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Permalink

<https://escholarship.org/uc/item/5w13p5b4>

Journal

Am J Vet Res

ISSN

0002-9645

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Publication Date

2026-10-05

DOI

10.2460/ajvr.26.05.0197

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Peer reviewed

Cytokine priming shifts mitochondrial architecture and extracellular vesicle cargo of feline placental mesenchymal stromal cells toward an immunosuppressive phenotype

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Objective

To characterize effects of inflammatory cytokine priming on viability, mitochondrial morphology, and extracellular vesicle (EV) cargo in feline placenta-derived mesenchymal stromal cells (MSCs).

Methods

Feline placenta-derived MSCs from 7 healthy cats were cultured under control, serum-deprived, or cytokine-stimulated (tumor necrosis factor- α , interferon- γ , IL-1, IL-6) conditions in vitro. Viability/apoptosis were assessed by flow cytometry and mitochondrial morphology by confocal microscopy with MicroP/MATLAB; EVs were characterized by surface markers, RNA sequencing, and microRNA (miR) profiling.

Results

Viability exceeded 96% across all conditions, with no significant intergroup differences. Kiel 67 proliferation marker and metabolic activity were significantly reduced under serum-free and cytokine-stimulated conditions versus controls (both $P < .001$). Cytokine priming increased branching and simple tubular mitochondria, whereas serum deprivation increased fragmented forms. Extracellular vesicle transcriptomics identified 329 differentially expressed genes, including upregulated immunosuppressive receptors C-C motif chemokine receptor 8 and poliovirus receptor-related immunoglobulin domain-containing protein and downregulated proinflammatory histone transcripts. microRNA (miR) profiling revealed 23 differentially expressed miRs, with enrichment of anti-inflammatory miR-204, let-7g, and miR-181b-2 and depletion of proinflammatory miR-34a and miR-320a.

Conclusions

Cytokine priming induces mitochondrial elongation and shifts EV cargo toward an immunosuppressive phenotype without compromising viability, supporting inflammatory priming as a strategy to optimize MSC-based therapies for T-cell-mediated inflammatory diseases.

Clinical Relevance

Inflammatory priming prior to MSC administration may enhance therapeutic efficacy in cats, supporting investigation of cytokine licensing as a translatable approach to improving MSC therapy outcomes in veterinary medicine.

Keywords: extracellular vesicle, feline, mesenchymal stromal cell, placenta, cytokine priming

Mesenchymal stromal cell (MSC) therapy has been used to treat cats with refractory feline chronic gingivostomatitis (FCGS) for over a decade, with approximately two-thirds of treated cats achieving successful clinical outcomes.¹⁻⁶ Mesenchymal stromal cells have been harvested from multiple sources,

including placenta (feline placenta-derived MSCs [fePDMSCs]), adipose tissue (feline adipose-derived MSCs [feAMSCs]), and uterine tissue (feline uterine-derived MSCs [feUMSCs]), with therapeutic success largely consistent across cell types.^{1-4,6} Despite the favorable long-term safety profile of MSC therapy, albeit with a notable rate of transient, manageable acute infusion reactions, administered cells undergo substantial pulmonary trapping and limited persistence following IV administration,³ and in vitro evidence suggests that activated natural killer cells are

Received July 14, 2026

Accepted September 9, 2026

Published online October 5, 2026

doi.org/10.2460/ajvr.26.05.0197

capable of killing MSCs,⁷ raising the possibility that immune-mediated clearance further limits cell survival in vivo. Treated cats are additionally exposed to an inflammatory cytokine milieu from the moment of administration given that FCGS is associated with systemic inflammation mirroring the chronic mucosal disease.^{2,8,9}

Feline adipose-derived MSCs are potent immunomodulators with relevance to both veterinary and translational medicine. Taechangam et al¹⁰ demonstrated that feAMSCs inhibit T-cell proliferation via prostaglandin E2 secretion and intercellular adhesion molecule 1/lymphocyte function-associated antigen 1 cell-cell interactions and subsequently showed that feAMSCs promote a terminally differentiated CD8⁺ T-cell effector phenotype with enhanced cytolytic capacity.¹¹ Clark et al¹² reported conserved phenotypic traits, immunomodulatory mechanisms, and transcriptional profiles between human and feAMSCs, affirming the translational relevance of feline MSC models.

The immunomodulatory effects of feline and human MSCs are mediated in part through extracellular vesicle (EV) secretion and paracrine signaling.¹³⁻¹⁸ Extracellular vesicles carry bioactive molecules, including cytokines and microRNAs (miRNAs; miRs), that regulate immune responses and tissue regeneration.¹⁹⁻²² Exposure to inflammatory cytokines, such as interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α), markedly alters MSC immunomodulatory capacity, upregulates immune-regulatory genes, and remodels EV cargo.²³⁻²⁹ A prior investigation of cytokine-primed feline MSCs demonstrated increased cellular projections and enhanced immunomodulatory EV capacity alongside enrichment of mitochondrial-related transcripts in EVs that suggested a compensatory cellular response.³⁰

We hypothesized that inflammatory priming would (1) alter fePDMSC viability, apoptosis, and mitochondrial dynamics relative to unprimed controls and (2) selectively enrich EV cargo with immunosuppressive receptors (C-C motif chemokine receptor 8 [CCR8] and poliovirus receptor-related immunoglobulin domain-containing protein [PVRIG]) and anti-inflammatory miRNAs (miR-204, let-7g, and miR-181b), yielding EVs with greater immunomodulatory potential than those derived from unprimed cells. This study tested these hypotheses by comparing cytokine-primed and unprimed fePDMSCs and their EVs across cellular, mitochondrial, and molecular end points, with implications for optimizing MSC-based therapies in FCGS and beyond.

Methods

fePDMSC isolation and EV harvesting

With signed informed owner consent, placental tissues were obtained from 7 clinically healthy, pregnant queens undergoing routine ovariohysterectomy performed at owner request; tissue that would otherwise have been discarded was collected for this study. Tissue samples were rinsed in sterile PBS with 1% penicillin-streptomycin, minced, and enzymatically digested in 0.1 mg/mL collagenase type I (Sigma-Aldrich) for 40 minutes at 37 °C in 5%

CO₂. Digested material was filtered through a 70- μ m filter, centrifuged at 1,200 X *g* for 6 minutes, and washed twice in PBS. The fePDMSCs were cultured in low-glucose Dulbecco's modified Eagle medium (LG-DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in 5% CO₂, passaged every 3 days at 80% to 90% confluence using trypsin-EDTA.

Cell identity as fePDMSCs was inferred from tissue source, isolation methodology, plastic adherence, and morphological characteristics consistent with an MSC phenotype. Surface marker expression of multipotency-associated markers (CD44, CD90, and CD105) in fePDMSCs and feAMSCs from this cat cohort was previously characterized by our group,¹⁸ supporting baseline MSC identity of the source cell populations. However, this study did not reassess surface marker expression against the full International Society for Cell & Gene Therapy (ISCT) panel of positive (CD90, CD105, and CD73) and negative (CD34, CD45, CD14, CD19, and a component of the Major Histocompatibility Complex Class II [HLA-DR]) markers nor confirm trilineage differentiation potential (osteogenic, adipogenic, and chondrogenic) either at baseline or following cytokine priming.

