Targeting Fibrotic Scars with Extracellular Vesicles Extracted from Mature Aloe vera: Enhanced Antioxidant, Anti-inflammatory, and Antifibrotic Activity through M2 Macrophage Polarization and Myofibroblasts Inhibition

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Abstract

Background: Abnormal scarring and fibrotic skin disorders arise from dysregulated wound healing processes. While Aloe vera is widely recognized for its therapeutic properties, the potential of its extracellular vesicles (Av-EVs) remains underexplored.

Objective: This study aimed to isolate and characterize Av-EVs and evaluate their antioxidant, anti-inflammatory, and antifibrotic properties *in vitro*, focusing on the impact of extraction method and plant maturity.

Methods: Av-EVs were isolated from mature and young Aloe vera leaves using manual (NB) or blender-based (B) homogenization. Vesicles were characterized by nanoparticle tracking analysis, transmission electron microscopy, and protein quantification. Antioxidant and cytotoxicity assays (DPPH, alamarBlue) were followed by functional anti-inflammatory and antifibrotic analyses respectively in LPS-stimulated THP-1 macrophages and TGF-β1/Vitamin C-activated human dermal fibroblasts (RT-qPCR, immunofluorescence, proteomics).

Results: NB-derived EVs from mature leaves exhibited the most potent activity across all assays, showing superior antioxidant capacity, greater suppression of pro-inflammatory cytokines, enhanced M2 macrophage polarization, and significant downregulation of COL1A1 and α-SMA. In contrast, B-derived and young leaf-derived EVs showed reduced bioactivity, with young EVs failing to inhibit fibrotic markers.

Conclusion: Manually extracted Av-EVs from mature leaves demonstrate superior multifunctional bioactivity, highlighting their potential as plant-derived nanotherapeutics for fibrotic scar modulation.

Keywords
Wound healing, oxidative stress, inflammation resolution, myofibroblast differentiation, regenerative skin therapy

1. Introduction

Disruptions in the tightly regulated wound healing process – hemostasis, inflammation, proliferation, and remodeling – can lead to pathological outcomes, ranging from chronic nonhealing wounds to excessive fibrotic scarring. The latter is characterized by aberrant collagen accumulation, disorganized extracellular matrix (ECM) remodeling, and persistent myofibroblast activation driven by abnormal fibroblast proliferation and transdifferentiation.[1] Among the principal causes of abnormal wound healing are chronic inflammation and oxidative stress, which induce intracellular overproduction of reactive oxygen species (ROS).[2] Excess ROS not only damage cellular macromolecules such as lipids, proteins, and DNA, but also perpetuate pro-inflammatory signaling and impair tissue regeneration.[3] In this dysregulated microenvironment, ROS activate latent transforming growth factor-beta (TGF-β), which in turn promotes fibroblast-to-myofibroblast differentiation and excessive secretion of collagen and α-smooth muscle actin (α-SMA) – a critical protein for wound contraction. These molecular events ultimately result in the formation of fibrotic scar tissue, clinically recognized as hypertrophic scars and keloids.[4, 5]

To mitigate such outcomes, Aloe vera has been extensively studied for its diverse pharmacological properties and high concentration of bioactive constituents.[6-8] This succulent plant harbors over 200 compounds and 75 nutrients, including soluble sugars, lipids, proteins, polysaccharides and lignin, polyphenols, enzymes, vitamins (e.g., A, C, E, B_1, B_2, B_{12}) and minerals (e.g., calcium, potassium, sodium, magnesium),[9] which collectively confer its wound healing, anti-inflammatory, antioxidant, antimicrobial, immunomodulatory, analgesic, and moisturizing benefits.[10, 11]

Historically, Aloe vera research has centered on topical and oral formulations. A large body of evidence, including *in vitro* studies, preclinical models, and human clinical trials, has consistently demonstrated the therapeutic efficacy of Aloe vera-based products.[12-29] However, a promising new frontier in this research has emerged with the exploration of plant-derived extracellular vesicles (PDEVs) – nanoscale membranous particles secreted by plant cells that not only retain the therapeutic attributes of their source tissue but also offer target delivery capabilities. By delivering a diverse cargo of lipids, nucleic acids, proteins, metabolites, and even pharmacological compounds, PDEVs can mediate intracellular and intercellular communication, maintain tissue homeostasis, and support regenerative processes.[30]

Compared to their mammalian counterparts, PDEVs offer multiple advantages beyond their typical mechanism of action, such as being ethically uncontroversial, cost-effective and scalable, and evidence thus far suggests that they do not pose risk of carcinogenesis, immunogenicity, or tissue rejection. Additionally, they overcome key limitations of synthetic and mammalian EV-based drug delivery systems, including low yield, short bioavailability, poor loading efficiency, and limited tissue targeting.[30-34] For these reasons, PDEVs are now considered as a new class of cell-free therapeutic platforms with high potential for clinical translation and preventive healthcare.

Among PDEVs, Aloe-derived EVs have recently moved into the spotlight for their ability to resemble the plant's therapeutic properties while acting as efficient, biocompatible nanocarriers. Preliminary studies suggest that they can regulate inflammatory signaling, activate antioxidant defenses, and prevent the differentiation and contractile activity of myofibroblasts.[35-37] Their versatility is further exemplified by

their applications in indocyanine green encapsulation for targeted melanoma therapy.[38] However, there remains a significant lack in the literature regarding the influence of extraction methodology and plant maturity on the biological efficacy of Aloe-derived EVs, and no *in vitro* study has yet evaluated their concurrent ability to modulate oxidative stress, inflammatory responses, and fibrotic remodeling within the context of abnormal skin scarring.

Therefore, in this study, we report a comprehensive biological characterization of Aloe vera gel-derived extracellular vesicles (Av-EVs), with a focus on their capacity to modulate key pathological mechanisms underlying abnormal scar formation. Specifically, we investigated the ability of Av-EVs to attenuate pro-inflammatory cytokine expression in M1-polarized macrophages while promoting M2-like phenotype, suppress myofibroblast differentiation in human dermal fibroblasts, and reduce intracellular ROS levels – three interrelated processes central to fibrotic remodeling. To deepen our understanding of their therapeutic value, we systematically compared Av-EVs isolated via manual extraction (no blender, NB) versus shear-force homogenization (blender, B) and evaluated the influence of leaf maturity by comparing EVs derived from mature Aloe vera leaves (commercially-grade) and younger leaves from nursery-grown plants. This comparative framework provides critical insights into how source material and processing techniques affect the physicochemical and functional properties of Av-EVs, and positions them as promising, plant-based nanotherapeutics for the prevention and treatment of fibrosis-related skin disorders.

2. Materials and Approach

2.1 Av-EVs Isolation and Characterization

2.1.1 Av-EVs Isolation from Aloe vera Gel

Fresh Aloe vera *barbadensis miller* leaves from mature plants (Melissa's/World Variety Produce, Inc., USA) with similar appearance and dimensions (~67 cm long x ~9 cm wide x ~3 cm thick) were purchased from a local market. Note that the comparison between mature and young plants is described under 2.3.7. These mature leaves were cut and rinsed with distilled water until the mucilage was removed. Subsequently, the pointed ends and the anterior leaf skin were chopped with a sterilized knife to separate it from the transparent inner gel. After peeling, the extraction and homogenization of the Aloe vera gel was carried out employing a manual method (no blender, NB Av-EVs) and a shear force-based (blender, B Av-EVs) method. For the manual method, the gel was meticulously scooped out using a sterilized spoon, avoiding any inclusion of the yellow latex. A total of 400 mL of the extracted gel was transferred into a clean glass container where it was mixed with Dulbecco's phosphate-buffered saline (DPBS, Cytiva, USA, SH30028.02) in a 1:1 v/v ratio and homogenized using an orbital shaker (Chemglass CLS-4021-100 Versa-Orb, USA) at 100 rpm and 4 °C for 1 h. Then, the remaining solid residues were separated with a sterilized fine-mesh sieve. On the other hand, the entire transparent inner portion was removed from the posterior leaf skin and homogenized with 1:1 v/v ratio of DPBS using a commercial blender for the shear force-based method to obtain 800 mL of Aloe vera juice. In both cases, the soluble fractions were sequentially centrifuged (Centrifuge 5810, Eppendorf, USA) at 1,000 g for 10 min, 2,000 g for 20 min, 3,000 g for 30 min, and 15,000 g for 35 min at 4 °C to remove debris and larger particles. The supernatants were then ultracentrifuged (Optima™ MAX-XP Ultracentrifuge, Beckman Coulter®, USA) at 100,000 g

for 60 min at 4 °C and the resulting 6 pellets were resuspended and pooled in 10 mL of DPBS. The final suspensions containing the Av-EVs were filtered through a 0.22 µm filter (GVS, USA, FJ25BSCCA002AL01) and stored in aliquots of 1 mL at −80 °C until further use. The entire process was performed four times for each method, processing a total of 400 mL of Aloe vera gel and yielding four 10 mL batches of purified Av-EVs per ultracentrifugation cycle.

2.1.2 Nanoparticle Tracking Analysis

Particle concentration and size distribution were measured using nanoparticle tracking analysis (NTA, NanoSight NS300, Malvern Panalytical, UK). The samples were diluted 1:50 with DPBS to reach an optimal concentration for instrument linearity. Three videos of 30 s were captured with a camera level of 14 and measured with a detection threshold of 5. Data was processed with the NanoSight NTA 3.4 software.

2.1.3 Transmission Electron Microscopy

Transmission electron microscopy (TEM) was employed to verify vesicle structure, for which 3 mL of the Av-EVs preparation were spotted on formvar/carbon-coated copper grids with a 200-mesh size (Electron Microscopy Science, USA) following glow discharge for 30 s at 30 mA and incubation for 30 s. The excess sample was blotted, and grids were rinsed sequentially with molecular grade water and counterstained with 0.75% uranyl formate solution for 1 min. Grids were allowed to air dry before imaging them on a Talos L120C TEM equipped with a 16 MP CETA camera at 8,500 and 22 k magnification using the TIA software (Thermo Fisher Scientific, USA).

2.1.4 Protein Quantification

Pierce™ BCA protein assay kit (Thermo Scientific, USA, 23225) was used to quantify protein concentration in Av-EV samples following the manufacturer's instructions for the standard protocol and microplate procedure. For each protein quantification assay, a single EV pellet – obtained from one of the six ultracentrifugation pellets produced during one Av-EV isolation cycle – was individually resuspended in 300 µL of Pierce™ RIPA lysis buffer (Thermo Scientific, USA, 89901). No pooling of multiple pellets was performed prior to protein analysis to ensure that concentration measurements reflected the yield per individual pellet.

Briefly, the diluted bovine serum albumin (BSA, 23209) standards were prepared using 0 – 400 µL of DPBS for a working range of 2,000 – 25 µg/mL. The working reagent (WR) was prepared by mixing reagent A (23228) and B (1859078) at a 50:1 ratio until obtaining a clear, green WR for a total of 9 standards, 6 Av-EV samples and 2 replicates. 10 µL of each standard and Av-EVs samples were pipetted into a clear 96-well plate and 200 µL of the WR were added to each well. The plate was thoroughly mixed on an orbital shaker for 30 s, covered with aluminum foil to protect it from direct light, and incubated at 37 ºC for 30 min. Afterward, the absorbance was read at 562 nm on a SpectraMax iD3® spectrophotometer equipped with the Softmax Pro 7 software (Molecular Devices, USA). Results were normalized to the blank standard measurement of DPBS, and the protein concentration of each Av-EV sample was determined based on the BSA standard curve. Statistical difference was assessed with unpaired student's t-test.

2.2 Cell Cultures

2.2.1 THP-1 Culture and Differentiation into M0 Macrophages

Human leukemia monocytic type 1 (THP-1) cells (The American Type Culture Collection (ATCC), USA) were cultured in Roswell park memorial institute medium (RPMI 1640, Cytiva, USA, SH30255.01) supplemented with 10% fetal bovine serum (FBS, Cytiva, USA, SH30070.03), 1% anti-anti (A/A, Gibco™, USA, SV30079.01), and 1% sodium pyruvate (Gibco™, CN, 11360-070) at 2×10^5 cells/mL concentration under standard conditions (37 ºC with 5% CO_2). The cells were allowed to grow for two weeks with medium change every 2 – 3 days. Differentiation to macrophages (M0) was done by seeding the THP-1 cells into 24-well plates at 2×10^5 cells per well and adding 100 ng/mL of phorbol 24-myristate 13-acetate (PMA, Cayman Chemical, USA, 16561-29-8) with standard medium for 24 h until the cells were attached and attained a macrophage-like morphology. Following adhesion, the corresponding treatment was added, and the specific assay was performed.

2.2.2 hDFs Culture

Primary human dermal fibroblasts (hDFs), lot numbers 80405992 and 80124232 (ATCC, USA), were cultured in Dulbecco's modified eagle medium (DMEM, Cytiva, USA, SH30023.01) supplemented with 10% FBS and 1% A/A under standard conditions. To support cell proliferation and maintenance, 5 ng/mL fibroblast growth factor (FGF, PeproTech, USA, 100-18B) was freshly added to the culture medium with each media change. Cells were fed every 2 – 3 days and passaged using 1X Trypsin-EDTA (Gibco™, CA, 15400-054) upon reaching approximately 80% confluence, and only passages ≤ 5 were used for experiments. For all assays, 1.5×10^4 cells per well were seeded into 24-well plates and allowed to adhere for 24 hours. Following adhesion, the corresponding treatment was added, and the specific assay was performed.

2.3 Biological Effects of Av-EVs Cargo

2.3.1 Antioxidant Capacity

The antioxidant activity of Av-EVs was evaluated by measuring their ability to scavenge the free 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, using a DPPH antioxidant assay kit (Dojindo, USA, D678) following the manufacturer's protocol. Identical to the protein quantification assay, for each antioxidant analysis, a single EV pellet – obtained from one of the six ultracentrifugation pellets produced during one Av-EV isolation cycle – was individually resuspended in 300 µL of DPBS. No pooling of multiple pellets was performed.

Briefly, 20 µL of three (n=3) NB and B Av-EV samples obtained from separate isolation experiments under the same conditions were prepared at different concentrations (5×10^8, 1×10^9, 5×10^9, and 2×10^{10} EVs/mL) and incubated at RT in the dark with 80 µL of assay buffer and 100 µL of DPPH working solution (WS) diluted in 99.5% molecular grade ethanol (Sigma-Aldrich, USA, SHBS1884). Additionally, DPPH + ethanol was used as negative control, and EVs in DPBS as sample blank. At time intervals of 1, 24, 48, and 72 h, absorbance was measured at 517 nm using the SpectraMax iD3® spectrophotometer.

The DPPH radical scavenging activity (inhibition ratio %) of the samples was calculated using the following equation:

$$Inhibition\ ratio\ (\%) = \frac{A_{control} - (A_{sample} - A_{blank})}{A_{control}} \times 100$$

Statistical difference was assessed with two-way ANOVA followed by Geisser-Greenhouse's epsilon correction with the concentrations and time points as the factors.

2.3.2 Cytotoxicity Assessment

Cell viability was assessed using alamarBlueTM cell viability reagent (InvitrogenTM by Thermo Fisher Scientific, USA, DAL1025). Six biological replicates (n=6) of THP-1 and hDFs were seeded in their respective standard medium and left to adhere for 24 h, as described in sections 2.2.1 and 2.2.2. Following attachment, cells were stimulated according to the protocols detailed in sections 2.3.3 and 2.3.4. to induce either M1 macrophage polarization (via LPS) or fibroblast to myofibroblast differentiation (via TGF-β1/vitamin C), respectively. Then, 350 µL of freshly made working alamarBlue solution diluted at 10% in each corresponding standard medium was added to each well at the indicated time points and allowed to incubate under standard conditions for 3 h. The plate was wrapped with aluminum foil to protect it from direct light. Next, 200 µL of the supernatant from each well was transferred to a 96-well black plate with clear bottom (Corning Incorporated, USA, 3603) and fluorescence levels of the reduced resazurin were read on a SpectraMax iD3® spectrophotometer equipped with the Softmax Pro 7 software (Molecular Devices, USA). The excitation/emission wavelengths were set at 560/590, respectively. Results were normalized with a blank of 10% alamarBlue without cells and the statistical analysis was performed using the respective untreated conditions (i.e., –LPS/–EVs for macrophages or –TGFβ1–VitC/–EVs for fibroblasts) as controls. Statistical difference was assessed with one-way ANOVA followed by Tukey's post-hoc comparison.

2.3.3 Anti-inflammatory Response (Macrophages)

M0 macrophages were polarized to M1 macrophages through lipopolysaccharide (LPS, Sigma-Aldrich, USA, L6529-1MG) treatment. Fresh NB or B Av-EVs were added to serum-free medium simultaneously with 500 ng/mL of LPS and the treatment continued for 24 h, 72 h or 7 days. The experimental setup is specified in **Table 1**. D indicates different doses of EVs, where D1, D2, and D3 correspond to 1,000, 2,500, 5,000 Av-EVs per target cell, respectively. The term "EVs" is used throughout to describe particle concentrations measured by nanoparticle tracking analysis (NTA); however, it is important to note that not all particles quantified by NTA are necessarily extracellular vesicles. These measurements may include co-isolated nanoparticles such as protein aggregates or other vesicle-like structures. C-X-C motif chemokine ligand 10 (CXCL10) also known as Interferon gamma-induced protein 10 (IP-10), C-C motif chemokine ligand 2 (CCL2) also known as monocyte chemoattractant protein-1 (MCP-1), interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-13 (IL-13), and arginase 1 (ARG1) expression was quantified by RT-qPCR to analyze the anti-inflammatory potential of Av-EVs and their capacity to modulate macrophage polarization from M1 to M2 phenotype (see details in 2.4.1).

