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Immunomodulation of mesenchymal stem cells in discogenic pain

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Abstract

BACKGROUND CONTEXT: Back pain is a highly prevalent health problem in the world today and has a great economic impact on health-care budgets. Intervertebral disc (IVD) degeneration has been identified as a main cause of back pain. Inflammatory cytokines produced by macrophages or disc cells in an inflammatory environment play an important role in painful progressive degeneration of IVD. Mesenchymal stem cells (MSCs) have shown to have immunosuppressive and anti-inflammatory properties. Mesenchymal stem cells express a variety of chemokines and cytokines receptors having tropism to inflammation sites.

PURPOSE: This study aimed to develop an in vitro controlled and standardized model of inflammation and degeneration of IVD with rat cells and to evaluate the protective and immunomodulatory effect of conditioned medium (CM) from the culture of MSCs to improve the conditions presented in herniated disc and discogenic pain processes.

STUDY DESIGN: This is an experimental study.

METHODS: In this study, an in vitro model of inflammation and degeneration of IVD has been developed, as well as the effectiveness of CM from the culture of MSCs.

RESULTS: Conditioned medium from MSCs downregulated the expression of various proinflammatory cytokines produced in the pathogenesis of discogenic pain such as interleukin (IL)-1β, IL-6, IL-17, and tumor necrosis factor (TNF).

CONCLUSION: Mesenchymal stem cells represent a promising alternative strategy in the treatment of IVD degeneration inasmuch as there is currently no treatment which leads to a complete remission of long-term pain in the absence of drugs.

Keywords: Conditioned medium; Cytokines; Discogenic pain; Immunomodulation; Mesenchymal stem cells; Rat model

FDA device/drug status: Not applicable.

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Introduction

Back pain is a highly prevalent worldwide condition. At least 84% of individuals suffer from back pain at some time or other in their lives, resulting in a costly health problem [1]. Back pain is associated with degeneration of the intervertebral disc (IVD) and herniation [2–4], although the pathogenesis is not fully understood. The IVD is composed of the central gelatinous nucleus pulposus (NP), the surrounding elastic annulus fibrosus (AF), and the cartilaginous end plates. Nucleus pulposus exerts a hydrostatic pressure resisted by the AF and absorbs shock [5]. Vertebral stability partly depends on the balance of these two forces and the balance between the anabolic and the catabolic processes [6,7].

During disc degeneration, levels of proinflammatory cytokines and degradation of collagen and aggrecan from the extracellular matrix increase. Changes also occur in the disc tissue phenotypes [8,9]. The progressive hydrophilic molecules' loss of extracellular matrix, which causes structural changes as well as spinal instability, produces disc herniation [3]. In the final stages of disc degradation, the NP extrusion through the AF fissures causes pain owing to compression of nerves and the recruitment of immune cells to the disc, thus triggering off more pain [10,11]. However, pain can also occur in the early stages of disc degeneration and in the absence of nerve compression and is associated to the production of inflammatory cytokines [12].

It is clear that in the disc degeneration, inflammatory cytokines produced by macrophages or disc cells play an important role [13–16]. Various inflammatory mediators have been suggested to play a main role in the catabolic processes in human IVD [17,18]. Secreted proinflammatory mediators that increase their expression in IVD degeneration include tumor necrosis factor (TNF), interleukin (IL)-1α, IL-1β, IL-6, IL-17, IL-8, IL-2, IL-4, IL-10, interferon gamma, chemokines, and prostaglandin E_2 [19]. Of these, TNF, IL-1β, IL-6, and IL-17 have a greater involvement [20,21]. Tumor necrosis factor and IL-1β play a key role in triggering of inflammation in the herniated disc. They are produced by the IVD cells and the immune system cells [6]. Both of them are involved in matrix degradation [22] and are the most studied. Tumor necrosis factor is involved in disc herniation and nerve irritation [12], whereas IL-1β is implicated in disc degeneration [23]. Interleukin-6 contributes to the inflammatory process after disc herniation [24]. Interleukin-17 is a cytokine produced by Th17 cells and is associated with the inflammatory and the autoimmune process [25]. In the herniated disc, IL-17 promotes autoimmune inflammation, chemotaxis, and angiogenesis [26].

Currently, there are treatments such as anti-TNF, that is, infliximab, adalimumab, and etanercept [27]. Other cytokines such as IL-6 and IL-1β have been investigated as therapeutic targets. There are specific anti-inflammatories in experimental phase, such as IL-1β inhibitors (Kineret) and IL-6 inhibitors (Tocilizmab). Many more treatments are known; however, these treatments are very expensive, unspecific, and none of the biological agents used at present lead to a remission of long-term pain in the absence of drugs. Several cell-based therapies have been proposed in recent years [28]. Multipotent mesenchymal stromal cells or mesenchymal stem cells (MSCs) are the most interesting candidate cells for treating IVD degeneration and herniation [29].

According to the International Society of Cellular Therapy, MSCs are defined by the following: their ability to adhere to plastic under standard culture conditions; express cell surface markers CD105, CD73, and CD90; lack expression of other markers; and differentiate into osteoblasts, adipocytes, and chondrocytes under in vitro conditions [30]. In addition to displaying multipotentiality and having self-renewal capacity, MSCs have immunomodulatory and anti-inflammatory properties [29] and also have the capacity to modulate the activity of macrophages [31,32]. When macrophages are stimulated with proinflammatory substances, they secrete inflammatory factors such as TNF, IL1β, IL6, and reactive oxygen species. However, MSCs are capable of secreting factors which attenuate the inflammatory effects of activated macrophages [33,34]. The initial action model of MSCs was based on migration to damaged tissue, insertion and differentiation into functional cells regenerating the tissue. However, MSCs are not engrafted in sufficient number or with enough time to explain the results of regeneration of tissue which are reported in recent studies [35–37]. Therefore, new MSCs regeneration mechanisms have been put forward. MSCs are capable of secreting a large range of trophic mediators that can exert paracrine effects on other cell types. Hence, MSCs conditioned media have shown useful results in reducing inflammation [38]. The MSCs-secreted factors comprise a diverse group of soluble peptides and proteins with different biological activities [39,40]. Some of these cytokines and factors are transforming growth factor beta, indolamine 2,3-dioxygenase, IL-6, which are implicated in MSCs immunomodulation [41], as well as others that are also implicated in the renewal of the extracellular matrix, such as collagen II [42]. MSCs obtained from bone marrow (BMSCs) represent a standard in the field of Adipose stem cells (ASC) biology. However, stromal cells obtained from adipose tissue (ASCs) are a good alternative due to their less invasive accessibility and abundant availability [43].

