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Case report

Cartilage responses to inflammatory stimuli and adipose stem/stromal cell-derived conditioned medium: Results from an ex vivo model

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ABSTRACT

Introduction: Osteoarthritis (OA), a chronic inflammatory joint disorder, still lacks effective therapeutic interventions. Consequently, the development of convenient experimental models is crucial. Recently, research has focused on the plasticity of Mesenchymal Stem/stromal Cells, particularly adipose-derived ones (ASCs), in halting OA progression. This study investigates the therapeutic potential of a cell-free approach, ASC-derived conditioned medium (CM), in reversing cytokine-induced OA markers in an *ex vivo* model of human cartilage explants.
Methods: 4 mm cartilage punches, derived from the femoral heads of patients undergoing total hip replacement, were treated with 10 ng/ml TNFα, 1 ng/ml IL-1β, or a combination of both, over a 3-day period. Analysis of OA-related markers, such as MMP activity, the release of NO and GAGs, and the expression of *PTGS2*, allowed for the selection of the most effective inflammatory stimulus. Subsequently, explants challenged with TNFα+IL-1β were exposed to CM, consisting of a pool of concentrated supernatants from 72-h cultured ASCs, in order to evaluate its effect on cartilage catabolism and inflammation.
Results: The 3-day treatment with both 10ng/ml TNFα and 1ng/ml IL-1β significantly increased MMP activity and NO release, without affecting GAG release. The addition of CM significantly downregulated the abnormal MMP activity induced by the inflammatory stimuli, while also mildly reducing *MMP3*, *MMP13,* and *PTGS2* gene expression. Finally, *SOX9* and *COL2A1* were downregulated by the cytokines, and further decreased by CM.
Conclusion: The proposed cartilage explant model offers encouraging evidence of the therapeutic potential of ASC-derived CM against OA, and it could serve as a convenient *ex vivo* platform for drug screening.

1. Introduction

Osteoarthritis (OA) is a complex musculoskeletal disorder marked by persistent joint degeneration, resulting in irreversible damage and functional limitations. The interplay of intrinsic factors such as advancing age, genetic predispositions [1], and obesity [2], contributes to the onset of OA, while external influences, including insults and traumas from accidents or sport-related wear and tear, can trigger and expedite the degenerative process. Early stages of OA are first characterized by mild inflammatory symptoms that progress to joint swelling and pain, ultimately contributing to cartilage erosion [3]. Cartilage is subjected to the negative influence of IL-1β and TNF-α, produced after the activation of NF-κB pathway in response to stimuli like other pro-inflammatory cytokines and mechanical stress [4]. Released cytokines further exacerbate inflammation by ulteriorly activating NF-κB and MAPK signaling, including ERK, JNK, and p38 MAPK pathways. The combination of these events contributes to chondrocyte apoptosis, inflammasome activation, oxidative stress, and ROS production, fueling inflammation and joint damage [4]. These interconnected pathways

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Abbreviations

APMA	4-aminophenyl mercuric acetate
ASC	Adipose-derived Stromal Cell
CM	Conditioned Medium
COL2A1	Collagen Type II Alpha 1 Chain
COX2	Cyclooxygenase 2
DMMB	Dimethyl Methylene Blue
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
IL-1β	Interleukin-1 β
MMP	Matrix Metalloproteinases
MSC	Mesenchymal Stem/Stromal Cell
NO	Nitric Oxide
NTA	Nanoparticle Tracking Analysis
OA	Osteoarthritis
PTGS2	Prostaglandin-Endoperoxide Synthase 2
SOX9	SRY-Box Transcription Factor 9
TBP	TATA-binding Protein
TNFα	Tumour Necrosis Factor α
TIMP	Tissue Inhibitor of Metalloproteinases

collectively sustain chronic inflammation in OA. The first steps of OA management involve lifestyle modifications, pain control, and physiotherapy within a patient-centered approach. Over time, available treatments allow to alleviate symptoms, control inflammation, and preserve the remaining joint function. Ultimately, a significant number of patients may undergo prosthetic interventions as the last chance [5]. In the dynamic landscape of regenerative approaches, orthobiologics, particularly Mesenchymal Stem/stromal Cells (MSCs) and their derivatives, have emerged as promising tools [6]. MSCs, with their unique properties encompassing multilineage differentiation and immunomodulation, exert their main mechanism of action through paracrine signaling [7]. As part of the evolving field of cell-free therapies, conditioned medium (CM), the complex mixture of bioactive molecules, growth factors, and extracellular vesicles (EVs) released by the cells during *in vitro* culture, has gained attention. Indeed, it has been proven to exert positive effects due to the natural presence of EVs and soluble factors [8,9]. However, the absence of clear guidelines for CM manufacturing and usage, coupled with the necessity to develop suitable models to evaluate its efficacy, has hindered its translation into clinical practice. To start addressing this gap, we propose human cartilage explants as a fitting experimental model to evaluate CM efficacy in the context of OA. The use of cartilage explants, as opposed to cell cultures, ensures the retention of physiological chondrocyte phenotypes due to the intrinsic tridimensionality and extracellular matrix (ECM) preservation. This *ex vivo* model has already been employed in various settings, including OA-cartilage metabolomics and proteomics [10], drug testing [11], and testing cell therapies [12]. In this work, human cartilage explants offer an interesting platform for testing the potential of our novel anti-OA biotherapeutic.