EVs were isolated as previously described.³⁰ The fePDMSCs at passages 2 and 3 were cultured until optimal confluence, medium was replaced with serum-free LG-DMEM containing 1% insulin-transferrin-selenium (ITS) (Thermo Fisher Scientific) for 48 hours. Conditioned medium was filtered (0.22 μ m), centrifuged at 300 X *g* (10 minutes, 4 °C), and ultracentrifuged at 22,000 X *g* for 2 hours. The EV pellets were resuspended in PBS and stored at -80 °C.

Inflammatory priming

At approximately 80% confluence and passages 2 and 3, medium was replaced with serum-free DMEM/ITS supplemented with recombinant human TNF- α (15 ng/mL; Sigma-Aldrich), recombinant human IFN- γ , recombinant human IL-1, and recombinant human IL-6 (10 ng/mL each; Sigma-Aldrich) for 72 hours.³⁰⁻³² Recombinant human cytokines were used given the limited commercial availability of feline-specific recombinant cytokines; crossreactivity and bioactivity of human TNF- α and IFN- γ in feline cells have been established in prior feline MSC priming work, including our related companion study of cytokine-primed feAMSCs.³⁰

Serum-free medium was used during priming because conditioned medium was collected from the same cultures immediately afterward for EV isolation, and FBS contains bovine-derived EVs that would confound analysis of fePDMSC-derived EV cargo; priming and EV collection therefore necessarily occurred within the same defined, serum-free window. Because serum withdrawal is itself a cellular stressor independent of cytokine exposure, a serum-free, non-cytokine-primed control arm was included alongside standard-serum control and cytokine-primed conditions throughout this study, allowing the effects of serum deprivation and cytokine priming to be distinguished. The EV isolation from

conditioned supernatants followed the same protocol described above.

Morphological and ultrastructural characterization

The fePDMSC morphology was assessed by inverted light microscopy (AxioObserver A1; Carl Zeiss AG). For confocal imaging, cells were fixed in 4% paraformaldehyde; permeabilized with 0.1% Triton X-100; stained with phalloidin-Atto488 (actin), 4',6-diamidino-2-phenylindole (nuclei), and MitoRed (mitochondria); and imaged on a Zeiss Observer Z1 Spinning Disc confocal at the University of California-Davis. Mitochondrial morphology was quantified using MicroP software (version 1.1.11b; Biomedical Image Informatics Lab, Institute of Biomedical Informatics, National Yang-Ming Chiao Tung University) integrated with MATLAB (version R2010b; The MathWorks, Inc). The EV ultrastructure was assessed by transmission electron microscopy following standard fixation, dehydration, and epoxy embedding protocols.

RNA isolation, sequencing, and transcriptomic analysis

Total RNA was extracted using EXTRAzol (Blirt SA) from 3 biological replicates/condition; only

samples with an RNA integrity number > 7.0 were included. Libraries were prepared with the Illumina TruSeq Kit (strand specific and deoxyuridine triphosphate [dUTP]-based) and sequenced on the NovaSeq X platform (paired end; 100 bp) at the University of California-Davis Genome Center DNA Technologies and Expression Analysis Core. Reads were quality checked with FASTQC, trimmed, and aligned to the feline reference genome (felCat9.2_X; GCA_000181335.6) using STAR, and differential expression was analyzed with DESeq2.³³ Significance thresholds were set at Benjamini-Hochberg adjusted P (p_{adj}) $\leq .05$ and $|\log_2$ fold change| ($|\log_2FC|$) ≥ 1 . For quantitative PCR validation, cDNA was synthesized using PrimeScript RT Kit (Takara Bio) and amplified with SensiFast SYBR (Bioline) on a CFX Connect system (Bio-Rad Laboratories Inc); glyceraldehyde 3-phosphate dehydrogenase served as the reference gene, and relative expression was calculated using the $2^{-\Delta\Delta C_q}$ method.³⁴ microRNA profiling used the Mir-X First-Strand Kit (Takara Bio), with U6 as the endogenous control.

Flow cytometry and functional assays

Viability, apoptosis (MultiCaspase Kit; Luminex Corp), and mitochondrial membrane potential

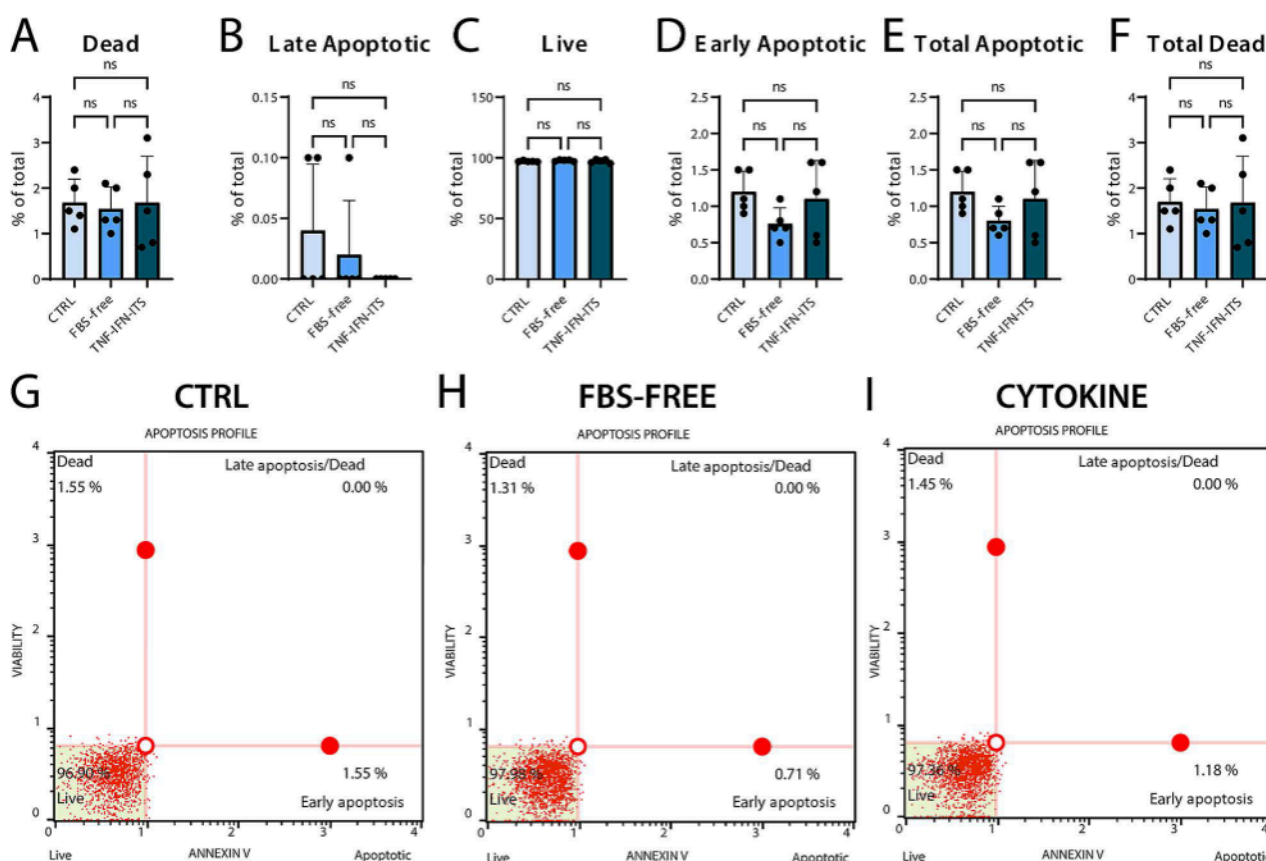


Figure 1—Cell viability assessment by annexin V/propidium iodide flow cytometry across feline placenta-derived mesenchymal stromal cell (fePDMSC; MSC) culture conditions. Live cell fractions: control (CTRL; 96.90%), fetal bovine serum (FBS)-free (97.98%), and cytokine stimulated (97.36%). Early apoptotic populations remained below 1.6% in all groups; late apoptotic fractions were undetectable (0.00%). No statistically significant differences in viability were observed between conditions. Data are presented as mean $\pm$ SD from 3 independent experiments. ns = Not significant.