Table 1. *Experimental setup for the anti-inflammatory response.*

Condition	Explanation

–LPS / –EVs	Untreated control
+LPS / –EVs	Pro-inflammatory positive control
–LPS / +NBEVs	NB Av-EVs control
–LPS / +BEVs	B Av-EVs control
+LPS / +NBEVs D1	LPS and 1,000 NB AV-EVs / cell
+LPS / +NBEVs D2	LPS and 2,500 NB AV-EVs / cell
+LPS / +NBEVs D3	LPS and 5,000 NB AV-EVs / cell
+LPS / +BEVs D1	LPS and 1,000 B AV-EVs / cell
+LPS / +BEVs D2	LPS and 2,500 B AV-EVs / cell
+LPS / +BEVs D3	LPS and 5,000 B AV-EVs / cell

2.3.4 Antifibrotic Response (Dermal Fibroblasts)

TGF-β1 (PreproTech, USA, 100-21) and vitamin C (L-Ascorbic acid, Sigma-Aldrich, USA, A8960-5G) treatment was used to induce healthy hDFs transdifferentiation of fibroblasts to myofibroblasts, a recognized indicator of fibrosis. In brief, healthy hDFs were expanded and seeded as described in section 2.2.2. Once the cells were attached to the wells, the standard medium was replaced with fresh medium with 2.5% FBS + 10 ng/ml TGFβ1 + 100 μM vitamin C. Fresh NB or B Av-EVs were added simultaneously and the treatment continued for 72 h. The experimental setup is specified in **Table 2**. Identical to macrophages, D1, D2 and D3 were 1,000, 2,500, 5,000 NB or B Av-EVs per target cell, respectively. The expression of collagen type I (COL1A1) and α-SMA, also known as actin alpha 2 (ACTA2) was quantified by RT-qPCR to analyze the antifibrotic potential of Av-EVs and their capacity to modulate myofibroblast differentiation. Additionally, immunofluorescence microscopy was used for α-SMA protein visualization (see details in 2.4.1 and 2.4.2).

Table 2. *Experimental setup for the antifibrotic response.*

Condition	Explanation
–TGFβ1-VitC / –EVs	Untreated control
+TGFβ1+VitC / –EVs	Fibrotic positive control
–TGFβ1–VitC / +NBEVs	NB Av-EVs control
–TGFβ1–VitC / +BEVs	B Av-EVs control
+TGFβ1+VitC / +NBEVs D1	TGFβ1/VitC and 1,000 NB AV-EVs / cell
+TGFβ1+VitC / +NBEVs D2	TGFβ1/VitC and 2,500 NB AV-EVs / cell
+TGFβ1+VitC / +NBEVs D3	TGFβ1/VitC and 5,000 NB AV-EVs / cell
+TGFβ1+VitC / +BEVs D1	TGFβ1/VitC and 1,000 B AV-EVs / cell
+TGFβ1+VitC / +BEVs D2	TGFβ1/VitC and 2,500 B AV-EVs / cell
+TGFβ1+VitC / +BEVs D3	TGFβ1/VitC and 5,000 B AV-EVs / cell

2.3.5 Cellular Uptake of Av-EVs

To visualize the cellular uptake of Av-EVs, vesicles were labeled using the PKH26 red fluorescent membrane kit (Sigma–Aldrich, USA, 4103849473) (panel: red). After isolation, 10 mL of purified Av-EVs in DPBS were stored in aliquots at −80 °C as previously described. For PKH26 labeling, one 1 mL aliquot was thawed and ultracentrifuged at 100,000 g and 4 °C for 60 min. The pellet was then resuspended in 1 mL of Diluent C. Separately, 4 μL of PKH26 ethanolic dye solution was mixed in 1 mL of Diluent C until complete dispersion. Then, the Av-EVs suspension was mixed in the dye solution and incubated at room temperature (RT) for 10 min. An equal volume (2 mL) of 10% FBS in DMEM was added to stop

the labeling reaction. Finally, the solution was ultracentrifuged, and the resulting pellet was washed with DPBS and ultracentrifuged one more time to remove free dye aggregates. The final pellet containing the labeled Av-EVs was resuspended in the initial volume of DPBS (1 mL), wrapped in aluminum foil to protect from light and stored at −20 °C until further use.

THP-1 and hDFs were seeded in their respective standard medium and left to adhere for 24 h, as described in sections 2.2.1 and 2.2.2. Following attachment, cells were stimulated according to the protocols detailed in sections 2.3.3 and 2.3.4. to induce either M1 macrophage polarization (via LPS) or fibroblast to myofibroblast differentiation (via TGF-β1/vitamin C), respectively. Simultaneously, cells were treated with PKH26-labeled Av-EVs at the highest dose (D3: 5,000 EVs/cell) and incubated for an additional 24 h to allow for vesicle uptake. Subsequently, the cells were fixed with 4% paraformaldehyde solution (Thermo Scientific, USA, J19943-K2) at RT for 15 min, permeabilized with 10X PBS-Triton X-100 (Alfa Aesar, USA, J63521) at RT for 15 min, and blocked with 4% BSA (Thermo Scientific Chemicals, USA, AAJ6410009) in DPBS at 4 ºC for 2 h. From this point on, all procedures were carried out under low-light conditions to protect the fluorescent dyes. The Alexa Fluor™ 488 phalloidin (Invitrogen, USA, A12379) was diluted 1:400 in 4% BSA and added to the cells to stain the actin filaments (F-actin) at RT for 30 min (panel: green), followed by Hoechst 33342 (Thermo Scientific, USA, H3570) staining to label cell nuclei at a dilution of 1:3,000 in DPBS at RT for 5 min (panel: blue). Finally, the cells were washed and visualized under fluorescence microscopy (Olympus IX81, USA) to confirm the cellular uptake of PKH26-labeled Av-EVs and evaluate cytoskeletal and nuclear features. The images were captured at 20X and 40X magnifications and processed using FIJI (Image J) software.

2.4 Analysis of Cell Responses to Av-EVs from Mature Aloe vera Leaves

2.4.1 Gene Expression

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was employed to investigate the anti-inflammatory potential of Av-EVs using THP-1 macrophages stimulated with LPS by analyzing the gene expression of human pro-inflammatory (CXCL10/IP-10, CCL2/MCP-1, IL-1β, TNF-α, IL-6) and anti-inflammatory (IL-10, IL-13) cytokines, as well as the expression of ARG1, an enzyme highly expressed in M2 macrophages. Additionally, RT-qPCR served to investigate the hDFs transdifferentiation into myofibroblast after treatment with TGF-β1 and vitamin C by analyzing the gene expression of COL1A1 and α-SMA/ACTA2. The ID of each primer can be found in **Table 3**.

Total RNA was extracted from THP-1-derived macrophages and hDFs using the RNeasy mini kit (Qiagen, DE, 74106) according to the manufacturer's protocol. RNA concentration and purity were assessed spectrophotometrically using a NanoPhotometer® N50 (Implen, DE), and yields were expressed in ng/µL. Subsequently, RNA was reverse transcribed into complementary DNA (cDNA) using the high-capacity cDNA reverse transcription kit with RNase inhibitor (Applied Biosystems™ by Thermo Fisher Scientific, USA, 4374967) on a T100™ thermal cycler (Bio-Rad, USA). Quantitative real-time PCR (qPCR) was then performed using TaqMan™ fast advanced master mix (Applied Biosystems™, USA, 4444557) with gene-specific primers (Applied Biosystems™, USA, 4331182) on a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems™, USA). Gene expression levels were quantified by the ΔΔCt method represented in terms of fold change ($2^{-\Delta\Delta Ct}$). The TATA-binding protein (TBP) gene served as the endogenous reference. Experimental conditions were normalized to their respective stimulated controls: +LPS/–EVs for pro-inflammatory assays and +TGF-β1+VitC/–EVs for fibrotic assays. All qPCR

reactions were conducted in duplicate for each gene and treatment group. Statistical difference was assessed with one-way ANOVA followed by Tukey's post-hoc comparison.

Table 3. *TaqMan™ gene expression assays used for RT-qPCR analysis.*

Primer Name	Primer ID
TBP	Hs00427620_m1
CXCL10	Hs00171042_m1
CCL2	Hs00234140_m1
IL-1β	Hs01555410_m1
TNF-α	Hs00174128_m1
IL-6	Hs00174131_m1
IL-10	Hs00961622_m1
IL-13	Hs00174379_m1
ARG1	Hs00163660_m1
COL1A1	Hs00164004_m1
ACTA2	Hs00426835_g1

2.4.2 Smooth Muscle Actin Immunofluorescence Analysis

To analyze α-SMA protein expression in fibrotic hDFs treated with the intermediate dose (2,500 EVs/cell) of either NB- or B-derived Av-EVs (as described in section 2.3.4), immunofluorescence staining was performed. The cells were seeded, differentiated, fixed, permeabilized and blocked as described in section 2.3.5. Then, the cells were incubated with alpha-smooth muscle actin monoclonal antibody (1A4), eBioscience™ (Invitrogen, USA, 14-9760-82) at 1 µg/mL in 0.1% BSA at 4 ºC overnight and then labeled with goat anti-mouse IgG (H+L) superclonal™ secondary antibody, Alexa Fluor 488™ conjugate (Invitrogen, USA, A28175) at a dilution of 1:2,000 at RT for 45 min (panel: green). Afterward, F-actin was stained with Alexa Fluor™ 594 phalloidin (Invitrogen, USA, A12381) diluted 1:400 in 4% BSA at RT for 30 min (panel: red), and nuclei were stained with Hoechst 33342 (panel: blue). For each condition, three independent images were acquired at 20X magnification under identical exposure settings to enable objective comparison of fluorescence intensity. From each image, two regions of interest were analyzed, yielding a total of six α-SMA and six background pixel intensity measurements per group. Quantification was performed using FIJI (ImageJ) software by calculating net α-SMA signal intensity as the difference between each α-SMA measurement and the average background value. To enhance visualization while preserving quantitative accuracy, uniform brightness and contrast adjustments were applied post-capture across all images. Statistical difference was assessed with one-way ANOVA followed by Tukey's post-hoc comparison.

2.5 Comparative Analysis of NB Av-EVs Isolated from Mature and Young Aloe vera Leaves

2.5.1 Anti-inflammatory (Macrophages) and Antifibrotic (Dermal Fibroblasts) Responses

To assess the influence of plant maturity on the biological activity and protein composition of Av-EVs, we extended our investigation to include EVs manually extracted from young Aloe vera *barbadensis miller* leaves. Six leaves (n=6) were harvested from live potted plants purchased at a local plant shop and the EVs were isolated using the same manual protocol previously described in section 2.1.1. Following

isolation, the anti-inflammatory potential of young versus mature NB Av-EVs was evaluated in THP-1-derived macrophages after 24 hours of co-treatment, and the antifibrotic response was assessed in hDFs (see details in 2.3.3 and 2.3.4, respectively). Gene expressions of CXCL10/IP-10, CCL2/MCP-1, IL-1β, TNF-α, IL-6, IL-10, COL1A1 and α-SMA/ACTA2 were analyzed by RT-qPCR (see details in 2.4.1). All qPCR reactions were conducted in duplicate for each gene and treatment group. The ID of each primer can be found in **Table 3**. Importantly, due to the independent experimental setup for mature and young Aloe vera EVs, separate untreated control groups are shown. Statistical difference was assessed with one-way ANOVA followed by Tukey's post-hoc comparison.

2.5.2 Proteomic Characterization via Label-Free Data-Dependent Acquisition Mass Spectrometry

To investigate molecular differences in vesicle cargo, label-free data-dependent acquisition (DDA)-based proteomic profiling was carried out on Av-EVs derived from both mature and young Aloe vera leaves. Each group consisted of three biological replicates (n=3), where the mature EV samples were isolated from three independent leaves, and the young EV samples were obtained from three separate potted plants. Following ultracentrifugation, EV pellets were directly resuspended in Pierce™ RIPA lysis buffer (Thermo Scientific, USA, 89901), and protein concentration was determined using the BCA assay as described in section 2.1.4.

Samples were submitted to MtoZ Biolabs (USA) for protein extraction, LC-MS/MS analysis, and downstream bioinformatics employing a transcriptome-informed Aloe vera database (~77,000 protein sequences)[39]. In their facilities, proteins were reduced with 10 mM dithiothreitol at 56 °C for 1 h, alkylated with 55 mM iodoacetamide at RT in the dark for 1 h, and digested overnight with sequencing-grade trypsin (Promega) in 50 mM NH_4HCO_3 at 37 °C, using the previously described SP3 workflow.[40] Peptides were desalted using C18 columns, vacuum-dried at 45 °C, and resuspended in 0.1% formic acid. Peptide separation was carried out using an Easy-nLC 1200 system coupled to an Orbitrap Exploris 480 (Thermo Fisher Scientific) with a 66-minute gradient on a C18 column (150 µm i.d. × 170 mm, 1.9 µm, 100 Å). Mass spectrometry was performed in positive ion mode using a top-20 data-dependent acquisition method with a full scan range of m/z 400–1200 at 60,000 resolutions. Peptide fragmentation was achieved using high-energy collision dissociation at a normalized collision energy of 30%. Raw MS/MS data were analyzed against Aloe vera specific database that was generated based on the transcriptomics data described by Choudhri et al.[39] Briefly, unigenes were downloaded from Bioproject PRJNA359629, and coding sequences were predicted using TransDecoder[41] implemented in Galaxy,[42] resulting in 76,958 protein sequences. Search parameters included up to two missed cleavages, fixed carbamidomethylation of cysteines, and variable modifications including methionine oxidation and N-terminal acetylation. A 1% false discovery rate was applied at both peptide and protein levels. Quantitative data were median-normalized and missing values imputed using the K-nearest neighbor (KNN) algorithm. Bioinformatics analyses included differential protein expression, principal component analysis (PCA), hierarchical clustering, gene ontology (GO), Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment, and protein–protein interaction (PPI) network mapping.

2.6 Statistical Analysis

Experiments were performed in six biological replicates (n=6) for the protein quantification, cytotoxicity assessment, gene expression, and α-SMA immunofluorescence analyses, and three biological replicates (n=3) for the antioxidant capacity. The data were represented as means ± standard deviations (SD).

Normality was evaluated with the Shapiro-Wilk test. Statistical difference was assessed with unpaired student's t-test for protein quantification, two-way analysis of variance (ANOVA) followed by Geisser-Greenhouse's epsilon correction for the antioxidant capacity, and one-way ANOVA followed by Tukey's post-hoc comparison for the rest of the assays. Significance was considered for p values < 0.05. Analyses were performed using GraphPad Prism version 10.6.0 (GraphPad Software, USA).

3. Results and Discussion

3.1 Av-EVs Isolation and Characterization

Extracellular vesicles from Aloe vera gel (Av-EVs) were isolated using two protocols: a gentle manual homogenization approach (NB Av-EVs) and a shear force-based method using a commercial blender (B Av-EVs), as illustrated in **Figure 1A**. All vesicle preparations were characterized in accordance with the MISEV2023 guidelines established by the International Society for Extracellular Vesicles (ISEV).[43] Characterization was conducted on four independent replicates for each method.

Nanoparticle tracking analysis (NTA) established that all final suspension samples consistently yielded concentrations ranging from 1.4 to 2.0×10^{10} particles/mL, for an average total of 5.6 to 8×10^{12} particles per leaf. Furthermore, they displayed heterogeneous size distributions with mean diameters between 150 – 250 nm [**Figure 1B**], consistent with previous reports on Aloe-derived EVs.[35-37, 44] While this overall range was reproducible across both methods, NB EVs typically clustered between 1.4 to 1.6×10^{10} particles/mL, whereas B-derived EVs approached 1.8 to 2.0×10^{10} particles/mL. The disparity may reflect the influence of shear stress introduced by blender homogenization, which could not only liberate vesicles already present in the inner parenchyma but also mobilize vesicles from within vacuoles or induce vesiculation through mechanical disruption. This hypothesis is supported by literature describing how laminar or turbulent shear flow can destabilize lipid membranes, promoting membrane budding or fragmentation. Such mechanical stress can cause intracellular content leakage and drive the spontaneous formation of EV-like vesicles, often diverse in size and composition.[45]

Transmission electron microscopy (TEM) verified the presence of rounded, membrane-bound vesicles with well-defined lipid bilayers – characteristic of EVs. The morphology observed in both NB and B Av-EVs groups supports the preservation of vesicle integrity following isolation, regardless of the extraction method used. Nonetheless, notable differences in size distribution were evident as expected: B-derived Av-EVs displayed a broader range of diameters (approximately 70 to 170 nm), whereas NB-derived EVs showed a more uniform morphology, with most imaged vesicles falling near the upper size range observed by NTA (~250 nm). While some vesicles measured closer to 300 nm in TEM, this discrepancy may be attributed to swelling or flattening during sample preparation and imaging, a known artifact in conventional TEM workflows. These observations align with NTA results and further support the idea that blender-induced shear stress promotes vesicle heterogeneity, potentially generating a mixture of natural and mechanically derived vesicles.