2. Materials and methods

Materials were purchased from Merck Life Science (Milan, IT) unless otherwise specified.

2.1. Collection of human specimens

All the human-derived materials mentioned in this work are from consentient subjects undergoing total hip replacement or aesthetic surgery at the IRCCS Ospedale Galeazzi-Sant'Ambrogio (Table 1).

2.1.1. Harvest of cartilage explants

Cartilage explants were obtained from 14 patients enrolled in the TENET study, approved by the Ethical Committee of IRCCS Ospedale San Raffaele (approval number 38/int/2022). All participants had been diagnosed with progressive hip arthritis unresponsive to conservative therapy and were classified as grade III osteoarthritis according to the Kellgren-Lawrence classification system [13]. The cartilage specimens were excised using a scalpel from macroscopically preserved regions of the femoral head, avoiding arthritic lesions. The selected portions exhibited a polished surface, translucent appearance, and semi-white cartilage. To mitigate any potential bias linked to severe tissue damage, areas displaying visible deformities and calcified cartilage were deliberately excluded. Following extraction, the samples underwent a thorough washing in phosphate-buffered saline (PBS). Subsequently, 4 mm biopsy punch explants (a minimum of 10 per group) were obtained and cultured for one week. The culture medium comprised high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS HyClone, Euroclone, Pero, IT), 2 mM L-Glutamine, 150 U/ml Penicillin, 150 μg/ml Streptomycin, 2.5 μg/ml Amphotericin-β, and 110 μg/ml of Sodium Pyruvate.

2.1.2. ASC isolation and characterization

The adipose-derived stem/stromal cells employed in this study were cryopreserved. These cells were originally isolated from waste subcutaneous adipose tissue collected from 5 donors at IRCCS Istituto Ortopedico Galeazzi upon Institutional Review Board approval (PQ 7.5.125, version 4). The isolation followed standard protocols [14].

To validate the stemness potential of the ASCs, various assessments were conducted in accordance with established guidelines [15]. Clonogenicity was determined using Crystal Violet staining. Multilineage differentiation towards the adipogenic and osteogenic lineages were evaluated through Oil Red O staining of fatty vacuoles, and Sirius Red staining of collagen respectively, following standard procedures [16]. Immunophenotyping was carried out using flow cytometry (CytoFLEX, Beckman Coulter, Brea, CA, USA), confirming positivity to CD90 and CD73, and negativity to CD14 and CD45 (BioLegend, San Diego, CA, USA).

2.2. CM production and quality assessment

ASCs ranging from the fifth to the seventh culture passage were employed for CM production, following established laboratory protocols [17]. In summary, cells underwent two washes with PBS and were subsequently cultured for three days under starving conditions. The starvation medium consisted of high-glucose DMEM without phenol red, 50 U/ml Penicillin and 50 μg/ml Streptomycin, and 2mM L-Glutamine. After three days, the media were collected and subjected to centrifugation at 2500*g* for 10 minutes at 4 °C to remove cell debris. The supernatant was concentrated utilizing Amicon filtering units (Merck Millipore, Burlington, MA, USA) with 3kDa cut-off membranes, following the

Table 1
Summary of donor characteristics for ASCs and cartilage explants. BMI ranges follow the Italian Ministry of Health indications (https://www.salute.gov.it/portale/nutrizione/).

	ASCs ($n = 5$)				Cartilage explants ($n = 14$)							
	N. of donors	Age (years)			N. of donors	Age (years)			BMI range (kg/m^2) - N. of donors			
		Mean	Median	Range		Mean	Median	Range	<16	18,5–24,9	25,0–29,9	30,0–34,9
Female	5	47	56	26–70	8	71	75	51–81	/	2	5	1
Male	/	/	/	/	6	66	65	49–76	1	2	3	/

manufacturer's instructions. The concentrated CM was then aliquoted and stored at −80 °C. Concurrently, ASCs were detached and counted to establish a correlation between the number of donor cells and the volume of the retrieved CM. As part of our standard quality assessment, all CM underwent protein dosage testing using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA), and the content and size of extracellular vesicles were assessed using Nanoparticle Tracking Analysis (NTA; Malvern PANalytical, Salisbury, UK). For the experimental treatments, the CM derived from five distinct cell populations were pooled to minimize inter-donor variability, ensuring a consistent and standardized approach in the study.

2.3. Treatment with inflammatory cues and CM

After a week in standard conditions, cartilage explants were shifted to a culture medium containing 1% FBS and treated as reported below.

