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Dynamic Compression Reveals Donor-Age-Associated Inflammaging in Human MSC Spheroids

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Abstract

Mechanical cues play a critical role in musculoskeletal homeostasis and disease pathophysiology, where excessive loading induces tissue damage and promotes inflammation. Aging further exacerbates this process, particularly in age-related diseases, such as osteoarthritis (OA). Although both hyper-mechanical loading and aging are known to contribute to chronic inflammation in OA and other age-related diseases, their combined effects remain poorly modeled in vitro. Here, we developed a dynamic 3D culture platform integrating gelatin hydrogels, human bone marrow-derived mesenchymal stromal cell (MSC) spheroids, and a compressive bioreactor to recapitulate joint-like mechanical environments. Gelatin hydrogel-encapsulated MSC spheroids maintained mechanical stability under repeated loading (10-20 kPa) while preserving cell viability and spreading. Aged MSC spheroids exhibited metabolic reprogramming toward glycolysis, elevated secretion of pro-inflammatory cytokines (IL-6 and IL-8), and increased *PIEZO1* expression. Compressive loading further amplified inflammatory signaling and promoted M1-like macrophage polarization, particularly in aged donor-derived MSCs, modeling key features of inflammaging. Dexamethasone suppressed inflammatory cytokine secretion and promoted M2-like macrophage polarization, whereas tocilizumab modulated immune responses without reducing cytokine production. This platform establishes a donor-specific, mechanobiological model of inflammaging for precision therapeutic screening in osteoarthritis and related diseases.

Keywords

Compression, gelatin hydrogels, aging, immunomodulation, mesenchymal stromal cell

1. Introduction

Osteoarthritis (OA) is a chronic degenerative disease affecting the entire joint.[1] It is characterized by deterioration of articular cartilage, increased synovial inflammation, and varied pain.[2,3] OA affects more than 500 million people worldwide[1], is strongly associated with aging, and as the global population continues to age, its burden is expected to rise substantially. Recent studies suggest that age-related changes in the immune system contribute to OA pathogenesis. Elderly individuals exhibit elevated circulating inflammatory markers compared to younger individuals, largely due to immune aging and immunosenescence.[4] Within the joint, early in vivo studies revealed that synovial lining macrophages contribute to cartilage damage[5] and osteophyte formation.[6] In particular, they shift toward a pro-inflammatory phenotype under pathological conditions. Although OA is a heterogeneous disease with multiple phenotypes, such as bone-driven, pain-dominant, and inflammatory phenotypes,[7] inflammation is a critical feature.[7] Consequently, modulation of macrophage polarization represents a promising therapeutic strategy for OA. Targeting macrophage-mediated inflammation may therefore provide an effective approach to mitigate disease progression and improve joint function.

Mesenchymal stromal cells (MSCs) are promising therapeutic candidates to modulate the inflammatory microenvironment and are widely investigated for regenerative medicine due to their potent immunomodulatory properties, capacity to promote tissue repair, and therapeutic potential across diverse diseases.[8] However, the therapeutic efficacy of transplanted MSCs is strongly influenced by the local microenvironment (including inflammatory, biochemical, and mechanical factors) and donor age. Insights from OA pathogenesis have highlighted abnormal mechanical loading as one of the major contributors to OA initiation and progression, triggering degenerative and inflammatory cascades.[9] Repetitive and/or excessive mechanical stress can alter MSC

immunomodulatory function and enhance the secretion of pro-inflammatory cytokines.[10] Bioreactors capable of controlling mechanical stimuli have emerged as essential tools for recapitulating these relevant loading conditions to investigate MSC behavior.[11] Moreover, donor age is a well-recognized determinant of MSC properties.[12] Aging is associated with chronic systemic inflammation, resulting in cellular senescence, mitochondrial dysfunction, and elevated pro-inflammatory mediators.[12] These changes shift MSCs toward a more pro-inflammatory phenotype. Despite increasing evidence that both aging and mechanical stimulation influence MSC function, their combined effects on MSC immunomodulatory behavior remain poorly understood. Addressing this gap is important for the development of cell-based therapies intended for aged patients and mechanically active tissues such as joints. Furthermore, there is a lack of human-relevant in vitro platforms capable of recapitulating both the age-associated inflammatory microenvironment and the dynamic mechanical loading experienced in musculoskeletal tissues.

In this study, we developed a dynamic MSC spheroid culture system using a compressive bioreactor. We hypothesized that secretome profiles differ between young and aged donor-derived MSCs and are more accurately captured under dynamic culture conditions, with aged spheroids exhibiting increased secretion of pro-inflammatory cytokines. Because this imbalance in the pro-inflammatory milieu is closely linked to the progression of age-related diseases,[13] our platform may serve as a valuable tool for studying inflammatory mechanisms and evaluating therapeutic interventions. To validate this system, we applied anti-inflammatory drugs and assessed changes in key inflammatory cytokines under compression. This platform enables the evaluation of donor-dependent MSC responses under controlled mechanical loading and serves as a human-relevant model of inflammaging.

2. Materials and methods

2.1 MSC spheroid formation and scaffold fabrication

Human bone marrow-derived mesenchymal stromal cells (MSCs) from a young male donor (25-year-old, RoosterBio, Frederick, MD, USA) and an aged female donor (55 years old, CGT Global, Folsom, CA, USA) are referred to as young MSCs (Y) and aged MSCs (A), respectively. MSCs were expanded in growth medium consisting of minimum essential alpha medium (α-MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma) and 1% penicillin/streptomycin (P/S; Gemini Bio-Products, Sacramento, CA, USA). Cells were used at passage 4-6 for all experiments.

MSC spheroids were generated using a forced aggregation approach.[10, 14] Briefly, a 1.5% (w/v) agarose solution was prepared in distilled water (DIW) and autoclaved at 250°C for 1 h to ensure complete dissolution. Positive molds containing 29 microwells were placed into each well of a 24-well plate. Agarose solution (800 μL) was added to each well and allowed to cool at room temperature for 15 min. After removing the molds and cleaning residual agarose from the well edges, phosphate-buffered saline (PBS; 1 mL) was added to each well, followed by centrifugation at 1200 rcf for 3 min. PBS was then aspirated, and MSC suspension (232,000 cells/mL, 1 mL) was added to each well and centrifuged at 800 rpm for 9 min. The plate was incubated at 37°C with 5% CO_2 for up to 48 h to allow spheroid formation (8,000 cells/spheroid). Phase-contrast images of spheroids were captured using an ECHO Revolve microscope (ECHO, CA, USA). Spheroid diameters were quantified using FIJI software.

