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BASIC SCIENCE

Immunomodulation of Human Mesenchymal Stem/Stromal Cells in Intervertebral Disc Degeneration

Insights From a Proinflammatory/Degenerative Ex Vivo *Model*

Graciosa Q. Teixeira, PhD,[*,†,‡,§] Catarina Leite Pereira, PhD,[*,†,§] Joana R. Ferreira, MSc,[*,†,§] André F. Maia, PhD,[*,¶] Maria Gomez-Lazaro, PhD,[*,†] Mário A. Barbosa, PhD,[*,†,§] Cornelia Neidlinger-Wilke, PhD,[‡] and Raquel M. Goncalves, PhD[*,†,§]

Study Design. *Ex vivo* experimental study.

Objective. To investigate the effect of proinflammatory/degenerative intervertebral disc (IVD) microenvironment on the regenerative and immunomodulatory behavior of mesenchymal stem/stromal cells (MSCs), using an *ex vivo* model from bovine origin.

Summary of Background Data. Low back pain is a cause of disability worldwide, most frequently associated with IVD degeneration and inflammation, and characterized by increased levels of inflammatory mediators, often disregarded. MSC-based therapies to low back pain have been advocated, but the involvement of inflammation in IVD remodeling mechanism, promoted by MSCs has not yet been explored.

Methods. Bovine IVD organ cultures of nucleus pulposus punches were stimulated with needle puncture and culture medium supplementation with 10 ng/mL of interleukin (IL)-1β, to induce a proinflammatory/degenerative environment, as previously established. Human bone marrow–derived MSCs were cultured on top of transwells, placed above nucleus pulposus punches, for up to 16 days. MSCs were analyzed by screening cell viability/apoptosis, metabolic activity, migration, and inflammatory cytokines production in response to the proinflammatory environment. IVD extracellular matrix (ECM) remodeling, gene expression profile of IVD cells, and inflammatory cytokine profile in the presence of MSCs in basal *versus* proinflammatory conditions were also evaluated.

Results. Proinflammatory/degenerative IVD conditions did not affect MSCs viability, but promoted cell migration, while increasing IL-6, IL-8, monocyte chemoattractant protein-1, and prostaglandin E_2 and reducing transforming growth factor-β1 production by MSCs. MSCs did not stimulate ECM production (namely type II collagen or aggrecan) in neither basal nor inflammatory conditions, instead MSCs downregulated bovine proinflammatory *IL-6*, *IL-8*, and *TNF-α* gene expression levels in IL-1β-stimulated IVDs.

Conclusion. The present study provides evidence for an immunomodulatory paracrine effect of MSCs in degenerated IVD without an apparent effect in ECM remodeling, and suggest an MSCs mechanism-of-action dependent on a cytokine feedback loop.

Key words: inflammation, interleukin-1β, intervertebral disc, mesenchymal stem/stromal cells, organ culture.

Level of Evidence: 5

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From the [*]i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal; [†]INEB - Instituto de Engenharia Biomédica, Universidade do Porto, Porto, Portugal; [‡]Institute of Orthopaedic Research and Biomechanics, Center for Musculoskeletal Research, University of Ulm, Ulm, Germany; [§]ICBAS - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal; and [¶]IBMC - Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal.

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Address correspondence and reprint requests to Raquel M. Goncalves, PhD, Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Rua Alfredo Allen 208, 4200-135 Porto, Portugal; E-mail: raquelg@ineb.up.pt

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The potential of intervertebral disc (IVD) to regenerate benefits from the presence of cells capable of proliferating and differentiating into nucleus pulposus (NP)–like cells.[1–3] Although IVD progenitor cells have been found in the human IVD, their number decreases very rapidly after birth.[1–3] These cells become exhausted with ageing, limiting IVD's potential to counteract degeneration.[4,5] Therefore, cell-based therapies to low back pain, with the purpose to stimulate regeneration of the IVD, are being increasingly used.[5] Although the hypoxic IVD environment, together with mechanical load, high osmolarity and low pH may impair cell survival and

function,[6] autologous or allogeneic mesenchymal stem/stromal cells (MSCs) transplantation has shown a high therapeutic potential.[5,7] MSCs have been shown to differentiate into an NP-like phenotype,[8–10] and to enhance IVD matrix production *in vivo*.[11,12] Moreover, MSCs are recognized as immunomodulatory cells,[13] and contribute to IVD immune privilege maintenance by the expression of Fas ligand.[12] Nonetheless, MSCs potential to modulate the inflammatory milieu in IVD-associated inflammation scenarios remains largely unknown.