(MitoPotential Kit; Luminex Corp) of control and primed fePDMSCs were assessed using the Muse Cell Analyzer (Luminex Corp) per manufacturer instructions.³⁵

Statistical analysis

For flow cytometric, metabolic, and mitochondrial morphology outcomes (viability, apoptosis, mitochondrial membrane potential, Kiel 67 proliferation

marker (Ki-67) mean fluorescence intensity (MFI), and mitochondrial subtype quantification), data from control, serum-free, and cytokine-primed fePDMSCs were compared using 1-way ANOVA with the Tukey post hoc test (Prism, version 8; GraphPad Software Inc), with results presented as mean $\pm$ SD from ≥ 3 independent biological experiments, each performed in technical triplicate. Formal normality

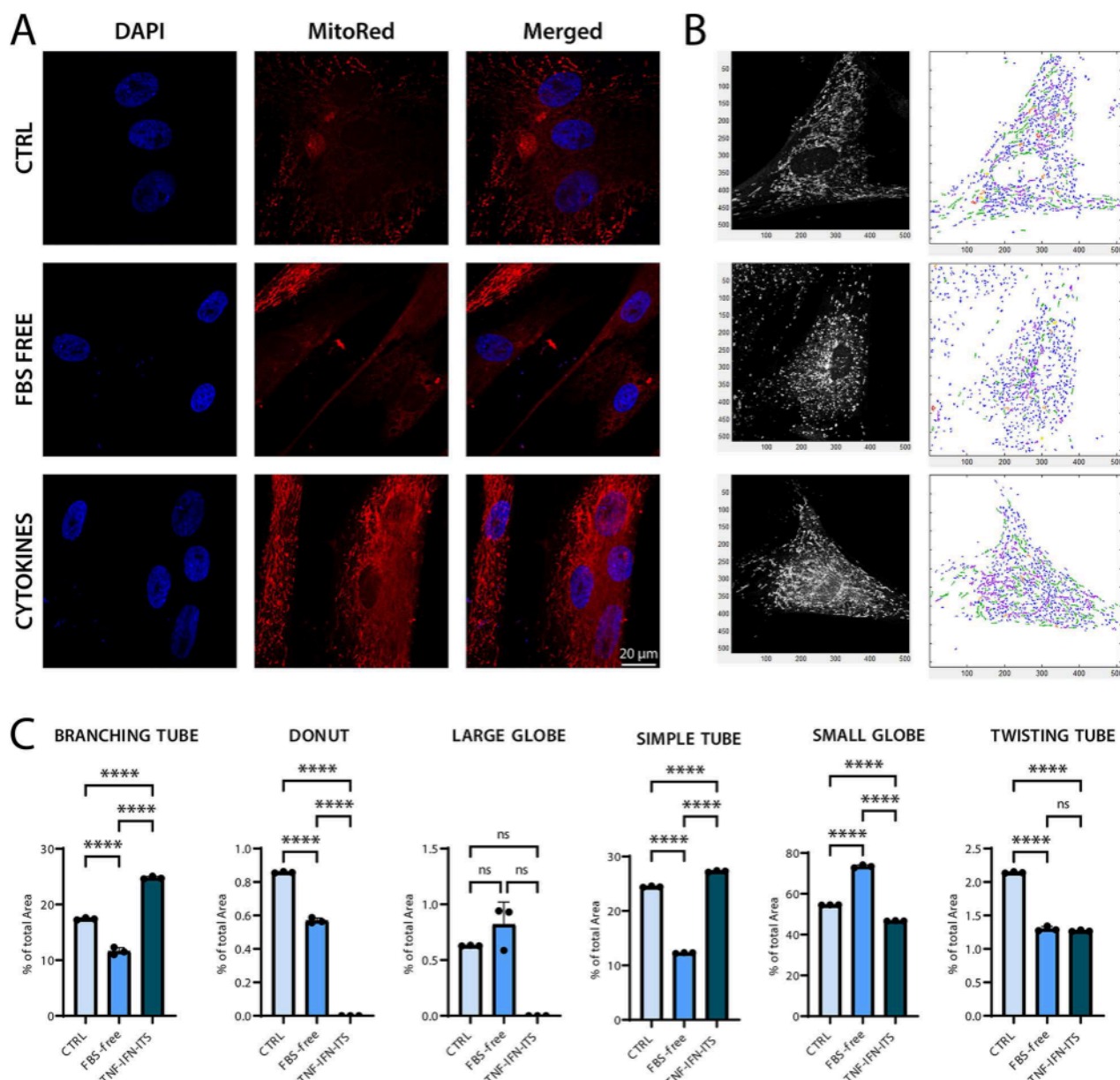


Figure 2—Mitochondrial network architecture and morphological subtype distribution in fePDMSCs across culture conditions. A and B—Representative confocal immunofluorescence images (mitochondrial marker, red; nuclei, 4',6-diamidino-2-phenylindole, blue) of CTRL, FBS-free, and cytokine-stimulated fePDMSCs. Control cells show a dense, perinuclear reticulated mitochondrial network, whereas FBS-free and cytokine-stimulated cells display more elongated, dispersed mitochondria. Binary and trajectory maps confirm these differences. Scale bar = 20 μ m. C—Quantification of mitochondrial morphological subtypes—branching tubular, simple tubular, donut shaped, twisting tubular, small globular, and large globular—across CTRL, FBS-free (starved), and cytokine-stimulated fePDMSCs. Cytokine-primed cells show a significant increase in branching and simple tubular forms. Donut-shaped and twisting tubular mitochondria are most prevalent in CTRL. Starvation increases small globular mitochondria consistent with fragmentation. Data are presented as mean $\pm$ SD. * $P < .05$. *** $P < .001$.

testing (eg, Shapiro-Wilk) was not performed given the limited number of biological replicates per condition ($n = 3$), at which such tests have very limited

power to detect departures from normality; ANOVA was applied as the standard approach for this experimental design and replicate structure, consistent