To assess the susceptibility to inflammatory cues:

- Control (CTR)
- 10 ng/ml TNFα (TNF) (DBA Italia Srl, Segrate, IT)
- 1 ng/ml IL-1β (IL) (DBA Italia Srl, Segrate, IT)
- 10 ng/ml TNFα + 1 ng/ml IL-1β (TNF+IL)

To evaluate CM action:

- Control (CTR)
- 10 ng/ml TNFα + 1 ng/ml IL-1β (TNF+IL)
- 10 ng/ml TNFα + 1 ng/ml IL-1β + CM from 1 × 10^6 ASC (TNF+IL+CM)

After a 3-day period, supernatants were collected, subjected to centrifugation at 2000*g* for 10 minutes at 4 °C, aliquoted, and stored at −20 °C. Concurrently, cartilage explants were washed twice with PBS and preserved at −80 °C for a minimum of overnight before subsequent mechanical and chemical digestion procedures.

2.4. Supernatant analysis

2.4.1. MMP activity assay

The general activity of extracellular metalloproteinases (MMPs) was assessed using SensoLyte 520 Generic MMP Assay Kit (AnaSpec Inc., Fremont, CA, USA) following manufacturer's protocol. Undiluted samples underwent pre-activation with 4-aminophenyl mercuric acetate (APMA), for 40 minutes at 37 °C. After 45 minutes incubation with substrate, fluorescence was read at Ex/Em = 490 nm/520 nm using a Wallac Victor II microplate reader (Perkin Elmer, Milan, IT).

2.4.2. GAG release

The levels of released glycosaminoglycans (GAGs) were evaluated through the dimethyl methylene blue (DMMB) assay [18]. Briefly, 50 μl of each undiluted sample were tested in duplicates. The absorbance was measured at 500 nm with Wallac Victor II spectrophotometer, and GAG concentrations were extrapolated using a chondroitin sulphate standard curve.

2.4.3. NO assay

The quantification of nitric oxide (NO) levels was performed utilizing the Nitric Oxide Assay kit from Abcam (ab272517, Abcam, Cambridge, UK). Undiluted samples underwent processing in accordance with the provided protocol. Samples were deproteinized, incubated for 10 minutes at 60 °C, and finally the oxidized NO content was quantified by measuring the optical density at 540nm, with Wallac Victor II spectrophotometer. Extrapolation of NO levels was derived from a nitrite standard curve (0–200 μM).

2.5. Gene expression

Cartilage explants were subjected to RNA extraction by the TRIzol-Chloroform extraction method, utilizing PreCellys Mini Tubes (Bertin Technologies, Montigny-le-Bretonneux, FR). The resulting RNAs were then retrotranscribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol. Subsequently, the expression levels of different genes were quantified through reverse transcription quantitative polymerase chain reaction (RT-qPCR) employing TaqMan technology using the following probes: *PTGS2* (hs00153133_m1), *MMP3* (hs00968305_m1), *MMP13* (hs00233992_m1), *SOX9* (hs00165814_m1), *COL2A1* (hs10060345_m1), and *TBP* (hs00427600_m1) (Thermo Fisher Scientific, Waltham, MA, USA). The RT-qPCR assays were conducted on the QuantStudio Real-Time PCR systems (Thermo Fisher Scientific, Waltham, MA, USA). Data normalization was performed using *TBP* as a reference, and relative quantification was determined employing the 2^{-ddCT} method.

2.6. Statistical analysis

All data were processed using GraphPad Prism 10, either by one-way ANOVA (when the dataset had no missing values) or mixed-effect analysis (in the presence of missing values). Subsequently, Tukey post-hoc test was used to compare means across multiple groups. The results were considered statistically relevant with a p-value ≤0.05. Significant differences among groups are shown in the graphs as * (significance compared to the CTR group, Figs. 2 and 3, and Supplementary 1, 3 and 4) or $ (compared to the TNF+IL group, Fig. 3).

3. Results

3.1. Characterization of ASCs and CM

The ASC populations selected for the CM production were characterized as outlined by *Dominici* et al., in 2006 [15]. Their clonogenicity (Fig. 1a) and their ability to differentiate towards adipogenic and osteogenic lineages were confirmed (Fig. 1b, left to right). In addition, the immunophenotype of ASCs, assessed by flow cytometry, revealed a strong positivity for the expression of