Yoshikawa *et al*[7] reported two case studies of autologous bone marrow–derived MSCs implantation in degenerated IVD during fenestration surgery on stenosed spinal canal. At 2 years after surgery, symptomatic and radiological improvement was observed without significant adverse effects.[7] A larger pilot study performed afterwards, included 10 patients suffering from low back pain associated with IVD degeneration.[14] These patients were transplanted with autologous bone marrow–derived MSCs. After 1 year of follow-up, results indicated similar outcome improvements as other procedures such as spinal fusion or total disc replacement.[14]

Moreover, MSCs have demonstrated anticatabolic properties, as well as anti-inflammatory and immunomodulatory paracrine effects in several studies of osteoarthritis[15–17] and rheumatoid arthritis.[18] MSCs were shown to secrete anti-inflammatory factors, and influence matrix turnover, in short-term osteoarthritic synovium and cartilage explant cultures,[19] by acting on chondrocytes *via* the cyclooxygenase (COX)-2/prostaglandin E_2 (PGE_2) pathway.[20]

Although the number of clinical trials proposing MSC-based therapies for articular cartilage and intervertebral disc regeneration are increasing,[15,21] the studies published so far fail to properly address the inflammatory environment characteristic of the degenerated IVD, and how this milieu can influence the MSCs response. Therefore, this study hypothesizes that the "proinflammatory/degenerative" IVD microenvironment can affect MSCs, exploring the proregenerative and immunomodulatory potential of these cells. Herein, we hope to highlight the importance of analyzing MSCs immunomodulatory effect on IVD degeneration, at the initial stage after transplantation. Nonetheless, before they reach daily clinical practice, further studies must be performed, namely adequate clinical trials.

MATERIALS AND METHODS

Culture of Human Mesenchymal Stem/Stromal Cell

Human MSCs harvested from bone marrow were obtained from different donors who underwent bone marrow donation, hip replacement, or knee joint surgery, with informed consent and following the rules of the ethical commission of the University of Ulm (Ulm, Germany) and the Portuguese authorities (*Direcção-Geral da Saúde*, Porto, Portugal). MSCs phenotypic profile and multilineage differentiation potential were previously accessed.[22,23] Cells were expanded as reported in Almeida *et al*.[23] Experiments were performed with MSCs from seven donors in passages 3 to 7 (Supplementary Table S1, http://links.lww.com/BRS/B317).

Proinflammatory Intervertebral Disc Organ Culture Model and Coculture With Mesenchymal Stem/Stromal Cell

IVDs were isolated from bovine tails (mean age: 26 mo) within 3 hours' post-slaughter, with the ethical approval of Portuguese authorities. Caudal discs were isolated and cultured as described by Teixeira *et al*[24] (Figure 1). Briefly, the IVDs were maintained for 5 days in six-well tissue culture plates, with membrane filter inserts on top and under 0.46 MPa static loading. IVDs' culture medium was Dulbecco's Modified Eagle's Medium with low glucose (DMEM, Biochrom, Berlin, Germany), supplemented with 5% v/v fetal bovine serum (FBS, HyClone, GE Healthcare, Logan, UT), 1% v/v penicillin/streptomycin (10,000 U/mL-10,000 μg/mL, Biowest, Nuaillé, France), 0.5% v/v amphotericin B (Capricorn Scientific, Ebsdorfergrund, Germany) and with the osmolarity adjusted to 400 mOsm by addition of 1.5% v/v of a 5 M NaCl/0.4 M KCl solution. Samples were incubated at 37°C, 6% O_2 and 8.5% CO_2. Culture medium was replaced every second day. At day 5 IVDs were needle-punctured (21G), 10 ng/mL of recombinant human interleukin (IL)-1β (PeproTech, London, UK) was added to the medium, and 3 hours later, 1×10^6 MSCs were seeded on top of each IVD punch. MSCs were also cultured alone in six-well plates, with IVDs' medium supplemented with 10 ng/mL IL-1β, under 6% O_2 and 8.5% CO_2, and at 37°C. The experimental groups and respective controls are presented in Figure 1.

Apoptosis and viability of the IVDs and MSCs were accessed by flow cytometry at day 7. Gene expression profile of IVD cells and inflammation factors production by both cell types were also analyzed at day 7. Extracellular matrix (ECM) components were analyzed at gene expression and at protein levels at days 7 and 21, respectively. The detailed assays are described in Supplementary Materials and Methods.

Statistical Analysis

Results are presented as median ± interquartile range (IQR) in box and whiskers plots, and in bar plots for the inflammation factors. Data normality was first analyzed by D'Agostino and Pearson normality test after which statistical analysis was performed with nonparametric Kruskal-Wallis test and Dunn multiple comparison test as *post hoc*. MSCs migration was compared with Wilcoxon test for paired analysis. Graph Pad v6.02 for Windows. Tests were 2 sided, and a confidence level of at least 95% ($P < 0.05$) was used.

RESULTS

Viability of Mesenchymal Stem/Stromal Cells and Intervertebral Disc

To investigate whether MSCs viability would be affected by the proinflammatory/degenerative IVD environment,