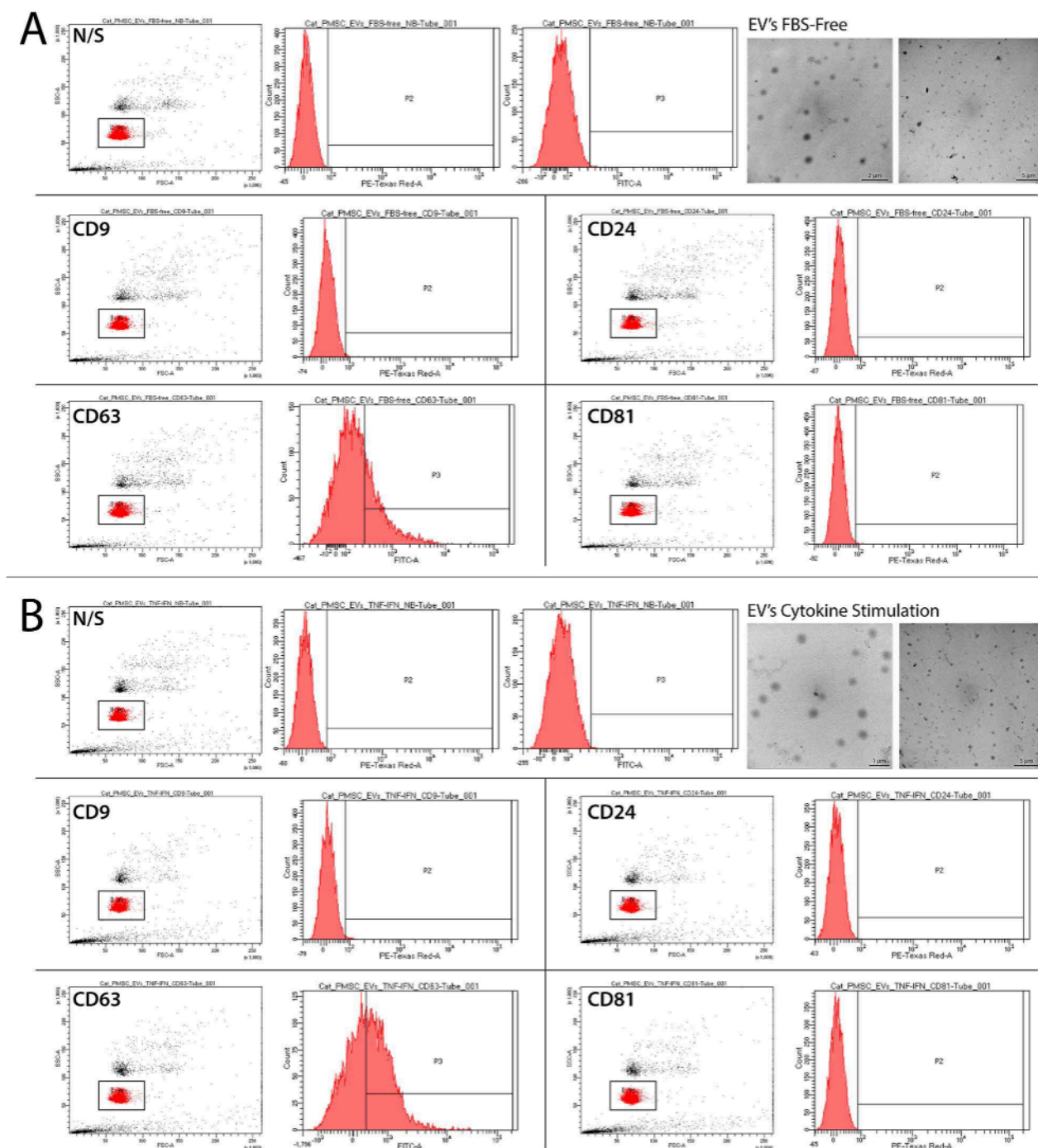


Figure 3—Flow cytometric surface marker profiling of fePDMSC-derived extracellular vesicles (EVs) under FBSfree and cytokine-stimulated conditions. A—Histogram overlays of unstained (gray) and stained EV populations for Cluster of differentiation (CD) 9, CD63, CD81, and CD24 under FBS-free conditions. CD9, CD63, and CD81 display distinct rightward shifts confirming tetraspanin expression consistent with a small EV/exosome identity. CD 24 overlaps with the unstained CTRL. B—Histogram overlays for CD9, CD63, CD81, and CD24 following priming with tumor necrosis factor- α , interferon- γ , IL-1, IL-6, and insulin-transferrin-selenium (ITS). CD9 and CD63 show clear positive shifts; CD63 exhibits the greatest fluorescence displacement. CD81 and CD24 show minimal to absent expression. Gating was established using unstained controls (CTRLs). N/S = Mean nonstimulated.