Protein quantification results provide further evidence of the impact of extraction methods [**Figure 1C**]. NB-derived EVs exhibited consistent protein concentrations across independently processed leaves, ranging from 159 to 198 µg/mL, corresponding to a total protein yield of 3,816 to 4,752 µg per leaf. In contrast, B-derived EVs showed substantial inter-replicate variability, with concentrations spanning 160 to 447 µg/mL and a total protein yield of 3,840 to 10,728 µg per leaf. Statistical analysis confirmed a

significant difference in total protein levels between the NB and B groups ($p < 0.05$), suggesting that the shear-based method may produce a more heterogeneous population of vesicles, potentially including non-physiological particles or protein-rich artifacts. However, when protein levels were normalized to the total EV yield per leaf, both methods exhibited relatively consistent protein-to-particle ratio. NB-derived EVs ranged from 6.81 to 7.43 × 10^{-9} µg/EV, while B-derived EVs ranged from 5.33 to 13.41 × 10^{-9} µg/EV. Despite the higher variability observed in the raw protein concentration of B samples, the differences between the two groups became less pronounced after normalization, indicating that the protein content per EV remains relatively similar across both EV types.

In summary, both isolation protocols were successful in yielding Av-EVs that meet established EV characterization criteria in terms of size, concentration, and morphology. However, the manual method yielded more uniform vesicle populations with slightly lower protein variability, suggesting greater preservation of native EV characteristics and reduced likelihood of shear-induced artifacts.

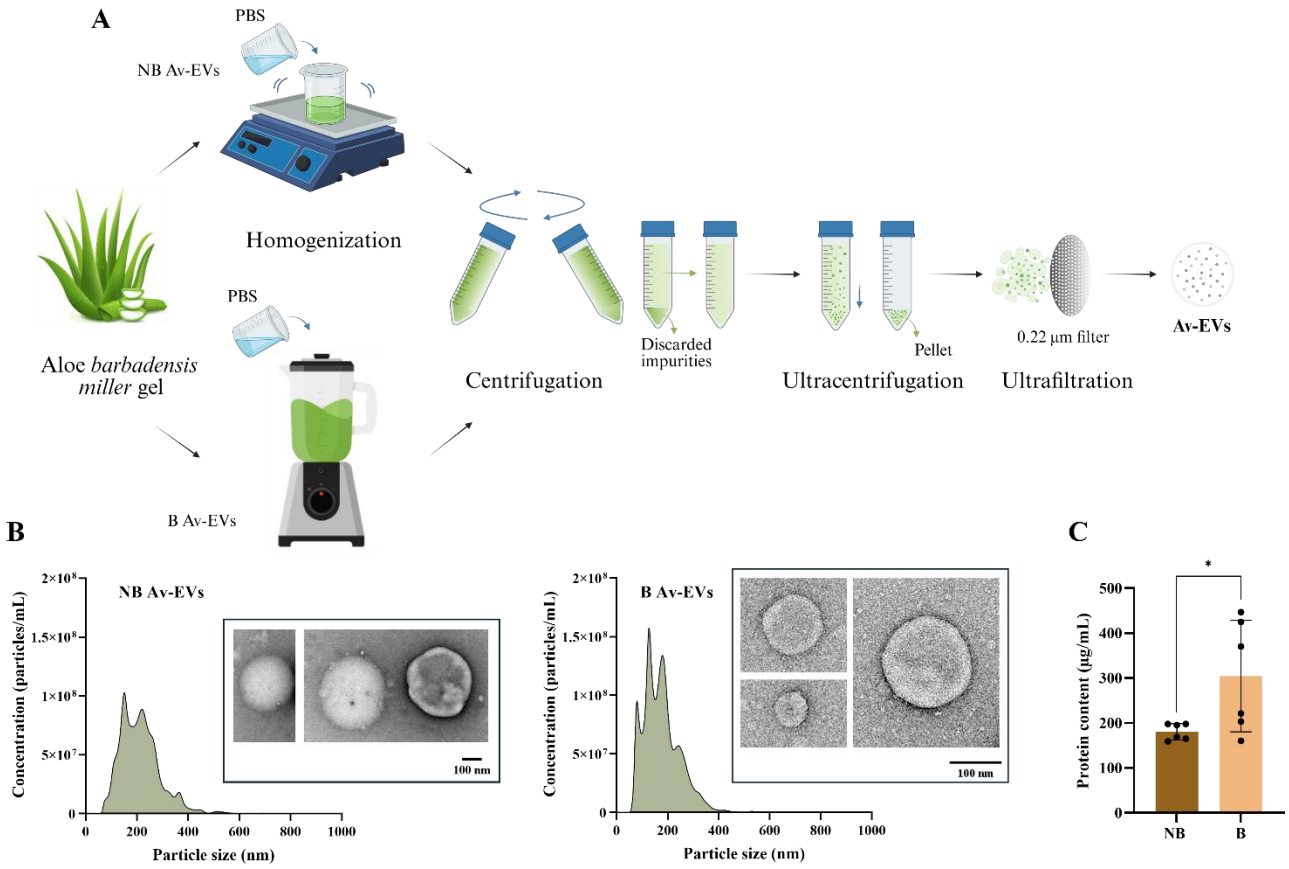

Figure 1. (A) *Schematic illustration of NB and B Av-EV isolation procedures from Aloe vera barbadensis miller leaves.* ***(B)*** *NTA data showing size distribution of typical NB and B Av-EV samples with their respective TEM images.* ***(C)*** *Protein content data represents the mean ± SD with n=6 and p < 0.05 *.*

Confirmation of Plant-specific Markers in Av-EVs

PDEVs resemble mammalian EVs in that they contain diverse biomolecules capable of reshaping the behavior and morphology of recipient cells; yet, compared with animal-derived EVs, PDEVs remain relatively underexplored, a comprehensive picture of their complete cargo is still emerging, and the vesicle composition and therapeutic performance appear to vary with the physiological and environmental

conditions under which vesicles are synthesized and released, as later outlined in section 3.3.3. However, according to previous literature, several marker families recur across species and preparations, including proteins like annexins, heat-shock proteins (HSP), aquaporins, proteins involved in cell-wall remodeling, actins, patellins, syntaxins, clathrin heavy chain, and RAS-related proteins, as well as lipids such as phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and plant sphingolipids (GIPCs). Finally, micro RNAs (miRNAs) and metabolites can also be informative.[46, 47]

Therefore, a label-free LC–MS/MS was performed to confirm the identity of a typical plant signature in our Av-EVs based on this limited but fundamental knowledge. The results showed that against that backdrop, our Av-EV successfully recovered some of the canonical plant-EV markers – HSP, actins, clathrin heavy chain, small GTPases, syntaxins, annexins, and cell-wall remodeling – providing convergent evidence that our isolates are bona fide plant EVs even in the absence of classical established surface marker western blot assays for Aloe-derived vesicles. A detailed characterization of the proteomic profile and differential protein expression patterns is presented in section 3.3.4, providing a comprehensive molecular framework that links Av-EV composition to their age-dependent bioactivity.

3.2 Biological Effects of Av-EVs Cargo from Mature Aloe vera Leaves

3.2.1 Antioxidant Capacity

The antioxidant potential of Av-EVs was evaluated using the DPPH radical scavenging assay, a widely accepted method that quantifies a compound's ability to neutralize free radicals via electron or hydrogen atom donation. In this assay, decreased DPPH absorbance correlates with increased antioxidant activity, expressed as the DPPH inhibition ratio (%). Both NB and B Av-EVs were tested at four concentrations (5×10^8, 1×10^9, 5×10^9, and 2×10^{10} EVs/mL) and assessed at four time points (1, 24, 48, and 72 hours) to evaluate both dose- and time-dependent antioxidant dynamics.

As shown in **Figure 2**, all Av-EV treatment groups demonstrated a clear time-dependent increase in DPPH inhibition, indicative of progressive and sustained antioxidant activity. During the first hour, all samples – regardless of dose or method – exhibited relatively uniform inhibition values (~20 – 40%), suggesting similar initial redox interactions. By 24 hours, B-derived EVs maintained modest inhibition levels (20 – 40%), whereas some NB-derived EV replicates began to display more pronounced scavenging effects, reaching inhibition values of up to ~80%. Notably, greater variability emerged across replicates between the 24 and 48 h window, potentially reflecting differences in cargo release kinetics, vesicle stability, or vesicle–radical interaction. Despite this fluctuation, the antioxidant response became more consistent by 72 hours, with nearly all treatment conditions – especially those involving NB EVs – stabilizing at high inhibition levels (> 90%).

Statistical analysis confirmed a significant increase in inhibition from 1 to 48 hours for NB EVs at both 5×10^9 and 2×10^{10} EVs/mL ($p < 0.01$ and $p < 0.05$, respectively), and from 1 to 72 hours ($p < 0.001$ and $p < 0.05$, respectively). In contrast, B-derived EVs at the same concentrations reached significance only between 1 and 72 hours ($p < 0.05$). Furthermore, even the lowest NB EV dose (5×10^8 EVs/mL) showed a significant increase between 24 and 48 hours ($p < 0.001$). These findings underscore the sustained redox-modulatory capacity of Av-EVs and suggest that NB-derived vesicles may deliver more timely and effective antioxidant action.

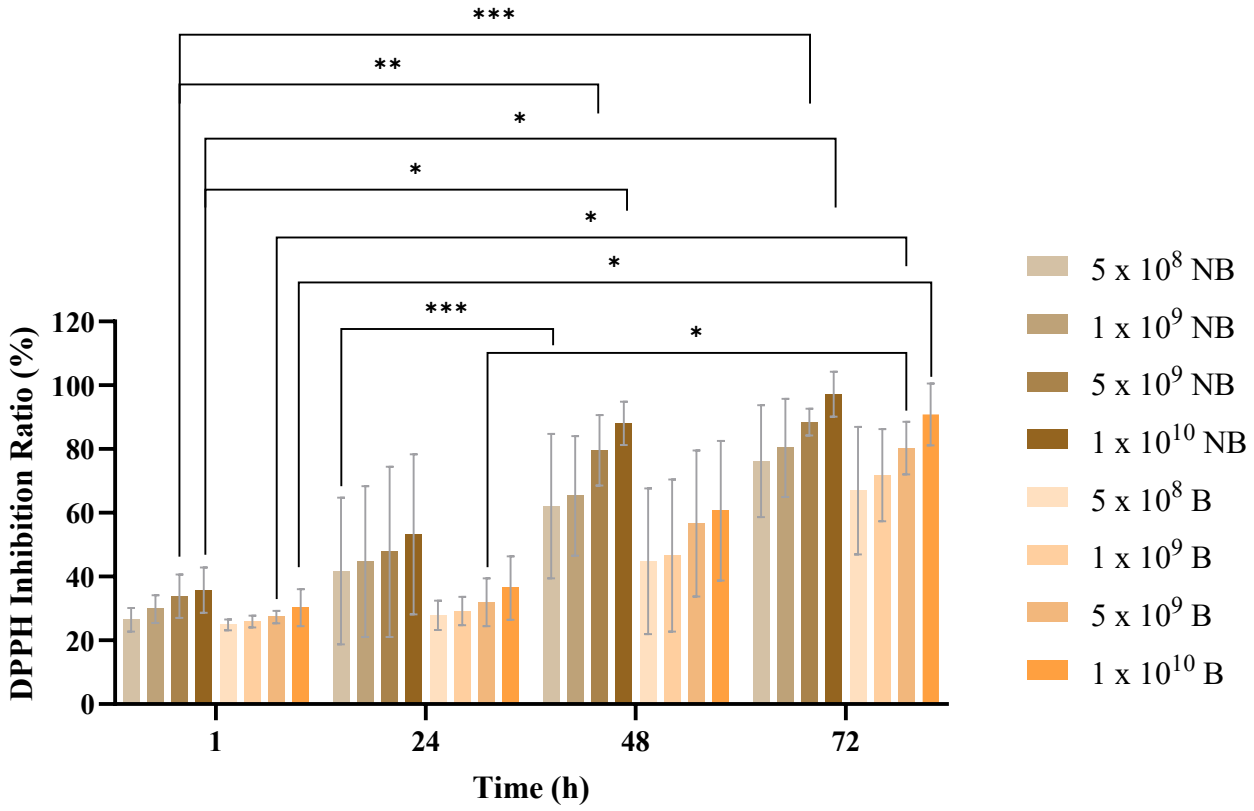

Figure 2. *DPPH inhibition ratio (%) over time for NB and B Av-EVs at four concentrations (5×10^8, 1×10^9, 5×10^9, and 2×10^{10} EVs/mL). Inhibition data represents the mean ± SD with n=3 and $p < 0.05$ *, < 0.01 **, < 0.001 ***.*

Additionally, although statistical significance was not observed across all comparisons, a consistent dose-dependent trend was observed: the highest concentration (2×10^{10} EVs/mL) yielded the strongest DPPH inhibition at each time point, whereas the lowest concentration (5×10^8 EVs/mL) produced the weakest response. This pattern reinforces the premise that the antioxidant efficacy of Av-EVs is proportional to the concentration of vesicle-delivered bioactive compounds. Interestingly, across all tested doses, NB-derived EVs achieved greater inhibition ratios than their B-derived counterparts. This superior performance, even at lower concentrations, highlights the importance of gentle manual extraction methods in preserving vesicle integrity and maximizing functional antioxidant potential.

This antioxidant efficacy aligns with the well-documented phytochemical profile of Aloe vera, which includes a variety of redox-active molecules such as α-tocopherol (vitamin E), ascorbic acid (vitamin C), carotenoids, polyphenols (flavonoids and tannins), indoles, alkaloids, and anthraquinones (e.g., aloin and aloe-emodin).[22, 48-50] The plant also contains enzymatic antioxidants (e.g., superoxide dismutase (SOD) and glutathione peroxidase (GPx)) that contribute to ROS detoxification.[22] Prior studies show Aloe gel scavenges DPPH, ABTS, and NO radicals in a concentration-dependent manner, limits lipid peroxidation, and protects against oxidative damage.[23] It can further modulate oxidative stress pathways, including inhibition of the NOX4/ROS/p38 axis,[51] preventing activation of latent TGF-β1 and the fibrogenic transition of fibroblasts and endothelial cells into α-SMA–positive myofibroblasts. Consistent with these reports, Aloe-derived EVs have been shown to delay skin photoaging via Nrf2/ARE activation, with

reductions in H_2O_2,[37] senescence-associated secretory phenotypes (SASP) factors, and malondialdehyde (MDA) *in vitro* and *in vivo*.[44]

Importantly, our proteomic profiling (section 3.3.4) corroborates the presence of a vesicle-encoded antioxidant program in mature Av-EVs. These vesicles exhibited enrichment of proteins involved in ether- and sphingolipid-metabolism pathways together with higher aldehyde dehydrogenase (NAD^+) activity, consistent with enhanced detoxification of lipid-peroxidation products such as 4-HNE. Collectively, these proteomic features provide molecular support for the sustained, dose-responsive reduction in intracellular ROS observed in our antioxidant capacity assay. Moreover, they help explain how the ability of Av-EVs to mitigate oxidative damage likely disrupts the ROS↔TGF-β1 positive-feedback loop, thereby facilitating the resolution of inflammation and preventing pathological scar formation (e.g., hypertrophic scars, keloids).

3.2.2 Cytotoxicity Assessment

Cell viability, defined as the ability of cells to maintain both functional integrity and proliferative potential, was assessed using the alamarBlue assay, which measures metabolic activity through the reduction of resazurin to fluorescent resorufin by intracellular oxidoreductases in metabolically active cells. This assay provides an integrative measure of cell viability by reflecting plasma membrane integrity, glycolytic flux, mitochondrial respiratory chain activity, and biosynthetic capacity. To evaluate the biocompatibility and potential cytotoxicity of NB and B Av-EVs, the alamarBlue assay was applied to both THP-1-derived macrophages and hDFs exposed to inflammatory or fibrotic stimuli, respectively. Untreated cells served as controls for statistical analysis (–LPS/–EVs or –TGF-β1–VitC/–EVs), and Av-EVs were administered at increasing doses (1,000, 2,500, and 5,000 EVs/cell) to assess their effects on cellular metabolism and viability across both the anti-inflammatory and antifibrotic experimental timelines.

THP-1-derived macrophages were activated using LPS, the major outer membrane component of Gram-negative bacteria, with or without Av-EV co-treatment. As shown in **Figure 3**, LPS alone significantly increased macrophage metabolic activity at 24 h, 72 h, and 7 days compared to the untreated control. Moreover, cotreatment with LPS and Av-EVs further enhanced metabolic activity at 24 and 72 hours across all doses and isolation methods, a period during which cells are expected to exhibit full M1 polarization and heightened inflammatory function. These findings confirm that Av-EVs do not exert cytotoxic effects and that macrophages remain metabolically viable under all treatment conditions.

The moderate decline in metabolic activity observed between 72 hours and day 7 suggests that Av-EVs facilitate a phenotypic transition from M1 to M2 macrophages, consistent with the expected resolution phase of inflammation as confirmed with the gene expression data later (see section 3.2.4). This phenotypic shift from a pro-inflammatory to a reparative state is not only defined by cytokine expression profiles but also by fundamental metabolic reprogramming. M1 macrophages depend on aerobic glycolysis for rapid ATP production and biosynthesis to support inflammatory signaling, ROS generation, and cytokine secretion.[52-55] In contrast, M2 macrophages rely on oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO), to support their anti-inflammatory and tissue-remodeling functions, which are energetically more efficient but generate fewer reduced equivalents.[52-55] Since the alamarBlue assay detects metabolic activity via NADH/NADPH-linked reduction of resazurin,[56] glycolytic M1 cells typically yield stronger signals than OXPHOS-driven M2 cells. Therefore, a decrease in alamarBlue signal at later time points under M2-polarizing conditions, such as Av-EV treatment, likely reflects a metabolic adaptation rather than cytotoxicity. Importantly, the dose-dependent nature of this effect – particularly the

notable reduction at 5,000 EVs/cell – suggests that higher concentrations of Av-EVs may more strongly influence macrophage polarization and promote the initiation of tissue repair.