Gelatin hydrogels were fabricated using a horseradish peroxidase-catalyzed crosslinking reaction. Gelatin (Type A, from porcine skin, bloom 300; Sigma-Aldrich, St. Louis, MO, USA) was modified with 3-(4-hydroxyphenyl)propionic acid using EDC/NHS chemistry following a previously reported protocol.[15] The resulting product was referred to as 3-(4-hydroxyphenyl)propionic acid-functionalized gelatin (GH). Gelatin hydrogels (Gel) were formed using GH (140 $\pm$ 5 µmol

phenol/g polymer) at 5.56 wt%, with horseradish peroxidase (HRP, type VI, 257.2 units/mg, Sigma-Aldrich) at 0.01 mg/mL, and hydrogen peroxide (H_2O_2) at 0.1%. Gelatin hydrogels (90 μL) encapsulating 50 spheroids were cast into silicon molds with an 8 mm diameter.[15] Gel(Y) and Gel(A) denote gelatin hydrogels encapsulating MSC spheroids derived from the young donor and aged donor, respectively.

2.2 Bioenergetics of MSC spheroids from young donor and aged donor

Mito Stress Test and Glycolysis Stress Test were performed using an Agilent Seahorse XFe96 Analyzer (Agilent, CA, USA) following the manufacturer's instructions. Briefly, spheroids were transferred to Agilent Seahorse XFe96 Spheroid Microplates (Agilent, CA, USA) pre-coated with 100 μg/mL poly-D-lysine hydrobromide (Sigma-Aldrich, MO, USA) and incubated overnight at 37°C with 5% CO_2. The plates were then switched into assay medium and incubated in a non-CO_2 incubator for 45 min prior to running an assay. An ECHO Revolve microscope was used to observe the spheroid in each well to verify its presence and central positioning within the well before and after running an assay.

For the Mito Stress Test, the assay medium was Seahorse XF RPMI medium pH 7.4 supplemented with 10 mM glucose, 1 mM pyruvate, and 2 mM glutamine (Agilent, CA, USA). Oligomycin, FCCP, and Rotenone & Antimycin A (Agilent, CA, USA) were respectively loaded into port A, B, and C, respectively. The final concentrations in the Seahorse plate were 1.5 μM oligomycin, 1.5 μM FCCP, and 0.5 μM rotenone/antymycin A. For the Glycolysis Stress Test, the assay medium was XF RPMI medium pH 7.4 supplemented with 2 mM glutamine (Agilent, CA, USA). Glucose, oligomycin, and 2-deoxyglucose (2-DG) were injected in port A, B, and C, respectively. The final concentrations in the Seahorse plate were 10 mM glucose, 1 μM oligomycin, and 50 mM 2-DG. After each compound injection, the mix-wait-measure cycle was set

to 1 min, 0 min, and 3 min, respectively, repeated for six cycles per injection.

2.3 Compressive bioreactor setup and MSC spheroid culture procedure

Mechanical stimulation was applied to Gel(Y) and Gel(A) using the FX-5000™ Compression System (Flexcell® International Corporation, Burlington, VT, USA). After fabrication, Gel(Y) and Gel(A) constructs were maintained in 1 mL of growth media for 1 d. Each sample was loaded into a BioPress™ culture plate with 3 mL of growth medium. Static samples (control) were cultured in 3 mL of growth medium without compression. For dynamic loading, positive air pressure was applied to generate compressive force on the samples positioned between a piston and stationary platen in a BioPress™ culture plate. The compression regimen was selected based on prior work[10] and was programmed to gradually increase to 10 kPa, hold for 30 s, and then slowly unload to 0 kPa and was denoted as L10H30. Similarly, L20H30 represents 20 kPa compression held for 30 s, and L20H100 represents 20 kPa compression held for 100 s. The compressive regimens were applied for 2 d, and the growth medium was replaced with fresh medium (3 mL). Compression was then continued for an additional day. Conditioned media were subsequently collected for ELISA (IL-6, IL-8, and IL-10) assays and macrophage polarization experiments.

2.4 Storage modulus of gelatin hydrogels encapsulating MSC spheroids

The mechanical properties of gelatin hydrogels, both prior to and following compression, were characterized using a Discovery HR2 Hybrid Rheometer (TA Instruments, New Castle, DE, USA) using an 8 mm parallel plate geometry. We performed an oscillatory strain sweep across a range of 0.004-4% at a fixed angular frequency (10 rad/s).

2.5 Cell response and secretome analysis

After 3 d of static and compressive culture, phase-contrast images of spheroids were captured using an ECHO Revolve microscope. Spheroid diameters were quantified using FIJI (ImageJ) software. Spheroid viability after compression and in static condition on day 3 was assessed using Calcein AM (1:500 dilution; Thermo Fisher) and propidium iodide (1:1000 dilution; Sigma-Aldrich) staining. Following a 30-min incubation at 37°C, samples were transferred to fresh medium. Fluorescence images were acquired using a Leica SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany). To visualize F-actin and nuclei, spheroid-encapsulated gelatin hydrogels were fixed in 10% formalin for at least 2 h at room temperature, then washed three times with PBS. Samples were then stained with Phalloidin-Alexa Fluor 488 (1:500 dilution) and Hoechst 33342 (1:1000 dilution) and incubated overnight. After three washes with PBS, fluorescent images were acquired using a Leica SP8 confocal microscope equipped with a 10x objective.

For DNA content and gene expression analysis, gelatin hydrogels encapsulating MSC spheroids were digested with collagenase type II (1 mg/mL; Worthington Biochemical, NJ, US) at 37°C for 45 min and then centrifuged at 900 rpm for 8 min to collect spheroids. After aspirating the supernatant, 500 µL of 1X Passive Lysis Buffer (PLB; Promega Corporation, Madison, WI, USA) or 200 µL of TRIzol Reagent (Thermo Fisher, Sacramento, CA, USA) was added to each tube for subsequent PicoGreen assay (Quant-iT PicoGreen dsDNA Assay Kit; Thermo Fisher) or RNA isolation, respectively.

