p can directly bind the *SOX4* 3'-UTR to regulate its expression. Collectively, our findings demonstrate that bovine colostrum exosomal bta-miR-30a-5p is a critical modulator in reducing inflammatory responses in macrophages, likely through targeting the *SOX4* gene. However, more studies should focus on other candidate miRNAs in milk exosomes to further understand the broad biological functions of bovine colostrum.

CONCLUSIONS

The anti-inflammatory potency of bovine colostrum exosomes peaks immediately after parturition and diminishes within 24 h. The superior activity of 2h-Exo is linked to a distinct miRNA profile, and we mechanistically demonstrate that one key component, bta-miR-30a-5p, alleviates inflammation by directly targeting *SOX4* mRNA in macrophages. These findings highlight early postpartum colostrum as a uniquely rich source of bioactive exosomes and define a specific miRNA-target axis that may be exploited for therapeutic immunomodulation. Future studies should explore the in vivo efficacy of these exosomes and the potential synergistic roles of other miRNAs within their complex cargo.

NOTES

This research was supported by the major scientific and technological projects of Bingtuan Science and Technology Program (Xinjiang, China; No. 2021AA004), International Science and Technology Cooperation Project of Shihezi University (Shihezi, China; No. GJHZ202307), National Key R&D Program of China (Beijing, China; No. 2024YFD1300105), National Natural Science Foundation of China (Beijing, China; No. 32060745), and the Open Project of State Key Laboratory of Animal Biotech Breeding (Beijing, China; No. 2024SKLAB 6-107). We thank the company Xinjiang Tianshan Junken Animal Husbandry Co. Ltd. (Shihezi, China) for their kind help in colostrum samples collection. This study was also supported by the Open Sharing Fund for the Large-scale Instruments and Equipments of Shihezi University. Supplemental material for this article can be found at <https://doi.org/10.6084/m9.figshare.31123519>. CRediT authorship contribution statement: M. Yang: Writing, review and editing the original draft, Methodology, Data

curation, Funding acquisition, Conceptualization, Formal analysis. H. Xu: Writing, editing the original draft, Methodology, Data curation, Formal analysis. Q. Q. Liang: Methodology, Data curation, Formal analysis. Y. Y. Wu: Methodology, Data curation, Formal analysis, Resources. W. J. Zhang: Methodology, Data curation, Funding acquisition. C. X. Nie: Methodology, Data curation, Formal analysis. B. L. Kurmanglievna: Methodology, Data curation, Formal analysis. Z. K. Toksanbaevich: Software, Methodology, data curation. B. T. Kulatayev: Methodology, data curation, review the original draft. G. Andersson: Writing, review and editing the original draft, Supervision. E. Bongcam-Rudloff: Writing, review and editing the original draft, Supervision. M. Rouzi: Methodology, Software, Resources and J. L. Han: Writing, review and editing the original draft, Supervision, Methodology, Data curation, Formal analysis, Funding acquisition, Conceptualization. The experimental protocol followed the Approval Letter of Biology Ethics Committee of Shihezi University (A2022-420). The authors have not stated any conflicts of interest.

Nonstandard abbreviations used: 2h-Exo = exosomes derived from 2-h postpartum colostrum; 24h-Exo = exosomes derived from 24-h postpartum colostrum; BCA = bicinchoninic acid; CCK8 = cell counting kit-8; CT = control; KEGG = Kyoto Encyclopedia of Genes and Genomes; M1 = proinflammatory; M2 = anti-inflammatory; miRNA = microRNA; MUT = mutant; NC = negative control; qPCR = quantitative PCR; RT = room temperature; UTR = untranslated region; WT = wild-type.

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