The main aims of this study are to develop an in vitro controlled and standardized model of inflammation and degeneration of IVD with rat cells and to evaluate the protective and immunomodulatory effect of conditioned medium (CM) from the culture of ASCs to improve the conditions presented in herniated disc and discogenic pain processes. To do so we proceeded to the isolation, culture, expansion, and characterization of ASCs, AF cells, and NP cells from rat. An in vitro herniated disc model was developed by culturing AF and NP cells with macrophages stimulated with TNF to create an inflammation environment as TNF plays a crucial role in the inflammatory response initiation [44]. Finally, the CM was added, and the expression of various

proinflammatory cytokines produced in the pathogenesis of discogenic
pain such as IL-1β, IL-6, IL-17, and TNF were determined.

Because of the similarities in the biological and biomechanical properties of the human lumbar disc and the concern on ethics, the rodent-tail model has become an excellent model for research [45] and the results obtained could be applied to humans.

Materials and methods

Animals

The protocols for this experimental study agree with the Guidelines of the Council of the European Union (86/609/EU) and have followed Spanish regulations (BOE 67/8509-12, 1998) for the use of laboratory animals.

In this study, 12 female Sprague-Dawley rats (*Rattus norvegicus*), aged 5–10 months old and weighing between 300 and 400 g were used. Immediately after euthanasia with pentobarbital (Dolethal, Vetoquinol, Madrid, Spain), tissues were obtained using sterile techniques and stored in a phosphate buffered saline (PBS, Sigma-Aldrich, St Louis, MO, USA) with 1% (v/v) penicillin-streptomycin (**GE-Healthcare** Hyclone, Marlborough, MA, USA).

Isolation of ASCs, NP, and AF cells

After collecting the tails each disc was transversely cut and the NP and the AF were separated from the vertebrae according to their distinct morphologic appearance. The NP was treated with 0.01% trypsin (DIFCO, Detroit, MI, USA) at 37°C for 20-minute shaking [46]. The AF was digested with 100 U/mL of type II collagenase (Gibco, Gaithersburg, MD, USA) at 37°C for 5-hour shaking.

To isolate ASCs, adipose tissue was obtained from abdominal and inguinal area. Approximately 5 g of fat were digested with 0.075% of type I collagenase (Sigma) at 37°C for 1-hour shaking. After centrifugation, the supernatant was removed and the pellet was resuspended in PBS (Sigma) and filtered (100 µm Nylon Mesh, Fisherbrand, Thermo Fisher Scientific, Loughborough, LE, UK) [47,48].

Murine macrophages cell line (Raw 264.7, ATCC, Teddington, Middlesex, UK) was provided by the Legal Medical Toxicology Department (University of León, Spain).

Expansion of cells

Isolated cells were expanded in monolayer cultures in low-glucose Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone) and 1% (v/v) penicillin/streptomycin (Gibco) at 37°C in a humid atmosphere containing 5% CO_2. Adherent cells were cultured to confluence, and medium were changed every 2–3 days.

Growth curve and population doubling time (PDT)

To evaluate the growth kinetics and to determine the PDT of the NP and AF cells, they were seeded into a 24-well culture plate at a density of 5×10^3 cells/well. The population doubling number (PDN) and PDT were calculated using the following formulae [49]:

$$PDT = CT / PDN$$

$$PDN = \log N / N_0 \times 3.31$$

CT: time of cultivation between days.
N: cell number at the end of the cultivation period.
N_0: cell number at culture initiation.

Characterization of ASCs, NP, and AF cells

To characterize ASCs, AF, and NP cells, expression of markers was determined by flow cytometry (all cellular types) and confocal microscopy (NP and AF).

Flow cytometry

The primary antibodies used to characterize ASCs, AF, and NP cells were mouse anti-CD73, anti-CD90, and anti-CD105 (1:100) (Abcam, Cambridge, UK); mouse anti-decorin (1:1000, Abcam), and mouse anti-cytokeratin 19 (1:100, ThermoFisher Scientific), respectively. The secondary antibody used was biotinylated anti-mouse IgG1 (1:400, Invitrogen, Carlsbard, CA, USA). Finally, cells were incubated with streptavidin-Alexa 488 antibody (1:100, Invitrogen). Cells were harvested using trypsinization and washed in cold buffer (pH = 7.4, 0.2% bovine serum albumin and 0.1% sodium azide in PBS [Sigma]). Standard labeling protocols and the suggestions by the manufacturers were followed. To conduct cytometry, 1×10^6 cells were used per sample. Three samples and a control (only secondary antibody) were used for each cell type. Immediately after staining, cells were acquired using a FACS Cyan ADP (Dako, Glostrup, Denmark). About 1×10^4 events (minimum) were used for fluorescence capture with Summit 4.3 (Cell Quest, BD Biosciences, East Rutherford, NJ, USA) software.

Confocal microscopy

Cells were cultured (2×10^5 per well) on two-well Chamber Slide System (Lab-Tek, Thermo Fisher Scientific). After 24 h, AF and NP cells were fixed with 2% paraformaldehyde (PFA, Merck, Darmstadt, DE, Germany) and permeabilized with 0.1% triton (Sigma) prior to incubation with primary rabbit anti-decorin (1:1000, ThermoFisher Scientific) and mouse anti-cytokeratin 19 (1:1000, ThermoFisher Scientific) antibodies, respectively, and incubated with secondary antibodies biotinylated anti-mouse IgG1 and biotinylated anti-rabbit IgG1 (1:400, Invitrogen). After this, cells were incubated with streptavidin-Alexa 488 and 568 antibodies (1:100, Invitrogen)

and then with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen). Finally, chamber slides were mounted using Fluoromount mounting medium (Sigma). Cells were observed using a confocal microscopy (Zeiss LSM 800, Oberkochen, DE, Germany).

Generation of cell-conditioned medium

To generate CM, MSCs were seeded at 5×10^5cells/well (6-well culture plates) in medium supplemented with 0.1% FBS and were cultured at 37°C and 5% CO_2. After 24 hours of cell culture, 0.1% FBS medium was replaced with serum-free medium and cultured for an additional 48 hours. The medium was filtered through a 0.2 μm filter and cryopreserved at −80°C.