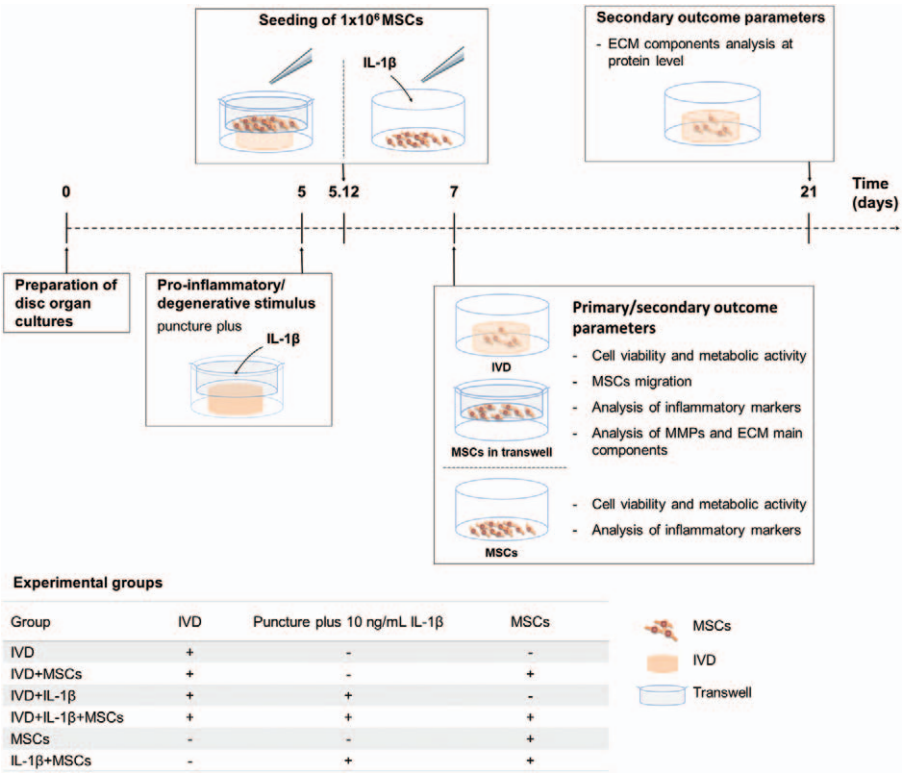

Figure 1. Experimental timeline and culture groups. ECM indicates extracellular matrix; IL, interleukin; IVD, intervertebral disc; MSC, mesenchymal stem/stromal cell.

1×10^6 MSCs were cocultured with IVD tissue in basal and proinflammatory (puncture + IL-1β) conditions, on top of 8-μm transwells. After 2 days of exposure to proinflammatory stimuli, both IVD and MSCs were analyzed separately for metabolic activity and cell apoptosis/death, as schematically represented in Figure 2A. The mitochondrial metabolic activity of conditions with IVD and with MSCs was similar in presence or absence of IL-1β (Figure 2B). Cell apoptosis/death was accessed by Annexin V (AnxV) and propidium iodide (PI) staining and overall staining levels were less than 20% (Figure 2C). No differences were observed in cell apoptosis ($AnxV^+PI^-$). On the contrary, the percentage of dead cells ($AnxV^+PI^+$) in the IVD ($6\% \pm 2\%$) increased in the presence of MSCs, either in the absence ($14\% \pm 7\%$) or presence ($15\% \pm 9\%$) of IL-1β, being this increase statistically significant for IVD groups ($P < 0.05$) (Figure 2D). MSCs collected from transwells maintained their viability in control and IL-1β-stimulated IVDs, which was expected, because IL-1β by itself did not affect MSCs metabolic activity or cell apoptosis/death (data not shown).

In addition, MSCs migration through the transwells was also analyzed. Representative images of IVD, IVD + MSCs, and IVD + IL-1β + MSCs conditions are depicted (Figure 3A, B, and C, respectively). MSCs migration was significantly increased in presence of IL-1β ($P < 0.05$). Nonetheless, flow cytometry analysis of cells in IVD digested tissue did not indicate presence of MSCs, suggesting that MSCs frequency was below detection limit (data not shown).

Inflammatory Factors Produced by Mesenchymal Stem/Stromal Cells

To evaluate whether proinflammatory/degenerative IVD conditions could influence MSCs cytokine profile, the protein content in the culture supernatants was evaluated using a human inflammatory cytokines array (Supplementary data, http://links.lww.com/BRS/B317). To validate the array, IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1 were quantified in the supernatants by ELISA (Figure 4). Moreover, the supernatant from MSCs cultures in basal (MSCs) or IL-1β-stimulated conditions (MSCs + IL-1β) was also analyzed by ELISA. Results showed a statistically significant increase of IL-6 (of about 6-fold, $P < 0.01$), and IL-8 (approximately 41-fold, $P < 0.05$) by MSCs in presence of IL-1β. In IVDs without MSCs, IL-6, IL-8, and MCP-1 were under the detection limit. Also in MSCs cultures, in presence of IL-1β, there was a significant increase of IL-6 ($P < 0.001$) and IL-8 ($P < 0.01$) production, as well as of MCP-1 ($P < 0.01$), when compared to MSCs cultured under control conditions. TNF-α, IL-10, indoleamine-2,3-dioxygenase, and TNF-α-induced protein 6 (TSG-6) were also analyzed by ELISA, but their values were also below the assays' detection limit.