Quantitative real time PCR (qRT-PCR) was performed to assess gene expression *PIEZO1*. After lysing spheroids in TRIzol Reagent, total RNA was extracted according to the manufacturer's protocol. RNA (800 ng) was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The resulting cDNA was diluted in RNase-free water

to a final concentration of 12 ng/μL. qRT-PCR reactions were carried out using Taq PCR Master Mix (Qiagen) on a QuantStudio 6 Pro Real-Time PCR System (Thermo Fisher). The following TaqMan gene expression assays were used: *PIEZO1* (Hs00207230-m1), and *GAPDH* (Hs02786624_g1) as a housekeeping gene. Threshold cycle (Ct) values were determined for each gene, and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. ΔCt values were obtained by normalizing *PIEZO1* to *GAPDH*, and ΔΔCt values were calculated relative to the average ΔCt of the control group.

IL-6, IL-8, and IL-10 secretions were determined by human-specific enzyme-linked immunoassay kits following manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

2.6 Tocilizumab and dexamethasone treatment

Tocilizumab (Selleck Chemicals, Houston, TX, USA), an antibody for the IL-6 receptor, was mixed 5.56 wt% GH solution to achieve a concentration of 5.56 μg/mL. This mixture was subsequently used to fabricate gelatin hydrogels encapsulating 50 spheroids. The final concentration of Tocilizumab (T) in the hydrogels was 5 μg/mL. Similarly, dexamethasone (Sigma-Aldrich), a broad-spectrum anti-inflammatory agent, was mixed with GH solution at initial concentration of 111 nM and used to fabricate gelatin hydrogels encapsulating 50 spheroids. The final concentration of dexamethasone (D) in the hydrogels was 100 nM.

After forming hydrogels encapsulating MSC spheroids loaded with tocilizumab or dexamethasone, each hydrogel was incubated in 1 mL of growth medium supplemented with either 5 μg/mL tocilizumab or 100 nM dexamethasone, respectively, for 1 d. After washing with PBS, hydrogel samples were then transferred to the compressive bioreactor and cultured in 3 mL of growth medium containing 5 μg/mL tocilizumab or 100 nM dexamethasone, respectively. The L10H30 compression regimen was applied for 2 d, after which the medium was removed, the

constructs were washed twice with PBS to ensure complete removal of residual drug, and fresh growth medium was added. Compression was subsequently continued for an additional day. Conditioned media were then collected for ELISA analysis and macrophage polarization studies.

2.7 Macrophage polarization

Human THP-1 monocytes were cultured in RPMI 1640 medium (ATCC Formulation containing L-glutamine, HEPES, sodium pyruvate, and high glucose) supplemented with 10% fetal bovine serum (FBS; SeraPrime, Fort Collins, CO, USA) in a humidified incubator at 37ºC with 5% CO_2 until the density reached approximately 1 x 10^6 cells/ml. THP-1 monocytes were then seeded at 75% confluency in 6-well plates and treated with 320 nM phorbol-12-myristate-13-acetate (PMA) for 36 h to induce adherence and differentiation into macrophages. Differentiated THP-1 macrophages were subsequently treated with a 1:1 mixture of conditioned medium and basal RPMI medium for 24 h. Polarization controls were treated with basal RPMI medium (M0), RPMI supplemented with 200 ng/mL lipopolysaccharide (LPS; M1), or RPMI supplemented with 20 ng/mL interleukin-4 (IL-4; M2). All media were then refreshed with basal RPMI medium for an additional 24 h.[10, 16]

Cells were harvested using cold 2.5 mM EDTA and gentle scraping, centrifuged, and resuspended in warm PBS containing 3% FBS. Cells were then stained for flow cytometric analysis. Following Fc receptor blocking (1:40, TruStain FcX, BioLegend), cells were stained with antibodies against CD11b (1:40, eBioscience #47-0118-42), HLA-DR (1:40, eBioscience, cat. 48-9956-42), CD163 (1:50, Invitrogen, Cat. MA5-17719), and CD206 (1:33, eBioscience, Cat. 12-2069-42). Cell viability was assessed using fixable Zombie Aqua dye (1:250, Life Tech). Cells were fixed with 2% paraformaldehyde. M1 macrophages were defined as $CD11b^+HLA\text{-}DR^+$ populations, while M2 macrophages were identified as $CD206^+CD163^+$ populations. Flow

cytometry was performed using an Attune™ NxT Flow Cytometer (Thermo Fisher Scientific, CA, USA), and data were analyzed using FlowJo software (v10). Representative flow cytometry scatter plots illustrating the gating strategy used to identify M1-like and M2-like macrophage populations are shown in Figure S1.

2.8 Statistical analysis

Data are reported as mean ± standard deviation (SD) from at least three independent experiments. Statistical analysis was performed using Welch's t-test to compare between young and aged spheroids. Comparisons among compression regimens within each donor group were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparisons test in GraphPad Prism 10. Statistical significance was defined as follows: ns, not significant ($p > 0.05$); $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); $p < 0.0001$ (****).