Co-cultures of macrophages and disc cells

Macrophages and NP or AF cells were cultured (ratio 1:1) in a 6-well plate; 6×10^6 total cells were seeded in each well. After 36 hours, when they reached confluence, the medium was removed and cultured under two different conditions: DMEM (without FBS) plus 5 μL of TNF (10 μg/mL) (Gibco) or the CM was collected without FBS plus TNF. Cells were incubated for 12 hours and then collected to analyze the effect on inflammation. Cultures of ASCs and macrophages plus and minus TNF were used as control to evaluate the cytokines expression when they were seeded without NP and AF cells.

Protein concentration in the CM at different times [12,24,36,48] was measured with NanoDrop, Thermo Fisher Scientific and these were visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of gene expression

Total RNA of co-cultured samples was extracted using the GeneMATRIX universal RNA purification kit (EURx, Sofia, Bulgaria) following the manufacturer's instructions. The quantity and quality ($OD_{260/280}\approx2.0$ and $OD_{260/230}=2.0–2.2$) of the extracted RNA was evaluated using a NanoDrop ND-1000 spectrophotometer. cDNA was obtained using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. A 600 ng of total RNA was used for synthesis of cDNA. Gene expression of IL-6, IL-1β, IL-17, and TNF were determined by RT-PCR. ACT-β was used as a housekeeping gene. Optimal concentration of each primer was determined (Sigma). Melting curve was performed, and only one peak appeared to confirm the specificity of the amplification products. Specific primer sequence, optimal primer concentration, and optimal PCR annealing temperatures are listed in the Table. The RT-PCR reactions were performed in a 25 μL volume with 350 ng of DNA using SG qPCR master mix (2×), plus ROX solution (EURx) following the manufacturer's instructions. Triplicate PCR reactions were performed for each sample and the internal control. Amplification was performed using a StepOne real-time PCR system (Applied Biosystems). Target gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and was normalized to ACT-β.

Statistical analysis

Each result of this study was expressed at the mean±SD and performed with three experimental replicates. Statistical analysis was performed using IBM SPSS Statistics 22 (IBM Corp, Armonk, NY, USA). Significant differences among the groups were determined using ANOVA followed by Tukey post hoc analysis for multiple group comparisons or Student *t* test for two group comparisons. Results with $p\leq.05$ were considered statistically significant.

Results

Culture characteristics of ASCs

ASCs were successfully isolated from inguinal rat adipose tissue using collagenase I digestion. Stromal vascular fraction obtained from isolation was cultured to obtain the minimum cell quantity to continue the assays. They approached confluency after 3 days and were passaged every 3–4 days for a maximum of 7 passages without major morphologic alterations. Cells displayed a spindle-shaped, fibroblast-like morphology similar to typical appearance of MSCs (Fig. 1, Top Left, Top Right). Expression of stem cells

Table
Gene primer sequences and conditions used for qRT-PCR

Gene	NCBI RefSeq	Primer sequence (5′-3′) (Forward/Reverse)	Melting Temperature (°C)	Optimal concentration
ACT-β	NM_031144	CTGAAGTACCCGGCAT	55.1	50 nM
		CATCTTTTCAAGGGTT	56.4	50 nM
IL-6	NM_012589	CCACTGCCTTCCCTACTTCACA	57.4	50 nM
		TTGTTTTCTGACAGTGCATCATCG	58.1	50 nM
IL-17	NM_001106897	GCCGAGGCCAATAACTTTCT	54.2	300 nM
		GAGTCCAGGGTGAAGTGGAA	54.1	300 nM
IL-1β	NM_031512	AGCCTTTGTCCTCTGCCAAGTCAGGT	63.3	50 nM
		TCGACAATGCTGCCTCGTGACCC	66.3	50 nM
TNF	NM_012675	CTACCCAGCCCCTGTCCCCGACTC	66.7	300 nM
		TCCAGGCCACTACTTCAGCGTCTCGT	65.0	300 nM

IL, interleukin; qRT-PCR, quantitative real-time polymerase chain reaction; TNF, tumor necrosis factor.