Extracellular Matrix Remodeling

In the IVD organ culture model previously established, *MMP1* and *MMP3* were shown to be upregulated, whereas ECM components, as type II collagen (*COL2A1*) and aggrecan (*ACAN*), were downregulated in the IVD tissue under degenerative/proinflammatory conditions.[24] Here,

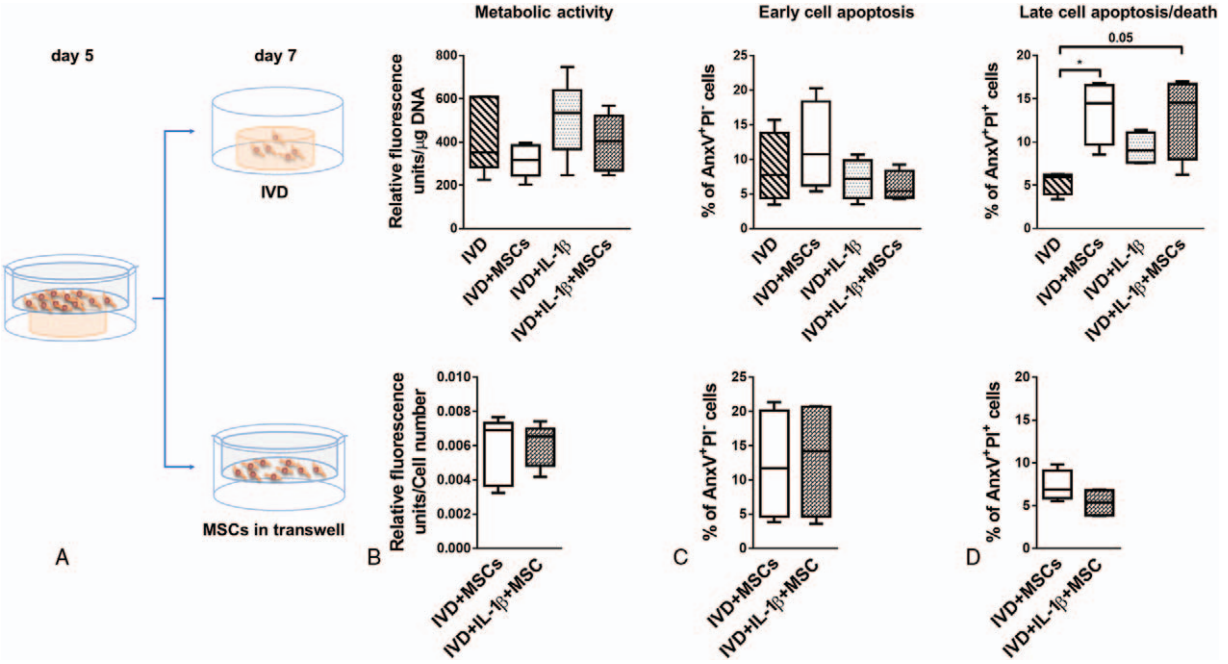

Figure 2. A, Layout of MSCs/ IVD cocultures. Samples were collected 2 days after proinflammatory stimulus. **B**, IVDs and MSCs mitochondrial metabolic activity. **C**, Percentage (%) of early cell apoptosis (annexin V positive [AnxV$^+$] and propidium iodide negative [PI$^-$]). **D**, Percentage of late cell apoptosis/death (AnxV$^+$PI$^+$ cells). Box and whiskers plots (n = 4–5). $^*P < 0.05$. IL indicates interleukin; IVD, intervertebral disc; MSC, mesenchymal stem/stromal cell.

metalloproteases (MMPs) and the IVD main ECM components were analyzed by gene expression 2 days after IL-1β stimulation and co-culture with MSCs (Figure 5A). The presence of MSCs did not induce upregulation of *MMP1* and *MMP3* by IVD cells. On the contrary, in IVD + IL-1β + MSCs, there was an upregulation of *MMP1* ($P < 0.05$) and of *MMP3* ($P < 0.05$), when compared to IVD + MSCs co-cultures. Also, *MMP13* expression was significantly