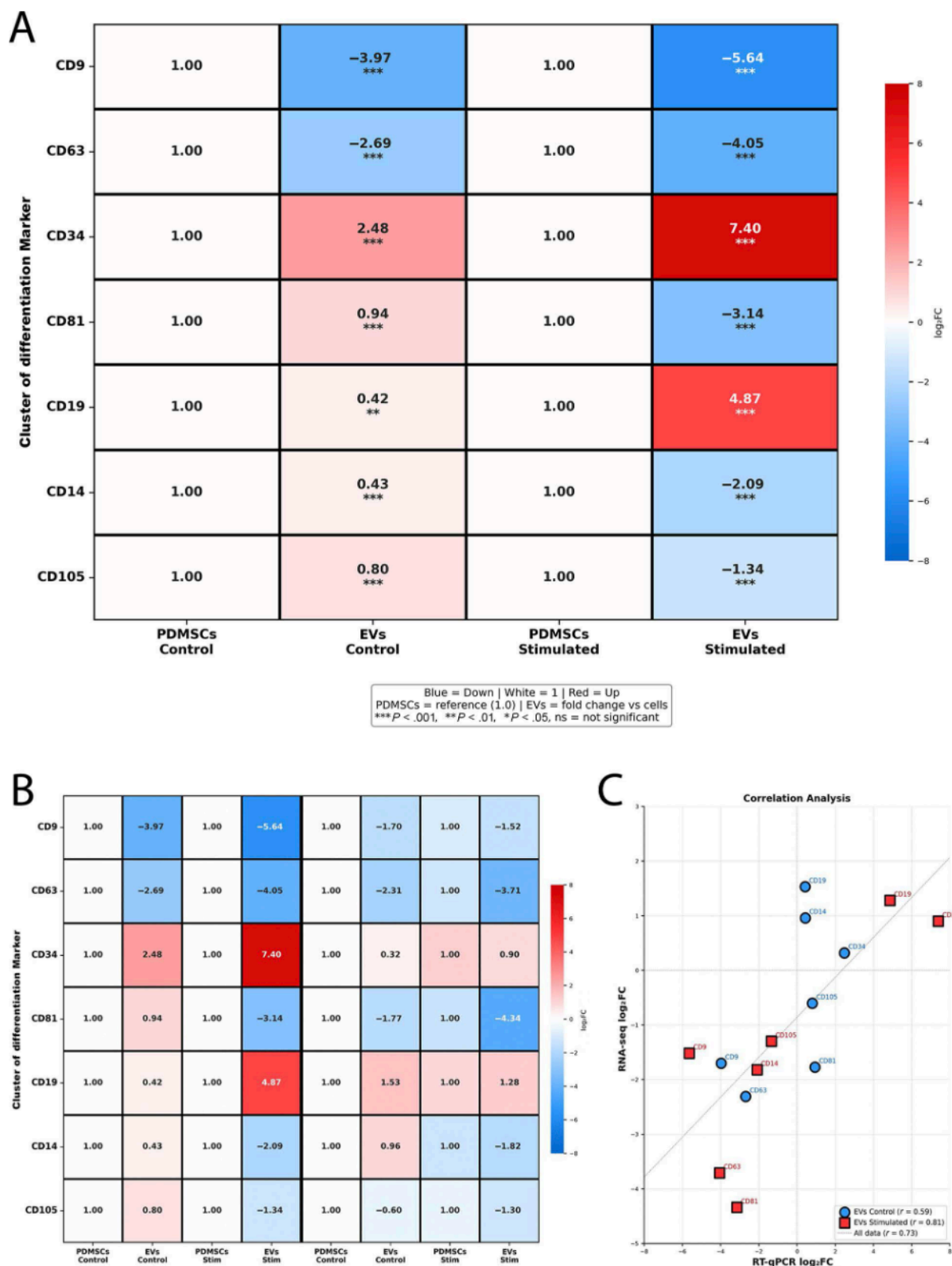


Figure 4—Heatmap of cluster of differentiation (CD) marker log₂ fold changes (log₂FCs) in fePDMSC-derived EVs relative to parental cells under CTRL and cytokine-stimulated conditions. Parental fePDMSC values are set as reference (1.0) per condition. Red indicates upregulation, and blue indicates downregulation. Tetraspanins CD9, CD63, and CD81 are consistently downregulated in EVs under both conditions, with greater reductions in cytokine-stimulated EVs. CD34 and CD19 are markedly upregulated exclusively in cytokine-stimulated EVs (log₂FC = +7.40 and +4.87, respectively). CD14 and CD105 are modestly reduced under both conditions. RNA-seq = RNA sequencing. RT-qPCR = Quantitative reverse transcription PCR.

with common practice in comparable in vitro MSC studies. Statistical significance was defined as $P < .05$. Differential gene and miRNA expression from RNA sequencing were assessed using DESeq2's

negative binomial model with Wald testing and Benjamini-Hochberg correction ($\text{padj} \leq .05$; $|\log_2\text{FC}| \geq 1$), as described above, independent of the ANOVA framework applied to the functional assays.