3 Results and Discussion

3.1 Development of a compressive bioreactor culture model

The inflammatory microenvironment is associated with various types of diseases, particularly aging and age-related conditions.[4] Therefore, *in vitro* models that mimic elevated inflammatory conditions using human cells are essential for evaluating anti-inflammatory drugs. To establish this model, we used gelatin hydrogels encapsulating human MSC spheroids under dynamic compression. Gelatin hydrogels formed via a horseradish peroxidase-catalyzed crosslinking reaction (**Figure 1a**) are biocompatible scaffolds that support cell migration, proliferation, and differentiation.[15, 17, 18] In this study, gelatin hydrogels with a storage modulus (G') of 1.83 ± 0.15 kPa were used to encapsulate 50 MSC spheroids under both static and compressive culture conditions. To apply compression, a Flexcell® Compression System was

used to regulate positive air pressure and compress gelatin hydrogels encapsulating MSC spheroids, which were sandwiched between an upper platen and a rubber bottom plate (**Figure 1b**). To model physiologically relevant joint loading conditions, compressive magnitudes were adjusted to subject MSC spheroids to stresses of 10 and 20 kPa, with holding durations of 30 and 100 s applied to mimic varying joint loading modes (**Figure 1c**). We applied a compressive bioreactor with controlled force and programmed cycles to mimic the dynamic mechanical environment of joint tissues. Although joint tissues are subjected to both compressive loading and shear stress,[19] we initially isolate compression as a single factor to better elucidate the specific mechanisms governing inflammation. Hydrogel deformation was assessed by measuring changes in diameter before and after compressive loading. No significant changes in diameter were observed after 3 days of compression (**Figure 1d**), and the storage modulus remained unchanged (**Figure 1e**). This behavior is consistent with the elastic nature of gelatin hydrogels, which exhibit a low loss modulus compared to storage modulus ($\tan\delta < 0.1$) and long stress relaxation time (**Figure S2**). Collectively, gelatin hydrogels exhibit predominantly elastic deformation, rapid recovery, and fatigue resistance, making them well-suited for dynamic compression in 3D culture systems. This behavior can be attributed to reversible physical interactions within the gelatin network, including hydrogen bonding among amine, amide, and carboxyl groups, as well as the presence of partially folded protein structures within the gelatin backbone. Moreover, gelatin hydrogels are formed from a polymer precursor derived from collagen – the main component of human tissues – and provide a supportive microenvironment for cells.

Figure 1: Gelatin hydrogels encapsulating MSC spheroids for compressive loading and mechanical characterization. (a) Schematic illustration of gelatin hydrogels encapsulating MSC spheroids. **(b)** Working principle of the compression bioreactor (adapted and modified from Flexcell International's BioPress™ compression system). **(c)** Experimental timeline and bioreactor operation cycle. **(d)** Representative images and **(e)** storage modulus (G') of gelatin hydrogels encapsulating MSC spheroids under static condition and compressive loading (n=3-4). Statistical significance was determined by one-way ANOVA with Tukey's multiple-comparisons test. L10H30, L20H30, and L20H100 denote three compressive loading regimens, where L represents the applied loading force and the following numbers (10 and 20) indicate the compression magnitude in kPa, and H represents the holding phase with the subsequent numbers (30 and 100) indicating the holding duration in seconds. ns = not significant.

Following validation of scaffold quality, we evaluated cell behavior within hydrogels and under loading by assessing DNA content, cell outgrowth from spheroids, live/dead staining, and F-actin/Hoechst staining. MSC spheroids were formed using bone marrow-derived MSCs obtained from one young (25 years old) donor and one aged (55 years old) donor. To determine the quantity of cells across conditions (young vs. aged; static vs. compressive culture), we quantified DNA content of each hydrogel sample after 4 days of culture. DNA content was comparable between young and aged spheroids under static control conditions and all three compressive regimens (**Figure 2a**), indicating a similar total cell number across those conditions. These results suggest that under both static and compressive culture, young and aged MSC spheroids exhibited comparable growth throughout the experimental period.

To examine cell spreading and migration, we quantified spheroid diameter after 3 days of culture. Spheroid diameter in both static control and compressive culture groups was significantly increased relative to Day −1 (immediately after formation) (**Figure 2b**). Compared to static controls, MSC spheroids subjected to compressive loading at 10 or 20 kPa with a 30 s hold (L10H30 and L20H30, respectively) exhibited comparable diameters, although spheroids under compression had a trend toward larger average diameters than those under static conditions. Notably, spheroids exposed to 20 kPa with a 100 s hold (L20H100) exhibited a larger diameter, suggesting that prolonged hold time may contribute to enhanced spheroid outgrowth. In terms of donor age, spheroids derived from the young donor had higher average diameters than those from the aged donor. However, this difference was not statistically significant. No strong red fluorescence (PI staining) was observed **(Figure 2c)**, indicating minimal cell death under all experimental conditions. Upon increasing compressive loading to 30 kPa, we observed more dead cells (**Figure S3**). Therefore, compressive loading up to 20 kPa was considered suitable for subsequent studies. In addition, to confirm and quantify the mechanical forces transmitted to MSC

spheroids embedded within gelatin hydrogels, we performed finite element analysis (**Figure S4a**). The model predicted that the spheroids underwent compression within the hydrogel matrix, with spheroids located near the hydrogel edge exhibiting greater displacement magnitude (**Figure S4b,c**) and effective stress (**Figure S4d,e**) than those located at the center. Increasing the applied compression from 10 to 20 kPa resulted in increased spheroid displacement magnitude and effective stress. However, the effective stress within the spheroids remained lower than the externally applied compressive pressure. For example, under applied compressive pressures of 10 and 20 kPa, the effective stress within the spheroids ranged from approximately 2.1 to 3.1 kPa and from 3.8 to 5.6 kPa, respectively. Altogether, the model predicted that embedded spheroids experienced lower compressive stress than the applied pressure, with stress and displacement increasing from the hydrogel center toward the edge.

F-actin/Hoechst staining (**Figure 2d**) revealed pronounced spheroid outgrowth with cells spreading into surrounding matrix. Importantly, cellular architecture was not disrupted by compression. Over the 3-day culture period, MSCs remained healthy, exhibiting anchorage and spreading within the gelatin hydrogels. Collectively, these results confirmed that gelatin hydrogels encapsulating MSC spheroids under dynamic compressive culture for 3 days maintain scaffold integrity and cell viability and architecture, thereby enabling the investigation of mechanotransduction-driven inflammatory responses without confounding effects from the hydrogel or cell damage.

Figure 2: Characterization of MSC spheroids in gelatin hydrogels after compressive loading. (a) Quantification of DNA content (n = 3) and **(b)** spheroid outgrowth (n = 30) under static and compressive conditions. Data are presented as mean ± SD. Statistical significance was determined by two-way ANOVA with Tukey's multiple-comparisons test. Statistical significance was defined as $p < 0.05$ with $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); and $p > 0.05$ (ns, not significant). **(c)** Representative fluorescence images of MSC spheroids stained with Calcein-AM (green) and propidium iodide (red) under static and compressive conditions. **(d)** Representative images of MSC spheroids stained with Phalloidin-488 (green) and Hoechst (blue) after static and compressive culture.