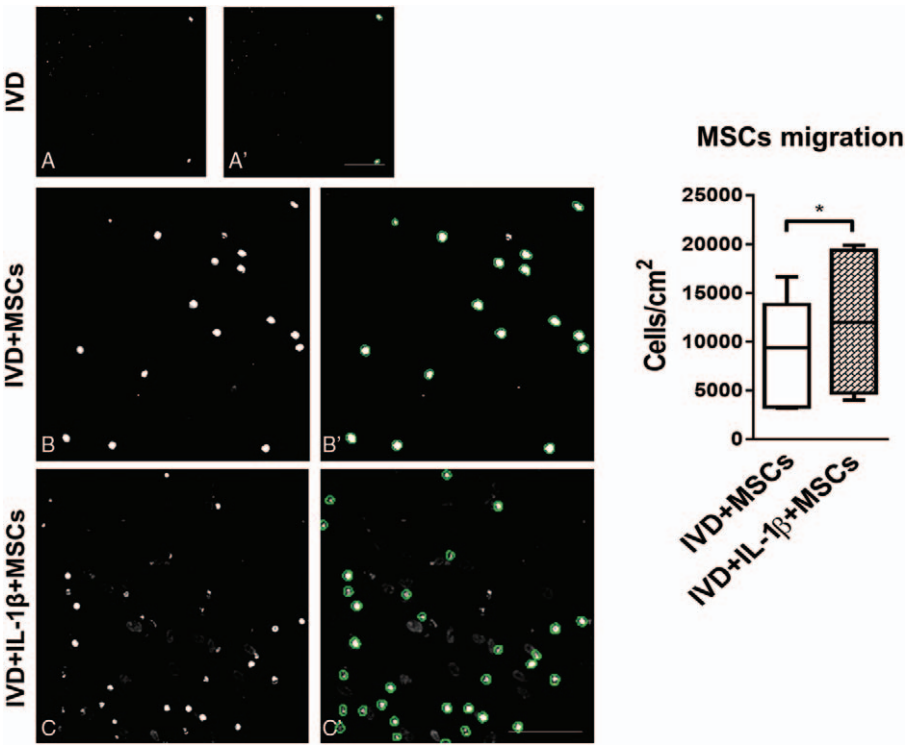

Figure 3. Quantification of human MSCs that migrated trough the transwells, after co-culture with IVD tissue for 2 days. High-throughput automated fluorescence wide field microscopy and a nuclear segmentation algorithm were used to identify and quantify the number of migrated cells on the bottom of transwells. Here it is shown representative micrographs for the control group IVD (**A**), and for IVD + MSCs (**B**), and IVD + interleukin IL-1β + MSCs (**C**), as well as their respective segmentation masks (**A′**, **B′**, and **C′**), for the automatic cell counting (counted cells delimited in green; scale bar, 100 μm). Data expressed as number of migrated cells per cm^2, normalized to the respective controls (n = 6). $^*P < 0.05$. IL indicates interleukin; IVD, intervertebral disc; MSC, mesenchymal stem/stromal cell.

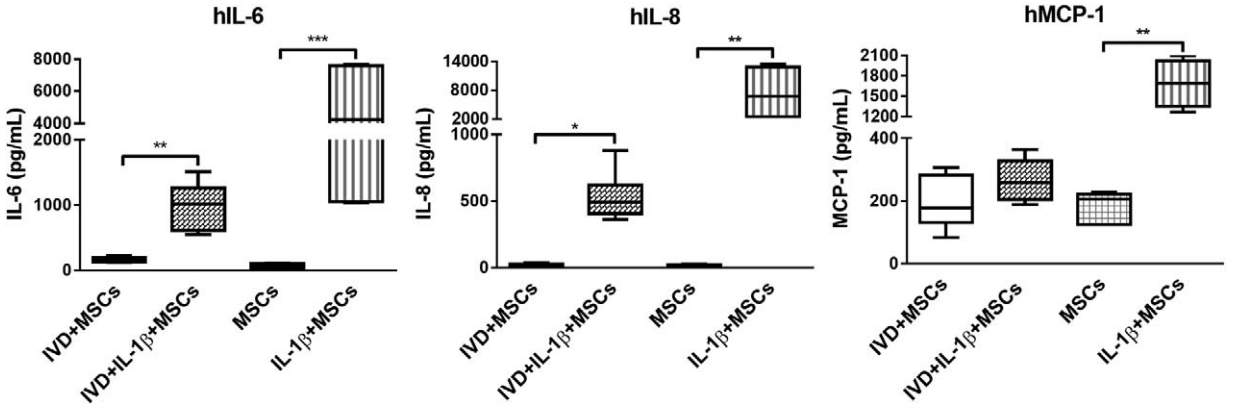

Figure 4. Human inflammatory factors produced by MSCs in basal or proinflammatory/degenerative conditions. Concentration of human IL-6 (hIL-6, pg/mL), hIL-8 (pg/mL), and hMCP-1 (pg/mL) in supernatants from MSCs cocultures with IVD tissue or cultured alone, under control or proinflammatory conditions, after 2 days (n = 4–13). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. IL indicates interleukin; IVD, intervertebral disc; MSC, mesenchymal stem/stromal cell.

downregulated in IVD + MSCs (0.3 ± 0.6), relative to the control IVD group ($P < 0.05$).

Concerning ECM proteins, *COL2A1* and *ACAN* were significantly downregulated in all the conditions tested, in comparison to control IVD. Moreover, a downregulation of *COL2A1* in IVD + IL-1β + MSCs was observed, when compared with both IVD + MSCs and IVD + IL-1β ($P < 0.01$).

To evaluate the effects of MSCs coculture on IVD ECM production, at protein level, the co-cultures were maintained for 16 days. A statistically significant decrease of sulfated glycosaminoglycan content (of about 0.3, $P < 0.01$) was observed in both IVD+IL-1β and IVD+IL-1β+MSCs groups, when compared to IVD (Figure 5B). Moreover, the fluorescence intensity of COL2A1 staining (Figure 5B: a–d) was quantified using the IntensityStatisticsMask software, and is presented as fold change to IVD. COL2A1 content did not seem to be altered. ACAN deposition (brown, Figure 5B: e–h), as well as cells negative ($ACAN^{-}$, Δ) and positive ($ACAN^{+}$, +) for ACAN deposition were also quantified for the different conditions and normalized to the IVD control condition. Overall, results indicated similar ACAN content in all conditions tested. Nonetheless, there was a significant increase of about twofold in the % of $ACAN^{+}$ cells/mm^2 for IVD+IL-1β, in contrast to IVD ($P < 0.05$). No significant effect of MSCs in ECM production was observed.