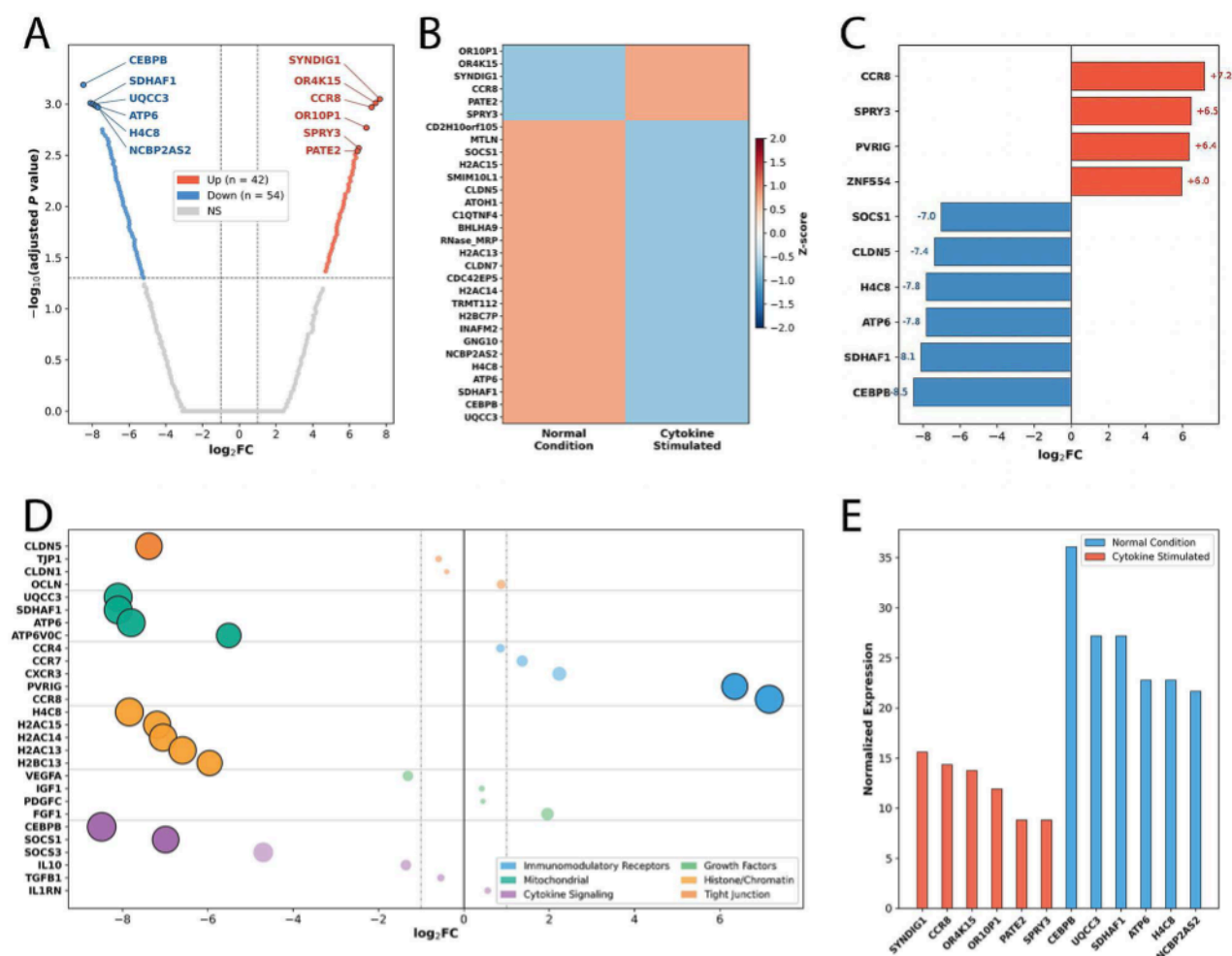


Figure 5—Differential mRNA cargo analysis of EVs from cytokine-stimulated versus control (CTRL) fePDMSCs. Volcano plot showing 329 significant differentially expressed genes (Benjamini-Hochberg adjusted P value $\leq .05$; $|\log_2\text{FC}| \geq 1$) from 17,690 analyzed transcripts: 231 upregulated (red) and 98 downregulated (blue). The top upregulated hits include immunomodulatory receptors C-C motif chemokine receptor 8 (CCR8; $\log_2\text{FC} = +7.18$; undetectable in CTRL EVs) and poliovirus receptor-related immunoglobulin domain-containing protein (PVRIG) ($\log_2\text{FC} = +6.37$). Histone transcripts are among the most substantially downregulated (mean $\log_2\text{FC} = -6.92$). Pathway enrichment analysis of downregulated genes highlights neutrophil extracellular trap formation and systemic lupus erythematosus pathways. ATOH1 = Atonal bHLH transcription factor 1. ATP6 = Mitochondrially encoded ATP synthase membrane subunit 6. ATP6V0C = ATPase H+ transporting V0 subunit c. BHLHA9 = Basic helix-loop-helix family member a9. C1QTNF4 = C1q and TNF related 4. C10orf105 = Chromosome 10 open reading frame 105. CCR4 = C-C motif chemokine receptor 4. CCR7 = C-C motif chemokine receptor 7. CCR8 = C-C motif chemokine receptor 8. CDC42EP5 = CDC42 effector protein 5. CEBPB = CCAAT enhancer binding protein beta. CLDN1 = Claudin 1. CLDN5 = Claudin 5. CLDN7 = Claudin 7. CXCR3 = C-X-C motif chemokine receptor 3. FGF1 = Fibroblast growth factor 1. GNG10 = G protein subunit gamma 10. H2AC13 = H2A clustered histone 13. H2AC14 = H2A clustered histone 14. H2AC15 = H2A clustered histone 15. H2BC13 = H2B clustered histone 13. H2BC7P = H2B clustered histone 7, pseudogene. H4C8 = H4 clustered histone 8. IGF1 = Insulin-like growth factor 1. IL10 = Interleukin 10. IL1RN = Interleukin 1 receptor antagonist. INAFM2 = InaF motif containing 2. MTLN = Mitoregulin. NCBP2AS2 = NCBP2 antisense RNA 2. OCLN = Occludin. OR10P1 = Olfactory receptor family 10 subfamily P member 1. OR4K15 = Olfactory receptor family 4 subfamily K member 15. PATE2 = Prostate and testis expressed 2. PDGFC = Platelet-derived growth factor C. PVRIG = Poliovirus receptor-related immunoglobulin domain containing. RMRP = RNA component of mitochondrial RNA processing endonuclease. SDHAF1 = Succinate dehydrogenase complex assembly factor 1. SMIM10L1 = Small integral membrane protein 10 like 1. SOCS1 = Suppressor of cytokine signaling 1. SOCS3 = Suppressor of cytokine signaling 3. SPRY3 = Sprouty RTK signaling antagonist 3. SYNDIG1 = Synapse differentiation inducing 1. TGFBI = Transforming growth factor beta 1. TJP1 = Tight junction protein 1. TRMT112 = tRNA methyltransferase activator subunit 11-2. UQCC3 = Ubiquinol-cytochrome c reductase complex assembly factor 3. VEGFA = Vascular endothelial growth factor A. ZNF554 = Zinc finger protein 554.

Results

Morphological stability and condition-dependent proliferative quiescence

Confocal microscopy of fePDMSCs at passage zero revealed a dense population with characteristic fibroblast-like morphology, prominent F-actin stress fiber networks, and extensively interconnected tubular mitochondrial networks spanning the cytoplasm, consistent with high mitochondrial membrane potential and active oxidative phosphorylation. By passage 2, actin organization was maintained, whereas mitochondrial signals became more perinuclear (**Supplementary Figure S1**).

Kiel 67 proliferation marker (Ki-67) immunofluorescence revealed a condition-dependent gradient in proliferative activity. Control fePDMSCs displayed active proliferation (mean fluorescence intensity [MFI], 232 ± 14 AU), significantly reduced in FBS-free cells (MFI, 126 ± 11 AU; $P < .001$ vs control) and further reduced in cytokine-stimulated cells (MFI,

50 ± 7 AU; $P < .001$ vs control). No significant difference was observed between FBS-free and cytokine-stimulated conditions. Metabolic activity assessed by In Vitro Toxicology Assay Kit, resazurin based (TOX-8) confirmed these findings (Supplementary Figure S1).

Cell viability is preserved across all culture conditions

Annexin V/propidium iodide flow cytometry confirmed high viability across all conditions, with live cell fractions exceeding 96% (control, 96.90%; FBS-free, 97.98%; cytokine stimulated, 97.36%) and no statistically significant intergroup differences. Late apoptotic fractions were undetectable, and early apoptotic populations remained below 1.6% (**Figure 1**). These findings indicate that altered EV cargo compositions reflect active transcriptional shift in viable, quiescent cells rather than apoptosis-driven release.