3.2 Metabolic activity of MSC spheroids derived from young donor and aged donor

MSC spheroids can exhibit variable metabolic functions depending on the donor, which can be observed by monitoring mitochondrial metabolism and changes in oxidative phosphorylation (OXPHOS) and glycolysis. To evaluate donor-dependent metabolic activity and dynamic changes in MSCs, we conducted mitochondrial and glycolysis stress tests (**Figure 3**) on MSC spheroids. These analyses were also conducted to determine whether aged-associated metabolic characteristics corresponded with established hallmarks of cellular aging. High proton leak suggests impaired mitochondrial efficiency, and proton leak was greater in MSC spheroids derived from the aged donor compared to those from the young donor (**Figure 3a**). Other parameters (non-mitochondrial oxygen consumption, basal respiration, maximal respiration, ATP production, spare respiratory capacity, and coupling efficiency) were similar in MSC spheroids regardless of donor age. These results indicate that mitochondrial health in aged donor-derived MSC spheroids is compromised compared to young donor-derived MSC spheroids, which is consistent with age-associated alterations in mitochondrial function.[20] Glycolytic capacity and glycolytic reserve of MSC spheroids derived from the aged donor were significantly higher than those from the young donor (**Figure 3b**), while non-glycolytic acidification and glycolysis were comparable between the two groups. Collectively, MSC spheroids derived from the aged donor exhibited better glycolytic activity but reduced mitochondrial respiratory efficiency compared to

MSCs from a young donor. This observation suggests a metabolic shift from mitochondrial oxidative phosphorylation (OXPHOS) toward glycolysis in MSC spheroids derived from the aged donor. This shift is consistent with age-associated declines in the expression of both mitochondrial- and nuclear-encoded components of the electron transport chain (ETC), leading to reduced OXPHOS capacity. Although cells generate energy through both OXPHOS and glycolysis, OXPHOS is more efficient in ATP production than glycolysis. Moreover, mitochondria play a critical role in maintaining cellular resilience and adaptability to changing microenvironments. Therefore, impaired mitochondrial function can contribute to metabolic dysfunction and the progression of degenerative diseases. Collectively, these results confirmed distinct age-associated metabolic phenotypes and supported the selection of representative young and aged donor-derived MSC spheroids for subsequent studies.

Figure 3: Comparison of the energy-producing pathways for MSC spheroids derived from a young donor and aged donor. (a) Oxygen consumption rates (OCR) of one spheroid.

Parameters of mitochondrial respiration included: **(i)** non-mitochondrial oxygen consumption, **(ii)** basal respiration, **(iii)** maximal respiration, **(iv)** proton leak, **(v)** ATP-linked respiration, **(vi)** spare respiratory capacity, (vii) spare respiratory capacity as a percentage, and **(viii)** coupling efficiency (n = 9-14 per group). **(b)** Extracellular acidification rate (ECAR) was normalized to one spheroid. Parameters of glycolytic function included: **(i)** non-glycolytic acidification, **(ii)** glycolysis, **(iii)** glycolytic capacity, and **(iv)** glycolytic reserve (n=13 per group). Statistical significance was determined by Welch's t-test. Statistical significance was defined as $p < 0.05$. ns, not significant ($p > 0.05$); $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

3.3 Cytokine production and macrophage polarization associated with MSC spheroids under dynamic compression

We applied elevated compressive loads (10 and 20 kPa) with holding times (30 and 100 s) to promote inflammatory responses and assess their impact on macrophage polarization. To determine differences in inflammatory cytokines/chemokines (IL-6 and IL-8) and the potent anti-inflammatory cytokine (IL-10) secreted by MSC spheroids under static and dynamic compression, we used ELISA to quantify IL-6, IL-8, and IL-10 in conditioned media. As expected, MSC spheroids from the aged donor secreted higher levels of IL-6 and IL-8 than those from the young donor under both static and compression conditions, whereas IL-10 levels were similar between the two groups **(Figure 4a** and **S5a**). Additionally, MSC spheroids from both young donor and aged donor secreted more IL-6 and IL-8 under compressive conditions compared to the static culture condition. Varying the compression magnitude between 10 kPa and 20 kPa with the same hold time of 30 seconds (L10H30 vs. L20H30) did not affect IL-6 or IL-8 secretion from either young or aged MSC spheroids **(Figure S5b**). When increasing the hold time from 30 to 100 seconds while maintaining compression at 20 kPa (L20H30 vs. L20H100), IL-6 and IL-8 secretion were higher, on average, at the longer holding time in aged donor MSC spheroids, although the difference was not statistically significant **(Figure S5b**). In young donor MSC spheroids, IL-8 secretion was also higher at the longer holding time **(Figure S5b**). Further Increasing the load or extending the loading duration did not result in significant differences in cytokine secretion. This

may indicate that the applied mechanical stimuli exceeded a threshold beyond which no further increase in inflammatory response was observed. This observation is consistent with previous studies using young MSC spheroids in alginate hydrogels.[10] In the case of IL-10, 20 kPa compression significantly decreased IL-10 secretion in MSC spheroids from both a young and aged donor **(Figure S5b**); the magnitude of these differences was small, indicating that IL-10 secretion remained largely unchanged overall. Altogether, MSC spheroids from the aged donor secreted higher levels of pro-inflammatory factors (IL-6 and IL-8) than those from a young donor under both static and compressive culture conditions. IL-10 secretion was largely unchanged in young and aged donor-derived MSCs in static and compressive culture conditions. These findings suggest that mechanical loading and donor age influence pro-inflammatory rather than anti-inflammatory factor production under the experimental conditions tested. Because IL-10 levels remained relatively unchanged, future studies using higher spheroid densities and optimized compression regimens may be necessary to better characterize potential changes in IL-10 secretion.