Inflammatory profile of Intervertebral Disc cells and Mesenchymal Stem/Stromal Cells

The proinflammatory gene expression profile of IVD cells was assessed by the expression of *IL-6*, *IL-8*, and *TNF-α*, 2 days after IL-1β stimulation and co-culture with MSCs (Figure 6A). In the IVD organ culture model an upregulation of *IL-6* and *IL-8* (14 ± 29- and 8 ± 8-fold, respectively, $P < 0.01$) were observed in the presence of IL-1β, in accordance with previous results.[17] *TNF-α* gene expression levels were similar between IVD + IL-1β and IVD. Interestingly, IVD cells from IVD + MSCs expressed similar levels of *IL-6*, *IL-8*, and *TNF-α*, compared to IVD group. In presence of IL-1β, MSCs significantly downregulated *IL-6* (from 14 ± 28- to 3 ± 2-fold, $P < 0.05$); in addition, MSCs also seemed to downregulate bovine *IL-8* levels (from 8 ± 8- to 2 ± 6-fold) and *TNF-α* (from 1 ± 5- to 0.3 ± 0.7-fold).

A commercially available array to detect 40 human inflammatory factors was used to evaluate the relative levels of cytokine production in the IVD culture supernatants (supplementary data, http://links.lww.com/BRS/B317). The most expressed proteins identified in the array (IL-1β and RANTES) were validated by ELISA, as well as other inflammatory mediators, PGE_2 and free-active transforming growth factor (TGF)-β1, previously identified by us in bovine IVD organ cultures.[24,25] Results are depicted in Figure 6B, and show that IL-1β was only detected in IL-1β-supplemented cultures as expected (supplementary data, http://links.lww.com/BRS/B317) and no differences were observed in the presence of MSCs. RANTES was not detected in IVD cultures (IVD and IVD + IL-1β), indicating that the quantification corresponds to RANTES produced by MSCs, but there were no significant differences between the different conditions analyzed. A significantly higher production of PGE_2 was detected in IVD + IL-1β samples compared to IVD ($P < 0.0001$), similarly to what was previous described.[24] PGE_2 production just slightly increased from 4 ± 3- to 13 ± 18-fold when MSCs were cocultured with IVD tissue in basal conditions, but without significant differences. Nonetheless, this production was significantly higher compared to the PGE_2 produced by MSCs alone ($P < 0.05$). Moreover, PGE_2 production by IVD + IL-1β + MSCs was significantly higher than in IVD + MSCs ($P < 0.001$), IVD + IL-1β and IL-1β + MSCs ($P < 0.05$). MSCs alone also produced significantly higher amounts of PGE_2 when stimulated with IL-1β ($P < 0.05$). In addition, although total TGF-β1 was not identified in the inflammation array, free active form of TGF-β1 was detected in all experimental conditions. MSCs in control IVDs contributed to an increase of TGF-β1 from 49 ± 89- to 277 ± 107-fold ($P < 0.01$), whereas in the IL-1β groups there were no significant differences. When comparing IVD + MSCs with MSCs cultures, a significantly higher production of free