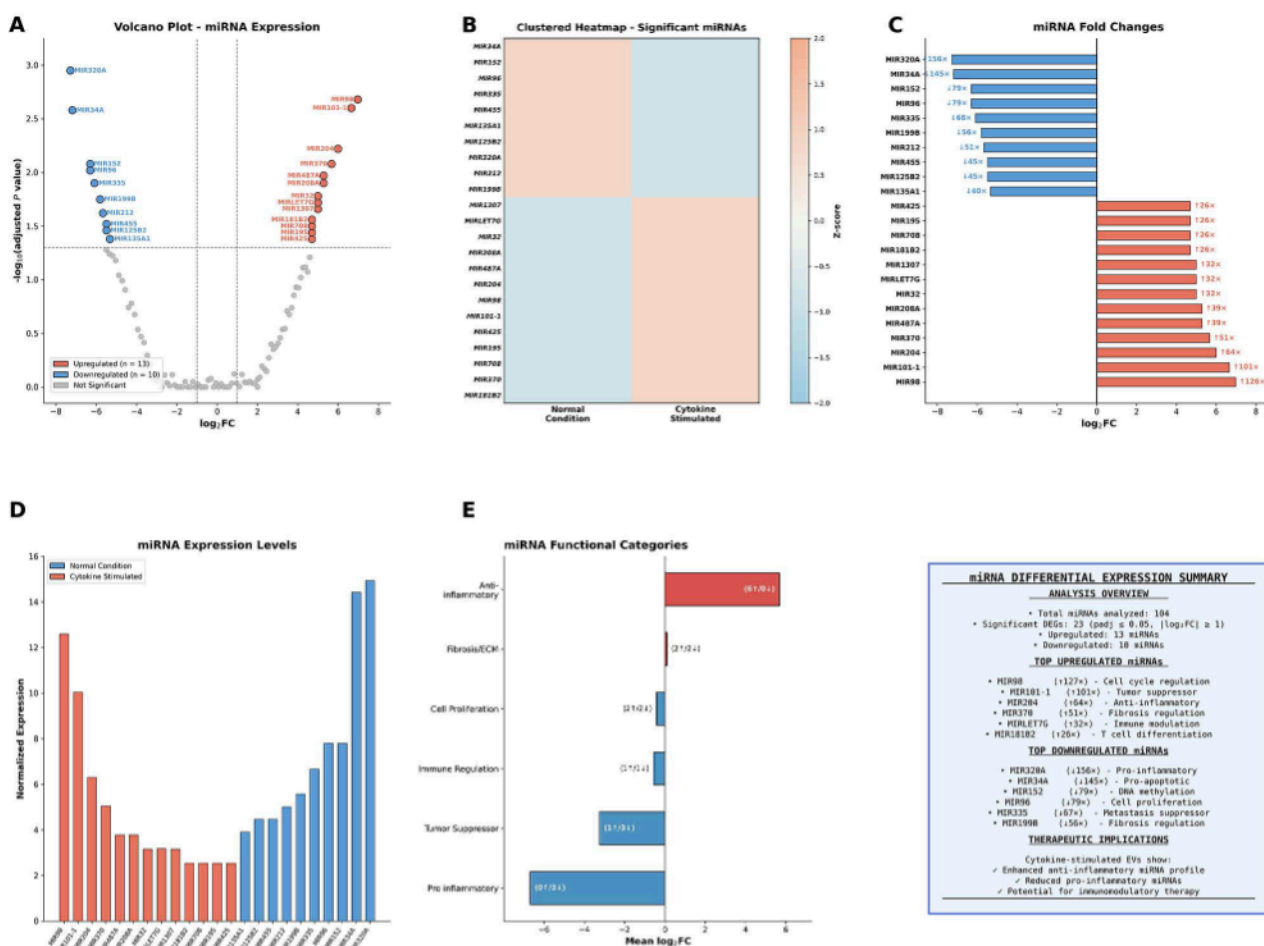


Figure 6—Differential microRNA (miRNA; MIR) cargo analysis of EVs from cytokine-stimulated versus CTRL fePDMSCs. A—Volcano plot of 23 significant differentially expressed miRNAs (Benjamini-Hochberg adjusted P value $\leq .05$; $|\log_2FC| \geq 1$): 13 upregulated (red) and 10 downregulated (blue). The top upregulated anti-inflammatory miRNAs: MIR204 (64-fold; nuclear factor κ B inhibition, antifibrotic), MIRLET7G (32-fold; inflammatory regulation), and MIR181B2 (26-fold; immune cell differentiation). The top downregulated proinflammatory miRNAs: MIR34A (145-fold decrease) and MIR320A ($\log_2FC = -7.29$). B—Hierarchical clustering heatmap confirming clear condition separation. C—Functional category analysis showing anti-inflammatory miRNAs with the highest positive FCs and proinflammatory miRNAs with the largest negative changes across all assessed categories. ECM = Extracellular matrix.

Cytokine priming induces mitochondrial elongation; serum deprivation promotes fragmentation

Confocal immunofluorescence revealed condition-dependent changes in mitochondrial network architecture. Control cells displayed a dense, perinuclear reticulated network consistent with an active, fused mitochondrial state. The FBS-free cells showed increased mitochondrial fragmentation with sparse trajectory patterns in binary mapping analysis. Cytokine-stimulated cells exhibited a significant increase in branching and simple tubular mitochondria, indicating enhanced elongation and network remodeling. Donut-shaped and twisting tubular forms were most prevalent under control conditions and markedly reduced in both stressed states. Starvation increased small globular mitochondria consistent with fragmentation, whereas large globular forms showed no statistically significant change across groups (**Figure 2**).

Surface marker profiling of fePDMSC-derived EVs

Flow cytometry confirmed canonical tetraspanin expression (CD9, CD63, and CD81) in EVs isolated under FBS-free conditions, consistent with a small EV/exosome phenotype. The CD24 expression was minimal, overlapping substantially with unstained controls (**Figure 3**). In EVs from cytokine-primed fePDMSCs, CD9 and CD63 expression was preserved, with CD63 demonstrating the strongest fluorescence shift; CD81 and CD24 remained largely absent.

Heatmap analysis of $\log_2$ FCs for 7 cluster of differentiation markers in EVs relative to parental fePDMSCs revealed condition-dependent surface marker remodeling. Classical tetraspanins CD9, CD63, and CD81 were consistently downregulated in EVs under both conditions, with greater reductions in cytokine-stimulated EVs (CD9, $\log_2$ FC = -5.64; CD63, -4.05; CD81, -3.14) than in control EVs (CD9, -3.97; CD63, -2.69; CD81, +0.94). Cluster of differentiation 34 and CD19 were markedly upregulated exclusively in cytokine-stimulated EVs ($\log_2$ FC = +7.40 and +4.87, respectively). Cluster of differentiation 14 and CD105 were modestly reduced under both conditions (**Figure 4**).

Cytokine priming alters EV mRNA cargo

Differential gene expression analysis of EV mRNA cargo from cytokine-stimulated versus control fePDMSCs identified 329 significantly differentially expressed genes from 17,690 analyzed ($\text{padj} \leq .05$; $|\log_2\text{FC}| \geq 1$), comprising 231 upregulated and 98 downregulated transcripts (**Figure 5**). The most pronounced upregulation involved immunosuppressive receptors CCR8 ($\log_2\text{FC} = +7.18$; undetectable in control EVs) and PVRIG/CD112R ($\log_2\text{FC} = +6.37$), whereas histone-encoding transcripts (mean $\log_2\text{FC} = -6.92$) and mitochondrial-associated mRNAs were substantially downregulated. Pathway enrichment analysis of downregulated genes identified neutrophil extracellular trap formation and systemic lupus erythematosus pathways.

Cytokine priming enriches EV miRNA cargo with anti-inflammatory regulators

Differential miRNA expression analysis identified 23 significantly differentially expressed miRNAs in EVs from cytokine-stimulated versus control fePDMSCs ($\text{padj} \leq .05$; $|\log_2\text{FC}| \geq 1$), comprising 13 upregulated and 10 downregulated transcripts (**Figure 6**). Hierarchical clustering confirmed coordinated rather than stochastic cargo shifts. Upregulated miRNAs included miR-204 (64-fold increase; $\log_2\text{FC}$ almost equal to 6.0), let-7g (32-fold), and miR-181b-2 (26-fold). Proinflammatory miR-34a (145-fold decrease; $\log_2\text{FC}$ almost equal to -7.2) and miR-320a ($\log_2\text{FC} = -7.29$) were markedly depleted. Functional category analysis confirmed a global shift toward an anti-inflammatory miRNA profile.

Discussion

This study investigated the effects of inflammatory cytokine priming on feline placental derived mesenchymal stromal cell (fePDMSC) viability, mitochondrial dynamics, and EV cargo composition, with implications for improving mesenchymal stromal cell (MSC)-based therapies in cats with feline chronic gingivostomatitis (FCGS) and related chronic inflammatory diseases. The principal findings were that (1) cytokine priming induces proliferative quiescence without compromising viability, (2) cytokine stimulation and serum deprivation exert divergent effects on mitochondrial architecture despite producing similar degrees of proliferative arrest, and (3) cytokine priming comprehensively remodels EV mRNA and micro RNA (miR) cargo toward an anti-inflammatory, immunosuppressive phenotype.

The fePDMSCs cells maintained characteristic morphology, phenotype, and viability across multiple passages and culture conditions, consistent with established criteria for multipotent MSCs.³⁶ Although both FBS-free and cytokine-primed conditions induced proliferative quiescence similarly described in hematopoietic stem cells,³⁷ evidenced by reduced Ki-67 expression and metabolic activity, cell viability remained exceptionally high (> 96%) with negligible apoptosis across all groups. This is an important distinction from conditions in which reduced proliferation is associated with cellular aging or apoptosis^{38,39} and confirms that the substantial EV cargo alterations reflect an active transcriptional shift in viable cells rather than stress-induced or apoptotic release.

The divergent mitochondrial responses to serum deprivation versus cytokine stimulation, fragmentation versus elongation and branching, respectively, represent a key mechanistic finding. Cytokine-primed cells demonstrated significant increases in branching and simple tubular mitochondria, morphologies associated with enhanced oxidative phosphorylation and immunomodulatory function in activated MSCs.⁴⁰⁻⁴⁴ Conversely, serum deprivation promoted small globular mitochondrial forms, consistent with fission, reduced membrane potential, and compromised oxidative capacity.⁴⁵⁻⁴⁷ The enhanced mitochondrial connectivity in cytokine-primed cells may support the energy demands of cytokine secretion,

EV biogenesis, and active immunomodulation,⁴⁸⁻⁵⁴ and prior studies⁵⁵⁻⁶⁰ have linked mitochondrial elongation to increased immunosuppressive capacity and paracrine signaling in MSCs.