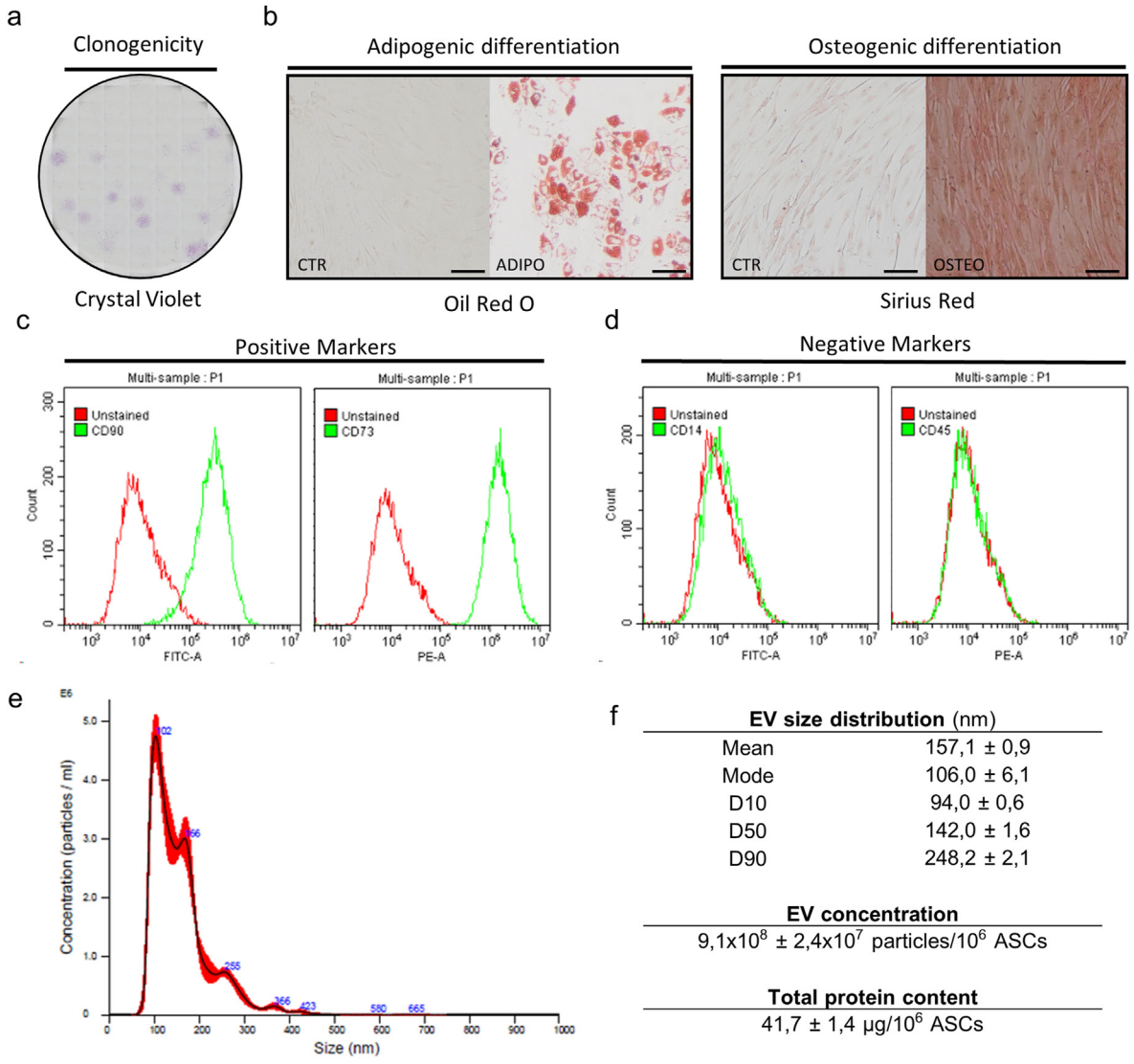

Fig. 1. Characterization of ASCs and CM. Representative characterization of ASC clonogenicity by Crystal Violet staining (clonogenic potential>20%) (a), adipogenic (ADIPO) and osteogenic (OSTEO) induction by Oil Red O and Sirius Red staining in comparison to their respective controls (CTR) (b, scale bars: 100 µm), and immunophenotype (c and d). Nanoparticle tracking analysis (e) of the pooled CM derived from 5 different ASC populations, together with EV size distribution, EV concentration, and quantification of CM total protein content (f). Data are expressed as mean ± SEM of technical replicates.

membrane markers CD90 and CD73 (Fig. 1c) and negativity for CD14 and CD45 (Fig. 1d), in accordance with standard guidelines (positivity to CD90 and CD73 > 95%; positivity to CD45 and CD14 < 2%). In the pooled CM, total protein content was quantified and EVs were analysed for their size distribution and count. In detail, we found a prevalence of small EVs, as the most abundant population showed a diameter around 100nm (Fig. 1e). Quantitative parameters were normalized based on the number of donor ASCs, resulting in 9.1 × 10^8 particles/10^6ASCs and a total protein content of 41.7 µg/10^6ASCs (Fig. 1f), in conformity with product internal standards [19].