Figure 4: Secretome analysis, macrophage polarization, and gene expression of MSC spheroids derived from a young donor and aged donor cultured under static and compressive conditions. (a) Heatmap illustrating cytokine secretion profiles of MSC spheroids derived from young donor and aged donor, encapsulated in gelatin hydrogels, and cultured under static or compressive conditions. IL-6, IL-8, and IL-10 levels were measured by ELISA in conditioned media collected on day 3 under static (control) and compressive conditions (L10H30, L20H30, and L20H100). Flow cytometric analysis of **(b)** M1- and **(c)** M2-like macrophage phenotypes from THP-1-derived macrophages treated with corresponding conditioned media. M1-like macrophages were identified as $CD11b^+HLA\text{-}DR^+$ cells, and M2-like macrophages were identified as $CD206^+CD163^+$ cells. Data are presented as mean ± SD (n=3-4). One-way ANOVA followed by Tukey's post hoc test was used to determine statistical significance. Statistical significance was defined as $p < 0.05$. ns, not significant ($p > 0.05$); $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***). **(d)** Heatmap of *PIEZO1* gene expression in MSC spheroids encapsulated in gelatin hydrogels under different compression regimens. Gene expression was quantified by real-time PCR, normalized to *GAPDH*, and expressed relative to the young static control group. Colors indicate the mean relative expression ($2^{-\Delta\Delta Ct}$) from 3-7 replicates.

Conditioned media collected from MSC spheroids encapsulated in gelatin hydrogels and

cultured under static or compressive bioreactor conditions were used to treat THP-1-derived macrophages. M1- and M2-like macrophage polarization was analyzed by flow cytometry by measuring the expression of phenotype-specific surface markers. M1-like macrophages were identified as $CD11b^{+}HLA\text{-}DR^{+}$ cells, whereas M2-like macrophages were characterized by $CD206^{+}$ and $CD163^{+}$ expression. Conditioned media from MSC spheroids derived from an aged donor induced significantly greater M1-like macrophage polarization (**Figure 4b**) and a lower proportion of M2-like macrophages (**Figure 4c**) compared to young donor MSC spheroids under static culture conditions. Under dynamic compression (L10H30), conditioned media from MSC spheroids derived from the young donor induced significantly greater M1-like macrophage polarization compared to static culture. Conditioned media from constructs under L20H30 and L20H100 conditions increased M1-like macrophage polarization, but the differences were not statistically significant. However, no differences were observed among the different compression magnitudes or holding times (**Figure 4b**). In contrast, conditioned media from aged donor MSC spheroids did not alter M1-like macrophage polarization across the compression regimens, although M1-like macrophage polarization under all compression conditions remained higher than that observed for young MSC spheroids under static conditions (**Figure 4b**). For M2-like macrophage polarization, no significant differences were observed between static and compression culture conditions in conditioned media derived from young and aged donor MSC spheroids (**Figure 4c**). These findings suggest that donor age has a significant influence on the immunomodulatory properties of MSC spheroids, with aged donor-derived MSC spheroids promoting a more pro-inflammatory macrophage phenotype compared to MSC spheroids from a young donor, evidenced by increased M1-like polarization. Compressive culture further enhanced the pro-inflammatory effects of young donor-derived MSC spheroids, whereas aged donor-derived MSC spheroids exhibited little change in their immunomodulatory profile in response to compression. Because aged donor-derived MSCs already induced higher levels of M1

macrophage polarization under static conditions, the additional effect of mechanical loading may have been attenuated.

PIEZO1 is mechanosensitive cation channel and can sense mechanical stress changes on the surface of various cell types.[21] *PIEZO1* activates mitochondrial fission leading to inflammation-induced cartilaginous endplate degeneration.[21, 22] Therefore, we hypothesized that donor age and compressive loading would differentially regulate *PIEZO1* expression in MSC spheroids. To test this hypothesis, we performed quantitative PCR (qPCR) to evaluate *PIEZO1* expression in MSC spheroids derived from a young donor and aged donor under static and compression culture conditions. *PIEZO1* was upregulated in aged MSC spheroids compared to MSCs from young donor under static conditions (**Figure 4d and S6a**). With aging, MSCs are exposed to a stiffer extracellular matrix due to age-associated fibrotic remodeling, which may alter mechanosensitivity and regulate *PIEZO1* expression. In young MSC spheroids, *PIEZO1* expression was upregulated when compressive forces were applied compared to the static condition **(Figure 4d and S6e)**. In contrast, in aged MSC spheroids, *PIEZO1* expression showed a slight downregulation under low compression (L10H30) and significant reduction under higher compression (L20H30 and L20H100) compared with static culture (**Figure 4d and S6f**). Under compressive loading, *PIEZO1* expression did not differ significantly between young and aged MSC spheroids at L10H30 or L20H30 (**Figure 4d and S6b-c**); however, *PIEZO1* expression was significantly lower in aged MSC spheroids than in young MSC spheroids under the L20H100 regimen (**Figure 4d and S6d**). These data suggest that aging alters *PIEZO1*-mediated mechanotransduction in MSC spheroids, characterized by elevated basal *PIEZO1* expression but a diminished transcriptional response to mechanical stimulation. Thai et al. also demonstrated that compressive loading disrupts the MSC actin cytoskeleton and downregulates *CTGF*

expression, which may compromise MSC phenotype and promote a more pro-inflammatory secretory profile.[10] Together, hyper-physiological compression may activate *PIEZO1*-mediated mechanotransduction in young MSCs, potentially contributing to cytoskeletal remodeling and downstream suppression of CTGF expression. In contrast, baseline *PIEZO1* expression was already elevated in aged MSC spheroids and not further increased by compression, which could explain their limited responsiveness to additional mechanical stimulation and their heightened pro-inflammatory profile. Consistent with the observed impairment in mitochondrial function and increased secretion of pro-inflammatory cytokines, aged donor-derived MSC spheroids exhibited altered *PIEZO1*-mediated mechanotransduction. These data suggest that mechanosensing, cellular metabolism, and inflammatory signaling are interconnected processes that contribute to the pro-inflammatory phenotype of aged donor-derived MSCs.