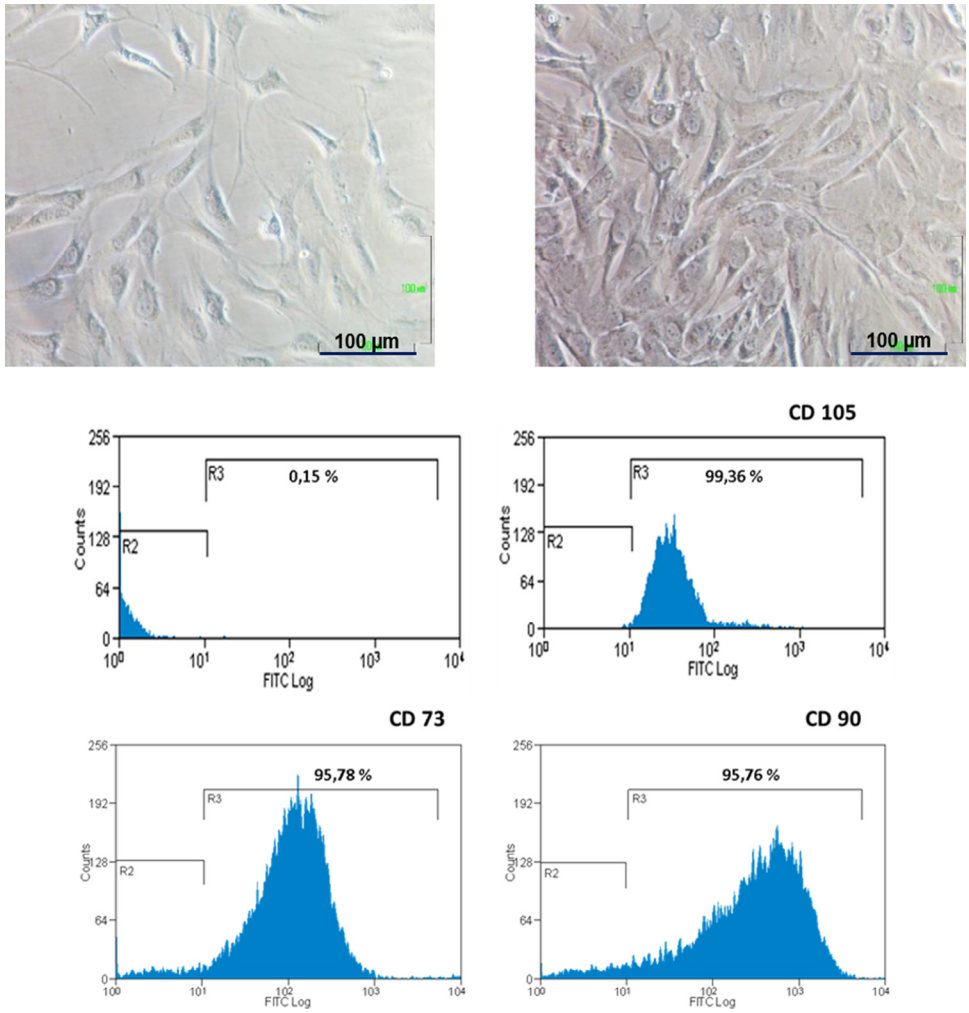

Fig. 1. Morphologic observation and immunophenotyping analysis by flow cytometry of ASCs. (Top Left) ASCs morphology after 48 hours of culture (magnification 40×, scale bar=100 µm). (Top Right) ASCs morphology after 3 days of culture (magnification 40×, scale bar=100 µm). ASCs show a confluence monolayer. (Bottom) Histograms show that ASCs were negative to non-positive control, CD105 (99.36% expression), CD73 (95.78% expression), and CD90 (95.76% expression).

markers CD105 (endoglin), CD73 (ecto-5′-nucleotidase) and CD90 (Thy1) of ASCs were determined. The percentages of positive cell markers and their histograms are shown in Fig. 1, Bottom. Immunophenotypic characterization was carried out by flow cytometry, using monoclonal antibodies.

Culture characteristics of AF and NP cells

Obtaining primary culture of AF and NP cells was relatively complicated. Cell density of the AF and NP was very low and also decreased with age. The number of endogenous cells obtained in the NP tissues for cell expansion was very small. After testing different protocols for NP cells and using young rats, we managed to obtain a sufficient number of viable cells for growth as this depended considerably from cell-to-cell contact. A higher cell density was found in the AF than in the NP.

After 24 hours of isolated cells by digestion with collagenase II and seeded cells, the primary AF cells were plastic-adherent with fibroblast-like morphology. About 8–10 days were needed for the primary AF cells to reach the 80%–90% confluence. Following cell expansion until passage 7, the morphology remained (Fig. 2A). Primary NP cells were isolated by trypsin digestion in a very short time and seeded in a small area. After 36 hours, primary NP cells had oval shapes and took almost 3 days to be plastic-adherent with a chondrocyte-like appearance (Fig. 2B), keeping these latest features throughout all cell expansion until passage 7. Primary NP cells reached confluence of 80%–90% after 17–19 days.

AF and NP cells growth kinetics

The behavior of the AF and NP cells in culture was performed using cellular growth curve analysis. Viable cell growth kinetics was determined by cell counting using the trypan blue exclusion method. AF cells showed an initial lag phase of 2 days, afterwards exponential growth of 4–9 days followed by the plateau phase. With regard to the growth of NP cells, a

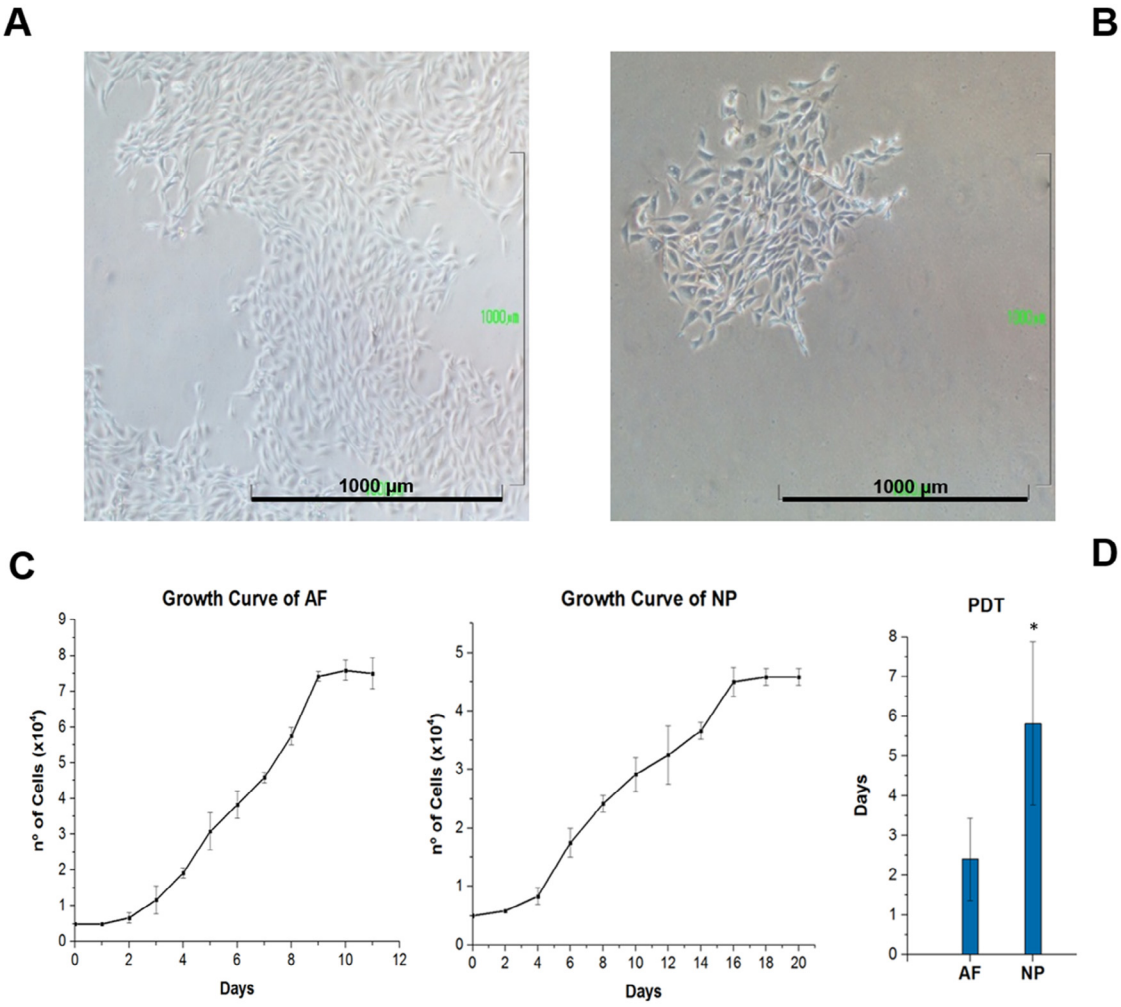

Fig. 2. Morphologic observation and growth kinetics of annulus fibrosus (AF) and nucleus pulposus (NP) cells. (A) Primary AF cells morphology after 6 days of culture (magnification 4×, scale bar=1000 µm). (B) Primary NP morphology after 6 days of culture. NP cells are greatly larger than AF cells (magnification 4×, scale bar=1000 µm). (C) Growth curves of AF and NP cells (passage 4–6). AF cells were counted every 24 hours and NP cells every 48 hours due to the slow cell growth previously observed. (D) Population doubling time (PDT). AF cells tended to double the population on an average of every 2 days, whereas NP cells exhibited a PDT every 5 days. * p≤.05.