3.2. Selection of the inflammatory prompt

To validate our *ex vivo* model and evaluate different markers involved in OA progression, human cartilage explants were treated for 3 days with different inflammatory stimuli. In detail, 10 ng/ml TNFα, 1 ng/ml IL-1β, or a combination of both, were administered to assess cartilage responses, focusing on markers of catabolism, inflammation, and cartilage health in comparison to unstimulated explants. The activity of matrix metalloproteinases (MMPs) was induced in all experimental groups, nonetheless it exhibited a significant increase only in response to the double treatment (TNF+IL) when compared to the control (CTR) (Fig. 2 a). This trend was also confirmed by the quantified levels of nitric oxide (NO) (Fig. 2 b). Conversely, quantification of glycosaminoglycan (GAG) release showed negligible differences among groups, with only a mild increment following exposure to cytokines (Fig. 2 c). Osteoarthritis-like phenotype was confirmed by the expected up-regulation of *PTGS2* and *MMP3* gene expression, coupled with the downregulation of *SOX9*, (Fig. 2 d-f). These modulations were more pronounced with the combination of TNFα and IL-1β, although already evident with the single insults. In conclusion, the higher efficacy of the TNFα and IL-1β combination in inducing an inflammatory phenotype guided us to apply the dual cytokine stimulus to our *ex vivo* model for the subsequent experimental design.

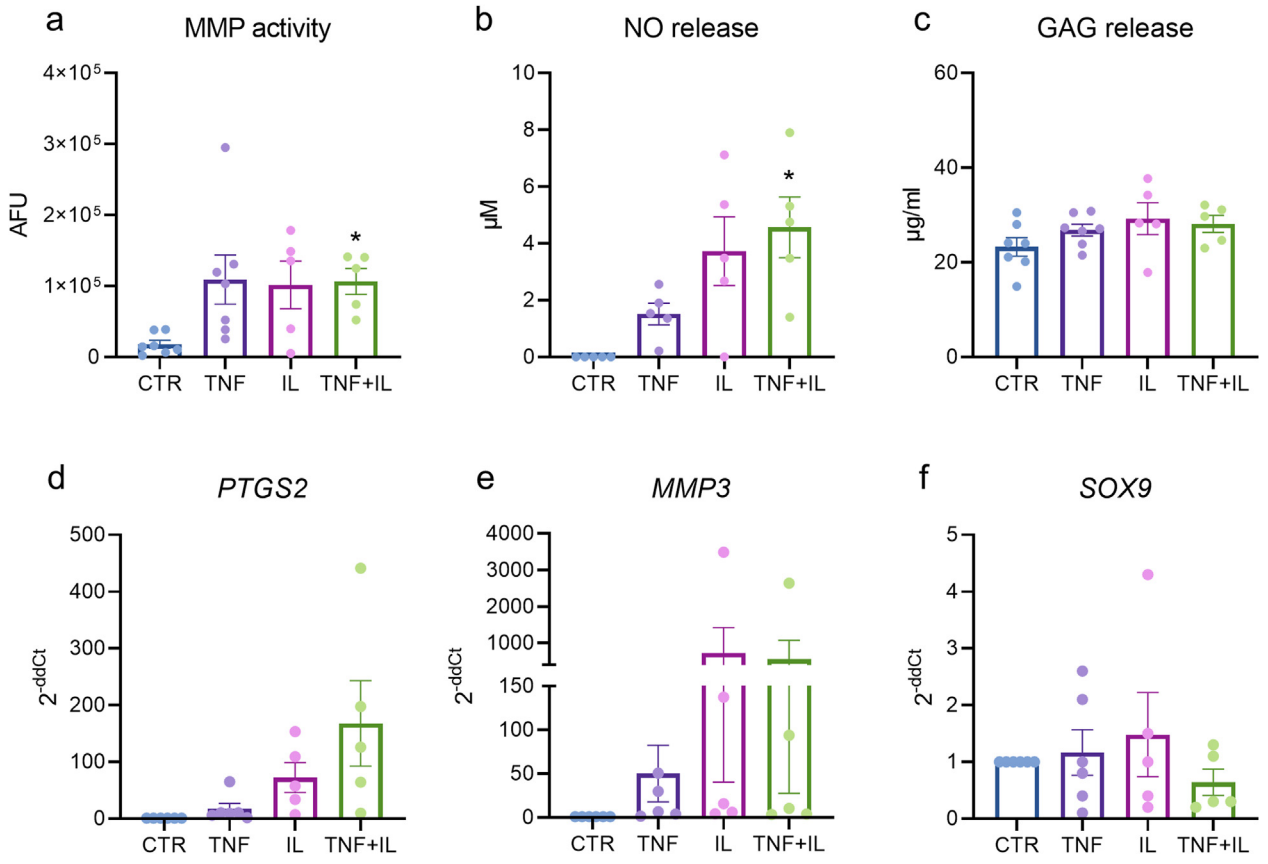

Fig. 2. Selection of the inflammatory prompt. MMP activity (a), NO (b) and GAG (c) release in the 3-day supernatants of cartilage explants treated with inflammatory stimuli. *PTGS2* (d), *MMP3* (e) and *SOX9* (f) relative gene expression using the 2^{-ddCt} method (*TBP* was used as housekeeping gene). Data are expressed as mean ± SEM of $n = 5–7$ independent experiments. Significance versus CTR is shown as * $p \leq 0.05$. AFU = arbitrary fluorescence unit.