3.4 Anti-inflammatory drug treatment on MSC spheroids is captured under dynamic compression

Regardless of donor age, MSC spheroids under compressive loading of 10 kPa for 30 s exhibited increased secretion of the inflammatory cytokine IL-6 and the chemokine IL-8 and enhanced M1-like macrophage polarization. This compressive regimen consistently elicited a pro-inflammatory response across donor groups. Therefore, L10H30 was used to model an inflammatory scenario in subsequent experiments. To evaluate the drug screening capability of our platform, tocilizumab and dexamethasone were selected as representative anti-inflammatory drugs with different targeting mechanisms. IL-6 is increasingly recognized as a key cytokine associated with chronic inflammation, which occurs in arthritic patients. Tocilizumab is a humanized monoclonal antibody that ameliorates autoimmune responses and inflammation by binding to both soluble IL-6 receptors and membrane-bound IL-6 receptors.[23] The efficacy of tocilizumab in treating moderate-to-severe rheumatoid arthritis in adults has been confirmed in

several Phase III clinical trials in Japan and worldwide.[23, 24] In addition, clinical practice in Japan has demonstrated that tocilizumab can prevent joint damage and improve anemia in patients with rheumatoid arthritis. In contrast, dexamethasone, a synthetic corticosteroid, has been widely used to treat OA by reducing chronic inflammation and pain.[25, 26] Dexamethasone is well known for its broad-spectrum anti-inflammatory therapeutic effects.

Based on previous studies using tocilizumab in microglial cells,[27] we selected concentrations ranging from 5 to 15 μg/mL to evaluate its cytotoxicity toward MSC spheroids under static culture conditions. Live/dead staining using Calcein AM and propidium iodide (PI) was performed to qualitatively determine whether these concentrations induced cell death. The screening showed that 15 μg/mL tocilizumab resulted in detectable cell death, whereas concentrations lower than 10 μg/mL were considered cytocompatible (**Figure S7**). For dexamethasone, 100 nM is a commonly used concentration for MSC differentiation, and previous studies have confirmed that this concentration was effective in promoting MSC osteogenic differentiation,[15] implying no cytotoxicity for MSC spheroids. Therefore, 5 μg/mL tocilizumab or 100 nM dexamethasone were incorporated into gelatin hydrogels and supplemented in the culture medium during the first 2 days. No significant cell death was observed under static and L10H30 compression culture in the presence of tocilizumab and dexamethasone treatment (**Figure 5a**). Although cell viability was confirmed by live/dead staining and cell spreading within the gelatin hydrogels, additional quantitative cytotoxicity assays, such as LDH release or DNA-content analysis, would further strengthen the evaluation of cytotoxicity and should be considered in future studies.

Figure 5: Tocilizumab and dexamethasone treatment altered the secretome profile and immunomodulatory effects of MSC spheroids. (a) Representative fluorescence images of MSC spheroids stained with Calcein-AM (green) and propidium iodide (red). **(b)** Heatmap showing cytokine secretion from mesenchymal stromal cell (MSC) spheroids derived from young donor and aged donor when encapsulated in gelatin hydrogels under static or compressive culture with and without tocilizumab (T, 5 μg/mL) or dexamethasone (D, 100 nM) treatment. IL-6, IL-8, and IL-10 secretion measured by ELISA in conditioned media collected on day 3. **(c-d)** Flow cytometric analysis of M1-like and M2-like macrophage phenotypes in THP-1-derived macrophages treated with conditioned media from MSC spheroids following tocilizumab or dexamethasone treatment under static culture conditions. **(e-f)** Flow cytometric analysis of M1-like and M2-like macrophage phenotypes in THP-1-derived macrophages treated with conditioned media from MSC spheroids derived from young donor and aged donor, encapsulated in gelatin hydrogels, and cultured under L10H30 compressive conditions following tocilizumab treatment. M1-like macrophages were identified as $CD11b^+HLA\text{-}DR^+$ cells, and M2-like macrophages were identified as $CD206^+CD163^+$ cells. Data are presented as mean ± SD (n=3-4). Statistical significance was determined by one-way ANOVA with Tukey's multiple-comparisons test. Statistical significance was defined as $p < 0.05$. ns, not significant ($p > 0.05$); $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

Under static culture (control), tocilizumab treatment did not alter the IL-6 and IL-8 secretion levels of MSC spheroids derived from both young donor and aged donor. Similarly, no change was observed under L10H30 compression culture (**Figure 5b, Figure S8**). In contrast, dexamethasone treatment decreased IL-6 and IL-8 secretion from MSC spheroids derived from both young donor and aged donor. Notably, dexamethasone reduced IL-6 and IL-8 secretion from the elevated inflammatory levels induced by compression to levels comparable to those observed under static culture (control) (**Figure 5b, Figure S8**). In the case of IL-10 secretion, although significant differences were observed in MSC spheroids derived from young donor under various conditions of drug treatment, the magnitude of these differences was small (**Figure S8)**. Interestingly, dexamethasone treatment led to an increase in IL-10 secretion from MSC spheroids derived from aged donor under static culture (**Figure 5b, Figure S8**). These findings are consistent with the known anti-inflammatory effects of dexamethasone.

Next, conditioned media collected from dexamethasone- and tocilizumab-treated MSC

spheroids cultured under static or L10H30 compressive conditions were used to assess their immunomodulatory effects on THP-1 macrophage polarization. Dexamethasone- or tocilizumab-treated young donor-MSC spheroids did not compromise the immunomodulatory effects under static culture (**Figure 5c**). In contrast, under the same static culture, dexamethasone- or tocilizumab-treated MSC spheroids derived from the aged donor significantly decreased the polarization of THP-1 macrophages toward an M1-like phenotype. M2-like macrophage polarization was similar when exposed to conditioned media from dexamethasone- or tocilizumab-treated MSC spheroids derived from a young donor compared to untreated control (**Figure 5d**), although dexamethasone treatment induced a non-significant increase in M2-like macrophage percentage. In the case of conditioned media from dexamethasone- or tocilizumab-treated MSC spheroids derived from aged donor, dexamethasone treatment led to a significant increase in M2-like macrophage polarization. Taken together, MSC spheroids from an aged donor were more sensitive to drug treatment and exhibited greater changes in immunomodulatory function compared to MSC spheroids from a young donor. Similarly, tocilizumab-treated MSC spheroids derived from aged donor reduced the pro-inflammatory effects, resulting in M1-like macrophage polarization levels comparable to MSC spheroids from young donor (**Figure 5e**). Interestingly, tocilizumab treatment significantly increased M2-like macrophage polarization in conditioned media from aged-donor MSC spheroids versus young-donor MSC spheroids. Overall, the elevated inflammatory environment created by L10H30 compressive bioreactor culture resulted in differential immunomodulatory responses to tocilizumab in MSC spheroids derived from the aged donor.