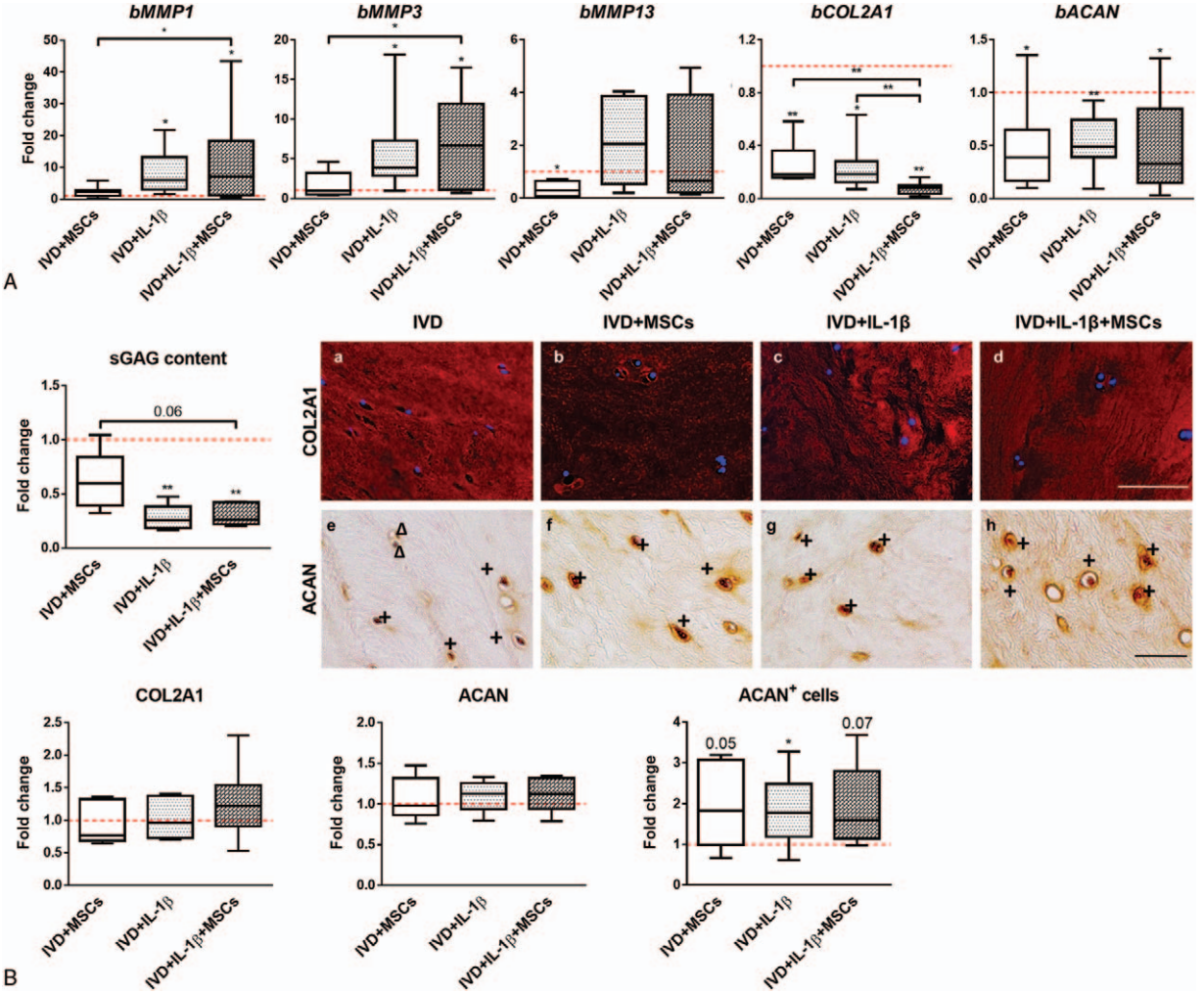

Figure 5. IVD ECM remodeling in the presence of MSCs in proinflammatory/degenerative stimulus. **A**, mRNA expression of bovine *MMP1 (bMMP1)*, *bMMP3*, *bMMP13*, *bCOL2A1*, and *bACAN*, after 2 days of coculture. mRNA levels were normalized to *bGAPDH* control gene and to the unstimulated discs (control level = 1; dashed line). **B**, Analysis at protein level of IVD ECM components, 16 days after coculture. Biochemical analysis of sGAG content of IVD punches, normalized to Ctr. Representative sagittal sections of disc punches stained for COL2A1 (**a**–**d**; scale bar, 100 μm) and ACAN (**e**–**h**; scale bar, 50 μm), displaying ACAN negative ($ACAN^-$, Δ) and positive ($ACAN^+$, +) cells. COL2A1 fluorescence intensity normalized to Ctr. ACAN intensity in the tissue, determined by measuring the optic density (OD) of the DAB staining, normalized to Ctr. Percentage of $ACAN^+$ cells normalized to the imaged area (mm^2) and to Ctr (n = 5–20). $^*P < 0.05$; $^{**}P < 0.01$. IL indicates interleukin; IVD, intervertebral disc; MSC, mesenchymal stem/stromal cell.

active TGF-β1 was detected in the coculture than in the MSCs alone ($P < 0.05$).

DISCUSSION

This study investigated the regenerative and immunomodulatory role of MSCs in a proinflammatory/degenerative bovine IVD *ex vivo* model, which had been formerly validated.[24] In human disc degeneration, IL-1 has been proposed as a key regulator of proinflammatory and matrix-degrading factors.[26–30] In the model used for this study, an upregulation of proinflammatory markers (*IL-6*, *IL-8*, *MMP1*, and *MMP3*), as well as downregulation of ECM components (*COL2A1* and *ACAN*) after IL-1β medium supplementation had been previously observed.[24]

MSC-based therapies aim to colonize and repopulate the degenerated IVD.[5] MSCs transplantation potential has been linked to their ability to differentiate into an NP cell phenotype, possibly acquiring NP cell–like function, producing IVD-like native ECM components, or promoting stimulation of endogenous IVD cells, and thus enabling anticatabolic and anti-inflammatory effects, as reviewed by Sakai and Anderson.[5] Nonetheless, the immunomodulatory role of MSCs and the communication/interplay between MSCs and IVD cells, in the context of IVD inflammation and degeneration, remains poorly understood. In this context, we cocultured 1×10^6 MSCs with IVD tissue, under the proinflammatory/degenerative conditions previously established.[24] Moreover, we chose the time point to begin the coculture based on studies in a rat disc stab injury model, which suggest that cell administration at an early stage of injury/disease progression might decrease matrix loss.[31] This was potentially linked to higher MSCs activity, due