It is also noteworthy that *in vitro*, THP-1-derived macrophages naturally undergo reduced viability over time due to nutrient depletion, lack of stromal survival factors, and activation of intrinsic apoptotic pathways.[57] Similarly, *in vivo* M1 macrophages are generally short-lived, undergoing apoptosis or efferocytosis during the resolution of inflammation, while M2-polarized macrophages tend to persist longer due to their reparative and homeostatic roles.[58]

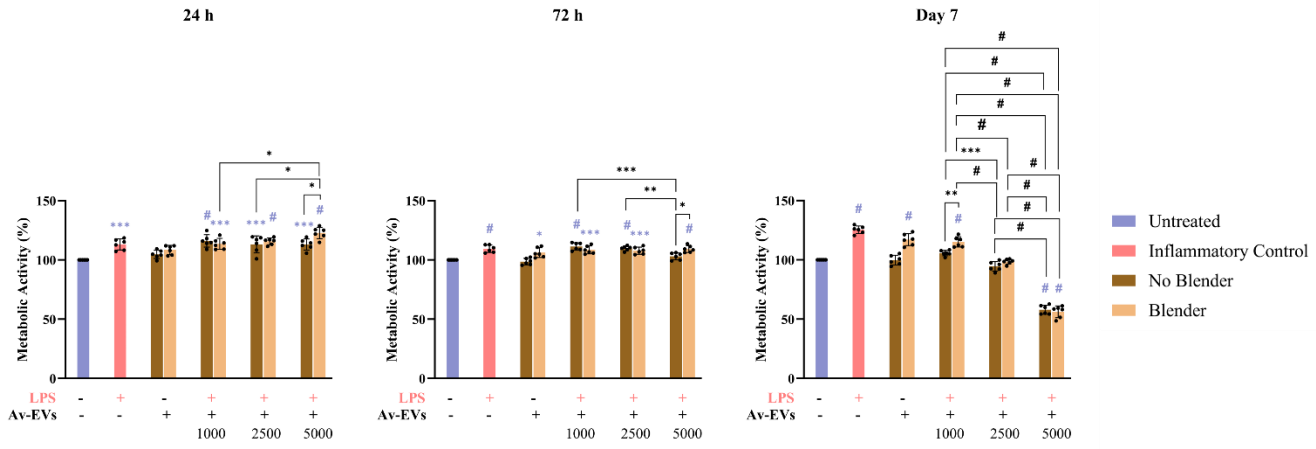

Figure 3. *Metabolic activity percentage in LPS-induced macrophages after 24 h, 72 h, and 7 days of co-treatment with No Blender or Blender Av-EVs at increasing doses (1,000, 2,500, 5,000 EVs/cell). Fluorescent data represents the mean ± SD with n=6 and p < 0.05 *, < 0.01 **, < 0.001 ***, < 0.0001 #. Statistical differences relative to untreated control (-LPS/-EVs) are shown in purple, while statistical differences between the isolation methods and doses are shown in black.*

To evaluate the metabolic impact and biocompatibility of Av-EVs on hDFs under fibrotic stimulation, the alamarBlue assay was performed following a 72-hour exposure to TGF-β1 and vitamin C, with or without Av-EV co-treatment. **Figure 4** shows that all groups exposed – including the fibrotic control and the Av-EV co-treated conditions – had a significant increase in metabolic activity compared to the untreated control. This increase reflects the transition from a quiescent fibroblast state to an activated phenotype. In this regard, resting hDFs exhibit low metabolic demand, relying mainly on basal mitochondrial respiration to support ECM maintenance and structural stability. Upon stimulation, activated fibroblasts display increased proliferation, migration, and biosynthesis – particularly of collagen and fibronectin – accompanied by enhanced glycolysis and mitochondrial oxidative metabolism.[59, 60] These shifts led to a measurable rise in cellular redox activity, as detected by the assay.

However, gene expression analysis and immunofluorescence later revealed that, despite similar metabolic activity across all conditions exposed to TGF-β1 and vitamin C, Av-EV co-treatment significantly suppressed the expression of key fibrotic markers such as COL1A1 and α-SMA, indicating a functional uncoupling between metabolic activity and fibrotic gene expression. This distinction is critical, as elevated metabolic activity alone does not necessarily equate to pathological transdifferentiation into myofibroblasts. Rather, Av-EVs may support a metabolically active yet functionally restrained fibroblast phenotype, in which the cells retain viability and activity but are prevented from progressing into a fully pro-fibrotic myofibroblast state, unlike those in the +TGFβ1+VitC/–EVs group.

Collectively, these findings confirm that Av-EVs are non-cytotoxic to both macrophages and hDFs across multiple time points and concentrations, validating their biocompatibility for immunomodulatory

applications and supporting their potential use in prolonged treatment regimens aimed at modulating inflammation and preventing fibrosis.

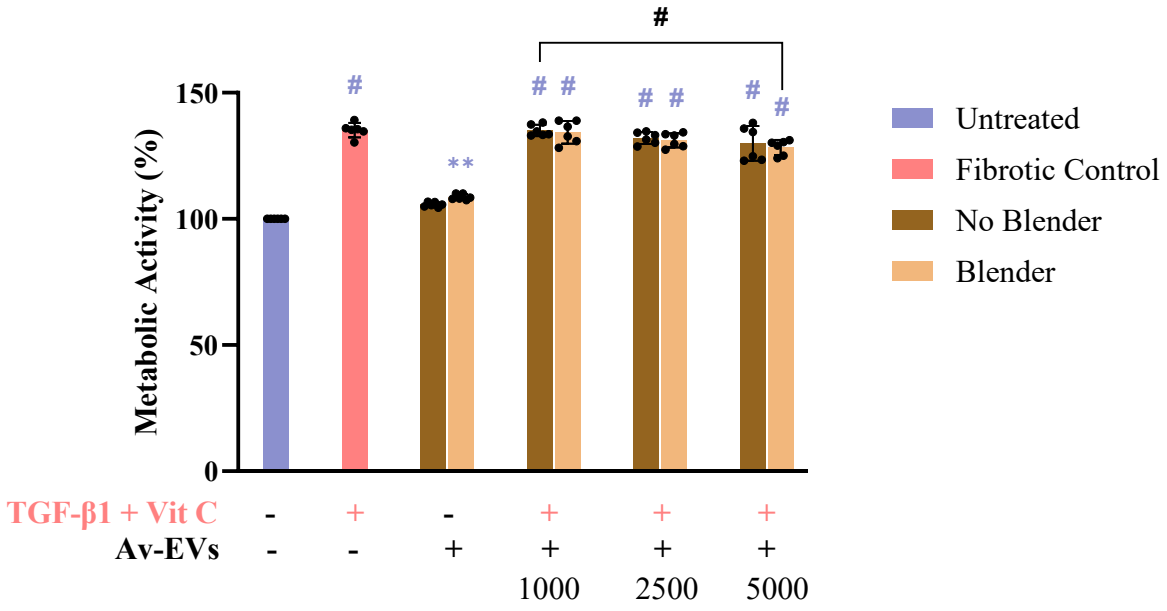

Figure 4. *Metabolic activity percentage in TGF- β1 + vitamin C- activated hDFs after 72 h co-treatment with No Blender or Blender Av-EVs at increasing doses (1,000, 2,500, 5,000 EVs/cell). Fluorescent data represents the mean ± SD with n=6 and p < 0.01 **, < 0.0001 #. Statistical differences relative to the untreated control (-TGFβ1-VitC/-EVs) are shown in purple, while statistical differences between the isolation methods and doses are shown in black.*

3.2.3 Cellular Uptake of Av-EVs

To confirm cellular uptake, THP-1 and hDFs cells were incubated with the highest dose (D3: 5,000 EVs/cell) of PKH26-labeled Av-EVs and subsequently analyzed by fluorescence microscopy. The results clearly demonstrate that both macrophages and dermal fibroblasts successfully internalized Av-EVs within 24 hours without compromising cell morphology, cytoskeletal organization, or viability, even under inflammatory or fibrotic activation.

Figure 5A presents the morphological features of macrophages across different conditions. M0 macrophages displayed a rounded shape, a diffuse cytoplasmic actin network, and clustered nuclei, consistent with a resting, non-polarized phenotype. F-actin was sparsely distributed and mainly localized at the cell periphery, indicating minimal cellular spreading. In contrast, LPS-stimulated macrophages (M1) showed a marked morphological shift, adopting an elongated, stellate shape with prominent stress fibers and lamellipodia, characteristic of M1 polarization. These cytoskeletal changes reflect enhanced motility and phagocytic readiness, driven by pro-inflammatory signaling. Macrophages treated with PKH26-labeled Av-EVs maintained this activated morphology but exhibited distinct red fluorescent signals localized within the perinuclear and cytoplasmic regions, confirming efficient uptake of the vesicles. This internalization, along with preserved cell structure and consistent metabolic activity from viability assays, indicates successful delivery of functional bioactive cargo capable of modulating the expression of both pro- and anti-inflammatory cytokines, as later confirmed in the anti-inflammatory response experiment (see section 3.2.4).

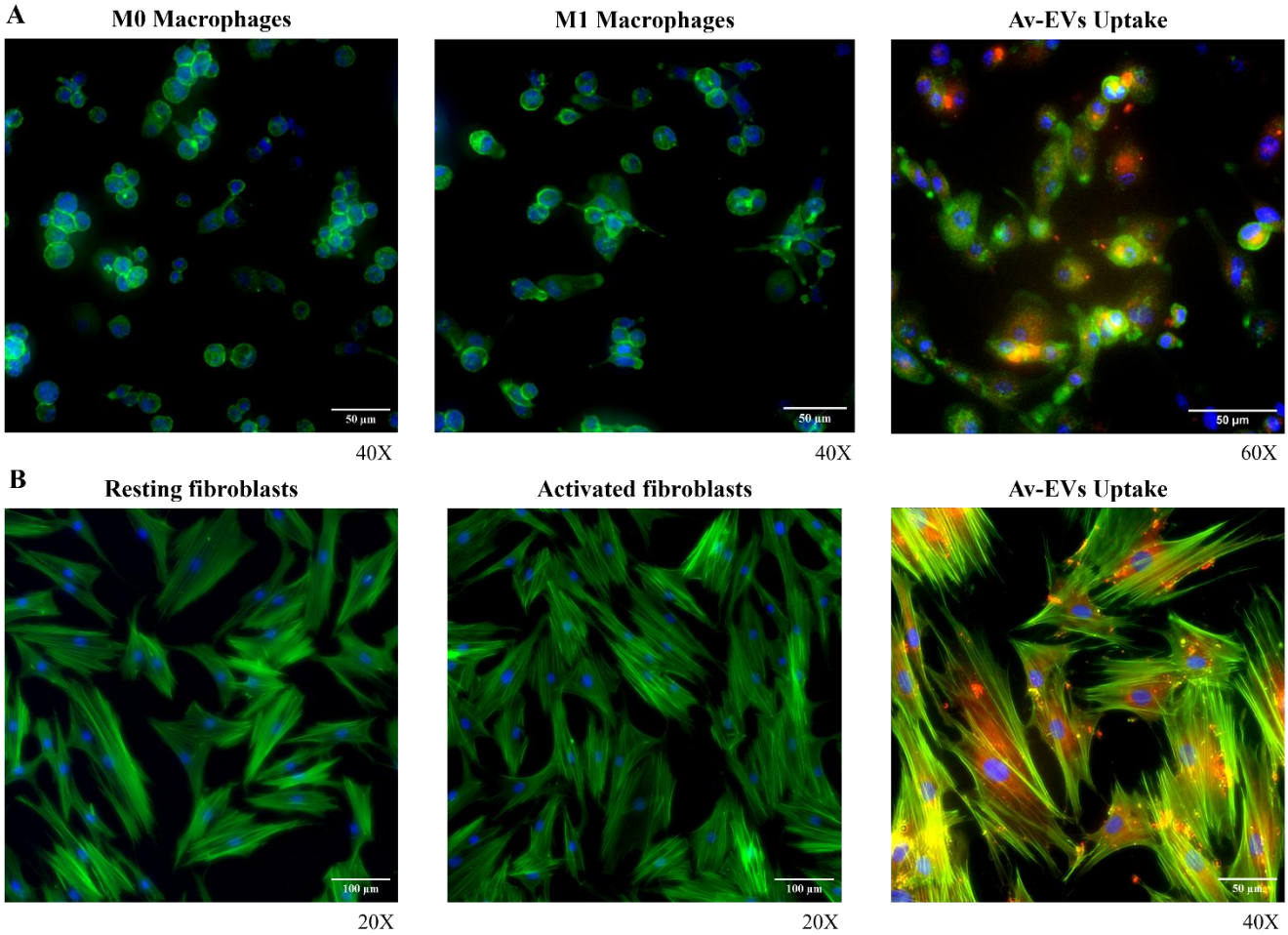

Figure 5. A. *From left to right: M0 macrophages, activation of M0 macrophages with LPS to induce M1 polarization, and cellular uptake of Av-EVs into M1 macrophages.* ***B.*** *From left to right: resting hDFs, activation of hDFs with TGF-β1 and vitamin C, and cellular uptake of AS-EVs into activated hDFs. PKH26 red fluorescent dye-labeled Av-EVs (red), F-actin (green), and nuclei (blue).*

Figure 5B shows the progression of fibroblast morphology following activation and Av-EV treatment. Resting fibroblasts exhibited the expected spindle-shaped appearance, with elongated nuclei and well-organized, parallel actin filaments aligned along the cell's longitudinal axis. These features are typical of quiescent fibroblasts that contribute to ECM maintenance under physiological conditions. After treatment with TGF-β1 and vitamin C, cells adopted a broader, more spread-out shape, with increased density and alignment and intensified F-actin staining, indicating the early stages of fibroblast-to-myofibroblast transdifferentiation. In cells exposed to PKH26-labeled Av-EVs, this activated morphology and cytoskeletal structure was retained, further confirming the non-cytotoxic and non-disruptive nature of Av-EV internalization. Importantly, bright red fluorescent signals were clearly visible, particularly in the perinuclear zones, indicating efficient vesicle uptake and intracellular delivery cargo capable of modulating gene expression of key fibrotic markers such as COL1A1 and α-SMA as later confirmed in the antifibrotic response experiment (see section 3.2.5).

3.2.4 Anti-inflammatory Response (Macrophages)

Two isolation protocols were compared to assess whether mechanical homogenization of Aloe vera gel alters the biological efficacy of the resulting vesicles. Both NB and B EVs were purified using identical

centrifugation and ultracentrifugation workflows, ensuring comparability. THP-1 monocytes were differentiated into macrophages and further induced with LPS to adopt a proinflammatory M1 polarity, while being exposed to cotreatment with Av-EVs. The experimental conditions were as stated in **Table 1**.

Suppression of Pro-inflammatory Cytokines Expression at 24 Hours

RT-qPCR analysis of LPS-induced THP-1-derived macrophages revealed a robust upregulation of pro-inflammatory cytokines, including CXCL10/IP-10, CCL2/MCP-1, IL-1β, TNF-α, and IL-6 compared to untreated controls, consistent with classical M1 macrophage polarization [**Figure 6**]. In contrast, cotreatment with Av-EVs significantly attenuated this pro-inflammatory profile within 24 h, in a dose- and preparation-dependent manner. Notably, Av-EV control groups did not induce cytokine expression, with levels remaining near baseline, confirming that Av-EVs do not activate macrophages under homeostatic conditions.

Among the suppressed genes, CXCL10/IP-10 – a chemokine induced by IFN-γ and LPS that promotes Th1 responses and recruits activated T cells and macrophages – was significantly downregulated. Similarly, CCL2/MCP-1, a critical chemokine for monocyte recruitment and a well-established marker of M1-type polarization, was also strongly inhibited. Both NB and B Av-EVs significantly reduced the expression of CXCL10 and CCL2 in a dose-responsive manner, with near-complete suppression observed at the highest dose (D3, 5000 EVs/cell). Specifically, CXCL10 expression was reduced to a fold change of ≤ 0.017 (NB) and 0.059 (B), while CCL2 was suppressed to ≤ 0.017 (NB) and 0.021 (B). Even at the lowest dose (D1, 1000 EVs/cell), strong inhibitory effects were observed, with CXCL10 expression reduced to ≤ 0.126 (NB) and 0.248 (B), and CCL2 reduced to ≤ 0.094 (NB) and 0.118 (B). These data highlight the potent gene-modulatory capacity of both EV preparations, even at lower concentrations. Notably, NB-derived Av-EVs consistently outperformed the B-derived Av-EVs across all doses for both gene targets, with a statistically significant difference at the lowest dose for CXCL10 expression.

In addition, the pro-inflammatory cytokines IL-1β and TNF-α were also evaluated. IL-1β is a canonical early-response cytokine, tightly linked to inflammasome activation, and is a major driver of tissue inflammation and fibrosis. TNF-α, a central cytokine in M1-driven inflammation, initiates and sustains pro-inflammatory cascades in response to endotoxins like LPS. As expected, both IL-1β and TNF-α were significantly upregulated in LPS-stimulated macrophages. Upon cotreatment with Av-EVs, both cytokines were strongly downregulated across all doses, with D3 (5000 EVs/cell) achieving the maximum suppression, regardless of the extraction method. Previous research in Aloe vera-EVs also demonstrated this dose-dependent effect.[35, 36] Interestingly, at lower and intermediate doses (D1 and D2), B Av-EVs exhibited superior suppressive effects compared to NB, with D2 (2500 EVs/cell) demonstrating comparable efficacy to D3 in both preparations.