lag phase of approximately 3 days was displayed, the exponential growth curve occurred from 4 to 16 days, and after that the stationary phase occurred (Fig. 2C). In either of the two cell types no significant difference in the growth rate between the different passages was observed. Analysis of the mean PDT of cells (Fig. 2D) showed that the NP cells had significantly higher PDT (*p<.05), showing better proliferative capacities in the same culture passage as AF cells. Despite this, both AF and NP have a slow cell proliferation.

Characterization of NP and AF by flow cytometry

Flow cytometric characterization showed a high expression of decorin in AF cells. In NP cells a very low or absent expression of cytokeratin 19 was shown; in this case the surface marker, which was not specific for rat species, was used. The percentages of positive cell markers and its histograms are shown in Fig. 3, Top, Middle.

Characterization of, NP, and AF by confocal microscopy

Fluorescence microscopy analysis revealed that AF cells displayed positive results for the surface marker decorin and NP cells displayed positive results for the surface marker cytokeratin 19. We used a different cytokeratin 19 antibody for immunocytochemistry; in this case cytokeratin 19 antibody was specific to rat. Confocal microscopy images are shown in Fig. 3, Bottom.

Action of TNF in each cell type and in disc cells co-cultures

The TNF concentration and incubation time for optimum response in vitro inflammation model have been determined by previous experiments in this laboratory. Different concentrations and action times were tested establishing a 10 µg/mL TNF concentration and 12 hours of incubation as parameters with better inflammatory response was

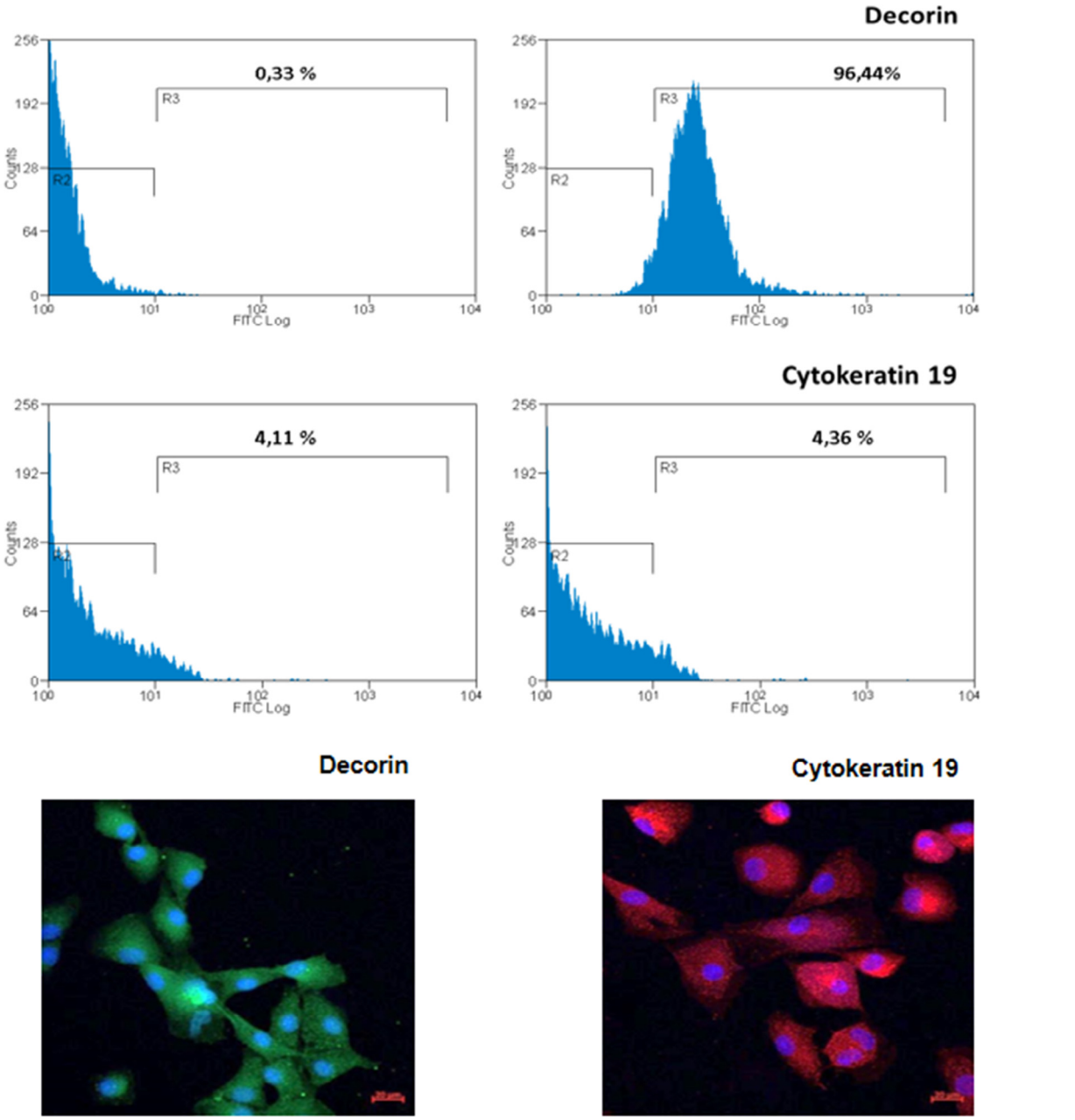

Fig. 3. Immunophenotyping analysis of annulus fibrosus (AF) and nucleus pulposus (NP) cells by flow cytometry and immune-fluorescence. (Top) AF cells were positive to decorin (96.44% expression). (Middle) NP cells were negative to cytokeratin 19 (4.36% expression). (Bottom) In the confocal analysis (magnification 40×, scale bar=20 μm), corresponding surface proteins to NP and AF were positive. AF cells were incubated with streptavidin-Alexa 488 (green) and NP cells with streptavidin-Alexa 568 (red) antibodies. Nuclei were stained with DAPI. DAPI, 4′,6-diamidino-2-phenylindole.