Flow cytometric characterization confirmed canonical tetraspanin expression (CD9, CD63, and CD81) in EVs under both conditions, consistent with an exosomal phenotype and in accordance with Minimal Information for Studies of Extracellular Vesicles (MISEV2023) guidelines to the extent permitted by currently available reagents.^{61,62} Full compliance with MISEV2023 recommendations for EV characterization could not be achieved, however, because validated antifeline antibodies targeting canonical EV transmembrane markers, including CD9, CD63, and CD81, are not currently available, precluding the transmembrane protein confirmation recommended as a minimum requirement for EV studies; this represents a broader methodological gap in feline EV research that warrants dedicated reagent development.⁶¹ The marked upregulation of CD34 and CD19 exclusively in cytokine-stimulated EVs ($\log_2FC = +7.40$ and $+4.87$, respectively) was unexpected and suggests that inflammatory priming may shift EVs toward enhanced angiogenic, vasculoprotective, or immunomodulatory functions,^{58,63} warranting further investigation.

Extracellular vesicle mRNA cargo shifts were characterized by strong upregulation of immunosuppressive receptors CCR8 and PVRIG/CD112R, both negative regulators of T-cell-mediated immune responses.^{64,65} C-C motif chemokine receptor 8, a chemokine receptor that preferentially recruits regulatory T cells and is associated with immunosuppressive microenvironments, was undetectable in control EVs but robustly expressed following cytokine priming.⁶⁶ Poliovirus receptor-related immunoglobulin domain-containing protein (PVRIG) engages PVRL2 to suppress T-cell activation and cytotoxicity, a mechanism directly relevant to FCGS, in which CD8⁺ T cells drive chronic mucosal inflammation.^{8,67} The coordinated downregulation of histone-encoding transcripts, which function as damage-associated molecular patterns when present extracellularly,⁶⁸ alongside neutrophil extracellular trap formation-associated genes suggests active quality-control mechanisms that prevent proinflammatory signal delivery to recipient cells.⁶⁹

The miRs cargo analysis revealed global alteration toward anti-inflammatory functionality. miR-204, the most substantially upregulated miR (64-fold), suppresses nuclear factor κ B signaling and exerts antifibrotic effects,⁷⁰⁻⁷³ whereas let-7g suppresses TLR4 signaling and inflammatory cytokine release in macrophages,⁷⁴ and miR-181b-2 regulates T-cell receptor reactivity and promotes regulatory T-cell development.⁷⁵ Proinflammatory miR-34a (145-fold decrease), linked to nuclear factor κ B activation and senescence-associated secretory phenotype,^{76,77} and miR-320a,⁷⁸ associated with endothelial dysfunction, were nearly completely depleted from cytokine-primed EVs, suggesting active sorting that prevents delivery of proinflammatory miR species.

It is important to note that the transcriptional and cargo changes observed here reflect a response to a specific, sustained proinflammatory cytokine exposure (72 hours) rather than a demonstrated permanent or systemic alteration of fePDMSC identity or function. Mesenchymal stromal cells are known to be highly plastic and transcriptionally responsive to their microenvironment; whether the shifts in gene expression and EV cargo composition described here persist, reverse, or are further modified following removal of the priming stimulus or upon exposure to a different microenvironment (eg, the *in vivo* inflammatory milieu of FCGS) was not evaluated in this study and represents an important direction for future work.

These findings have direct translational relevance to FCGS management. Approximately two-thirds of cats achieve successful outcomes with MSC therapy,¹⁻⁶ but response rates remain heterogeneous, and mechanisms of therapeutic efficacy are incompletely defined. Our data suggest that inflammatory priming prior to MSC administration may substantially enhance EV immunomodulatory potency. The finding that cytokine stimulation and serum deprivation both induce proliferative quiescence further suggests that the timing of MSC administration relative to disease chronicity warrants consideration; delayed administration into an established inflammatory niche may better replicate the cytokine conditions tested here and enhance EV-mediated immunomodulation.^{8,9,79,80}

Although this study was not designed to assess manufacturing feasibility, inflammatory cytokine priming represents a straightforward *ex vivo* preconditioning strategy that can be incorporated into existing MSC culture workflows.^{81,82} Importantly, cytokine exposure did not adversely affect fePDMSC viability, suggesting that this approach may be compatible with scalable cell manufacturing. Nevertheless, translation to clinical or commercial production will require optimization of cytokine concentrations, exposure duration, donor-to-donor reproducibility, large-scale culture methods, and Good Manufacturing Practices-compliant manufacturing processes.⁸³ Therefore, our findings establish a mechanistic rationale for inflammatory licensing as an approach to enhance MSC potency rather than demonstrating commercial readiness.

Several limitations warrant consideration. While surface marker expression supporting MSC identity of the parent fePDMSC and feAMSC populations used in this line of work was previously reported by our group,¹⁸ this study did not perform confirmatory flow cytometric assessment against the full ISCT minimal criteria³⁶ or trilineage differentiation assays and, most importantly, did not reassess surface marker phenotype following cytokine priming. This is a relevant limitation given that IFN- γ is known to upregulate major histocompatibility complex class II (HLA-DR) expression on MSCs, one of the International Society for Cell & Gene Therapy (ISCT)-defined negative markers, raising the possibility that priming altered the surface phenotype of these cells in ways not captured by the morphological, mitochondrial, and

transcriptomic assays performed here. Future studies should incorporate flow cytometric marker profiling and trilineage differentiation confirmation both before and after priming to formally establish MSC identity and characterize any priming-induced phenotypic drift in this study and in our related work on cytokine-primed feAMSCs. Functional consequences of EV cargo changes in recipient immune cells (eg, T-cell proliferation, macrophage polarization) were not assessed and represent an important next step; coculture and in vivo studies are needed to validate mechanistic interpretations. Whether transcriptomic changes translate into greater in vivo therapeutic efficacy in FCGS models also remains to be determined.⁸⁴ The mechanisms governing selective RNA sorting into EVs, including candidate RNA-binding proteins, such as heterogeneous nuclear ribonucleoprotein A2/B1, (hnRNP A2/B1), Argonaute 2 (AGO2), and Y-box binding protein 1 (YBX1), were not characterized.⁸⁵ Finally, full compliance with MISEV2023 EV characterization guidelines could not be achieved because validated antifeline tetraspanin antibodies are unavailable, representing a species-specific methodological gap that the field must address.⁶¹

Acknowledgments

None reported.

Disclosures

The authors have nothing to disclose. No AI-assisted technologies were used in the composition of this manuscript.

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Funding

This study was supported by the George and Phyllis Miller Feline Health Trust of the San Francisco Foundation, administered by the Center of Companion Animal Health, University of California-Davis. Library preparation and sequencing were performed at the DNA Technologies and Expression Analysis Cores at the University of California-Davis Genome Center, supported by NIH Shared Instrumentation grant No. 1S10OD010786-01. Dr. Soltero-Rivera was supported in part by the National Center for Advancing Translational Sciences, NIH (grant No. UL1 TR001860). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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