The observed suppression of these cytokines following Av-EV treatment demonstrates that Av-EVs act rapidly and effectively to modulate macrophage inflammatory responses. Upon LPS stimulation, Toll-like receptor 4 (TLR4) activation triggers the MyD88-dependent signaling cascade, leading to phosphorylation and degradation of IκBα, which permits nuclear translocation of NF-κB p65/p50 dimers and subsequent transcription of a wide array of pro-inflammatory genes, including CXCL10, CCL2, IL-1β, TNF-α, and IL-6. The downregulation of IL-1β and TNF-α, in particular, implies that Av-EVs could interfere with canonical TLR4/NF-κB signaling, potentially by inhibiting upstream kinases such as IKK, preserving IκBα stability, and ultimately preventing NF-κB nuclear localization.[61] This inhibitory effect may be

attributed to specific phytochemical and molecular cargos within Av-EVs – such as flavonoids and miRNAs – previously shown to antagonize NF-κB signaling and inflammatory gene expression.[62-64]

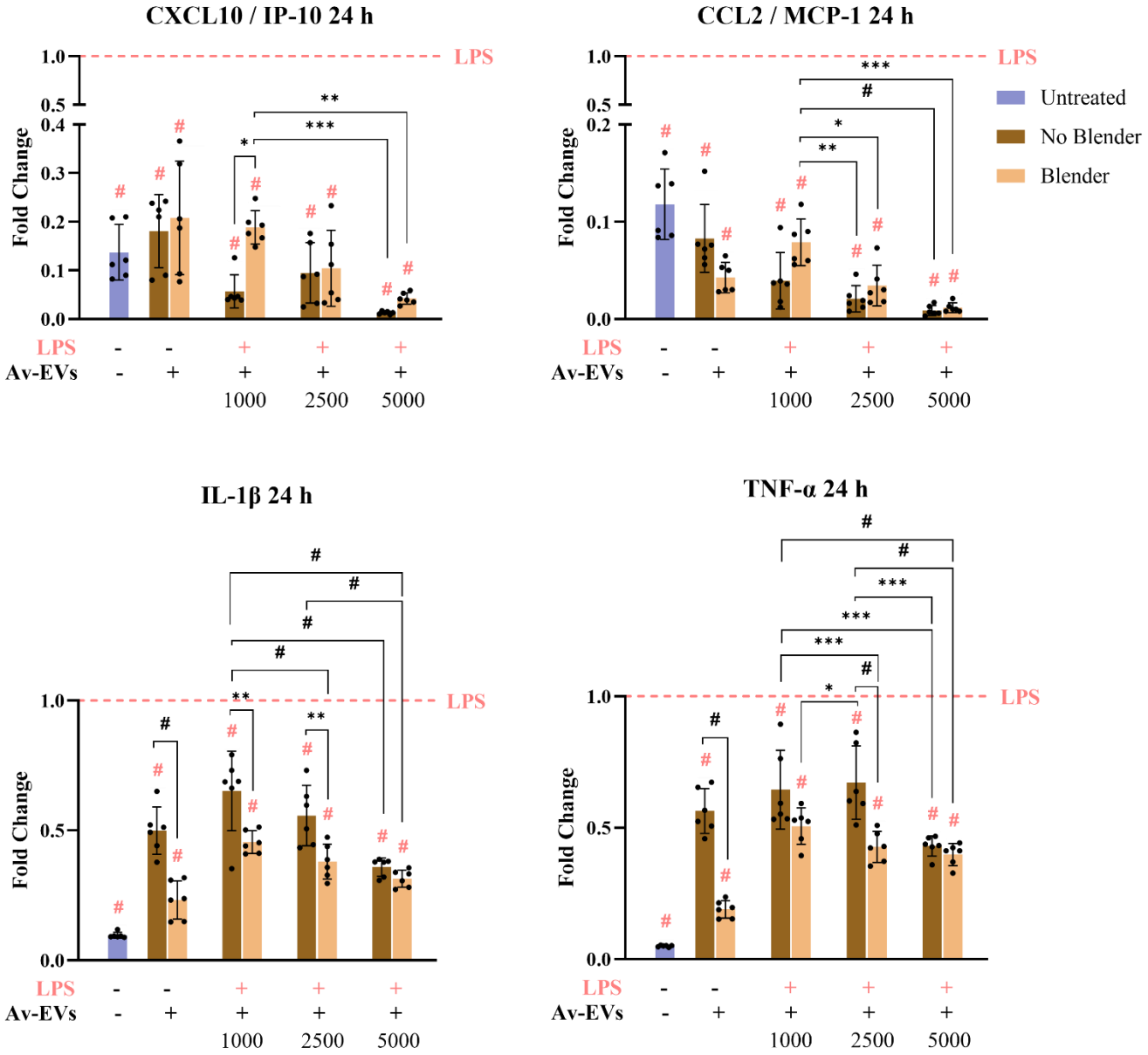

Figure 6. *CXCL10/IP-10, CCL2/MCP-1, IL-1β, TNF-α gene expressions in LPS-induced macrophages after 24 h of co-treatment with No Blender or Blender Av-EVs at increasing doses (1,000, 2,500, 5,000 EVs/cell). RT-qPCR data represents the mean ± SD with n=6 and p < 0.05 *, < 0.01 **, < 0.001 ***, < 0.0001 #. Statistical differences relative to the inflammatory control (+LPS/-EVs) are shown in pink, while statistical differences between the isolation methods and doses are shown in black.*

Consistent with these transcriptional findings, proteomic profiling (section 3.3.4) later confirmed a coordinated decreased abundance of TLR, NOD-like receptor, MAPK, PI3K–Akt, and JAK–STAT signaling components in mature Av-EVs, together with enrichment of proteins that are part of the lipid-remodeling and antioxidant-handling pathways (e.g., ether and sphingolipid metabolism). This proteomic pattern supports the notion that mature Av-EVs can suppress the MyD88/TRAF6→NF-κB/MAPK axis at multiple levels while reinforcing redox balance and membrane-dependent immunoregulation –

mechanisms fully aligned with the early reduction in CXCL10, CCL2, IL-1β, TNF-α, and IL-6 observed at 24 hours.

Modulation of M1-to-M2 Polarization from 24 Hours to 7 Days

IL-6 is a pleiotropic cytokine with complex roles in both pro-inflammatory signaling and tissue regeneration, making its behavior during macrophage activation highly context-dependent. During the early phase of activation (24 hours), IL-6 was significantly upregulated in LPS-treated macrophages [**Figure 7**]. At this point, Av-EV treatment did not result in a substantial modulation of IL-6 expression, regardless of dose or isolation method. This suggests that IL-6 expression during the acute inflammatory response is initially resistant to Av-EV-mediated suppression. After 72 hours, a notable shift occurred. IL-6 expression was significantly downregulated in Av-EV-treated groups compared to the LPS-only condition, with p-values reaching < 0.001 and < 0.0001 at intermediate and high doses for both NB and B EVs. This delayed suppression aligns with the early resolution phase of inflammation, during which IL-6 transitions from a pro-inflammatory effector to a mediator of immune regulation and repair and confirming that IL-6 elevation was part of a controlled resolution process rather than chronic inflammation.

Importantly, IL-6 appeared to play a transitional role in initiating macrophage phenotype switching from M1 to M2, a process supported by the concurrent rise in IL-10 expression.[65-69] This pattern is consistent with the activation of STAT3-mediated signaling as described in the literature, where both IL-6 and IL-10 are known to engage the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathway. Upon receptor engagement, these cytokines trigger JAK phosphorylation, which subsequently activates STAT3 through tyrosine phosphorylation. Activated STAT3 then dimerizes and translocates into the nucleus, promoting transcription of anti-inflammatory and reparative genes such as SOCS3, ARG1, and IL-10.[67, 68] In the context of Av-EV treatment, the initial persistence of IL-6, followed by its suppression alongside peak IL-10 expression, suggests that Av-EVs may support a controlled, pro-resolving environment possibly involving STAT3 activity. While the current study did not directly assess STAT3 phosphorylation or nuclear translocation, the observed gene expression kinetics align with previously reported STAT3-dependent resolution mechanisms. By day 7, IL-6 remained consistently suppressed across all Av-EV-treated conditions, regardless of dose or extraction method, indicating the establishment of a stable, anti-inflammatory macrophage state, likely corresponding to M2 polarization and resolution of the inflammatory response.

In parallel, IL-10 expression patterns strongly support this interpretation. At 24 hours, both NB and B Av-EVs induced a dose-dependent upregulation of IL-10, with a particularly strong response at the lowest dose (1000 EVs/cell) – inversely mirroring the IL-6 profile. Notably, NB EVs significantly outperformed B EVs in upregulating IL-10 at early and intermediate time points, suggesting superior early immunomodulatory potency. By 72 hours, IL-10 expression peaked in all EV-treated groups, coinciding with the sharp decline in IL-6 levels and further confirming the M1-to-M2 phenotypic transition. NB EVs again stood out, achieving up to a 5-fold increase in some replicates. By Day 7, IL-10 levels began to decline across all conditions, consistent with the transient nature of IL-10 in initiating the anti-inflammatory cascade and subsequent feedback mechanisms that restore immune homeostasis. This temporal profile underscores IL-10's role as an early switch that helps reset inflammatory macrophages toward a reparative fate which appeared later in the timeline.[68]

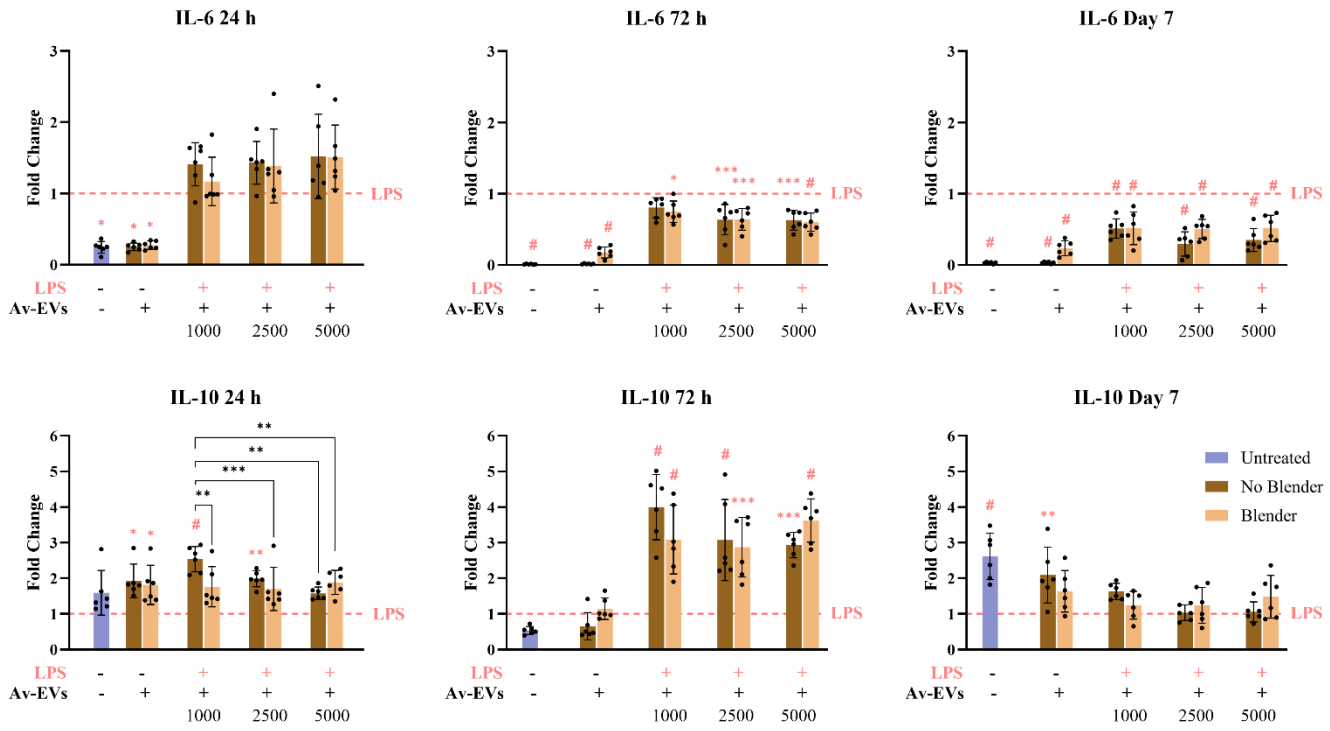

Figure 7. *IL-6 and IL-10 gene expressions in LPS-induced macrophages after 24 h, 72 h, and 7 days of co-treatment with No Blender or Blender Av-EVs at increasing doses (1,000, 2,500, 5,000 EVs/cell). RT-qPCR data represents the mean ± SD with n=6 and p < 0.05 *, < 0.01 **, < 0.001 ***, < 0.0001 #. Statistical differences relative to the inflammatory control (+LPS/-EVs) are shown in pink, while statistical differences between the isolation methods and doses are shown in black.*

Transition to an M2 Phenotype and Activation of Anti-inflammatory Markers in 7 Days

Although IL-10 expression declined by day 7, the combination of (i) sustained suppression of M1-associated cytokines during the critical 72-hour window and (ii) concurrent induction of M2-associated markers, supports the notion of an orchestrated immune transition toward inflammation resolution and tissue repair. These findings suggest that Av-EV treatment facilitates a durable shift toward an anti-inflammatory phenotype, not only by mediating early immunosuppression but also by reinforcing late-stage macrophage reprogramming, essential transition for initiating reparative processes such as wound healing, ECM remodeling, and fibrosis modulation. Aloe-derived vesicles have likewise been reported to modulate macrophage immunity *in vitro* and in clinical bronchoalveolar lavage fluid (BALF) samples *ex vivo*. The authors assessed macrophage M1-to-M2 reprogramming by flow cytometry of classic surface readouts: CD86 (M1) decreased while CD206 (M2) increased after vesicle uptake, accompanied by IL-10 elevation, shifting the overall M1/M2 balance toward an anti-inflammatory state. Although our study did not quantify CD86 or CD206, these observations outline practical endpoints that support our data and will guide future validation.[69]

Consistent with a pro-resolving program, ARG1 – a canonical M2 marker involved in proline synthesis and collagen stabilization –[70] was significantly upregulated in all Av-EV-treated groups compared to the LPS-only condition by day 7 [**Figure 8**]. While variability existed among replicates, several exhibited fold changes between 5 and 10, indicating a robust response. Although differences between EV doses and isolation methods did not reach statistical significance, a clear dose-dependent trend was observed in both types of EVs with a better response in NB Av-EVs with significance even at the lowest dose. The trend

aligns with the alamarBlue viability data, where reduced metabolic activity at higher Av-EV concentrations on day 7 may reflect enhanced polarization toward a reparative, quiescent M2 phenotype, rather than cytotoxicity. Additionally, the emergence of ARG1 expression may imply a sequential maturation from M2 into more specialized, tissue-repairing subtypes (e.g., M2a/M2c). However, further research is necessary to elucidate the specific contributions of Av-EV cargo in the modulation of cytokine signaling, receptor engagement, and downstream transcriptional programming involved in M2 macrophage polarization.

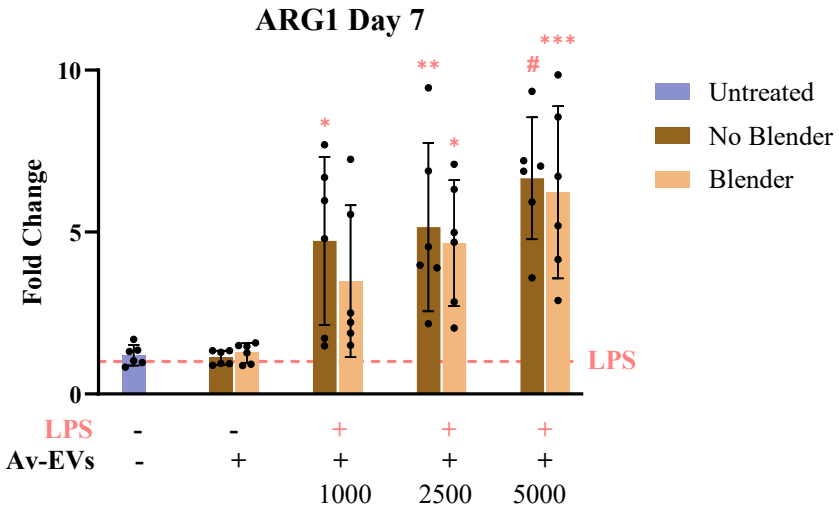

Figure 8. *ARG1 gene expression in LPS-induced macrophages after 7 days of co-treatment with No Blender or Blender Av-EVs at increasing doses (1,000, 2,500, 5,000 EVs/cell). RT-qPCR data represents the mean ± SD with n=6 and p < 0.05 *, < 0.01 **, < 0.001 ***, < 0.0001 #. Statistical differences relative to the inflammatory control (+LPS/-EVs) are shown in pink.*

It is worth noting that while M2 macrophages are known to facilitate tissue repair, they may contribute to pathological fibrosis through excessive collagen deposition under persistent inflammatory conditions.[71, 72] Favorably, in this study, Av-EV treatment also resulted in significant downregulation of fibrotic markers COL1A1 and α-SMA in fibroblasts (see section 3.2.5), indicating that matrix remodeling occurred without promoting fibrotic tissue accumulation. Moreover, we have already demonstrated that Av-EVs strongly suppressed key pro-inflammatory mediators at earlier time points, suggesting that the inflammatory stimulus had been resolved. In this context, M2 macrophage activity likely operates under homeostatic regulation, supporting tissue regeneration while minimizing the risk of fibrosis. Taken together, these findings highlight the therapeutic potential of NB and B Av-EVs as naturally derived nanocarriers capable of orchestrating both early and late phases of macrophage reprogramming.