Following treatment with dexamethasone, the pro-inflammatory secretome was reduced, while IL-10 levels were increased, particularly in MSCs from an aged donor. This shift may contribute to a decrease in M1-like macrophage polarization and increase in M2-like macrophage polarization. These findings are consistent with previous studies demonstrating that

dexamethasone exhibits anti-inflammatory and anti-catabolic effects while promoting synovial macrophage polarization toward an M2 phenotype.[2] The observed increased immunomodulatory effects in MSC spheroids from an aged donor may be due to increased baseline inflammation. Tocilizumab significantly decreased M1-like macrophage polarization, despite not reducing IL-6 and IL-8 secretion from MSCs. This observation is consistent with the mechanism of tocilizumab activity, which blocks IL-6 receptor signaling rather than inhibiting cytokine production, thereby preventing downstream inflammatory activation. Notably, tocilizumab also reduced M1-like macrophage polarization under compressive loading to levels comparable to static control conditions while promoting an increase in M2-like macrophage polarization. Although residual concentrations of dexamethasone and tocilizumab were not directly quantified following washing procedure, our previous study demonstrated that 0.1 mg/mL dexamethasone (255 μM) was released from gelatin hydrogels within approximately 30 h.[28] Herein, a substantially lower dexamethasone concentration (100 nM) was used, followed by 3 days of treatment, 2 PBS washes, and replacement with fresh growth medium before conditioned medium collection. Therefore, residual dexamethasone was expected to be minimal under the similar gelatin hydrogel formulation and culture conditions. Nevertheless, because residual dexamethasone and tocilizumab concentrations were not directly measured, potential drug carryover cannot be completely excluded. Future studies should directly quantify residual drug concentrations.

Together, the 3D scaffold generated from human stem cells provides a physiologically relevant system that enables patient-specific screening and allows evaluation of individual responses to anti-inflammatory therapies in aging and age-related diseases, particularly osteoarthritis. Mechanical loading enhances inflammatory signaling and better recapitulates the disease microenvironment compared to static culture using young or aged cells alone. These results validate our platform as a promising system for anti-inflammatory drug testing using patient-derived stem cells. This approach opens new avenues for personalized medicine by

enabling clinicians to identify the most effective therapies for individual patients. Moreover, the compression bioreactor can be applied to evaluate the responses of MSCs from different donors for transplantation purposes. MSCs derived from various donors may respond differently to compressive and dynamic mechanical cues present in the human body, potentially shifting toward a pro-inflammatory phenotype. By understanding these responses under controlled compression, MSCs can be preconditioned with agents such as dexamethasone, tocilizumab, or other therapeutics prior to transplantation to modulate their phenotype toward a regenerative state and minimizing undesirable inflammatory responses. In addition, the bioreactor platform may serve as a valuable tool for assessing biomaterial performance under dynamic mechanical loading before in vivo studies and serves as a complementary tool for investigating age-associated inflammatory mechanisms while reducing reliance on animal models. Despite these promising applications, the use of MSCs from one young donor (25-year-old male) and one aged donor (55-year-old female) is a limitation of this study. Future studies will include larger cohorts with multiple donors per age group and sex-matched donors to validate the generalizability of these findings.

4 Conclusion

MSC function differed between the young and aged donor examined in this study. Aged MSCs exhibited a metabolic shift characterized by reduced mitochondrial respiration and increased glycolysis. In addition, aged MSCs exhibited upregulation of *PIEZO1* and a more pro-inflammatory phenotype, as evidenced by increased secretion of inflammatory cytokines and enhanced M1-like macrophage polarization. The compressive bioreactor culture further established an elevated inflammatory milieu beyond the baseline differences observed between young and aged MSCs, thereby better recapitulating inflammaging compared to static systems. Two representative anti-inflammatory agents, dexamethasone and tocilizumab, were evaluated

using dynamic compression and demonstrated effects consistent with their known action. Notably, dexamethasone exhibited a greater suppressive effect on the inflammatory secretome compared to tocilizumab. This model enables anti-inflammatory drug screening in a more physiologically relevant context.

CRediT authorship contribution statement

TTTH: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Investigation.

KHG: Writing – review & editing, Methodology, Investigation, Formal analysis.

SM: Methodology.

AA: Methodology.

JKL: Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare they do not have any competing interests.

Supporting Information

Method of computational model to predict the compressive force transmitted to spheroids embedded in gelatin hydrogels. (**Figure S1**) Representative flow cytometry scatter plots with gating strategy for M1- and M2-like macrophages. (**Figure S2**) Rheometry and stress relaxation testing of gelatin hydrogels. (**Figure S3**) Live/dead staining of young and aged MSC spheroids under L30H30. (**Figure S4**) Finite element analysis of gelatin hydrogels containing MSC spheroids under compression. (**Figure S5**) IL-6, IL-8, and IL-10 secretion measured by ELISA in conditioned media collected on day 3 from MSC spheroids derived from young donor and aged

donor encapsulated in gelatin hydrogels and cultured under static (control) and compressive conditions (L10H30, L20H30, and L20H100). (**Figure S6**) Quantification of *PIEZO1* expression in MSC spheroids encapsulated in gelatin hydrogels by real-time PCR. (**Figure S7**) Live/dead staining of MSC spheroids encapsulated in gelatin hydrogels as a function of tocilizumab concentration under static culture conditions. (**Figure S8**) IL-6, IL-8, and IL-10 secretion measured by ELISA in conditioned media collected on day 3 from MSC spheroids derived from young donor and aged donor encapsulated in gelatin hydrogels and cultured under static and L10H30 (compressive) conditions with tocilizumab (5 μg/mL) and dexamethasone (100 nM) treatment.

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Graphical abstract