obtained. Each cell type (ASCs, macrophages, NP, and AF cells) and co-cultures (NP cells/macrophages and AF cells/macrophages) were seeded with and without TNF and incubated for 12 hours. After RNA extraction and transcribing to cDNA, four genes related to inflammation were analyzed to assess the TNF action. The relative expression of proinflammatory cytokines genes, including TNF, IL-1β, IL-6, and IL-17 was measured by qRT-PCR. Disc cells were seeded separately to study exactly what was occurring in each case.

The effects of the TNF addition and co-culture with macrophages in the AF cells were similar to NP cells, although with higher expression values in the AF cells, with regard to the TNF relative expression (Fig. 4, Top and Middle). In AF or NP cells with TNF, TNF expression showed the greatest increase (***p≤.005). In co-cultures AF or NP cells with macrophages, with and without TNF, TNF expression significantly increased (***p≤.005) less in co-culture NP cells with macrophages without TNF compared with unstimulated disc cells.

The relative expression of IL-1β was also similar in the AF and NP in each cell culture, with higher values in the AF case (Fig. 4, Top and Middle). IL-1β expression showed the greatest increase in co-culture AF or NP cells with

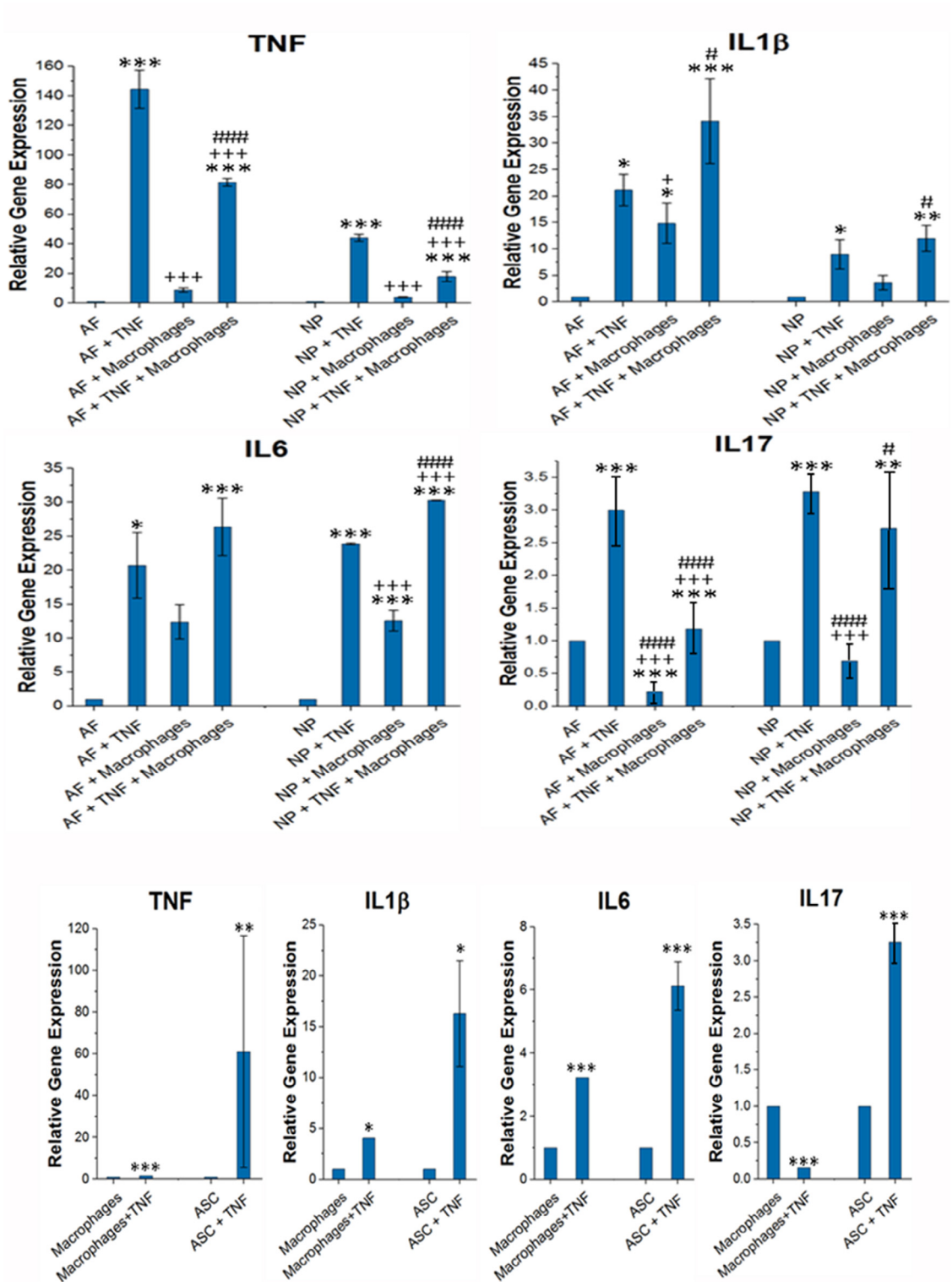

Fig. 4. (Top and Middle) Relative expression analysis of tumor necrosis factor (TNF), interleukin (IL)-1β, IL-6, and IL-17 genes in annulus fibrosus (AF) and nucleus pulposus (NP) cells and co-culture macrophages condition with or without TNF addition after 12 hours. (Bottom) Relative expression analysis of TNF, IL-1β, IL-6, and IL-17 genes in adult stem cells (ASCs) and macrophages with or without TNF addition after 12 hours. * p≤.05, ** p≤.01, *** p≤.005 compared with non-stimulated cells. + (p≤.05), ++ (p≤.01), +++ (p≤.005) compared with TNF stimulated cells. # (p≤.05), ## (p≤.01), ### (p≤.005) compared with macrophages stimulated cells.

macrophages with TNF addition (***p≤.005, **p≤05). In AF or NP cells with TNF or macrophages, IL-1β expression significantly increased (*p≤.05) compared with unstimulated disc cells.