3.2.5 Antifibrotic Response (Dermal Fibroblasts)

Myofibroblasts differentiation was induced in hDFs cultures exposed to 10 ng/mL TGF-β1, a potent pro-fibrotic factor that stimulates disorganized and excessive collagen deposition and enhances cell contractility and matrix tension by increasing the α-SMA stress fibers, mimicking the signaling observed in pathological scarring.[73] Additionally, vitamin C was used to enhance metabolic activity, growth and collagen production for a more robust fibrotic model.[74] Furthermore, the experiment investigated the potential of the three different doses of Av-EVs, isolated either manually or via shear force homogenization to attenuate this phenotype over a 72-hour exposure, by analyzing the gene expression of

COL1A1 and α-SMA, two canonical myofibroblast markers. The experimental conditions were as stated in **Table 2**.

Figure 9 presents the fold change in gene expression for all treatment conditions relative to the fibrotic positive control (+TGF-β1+VitC/–EVs), which was normalized to a fold change of 1. This control group exhibited a marked upregulation in the expression of both genes compared to the untreated hDFs and the EV-only controls, aligning with the expected profile of excessive ECM deposition and myofibroblast accumulation characteristic of fibrotic states. In contrast, untreated hDFs showed COL1A1 expression levels ranging from 0.280 to 0.409, and α-SMA levels from 0.092 to 0.216, indicating a baseline, non-fibrotic state. Similarly, NB Av-EV controls exhibited fold change values between 0.282 – 0.420 for COL1A1 and 0.081 – 0.216 for α-SMA, while B Av-EV controls ranged from 0.180 – 0.424 for COL1A1 and 0.066 – 0.229 for α-SMA. These findings confirm that Av-EVs alone do not promote fibrotic activation in resting fibroblasts and maintain gene expression levels within a physiological range comparable to untreated cells.

The addition of Av-EVs attenuated TGF-β1/VitC-induced gene expression of both COL1A1 and α-SMA, indicating reduced myofibroblast differentiation. Despite the higher inter-replicate variability inherent to *in vitro* models with primary cells, NB-derived Av-EVs achieved the strongest downregulation at intermediate dose (2,500 EVs/cell), reaching p values of $p < 0.0001$ and $p < 0.001$ for COL1A1 and α-SMA, respectively, and a reduction of COL1A1 employing 5000 EVs/cell with a $p < 0.01$. Moreover, there was a statistically significant difference between NB and B Av-EVs at the same dose for COL1A1 with a $p < 0.01$, indicating that the manual method of isolation counteracts more effectively TGF-β1-mediated transcriptional activation of collagen genes in comparison to those Av-EVs isolated via shear force homogenization.

These findings confirm the ability of Av-EVs to attenuate key markers of fibroblast-to-myofibroblast transdifferentiation and underscore their antifibrotic potential by inhibiting excessive ECM deposition and disrupting cytoskeletal reprogramming and mechanical force generation – both critical for myofibroblast function and fibrotic scar contracture.[35, 60] This parallel reduction of COL1A1 and α-SMA implies that Av-EVs may interfere with TGF-β1/SMAD signaling pathways or their downstream effectors involved in fibrotic transformation. This hypothesis is supported by the well-characterized canonical TGF-β1 pathway, wherein ligand binding to its receptor complex (TGF-βRI/II) leads to phosphorylation of SMAD2 and SMAD3, which then form a transcriptionally active complex with SMAD4. This complex translocates to the nucleus to drive expression of pro-fibrotic genes such as COL1A1 and α-SMA (ACTA2). Although direct inhibition of SMAD4 by Aloe vera components has not been demonstrated, several Aloe-derived bioactives, including acemannan, aloin and various flavonoids, have been shown to inhibit SMAD2/3 phosphorylation and nuclear translocation.[75, 76] This effectively disrupts the formation or function of the SMAD2/3/4 complex and impairs transcriptional activation of downstream fibrotic genes.

Consistent with this interpretation, proteomic enrichment in mature Av-EVs (section 3.3.4) highlighted modules that directly impinge on the contractile and matrix programs – regulation of actin cytoskeleton, Ca^{2+}-handling/channel activities, and lipid-remodeling pathways – all of which are predicted to reduce ERK, p38 MAPK, RhoA/ROCK, and PI3K/Akt/mTOR tone, limit stress-fiber assembly, dampen pro-contractile signaling, and temper redox-driven TGF-β amplification.[21, 61, 77] Moreover, proteomic signatures in mature Av-EVs reinforce the presence of non-canonical brakes on TGF-β signaling, evidenced by the relative depletion of translation-associated (ribosome) and proline-supply modules –

indicative of lower collagen biosynthetic/maturation throughput and congruent with the measured reduction in COL1A1 levels.

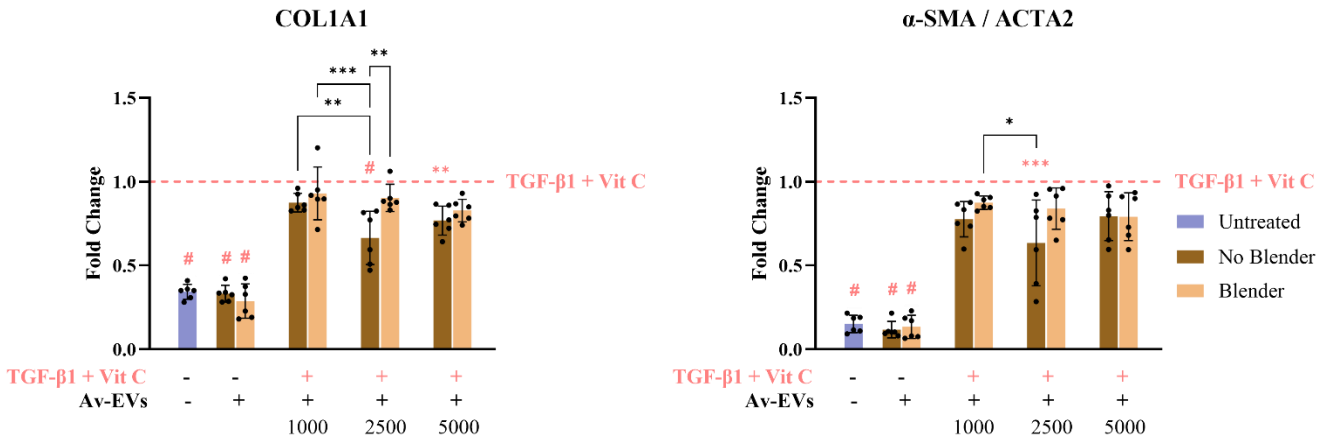

Figure 9. *COL1A1 and α-SMA/ACTA2 gene expressions in TGF-β1 + vitamin C- activated hDFs after 72 h co-treatment with No Blender or Blender Av-EVs at increasing doses (1,000, 2,500, 5,000 EVs/cell). RT-qPCR data represents the mean ± SD with n=6 and p < 0.05 *, < 0.01 **, < 0.001 ***, < 0.0001 #. relative to +TGFβ1+VitC/-EVs control. Statistical differences relative to the fibrotic control (+TGFβ1+VitC/-EVs) are shown in pink, while statistical differences between the isolation methods and doses are shown in black.*

Taken together, these combined mechanisms strongly support the interpretation that the bioactive cargo of NB Av-EVs functions as a potent modulator of fibrotic signaling, effectively reprogramming fibroblasts away from a pro-fibrotic trajectory toward a more quiescent, regenerative phenotype. Furthermore, the dual activity of Av-EVs – immune modulation and fibroblast reprogramming – exert a coordinated regulation of inflammation and fibrosis, two key phases that must be tightly balanced to ensure effective wound healing and tissue regeneration without abnormal scar progression.

3.2.6 Immunofluorescence Analysis of Smooth Muscle Actin

To further validate the antifibrotic properties of Av-EVs, α-SMA expression was assessed by immunofluorescence microscopy in hDFs cultured under healthy or fibrotic conditions, with or without Av-EVs co-treatment (2,500 EVs/cell). α-SMA was labeled in green, F-actin in red, and cell nuclei in blue. For each condition, six α-SMA and six background pixel intensity measurements were collected from three independent images. Quantification was performed by calculating net α-SMA signal intensity as the difference between each α-SMA measurement and the average background value.

As shown in **Figure 10**, untreated hDFs (far left) exhibited a classic quiescent fibroblast morphology with elongated spindle-shaped cells with aligned F-actin filaments as seen in the cellular uptake analysis. Image analysis revealed low α-SMA intensity values (817.2 to 1,090.2) consistent with a non-activated state. In contrast, fibrotic control cells (second panel), treated with TGF-β1 and vitamin C, displayed extensive cytoskeletal remodeling and strong colocalization of α-SMA with F-actin, forming dense contractile stress fibers – a hallmark of activated myofibroblasts. Fluorescence intensity dramatically increased to 2,403.3 and 2,649.3, representing a 2.5- to 3-fold rise over untreated cells ($p < 0.0001$). These results align with those reported by Ramirez et al., who observed a fivefold increase in α-SMA expression in myofibroblasts relative to untreated fibroblasts.[35]

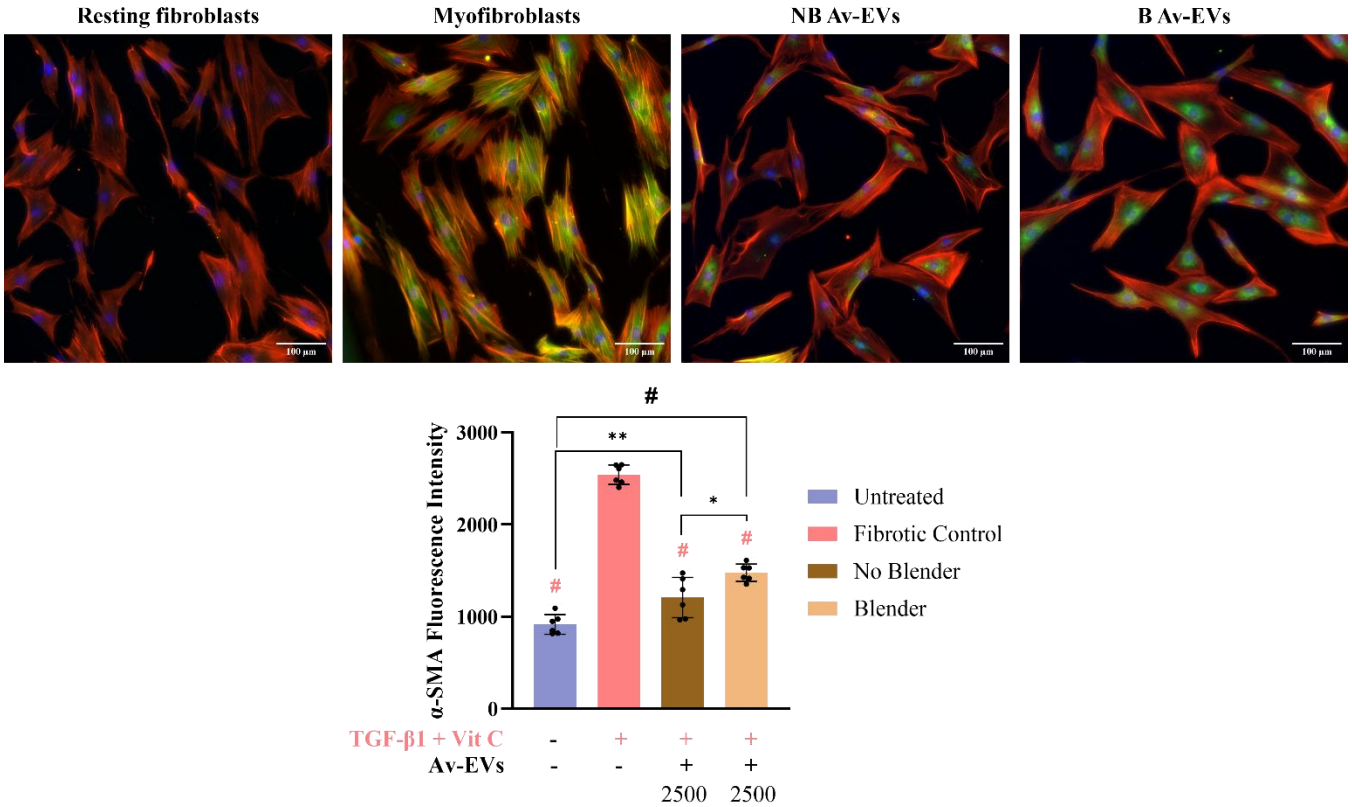

Figure 10. *Immunofluorescence staining of α-SMA (green), F-actin (red), and nuclei (blue) in hDFs. From left to right: resting hDFs, myofibroblast differentiation induced with TGF-β1 and vitamin C, and co-treatment with No Blender or Blender Av-EVs at 2,500 EVs/cell for 72 h. Images were captured at 20X magnification under identical exposure settings. α-SMA fluorescent intensity data represent mean ± SD with n=6 and p < 0.05 *, < 0.01 **, < 0.0001 #. Statistical differences relative to the fibrotic control (+TGFβ1+VitC/-EVs) are shown in pink, while statistical differences between the isolation methods are shown in black.*

Both Av-EV treatment groups significantly reduced α-SMA expression compared to the fibrotic control ($p < 0.0001$), with a significant difference between NB and B EVs ($p < 0.05$). NB Av-EV (third panel) reduced α-SMA levels to 966.8 – 1,474.8, effectively halving the fibrotic effect. Although the cells retained a spread morphology, they exhibited reduced colocalization and loss of organized stress fibers, suggesting diminished contractility and a shift toward a less fibrogenic state. B-derived EVs (fourth panel) also attenuated α-SMA expression (1,353.3 to 1,610.5), though less pronounced than NB-derived EVs. Notably, when compared to untreated cells, NB EV-treated hDFs showed only a modest difference ($p < 0.01$), whereas B EV-treated cells maintained a stronger divergence ($p < 0.0001$), further underscoring the superior efficacy of NB vesicles in restoring α-SMA expression toward baseline.

These findings support the gene expression data and reinforce the conclusion that the manual isolation method better preserves the functional vesicle cargo responsible for modulating fibroblast activation. Moreover, they confirm that Av-EVs suppress TGF-β1- driven fibrotic cascade at both transcriptional and protein levels. By downregulating α-SMA and disrupting associated cytoskeletal remodeling, Av-EVs emerge as promising nanotherapeutic agents capable of reducing tissue stiffness, contracture, and pathological ECM deposition, offering potential in the prevention of hypertrophic scars and keloids.

3.3 Comparative Analysis of NB Av-EVs Isolated from Mature and Young Aloe vera Leaves

All results discussed thus far were obtained using EVs isolated – either manually or mechanically – from mature Aloe vera leaves sourced from a supermarket supplied by a large-scale distributor in the United States. These leaves exhibited consistent morphological characteristics (~67 cm × ~9 cm × ~3 cm) and yielded approximately 400 mL per leaf. Given their robust and reproducible performance in reducing pro-inflammatory and fibrotic markers – particularly for NB-derived EVs – we extended our investigation to assess EVs derived from young Aloe vera leaves, manually harvested from live potted plants obtained from a local plant shop. These younger leaves were notably smaller (~45 cm × ~5 cm × ~1.5 cm) and produced a reduced gel yield of ~100 mL per leaf. NTA established that final suspension samples yielded concentrations ranging from 1.2 to 2.8 × 10^{10} particles/mL, for a total of 1.2 to 2.8 × 10^{12} particles per leaf. This large disparity in concentration is the first indication that even leaves of the same plant, at different stages of growth, produce and release different amounts of EVs.

This comparative analysis between NB Av-EVs derived from mature and young Aloe vera leaves is not only scientifically relevant but also holds important translational implications. Since Aloe vera plants require several years to reach full maturity – a factor that directly affects cultivation time, production scalability, and manufacturing costs –, understanding whether younger plants can yield vesicles with comparable therapeutic efficacy is critical for future commercialization efforts. Evaluating how plant age and extraction method influence EV composition and bioactivity may help streamline production strategies and inform selection criteria for cost-effective, large-scale Av-EV manufacturing.

3.3.1 Anti-Inflammatory Response (Macrophages)

Co-treatment with Av-EVs from both mature and young leaves resulted in attenuation of M1-associated pro-inflammatory cytokines (CXCL10/IP-10, CCL2/MCP-1, IL-1β, TNF, IL-6); however, mature leaf-derived EVs consistently outperformed young EVs across all measured markers [**Figure 11**]. Please note that separate untreated controls were included for the mature and young Aloe vera EV groups, as these samples were processed and analyzed in independent experimental runs.

CXCL10 and CCL2 were significantly downregulated by both EV types in a dose-dependent manner. Yet, NB-mature EVs achieved near-complete suppression at the lowest dose, while young EVs required the highest dose to reach comparable levels, highlighting a marked difference in potency. A significant difference ($p < 0.001$ for CXCL10; $p < 0.0001$ for CCL2) was observed between the two groups at the lowest dose. Furthermore, basal levels of these chemokines were elevated in young EV-only controls in comparison with the mature EV-only controls, with CCL2 showing statistical significance ($p < 0.01$), suggesting inherent differences in vesicle content or cell responsiveness.