3.3. Evaluation of CM effects

Further experiments aimed to elucidate CM influence on OA-like phenotypes. Interestingly, the introduction of CM led to a noteworthy reduction of MMP catabolic activity (−74% compared to TNF+IL), effectively mitigating any significant difference from the control (Fig. 3 a). However, no discernible effects of CM were observed on NO nor GAG release, with only the inflammatory cytokines significantly inducing the previously discussed effects (Fig. 3 b and c). To better evaluate the effect of CM on cytokine-induced inflammatory phenotype, we included the catabolic enzyme *MMP13* and the physiological marker *COL2A1* in the gene panel. Regarding the pathological markers, the double treatment confirmed the robust induction of their gene expression, whereas the presence of CM for 3 days produced a down-regulation of −54%, −21%, and −27% in *PTGS2*, *MMP3*, and *MMP13* levels, respectively (Fig. 3 d-f). Although not statistically significant, these reductions might represent a biologically relevant outcome. The susceptibility of cartilage to inflammatory stimuli is evidenced by a substantial increase in COX2 and MMP3 even at protein levels (Supplementary 1a-c). Notably, CM exerted a partial effect only on COX2 expression (Supplementary 1 a and b), while no impact was observed on MMP3 (Supplementary 1 a and c). At last, the cytokine treatment produced a significant reduction of *SOX9* and *COL2A1*, which was, to some extent, exacerbated by CM treatment (Fig. 3 g and h).

4. Discussion

4.1. Cartilage explants as an ex vivo model of osteoarthritis

In cartilage, inflammation induces physiological, metabolic, and molecular alterations, translating into increased expression of inflammatory mediators, enhanced release of matrix components, and, simultaneously, decreased levels of healthy cartilage markers [20]. In this study, we observed a robust response to cytokines in terms of increased expression of catabolic (MMP activity and expression, release of GAGs) and inflammatory (*PTGS2* expression and release of NO) markers and decreased expression of cartilage-specific ones (*SOX9* and *COL2A1)*, confirming the expected molecular changes induced by OA. *Ex vivo* models, while less standardized, provide a complex and realistic representation of matrix environment and pathological mechanisms [21]. This embodies an important advantage, since chondrocyte health relies on cartilage stiffness [22], underlining the major role of MMPs, zinc-dependent endopeptidases, in maintaining matrix homeostasis and therefore chondrocyte phenotype [23]. Using surgical waste materials from patients allows to better recapitulate physiological cartilage conditions. We collect explants only from macroscopically intact areas of the articular cartilage, presenting a smooth translucent appearance and lacking visible deformities or major signs of degeneration. Moreover, we culture the specimens for a one-week acclimatization period under standard conditions to recalibrate their inflammation