The IL-6 relative expression was also similar in the AF and NP in each cell culture (Fig. 4, Top and Middle). The IL-6 mRNA expression showed the largest increase in co-culture AF or NP cells with macrophages and TNF addition (***p≤.005). In AF or NP cells with TNF or macrophages, IL-6 expression significantly increased (*p≤.05, ***p≤.005) compared with unstimulated disc cells.

The TNF, IL-1β, IL-6, and IL-17 mRNA expression in controls (ASCs and macrophages) are shown in Fig. 4, Bottom.

Soluble factors in the CM

Concentration of protein in CM at 12 hours, 24 hours, 36 hours, and 48 hours of culture was measured to determine the appropriate time when ASCs were maintained in culture before collecting CM. NanoDrop results showed no significant differences at protein concentration at different times (Fig. 5, Top Left). Thirty-six hours of ASCs culture was chosen to collect the CM because, under this condition, the protein concentration was slightly higher. To verify that there were no proteins in DMEM and these proteins derived only from ASCs expression, an SDS-PAGE after concentrated proteins by centrifugation was performed (Fig. 5, Top Right).

Immunomodulatory effect of conditioned medium on inflammation model of intervertebral disc

To confirm the trophic effect of MSCs on NP and AF, CM was added to NP or AF co-cultures with macrophages and TNF and incubated for 12 hours. The relative expression of TNF, IL-1β, IL-6, and IL-17 was determined by qRT-PCR to study immunomodulatory effect of CM in an inflammatory environment (Fig. 5, Bottom). After adding the CM to cell cultures TNF, IL-1β, IL-6, and IL-17 expression significantly decreased both AF and NP co-cultures (***p≤.005, **p≤.05). The CM inhibits expression of proinflammatory molecules in inflamed disc cells, and the decrease in the expression of these factors may inhibit the degradation of ECM.

Discussion

This study showed that ASCs and CM collected from ASCs cultures were capable of inducing immunomodulatory effects in an in vitro model of disc degeneration.

A comprehensive characterization of ASCs, AF, and NP cells were performed prior to inflammatory model of disc degeneration. Immunofluorescent characterization and culture behavior of rat ASCs confirmed the MSC characteristics [47], and expression of all markers was clear. Positive expression of these surface markers agreed with the results from other studies into this cell type in humans [50,51]. To obtain primary culture of AF and NP cells, young rats were used and it was difficult to isolate sufficient number of cells due to low cell density in each sample [52,53]. The cellularity obtained in AF was higher than in the NP which agrees with previous reports [54–56]. As has been already reported by other authors [57], analysis of the mean PDT showed a slow cell proliferation in AF and NP cells.

Flow cytometric characterization showed a high expression of decorin in AF cells. Decorin antibody was chosen because studies have shown that decorin expression is confined to the AF [58]. Decorin is a member of a leucine-rich repeat class of proteins, which interact with specific regions on the surface of type I and II collagen fibrils [59]. In NP cells a very low or absent expression of cytokeratin 19 was shown; in this case the surface marker, which was not specific for rat species, was used. However, in other references, phenotyping for cytokeratin 19 provides positive results of expression [60,61]. Lee et al. [62] identified cytokeratin 19 as being a differentially expressed gene that also stained immunopositive in the NP but was negative in the AF. Fluorescence microscopy analysis revealed that AF cells displayed positive results for the surface marker decorin and NP cells displayed positive results for the surface marker cytokeratin 19 as was expected [63,64].

The TNF is an inflammatory cytokine which is important in immune response. One of its functions is to stimulate the inflammatory response and to produce other cytokines such as IL-1β, IL-6, IL-8, and IL-17 [65]. According to this study and our results of the relative gene expression including TNF, IL-1β, IL6, and IL-17, increased expression of inflammatory cytokines in an inflammatory environment was shown. An in vitro controlled and standardized model of inflammation and degeneration of IVD with rat cells was also developed. The expression levels of genes associated with TNF and IL-1β were higher in AF cells than in NP cells, conversely in a study by Shim et al. [19]. The TNF and IL-1β expression was similar in both cell types. The NP and AF layers have structural differences (cartilaginous and fibrous), and differences in cytokines expression might be caused by the diverse environmental effects from NP and AF cells, respectively. Deliverance of chemokines from degenerating discs promotes infiltration and activation of macrophages amplifying the inflammatory cascade [66]. We have observed that in the experimental model of inflammation, the expression of proinflammatory cytokines was significantly upregulated when AF and NP-cells were seeded in co-culture with macrophages and stimulated with TNF. This model of inflammatory disc degeneration has been widely used [67–69] and could prove highly appropriate to determine in vitro behavior of NP, AF, and macrophages in co-culture. However, this model is unlikely to mimic the complexity and heterogeneity of the immune response in vivo. For this reason, we believe that this in vitro model is essential to asses in vivo response, and the results obtained have provided the basis for further experiments which are being performed in our laboratory in order to analyze the immune response of ASCs and CM in an in vivo model.