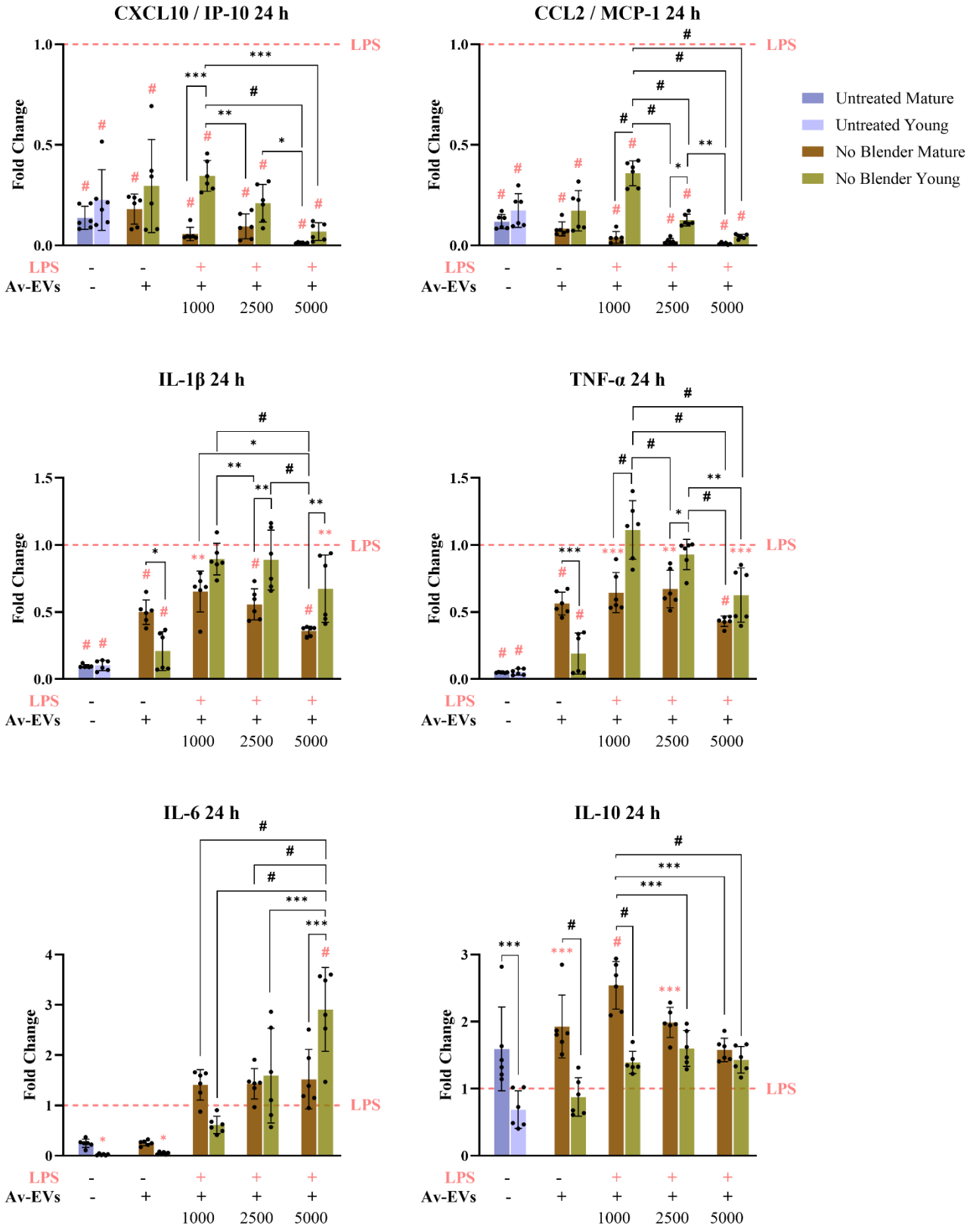

Figure 11. *CXCL10/IP-10, CCL2/MCP-1, IL-1β, TNF-α, IL-6 and IL-10 gene expressions in LPS-induced macrophages after 24 h of co-treatment with No Blender Av-EVs (isolated from mature or young Aloe vera leaves) at increasing doses (1,000, 2,500, 5,000 EVs/cell). RT-qPCR data represents the mean ± SD with n=6 and p value < 0.05 *, < 0.01 **, < 0.001 ***, < 0.0001 #. Statistical differences relative to the inflammatory control (+LPS/-EVs) are shown in pink, while statistical differences between the plant maturity and doses are shown in black.*

While IL-1β and TNF-α exhibited modest, dose-dependent downregulation in response to young EVs, their suppression remained consistently inferior to that achieved with mature EVs – with even the lowest dose of mature EVs outperforming the highest dose of young counterparts. Statistically significant differences between the two groups were evident across multiple doses, including $p < 0.01$ at D2 and D3 for IL-1β, and $p < 0.0001$ at D1 and $p < 0.05$ at D2 for TNF-α, underscoring the limited anti-inflammatory efficacy of young EVs. Interestingly, and unlike the trends observed for CXCL10 and CCL2, both IL-1β and TNF-α were significantly lower in the young EV-only control groups compared to the mature EV control groups, reaching $p < 0.05$ for IL-1β and $p < 0.001$ for TNF-α. This suggests different immune responses by vesicles from plants at different stages of development. Nevertheless, young EVs were unable to restore cytokine expression to baseline levels in the treated cells, in contrast to the superior immunomodulatory capacity of mature EVs.

Surprisingly, IL-6 expression exhibited a clear dose-dependent increase in cells treated with young Av-EVs, reaching fold changes of up to 4 at the highest concentration. This response stands in contrast to the stable IL-6 expression observed in mature EV-treated groups, resulting in a statistically significant difference between the highest dose groups ($p < 0.001$). These findings suggest that young EVs may not only lack the capacity to suppress IL-6-mediated inflammatory signaling, but at elevated doses, may in fact intensify the inflammatory response, highlighting a potential pro-inflammatory liability associated with immature vesicle cargo. In parallel, IL-10 expression was only modestly elevated in cells treated with young Av-EVs, displaying no clear dose-dependent trend and no significant difference from the LPS-only group, in contrast to mature Av-EVs, where induced IL-10 expression was in a consistent, dose-responsive manner opposite to IL-6 profile, with the most pronounced effect observed at the lowest dose, where expression was significantly higher than in the young EV group ($p < 0.0001$). Moreover, IL-10 levels in the young EV-only control group were markedly lower than those in the mature EV-only control ($p < 0.0001$), suggesting that the baseline immunomodulatory potential of vesicles from immature plants may be intrinsically limited.

3.3.2 Antifibrotic Response (Dermal Fibroblasts)

When evaluating fibrotic gene expressions (COL1A1 and α-SMA), young EVs again failed to match the efficacy of mature EVs [**Figure 12**]. Please note that separate untreated controls were included for the mature and young Aloe vera EV groups, as these samples were processed and analyzed in independent experimental runs.

Although a weak dose-response trend was observed, even the highest dose of young EVs did not reduce expression below that achieved by the lowest dose of mature EVs in any of the two targets. For COL1A1, significant differences were observed across all doses between the types of EVs ($p < 0.0001$ at D1 and D2; $p < 0.01$ at D3), and similar patterns were evident for α-SMA ($p < 0.001$ at D1 and $p < 0.0001$ at D2). These results highlight the superior antifibrotic potential of mature Av-EVs, which are more effective in preventing fibroblast-to-myofibroblast transdifferentiation, a key process in pathological scar formation.

Collectively, the results clearly highlight that EVs derived from mature Aloe vera leaves possess markedly superior anti-inflammatory and antifibrotic properties compared to those from younger plants. Mature Av-EVs consistently outperformed their younger counterparts, achieving stronger downregulation of inflammatory mediators and fibrotic markers, and demonstrating enhanced IL-10 upregulation. In contrast, young Av-EVs not only exhibited limited efficacy, but in some cases induced paradoxical pro-inflammatory effects, such as increased IL-6 expression at higher doses. These findings align with

previous work on orange-derived EVs, where the maturity of the fruit significantly influenced vesicle characteristics, including particle concentration and diameter, surface potential, lipid and protein content, and their ability to encapsulate and deliver bioactive compounds. Specifically, vesicles from ripe orange juice demonstrated superior curcumin delivery performance – enhancing its solubility, stability, bioaccessibility, and antioxidant activity – compared to those from unripe or overripe sources.[78] Given curcumin's well-established anti-inflammatory properties, this further supports the concept that plant maturity plays a crucial role in optimizing the therapeutic potential of EV-based delivery systems.

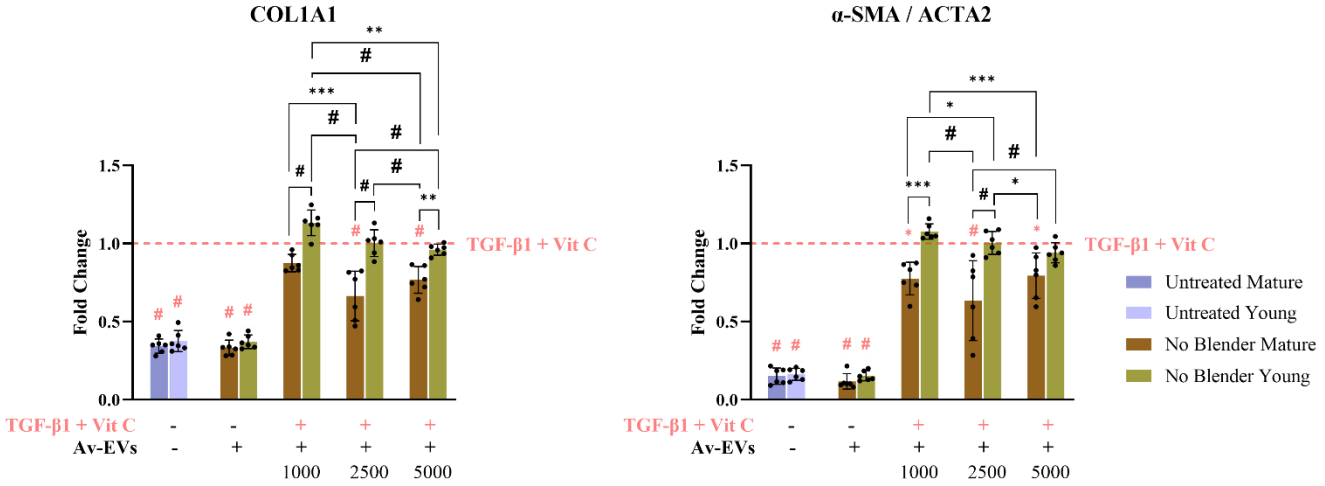

Figure 12. *COL1A1 and α-SMA/ACTA2 gene expressions in TGF-β1 + vitamin C- activated hDFs after 72 h co-treatment with No Blender Av-EVs (isolated from mature or young Aloe vera leaves) at increasing doses (1,000, 2,500, 5,000 EVs/cell). RT-qPCR data represents the mean ± SD with n=6 and p value < 0.05 *, < 0.01 **, < 0.001 ***, < 0.0001 #. Statistical differences relative to the fibrotic control (+TGFβ1+VitC/-EVs) are shown in pink, while statistical differences between the plant maturity and doses are shown in black.*

3.3.3 Biological Considerations

The disparity in the anti-inflammatory and antifibrotic efficacy observed between mature and young Av-EVs is likely rooted in biological differences in vesicle cargo and surface marker profiles, which are inherently influenced by the plant's age, developmental stage, and growing conditions.[79] Mature leaves from supermarket-grade Aloe vera *barbadensis miller* plants are typically cultivated under controlled agronomic environments optimized for commercial use. These conditions favor the accumulation of polysaccharides (e.g., acemannan), sterols, flavonoids, and other secondary metabolites that are well-documented for their anti-inflammatory, antioxidant, and immunomodulatory properties.[80, 81] In contrast, younger nursery-grown plants, especially those intended for ornamental purposes, are less metabolically mature. Their smaller biomass, lower gel yield, and underdeveloped biosynthetic pathways likely contribute to a less complex and less bioactive EV cargo as confirmed with the proteomics (section 3.3.4). This includes potential deficits in regulatory lipids, miRNAs, and proteins involved in modulating key inflammatory and fibrotic signaling cascades such as NF-κB and STAT3/STAT6 in macrophages, and TGF-β1/SMAD in fibroblasts. These molecular deficiencies may explain the reduced potency of young Av-EVs in downregulating pro-inflammatory cytokines or reversing myofibroblast-associated gene expression.

These findings highlight the critical role of plant maturity, cultivation purpose, and bioactive composition in determining the therapeutic potential of Aloe-derived bioproducts. In particular, the biochemical and functional properties of Av-EVs can vary substantially depending on factors such as Aloe species, plant

age, geographical origin, climate, soil type, and extraction methodology.[82-84] Importantly, the optimal harvest time for Aloe leaves is typically after three years of growth, when bioactive compound content peaks.[80] Furthermore, post-harvest handling, storage, and transport conditions play a pivotal role in preserving bioactivity, since exposure to excessive light, heat, or humidity can degrade key molecules before vesicle isolation.[30, 80, 82, 84] To address these limitations, the variables mentioned must be carefully controlled to ensure the reproducibility and potency of EV-based therapeutics derived from Aloe plant sources, streamlining both clinical translation and regulatory approval.

3.3.4 Differentially expressed proteins (DEPs) in Av-EVs based on Plan Maturity

After confirming plant-specific EV markers, a comparison of Av-EV protein cargo stratified by leaf age – mature (M1–M3) versus young (Y1–Y3) – was sought to provide mechanistic context for the performance gap between preparations.

Across all samples, 5,376 proteins (38,890 peptides) were identified. Label-free quantification followed by PCA revealed tight intragroup clustering with clear separation by leaf age [**Figure 13A**]. Statistical testing yielded 583 significantly differentially expressed proteins (DEPs), with 309 increased (red) and 274 decreased (blue) in mature EVs [**Figure 13B**]. This marker landscape suggests that leaf age is a key determinant of Av-EV protein cargo and supports the notion that mature Av-EVs are produced and trafficked through more robust secretory, cytoskeletal and stress-adaptive pathways than young Av-EVs; thus, providing a coherent explanation for their superior bioactivity in macrophage repolarization and suppression of fibroblast activation.

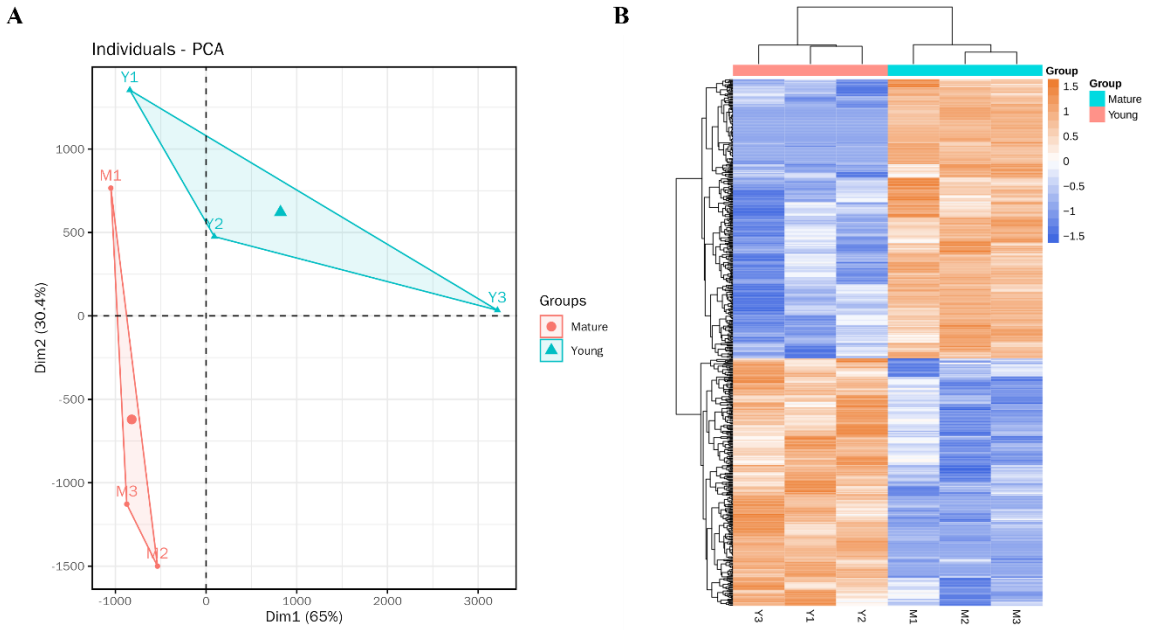

Figure 13. *Proteomic cargo comparison of Av-EVs isolated from mature (M1-M3) vs. young (Y1-Y3) leaves.* ***(A)*** *PCA.* ***(B)*** *Heatmap of DEPs.*

GO/KEGG Enrichment Related with the EV Biogenesis, Trafficking, and Uptake Sequence

GO/KEGG enrichment analysis revealed that the plant-specific marker families described above were among the DEPs, distinguishing EVs derived from mature versus young leaves. Indeed, higher

representation in mature Av-EVs of these terms help to support vesicle biogenesis and stabilize cargo during isolation and after uptake (HSP70/90);[85, 86] strengthen actin/clathrin-dependent budding and trafficking across the cell wall;[87-91] enhance targeting, docking, and fusion – and thus cargo delivery – via Rab GTPases and syntaxin/SNARE (soluble N-ethylmaleimide–sensitive factor attachment protein receptor) complexes;[92-94] [95-97] and provide Ca^{2+}/osmotic and redox-responsive protection and tuning of downstream signaling (annexins).[98-101] Taken together, this upregulated marker profile offers a mechanistic basis for the superior functional performance of mature Av-EVs.