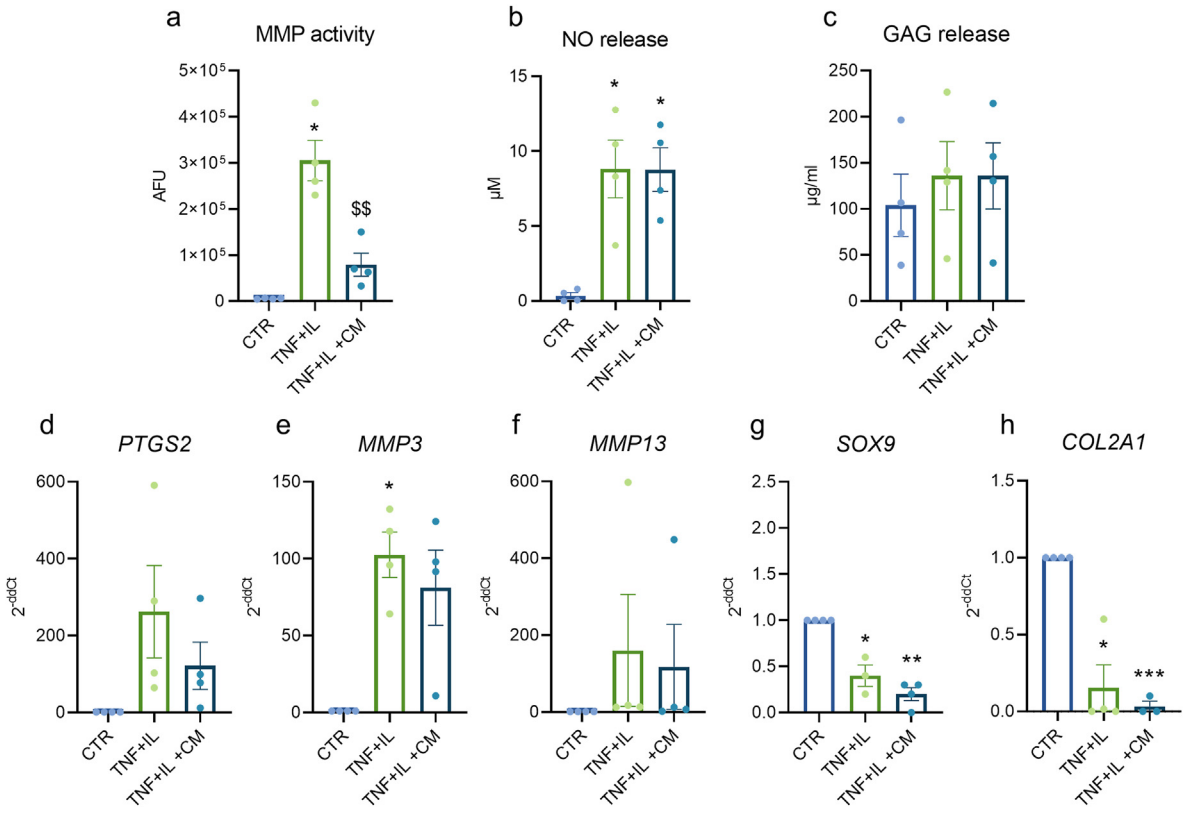

Fig. 3. Evaluation of CM effects. MMP activity (a), NO (b) and GAG (c) released by cartilage explants challenged with inflammatory cytokines for 3 days and either untreated or treated with CM. Relative gene expression analysis of *PTGS2* (d), *MMP3* (e), *MMP13* (f), *SOX9* (g) and *COL2A1* (h) using the 2^{-ddCt} method (*TBP* was used as housekeeping gene). Data are expressed as mean ± SEM of $n = 4$ independent experiments. Significance versus CTR is shown as * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.005$, versus TNF+IL as $^{\$\$}$ $p \leq 0.01$. AFU = arbitrary fluorescence unit.

levels and minimize any confounding factors linked to potential degenerative processes occurring *in vivo*. The histological evaluation of explants harvested from two donors and maintained in culture with or without TNFα and IL-1β for three days demonstrates the preservation of cartilage integrity (Supplementary 2). Notably, no substantial changes were observed following the inflammatory insults. This lack of discernible alteration can be attributed to the brief exposure to the cytokines: indeed, tissue remodeling is a complex process that requires time to manifest. To gain a more comprehensive understanding of the effects of inflammatory cytokines and ASC secretome on cartilage health, we plan to extend the experimental time points and capture both molecular and histological changes. The collection of preserved human cartilage represents a significant advantage considering that most used *in vitro* models are not representative of pivotal ECM properties, such as stiffness [24] and species-specific cellularity [25]. Nonetheless, caution is needed with severely OA-affected specimens, as heightened inflammation and tissue damage may be detected even without exogenously added cytokines. Ideally, sourcing cartilage from healthy joints, such as those from neck fractures, would be preferable. Overall, this cartilage explant model realistically depicts OA dynamics, supporting basic research and drug discovery.

4.2. CM anti-catabolic effect on osteoarthritic cartilage hallmarks

To evaluate the efficacy of our product, we investigated the modulations of several catabolic and inflammatory markers after CM administration to cytokine-treated explants. CM effectively hinders the activity of MMPs, as previously observed in other OA models, i.e. 2D chondrocyte cultures and an *ex vivo* osteochondral explants exposed to 10ng/ml TNFα and treated with different doses of CM over a 3-day period [26,27]. In this study, the influence of CM on extracellular MMPs is observed alongside a trend of down-regulation at gene expression level, with no discernible effect on MMP3 protein level. The major reduction affects the enzymatic activity, as demonstrated by *Giannasi* et al. in 2020 [9], and it is ascribed to the presence of high concentrations of tissue inhibitors of MMPs (TIMPs). These effectors, together with other MMP inhibitory molecules, such as α-1-antitrypsin and α-2-macroglobulin, were previously identified by differential proteomics as soluble or extracellular vesicle-embedded factors within the CM [28,29]. Direct quantification of the four human TIMPs in ASC-CM revealed their particularly abundant presence. Specifically, we observed mean levels of 1.8 ± 0.3 ng/ml for TIMP1 and 3.8 ± 0.5 ng/ml for TIMP2 (Supplementary Table 1 of [19]) while TIMP3 and TIMP4 levels were in the ng/ml and pg/ml range, respectively [26]. TIMPs act by binding to the active site of MMPs, thereby impeding their enzymatic activity through the formation of 1:1 covalent complex [30]. In addition to MMP activity, the release of GAGs serves as a well-established catabolic marker in OA cartilage, as it reflects the matrix remodeling activity [31]. Notably, CM did not exert any discernible effect on cytokine-induced GAG loss. This outcome partly contradicts the evidence from our *ex vivo* osteochondral model, where the reduction in MMP activity following CM treatment corresponded to a concurrent decrease in GAG loss [27]. This aspect warrants further investigation, including extended time points and continuous supernatant withdrawal and testing.