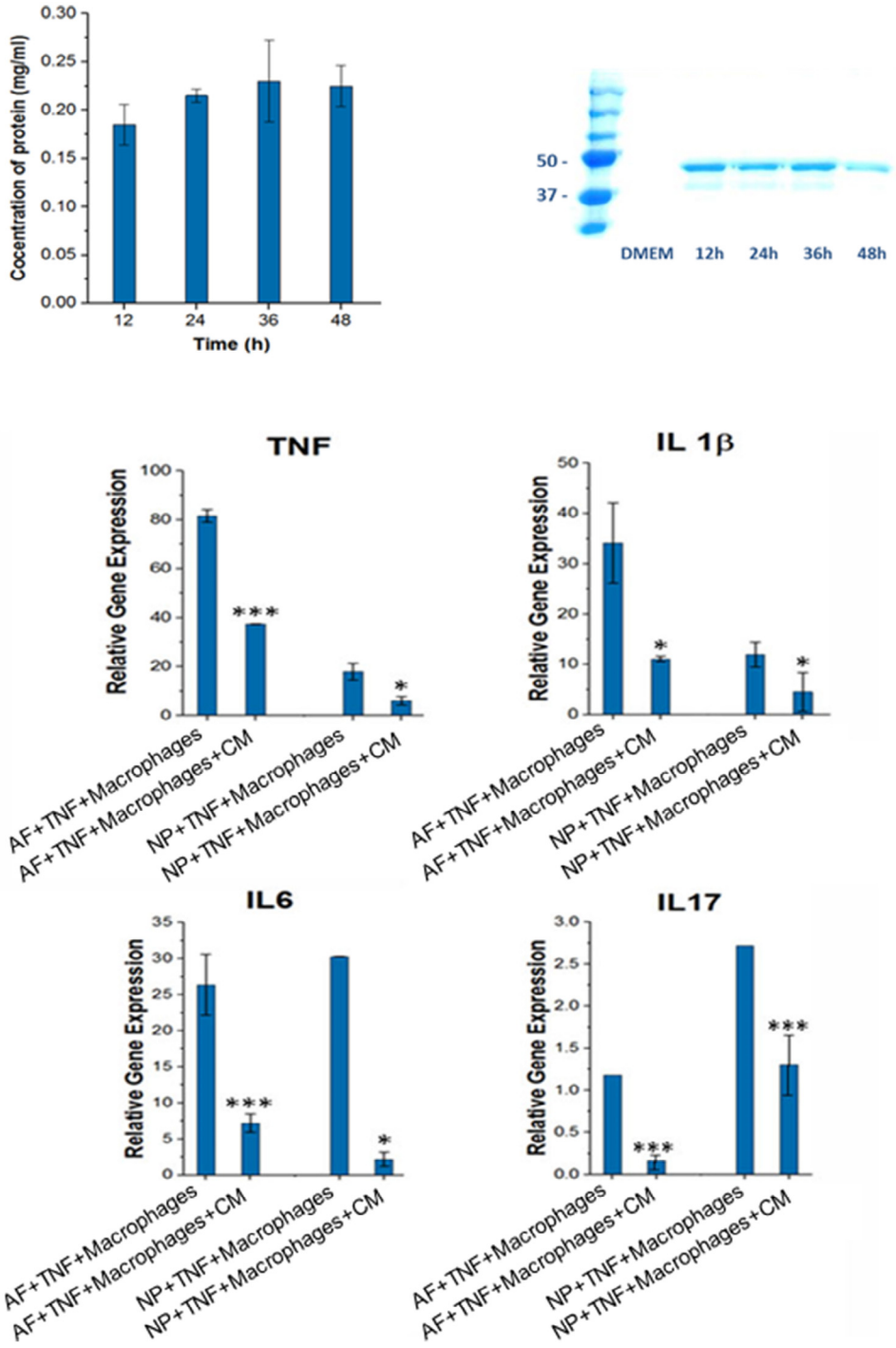

Fig. 5. (Top Left) NanoDrop protein concentration measures in the conditioned medium of adult stem cells (ASCs) at different times. (Top Right) Showing of free protein in Dulbecco's modified Eagle's medium (DMEM) and protein presence in conditioned medium of ASCs, separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). (Bottom) Relative expression analysis of tumor necrosis factor (TNF), interleukin (IL)-1β, IL-6, and IL-17 genes in annulus fibrosus (AF) and nucleus pulposus (NP) co-culture with macrophages and TNF after 12 hours of conditioned medium was added. * ($p \leq .05$), ** ($p \leq .01$), *** ($p \leq .005$).

Several studies have shown that MSCs have a trophic effect on cells by secretion of a variety of growth factors and cytokines that are capable of stimulating the cells [39]. MSCs starts several simultaneous actions, limiting inflammation and aiding healing through releasing cytokines and growth factors such as IL-10, transforming growth factor beta, prostaglandin E_2 (PGE_2); indolamine 2,3-dioxygenase, or hepatocyte growth factor, among others [70]. The mechanism is not exclusive to one factor but it is also anticipated that MSCs yield therapeutic effects by an orchestrated response which is

dictated by the unique pathophysiology of a given disease. The CM is the cell-free medium containing this variety of growth factors and cytokines, and it can be used for clinical application because of its therapeutic properties [71–73]. In this study we collected the CM after 36 hours of ASCs culture; under this condition, the protein concentration was slightly higher and the time period was similar to that of other studies [74].

After adding the CM to cell cultures TNF, IL-1β, IL-6, and IL-17 expression significantly decreased both AF and NP co-cultures (***p≤.005, **p≤.05). These results suggest an immunomodulatory effect of ASCs [75] and they also secrete bioactive factors [39]. CM inhibits expression of proinflammatory molecules in inflamed disc cells, and the decrease in the expression of these factors may inhibit the degradation of ECM. Our results also coincide with recent findings by Shim et al. [19] showing that ASCs prevent expression of proinflammatory molecules in degenerated disc cells. Organ healing by inducing a shift from proinflammatory to anti-inflammatory cytokine production at the site of injury is driven by the MSCs CM [76,77]. Previous studies focus on characterizing MSCs-produced soluble factors (ie, cytokines, chemokines, and growth factors). However, it is now clear that, apart from soluble factors, extracellular vesicles could be a key instrument in cell-cell communication [78].

Downregulating cytokine expression could be possible to regulate pain in several ways. Upregulated proinflammatory cytokines create an inflammation environment and causes an increase of catabolic molecules. These enzymes promote degradation of extracellular matrix molecules such as aggrecan and collagen II [79]. This process results in a decrease in weight-bearing capacity and in a loss of disc height [80]. Increasing grade of degeneration has been shown to be correlated with back pain [81]. Furthermore, proinflammatory cytokines released by the IVD cells in an inflammatory environment act on macrophages and mast cells to stimulate the production of nerve growth factor (NGF). The NGF increased levels lead to an increase of nociceptive nerve fibers within the IVD, an anterograde transport of molecules involved in IVD maintaining pain [82], and could stimulate vertebral nociceptors that border the cartilage end plate too [83,84].

Back pain has been frequently associated with the degeneration of the IVD. A large amount of research is being performed to identify the underlying mechanisms and to achieve a strategy for degeneration of IVD treatment. However, still there are no reliable solutions. Research in the field of cell therapy is rapidly progressing and supports a biological approach to solve discogenic pain. This new approach opens novel therapeutic perspectives of cell-free therapies based on the use of MSCs CM. Our study shows that the paracrine actions of ASCs may be useful in the immunomodulation of degenerative IVDs enhancing therapeutic efficacy producing anti-inflammatory effect. On the other hand, ASCs are excellent candidates for this cellular therapy as they are hypoimmunogenic, owing to their lack of expression of HLA class-II molecules, availability and abundance, and extensive proliferation ability in vitro [85].

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