GO/KEGG Directionality in the Context of Anti-inflammatory and Antifibrotic Functions

To relate proteomics to antifibrotic and anti-inflammatory functions, we categorized each GO term and KEGG pathway based on the differential abundance of the majority of its corresponding IDs. Terms whose constituent proteins were mostly increased in mature Av-EVs are discussed as "upregulated"; those whose IDs were mostly decreased in mature are discussed as "downregulated". The most representative GO molecular-function terms are summarized in **Figure 14**, and the KEGG pathways in **Figure 15**.

GO Enrichment Terms Upregulated in Mature Av-EVs:

- **Ceramidase axis (N-acylsphingosine amidohydrolase / ceramidase activity).** Ceramides are bioactive lipids that promote inflammation and apoptosis, whereas sphingosine-1-phosphate (S1P) supports cell survival and tissue repair. Enrichment of ceramidase activity in mature EVs is consistent with a shift from ceramide → sphingosine → S1P. Functionally, lower ceramide and appropriately tuned S1P would be expected to lessen RhoA/ROCK signaling and weakening stress-fiber formation, while in immune cells, this balance typically reduces NF-κB/NLRP3 activation and favors M2-like, IL-10/ARG1-positive phenotypes. [102-105]

- **Calcium handling (channel activity; calcium:proton antiporter activity; pyrophosphate-driven proton pumps).** Together these terms point to tighter cytosolic Ca^{2+} control. Limiting Ca^{2+} influx/redistribution would be expected to blunt Ca^{2+}-dependent myosin light-chain kinase activity (MLCK) and myocardin-related transcription factor–serum response factor (MRTF–SRF) pathways, reducing α-SMA assembly and contractile tone in fibroblasts, and to dampen Ca^{2+}-dependent steps of NF-κB/NLRP3 activation in macrophages – aligning with lower IL-1β/TNF and stabilized IL-6.[106-108]

- **Membrane remodeling (ATPase-coupled transmembrane transporter activity).** This term points to ABC transporters and P4-ATPase flippases that reset lipid asymmetry and lipid-raft architecture. In fibroblasts, such remodeling can reduce integrin clustering and focal adhesion kinase (FAK) activation, which in turn influences EV uptake, adhesion dynamics, and downstream cytoskeletal tension in recipient cells.[109] In macrophages, exposure of phosphatidylserine and raft reorganization can promote IL-10 production and temper TLR/NF-κB signaling.

- **Reactive-aldehyde detox (aldehyde dehydrogenase, NAD^+ activity).** Greater capacity to clear 4-HNE and related aldehydes would lower ROS-propagated NF-κB/TGF-β throughput, a common feed-forward driver of both myofibroblast differentiation and pro-inflammatory cytokine release.[110, 111]

GO Enrichment Terms Downregulated in Mature Av-EVs:

- **Proline supply (N2-acetyl-L-ornithine:2-oxoglutarate 5-aminotransferase activity).** This enzyme lies on the arginine→ornithine→proline axis, specifically in the acetylated branch of *de novo* arginine biosynthesis. Its depletion means that fewer mature-EV proteins map to this specific biosynthetic step than in young EVs. In other words, mature EVs are not enriched for an upstream enzyme that builds arginine from scratch, but rather skew toward arginine and proline metabolism as evidenced in the KEGG enrichment pathways, where it coupled to a more regulated proline supply in fibroblasts, moderating collagen synthesis and aligning immune resolution with controlled matrix deposition.[112-115]
  The ARG1 increase observed in macrophages (see section 3.2.4), therefore, likely reflects recipient-cell induction by mature-EV cues that dampen NF-κB signaling and enhance the pathways that bias macrophages toward an M2 phenotype, rather than direct enzyme delivery.

- **Protein-synthesis capacity (structural constituent of ribosome).** A young-skewed ribosomal signature suggests a higher translational drive that could support increased ECM production and cytokine synthesis; its attenuation in mature EVs is directionally consistent with less matrix output and a calmer cytokine milieu.[116-118]

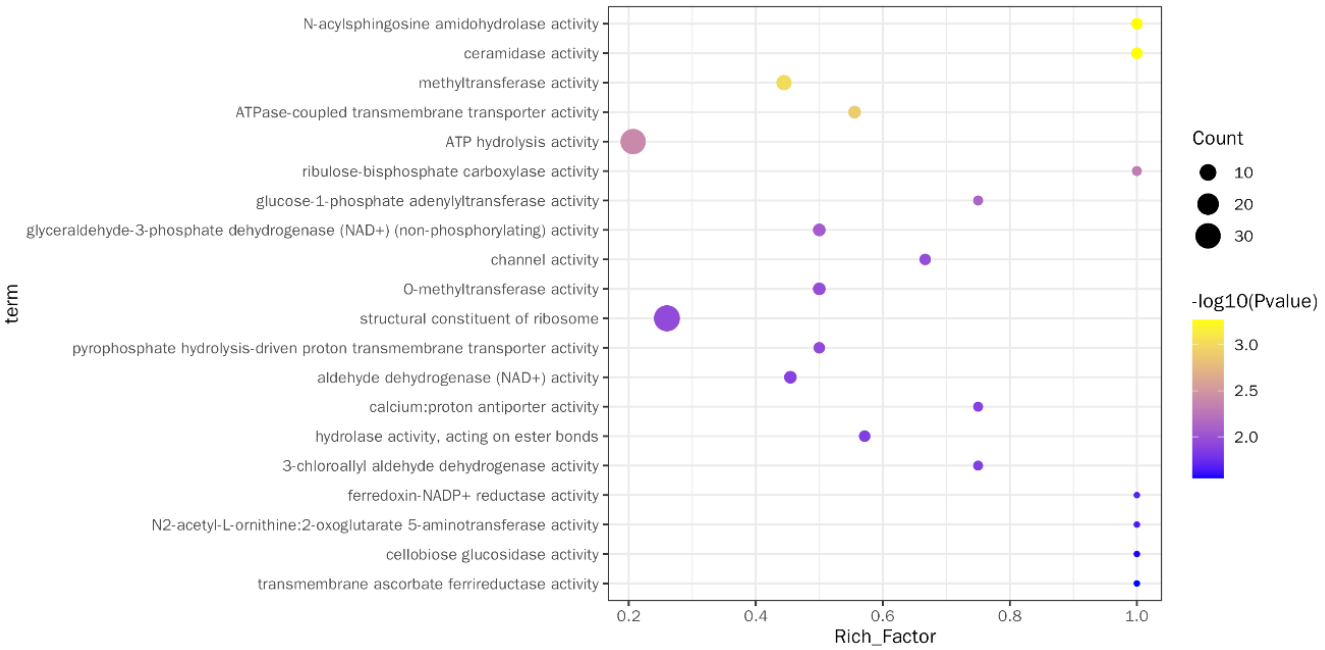

Figure 14. *GO molecular function enrichment for proteins in No Blender Av-EVs (isolated from mature and young Aloe vera leaves).*

KEGG Enrichment Terms Upregulated in Mature Av-EVs:

- **Ether lipid metabolism.** Plasmalogen-rich membranes bolster antioxidant buffering and stabilize lipid microdomains, thereby attenuating TGF-β–sensitized actomyosin stress-fiber assembly and blunting TLR–NF-κB signaling.[119-121]

- **Sphingolipid metabolism.** Re-tuning the ceramide → sphingosine → S1P pools away from ceramide is predicted to reduce inflammasome/NF-κB activation and soften cytoskeletal tension,

aligning with lower α-SMA and reparative macrophage phenotypes as described in the ceramidase GO enrichment term.[102-105]

- **Regulation of actin cytoskeleton.** Enrichment of actin-regulatory cargo is coherent with direct constraints on α-SMA assembly and myofibroblast contractility.[87-89]

- **Arginine and proline metabolism.** The enrichment suggests tuning of the arginine→ornithine→proline axis where mature EVs may facilitate controlled proline flux that supports tissue repair without excessive collagen deposition.[112-115]

- **IL-17 signaling pathway.** Several proteins mapping to nodes in IL-17 pathway function as modulators or brakes. Thus, their enrichment is compatible with a capacity to tune the IL-17 axis downward in recipient cells, helping to reduce the IL-17↔IL-6↔TGF-β1 feed-forward loop that sustains myofibroblast differentiation.[122, 123]

KEGG Enrichment Terms Downregulated in Mature Av-EVs:

- **Fluid shear stress and atherosclerosis.** Lower representation of this composite mechanotransduction/ROS/NF-κB module is consistent with less cytoskeletal tension and lower MAPK/NF-κB tone.[124, 125]

- **NOD-like receptor signaling.** Reduced NOD/NLR inputs predict less inflammasome activation and lower IL-1β maturation, matching the cytokine profile observed in the anti-inflammatory response.[126, 127]

- **Ribosome.** Depletion of translation machinery is coherent with lower ECM protein output and attenuated cytokine synthesis as also evidenced by the downregulation of the structural constituent of ribosome GO term.[116-118]

- **Ascorbate and aldarate metabolism.** A lower metabolism in mature EVs could limit effective endoplasmic reticulum ascorbate available for collagen prolyl/lysyl hydroxylases, thereby curbing collagen maturation.[128, 129]

- **Toll-like receptor, MAPK, PI3K–Akt, and JAK–STAT signaling.** Across these pathways, reduced representation in mature EVs would be expected to lower MyD88/TRAF6→NF-κB/MAPK throughput, temper AP-1–driven cytokines and reduce STAT-linked inflammatory transcription – all consistent with lower CXCL10/CCL2/IL-1β/TNF/IL-6 and increase of IL-10/ARG1.[130-134]

Viewed together, the enrichment landscape offers a unified mechanistic narrative. From a fibrosis-centric perspective, mature Av-EVs are enriched for modules that reduce contractility (actin regulation, Ca^{2+} control), reprogram membranes (ether/sphingolipid remodeling), and detoxify aldehydes, while being relatively depleted in proline-supply and ribosomal terms. This directional pattern bridges proteomics to phenotype by explaining the reductions in COL1A1 and α-SMA we observed. In parallel, an immune-

centric reading shows that terms increased in mature EVs converge on lipid signaling, Ca^{2+} control, redox detox, and tunable IL-17–pathway nodes, whereas terms decreased in mature EVs include NOD/TLR/MAPK/JAK–STAT modules. Together, that tilt rationalizes the lower chemokines/cytokines, stabilized IL-6, and higher IL-10/ARG1.

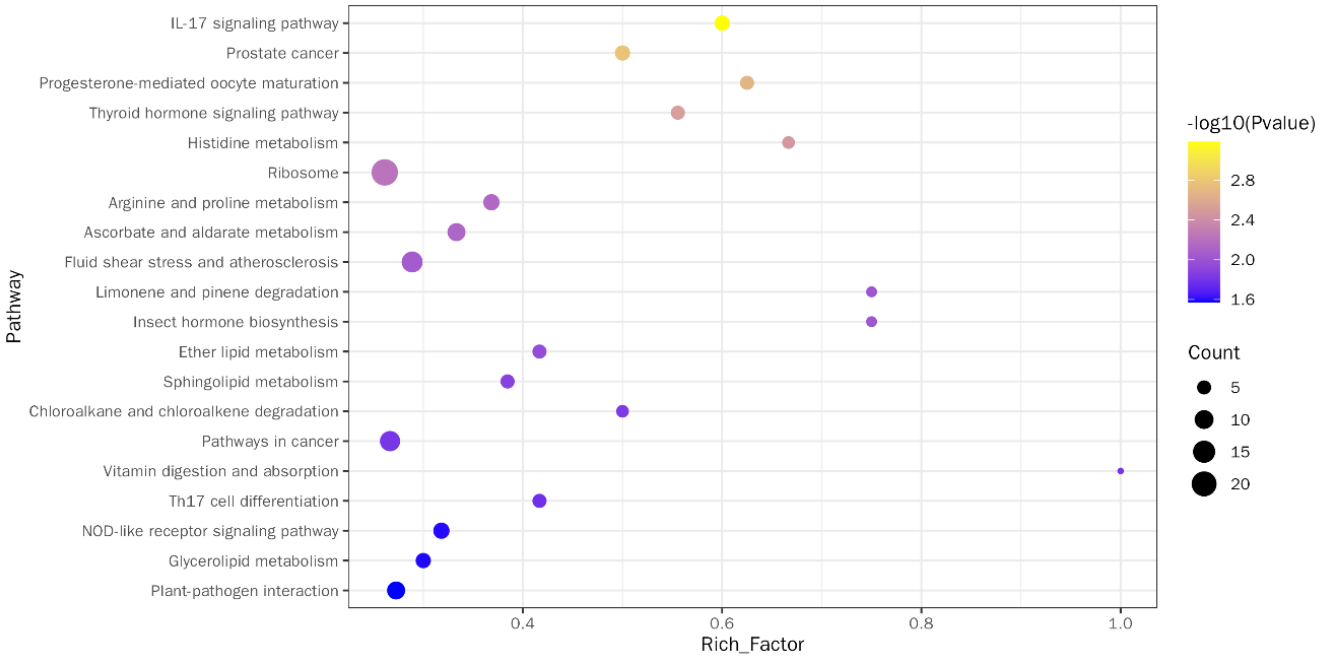

Figure 15. *KEGG pathway enrichment for proteins in No Blender Av-EVs (isolated from mature and young Aloe vera leaves).*

In sum, the proteomic signature of mature Av-EVs indicates that they are produced and trafficked through more robust secretory, cytoskeletal, and stress-adaptive pathways than vesicles from young leaves. This profile offers a coherent mechanistic link between EV cargo and redox control, immune modulation, and matrix regulation consistent with the age-dependent bioactivity. For instance, mature Av-EVs uniquely reduced α-SMA and COL1A1 in TGF-β1/vitamin C–stimulated fibroblasts and attenuated pro-inflammatory cytokines in M1 macrophages at markedly lower doses (1,000 vs 5,000 EVs per cell). Together, these findings identify leaf maturity as a decisive variable of Av-EV composition and function, and they motivate standardization of source maturity alongside targeted validation of candidate proteins to ensure reproducible therapeutic performance.

4. Conclusion

This study presents the first comprehensive characterization of Aloe vera gel-derived extracellular vesicles (Av-EVs), including the first proteomic atlas for this EV class, with emphasis on how isolation method and plant maturity shape their physicochemical and bioactive profiles, and thereby their capacity to modulate key pathological mechanisms implicated in abnormal scar formation: chronic inflammation, oxidative stress, and myofibroblast differentiation – three interlinked drivers of fibrotic remodeling.

Av-EVs were successfully isolated by both manual and shear-force-based methods and compared across mature and young Aloe vera leaves. Notably, Av-EVs manually extracted from mature leaves yielded superior vesicle bioactivity *in vitro* as evidenced in the attenuation of pro-inflammatory cytokine

expression in M1-polarized macrophages (CXCL10, CCL2, IL-1β, TNF-α, IL-6) alongside the promotion of an M2-like phenotype (IL-10, ARG1), time-dependent inhibition of intracellular reactive oxygen species (ROS) levels, and suppression of myofibroblast differentiation in activated human dermal fibroblasts (COL1A1, α-SMA). These results suggest that gentle extraction may have an advantage in retaining and preserving the bioactive components require to break the inflammation–oxidative stress–fibrosis cycle and restore tissue homeostasis.

Proteomic profiling established a plant-typical EV signature and mapped age-dependent mechanisms. Across samples we identified 5,376 proteins (38,890 peptides); PCA cleanly separated groups by leaf age, and 583 DEPs were detected (309 up, 274 down in mature EVs). Marker families repeatedly reported in plant EVs – HSP70/HSP90, actin-cytoskeleton machinery, syntaxin/SNARE, small GTPases, clathrin heavy chain, annexins, and cell-wall remodeling – were all present, confirming EV identity. KEGG/GO directionality then linked cargo to function: mature EVs were enriched in regulation of actin cytoskeleton and Ca^{2+}-handling/channel activities (reduced contractility), ether/sphingolipid/ metabolism (membrane remodeling and antioxidant buffering), ceramidase activity (S1P shift to support cell survival and tissue repair), and aldehyde dehydrogenase (NAD^+) (ROS detox). Conversely, mature EVs showed lower representation of ribosome (translation capacity), proline-supply nodes, and innate/inflammatory modules (NOD/TLR/MAPK/PI3K–Akt/JAK–STAT). Together with our gene-expression data, this maturity-biased proteome supports a model in which mature Av-EVs arise from more robust secretory, cytoskeletal, and stress-adaptive programs and deliver cargo that dampens fibroblast contractility and inflammatory throughput – molecular features absent from young counterparts.

While limited to *in vitro* contexts, these findings underscore the importance of standardizing the isolation protocol and source material for reproducible outcomes. Most importantly, they position Av-EVs as natural, scalable, cell-free nanotherapeutics that warrant further investigation in underlying mechanistic studies, *in vivo* validation, and advanced delivery systems to enable clinical translation for preventing and treating fibrosis-related skin disorders.

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Conflicts of Interest

The authors declare no conflict of interest.

Data Availability Statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD069742. All other datasets are available in the Figshare repository 10.6084/m9.figshare.29367212.

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