4.3. Reduction of inflammatory markers after CM treatment

To evaluate the potential anti-inflammatory effect of CM, we investigated its impact on the expression of *PTGS2* and its protein

product, COX2. Our findings reveal a decreasing trend in both markers following CM administration, with a more pronounced effect observed at gene level. This downregulation aligns with the previously characterized enrichment in CM of anti-inflammatory molecules [19,32]. Here, we also quantified the levels of released NO, a hallmark of OA-related inflammatory pathways. In OA pathophysiology, TNFα and IL-1 stimulate an upregulation in inducible NO synthase (iNOS) activity, that raises NO production, ultimately resulting in NF-κB activation, release of catabolic and inflammatory mediators, and cartilage degeneration [33]. Consistently with previous findings [27], we observed an increased NO concentration in the culture supernatants after cytokine stimulation, with no effect induced by CM. This result seems in contrast with what was seen by Simental-Mendía et al. in a cartilage-synovium co-culture model challenged with 10 ng/ml IL-1β and treated with ASC-derived CM, where the latter caused a significant reduction of NO after 3 days, compared to the inflamed group [34]. Of note, the study utilized a co-culture of cartilage explants with synovial membrane sections. This setup suggests, given the beknown presence of immune components within the synovial membrane [35], that the mode of action of CM may predominantly affect immune system cells rather than directly target stromal tissues, exposing our results under a new light. In fact, ASCs and their secretome are recognized for their anti-inflammatory and immunomodulatory properties, primarily through the recruitment of immune system components and the induction of pro-regenerative phenotypes [36,37], thus influencing the effectors of the inflammatory response. In this regard, our previous comprehensive CM characterization revealed that it contains molecules predominantly involved in immune system-related processes [32]. Therefore, more pronounced effects may be observed in a model that incorporates immune cells. As a proof of concept, synovial membrane explants were treated for 3 days with ASC-CM, either alone or in the presence of 10 ng/ml TNFα and 1 ng/ml IL-1β (Supplementary 3). As hypothesized, the anti-inflammatory effect of the ASC secretome becomes evident through the reduction of NO release and COX2 expression at both the gene and protein levels. Notably, the effect of the CM is particularly pronounced in the absence of inflammatory cytokines. Although promising, these preliminary data need to be validated with further experiments that consider an optimized experimental setting and a larger sample size. Finally, it is important to note that the reported data on both cartilage and synovial explants exhibit considerable variability due to inter-donor differences, therefore more experiments will be needed to define the postulated anti-inflammatory effect of our product more accurately.

4.4. Reduction of tissue-specific markers after CM administration

The effects of cytokines and CM on two cartilage-specific markers, *SOX9* and *COL2A1,* were also investigated. *SOX9*, also known as SRY-box 9, plays a crucial role in lineage-specific differentiation in cartilage. It acts as a pivotal transcriptional factor at various stages of chondrocyte development, and mutations in SOX9 have been linked to severe skeletal malformations, such as campomelic dysplasia [38]. *COL2A1* expression is an indicator of cartilage health, as it encodes the collagen type II pro-alpha1 chain, a major component of cartilage ECM. Proper expression of *COL2A1* is essential for correct matrix mechano-transduction and allocation of chondrocytes within the cartilage [39]. We observed a significant decrease in the expression levels of both *SOX9* and *COL2A1* following cytokine treatment, and CM appeared to exacerbate this effect. Notably, this trend is evident even when treating cartilage explants with CM in the absence of inflammatory stimuli (Supplementary 4). One potential explanation for this phenomenon could be the high presence of Vascular Endothelial Growth Factor (VEGF) in our product, with mean levels of 0.6 ± 0.2 ng/ml, as reported previously (Supplementary Table 1 of [19]). Although VEGF typically elicits anabolic responses in cells, a negative correlation between VEGF and levels of *SOX9*, *COL2*, and *ACAN* mRNAs, has been observed in chondrocyte treated with ASC-CM for 12 hours [40]. However, it has been demonstrated that VEGF can exert opposite effects on chondrogenic markers in a dose-dependent manner. Specifically, researchers have shown that a high ratio (greater than 10:1) of donor ASCs to recipient chondrocytes impairs the expression of *COL2A1*, *SOX9*, and other cartilage markers, whereas a lower ratio (1:1) increases their expression [41]. In our study the dose of CM is traceable to one million ASCs, therefore conclusive data on the impact of CM on cartilage health will require further investigation applying different doses.

5. Conclusion

The need for enhanced experimental models, mirroring *in vivo* structures and adhering to the principles of the 3Rs, has become increasingly urgent in different medical fields. Here we harnessed the *ex vivo* culture of cartilage explants as a tool to evaluate the anti-catabolic and pro-homeostatic properties of Mesenchymal Stem/stromal Cell secretome, revealing its efficacy in suppressing aberrant matrix-remodeling and mitigating inflammatory markers, after cytokine stimulus. This research lays the basis for a deeper investigation of the mode of action of this novel biotherapeutic approach, in the attempt to facilitate its way to the clinics.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.06.010. All data used to support the findings of this study are available at https://zenodo.org/records/11658062.

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