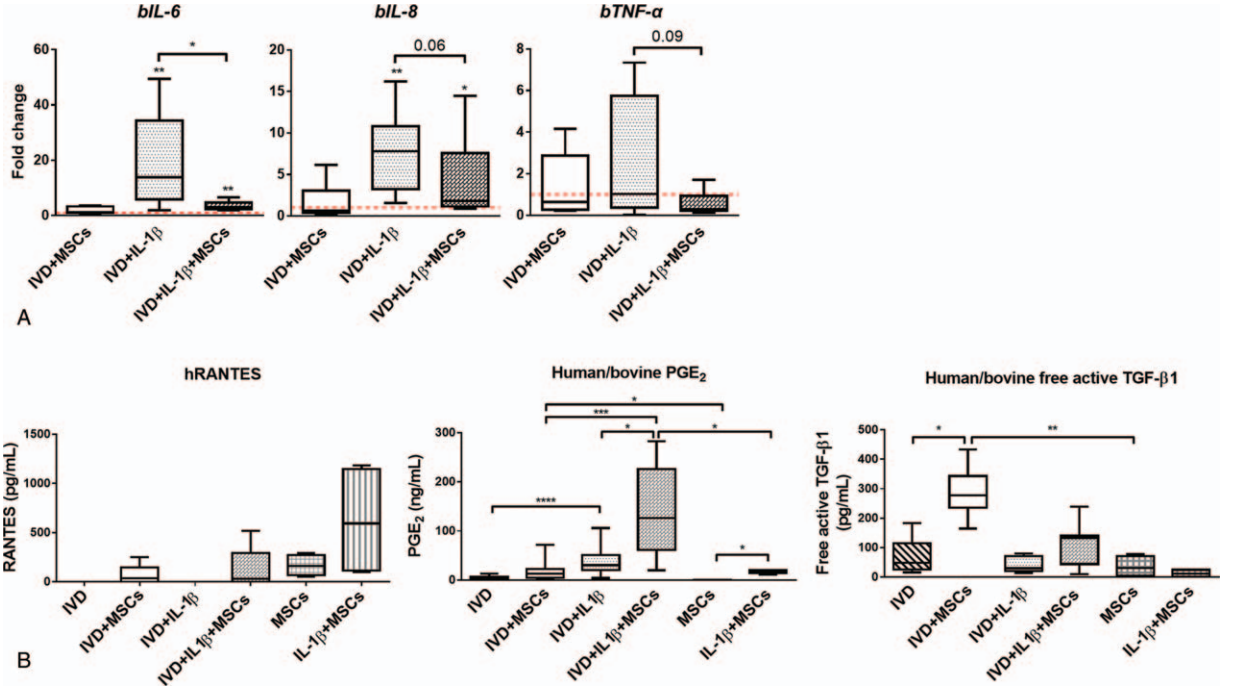

Figure 6. Inflammatory profile of bovine IVD cells and human MSCs, in control or proinflammatory/degenerative conditions. **A**, Two days after cocultures, mRNA expression of bovine *IL-6 (bIL-6)*, *bIL-8*, and *bTNF-α* by IVD cells. mRNA levels were normalized to *bGAPDH* control gene and to the unstimulated discs (control level = 1; dashed line). **B**, Human RANTES (hRANTES, pg/mL), bovine/human prostaglandin E_2 PGE_2 (ng/mL), and bovine/human transforming growth factor TGF-β1 (pg/mL) concentration in supernatants from MSCs cocultured with IVD tissue or cultured alone, under control, or proinflammatory conditions. Box and whiskers plots (n = 4–34). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

to the inflammatory microenvironment associated with injury.[31] In the present work, MSCs did not present an antiapoptotic effect, in contrast with findings from Yang *et al*.[32] Moreover, in presence of MSCs, there was an increase in late cell apoptosis/death for the control and IL-1β stimulated groups. Nonetheless, we hypothesize that this might be due to a higher consumption/less availability of nutrients, given the higher number of cells in culture. Of notice, there was higher MSCs migration in presence of IL-1β-stimulated IVDs, which might be due to the presence of IL-1β, known to be a chemotactic mediator for MSCs.[33] It has been reported that MSCs migration can be enhanced by degenerative cues and chemoattractor-delivery factors *ex vivo*, in bovine organ culture models,[34,35] as well as *in vivo* in a mouse tail-looping IVD degeneration model.[21]

Furthermore, in this proinflammatory/degenerative IVD model, MSCs exhibited an overall proinflammatory profile, producing higher amounts of IL-6, IL-8, and PGE_2, while free active TGF-β1 production seemed to have decreased. Nonetheless, MSCs contributed to a less proinflammatory profile of native IVD cells under proinflammatory/degenerative conditions. RANTES was previously described as key chemoattractant released by degenerative bovine IVD organ cultures, uncovering a relationship between concentrations of interleukin-1β and RANTES.[36] In our experiments, we did not observe differences in the concentration of human RANTES in culture media. However, the relative quantification of human and bovine RANTES by the array performed pointed out higher amounts of RANTES in the IVD culture medium in absence of MSCs. This suggests that RANTES might be consumed in the coculture, whereas the production of IL-6 and IL-8 is being further stimulated. On the contrary, Manferdini *et al*[20] observed a decrease in the production of inflammatory and chemotactic factors such as IL-6, IL-8, IL-1β, MCP-1, MIP-1α, and RANTES, when osteoarthritic chondrocytes or synoviocytes that produce high levels of inflammatory factors were cocultured with human adipose-derived MSCs.[20] In addition, this study also showed that COX-2/PGE_2 pathway may be one of the modulators of MSCs anti-inflammatory mechanism of action.[20] PGE_2 is known to be produced by both IVD cells[37] and MSCs,[38] in response to proinflammatory cytokine signaling, particularly IL-1β, as it was observed in this coculture model. Therefore, it has been proposed that MSCs can create a negative feedback loops as mode of action,[39] because the PGE_2 produced by MSCs will exert a regulatory influence on the activation status, proliferation, differentiation, and function of immune cells from adaptive and innate immunity.[40,41] MSCs have also been described to produce anti-inflammatory factors (IL-1 receptor antagonist [IL-1RA], TSG-6, IL-10, and IL-13), growth factors (TGF-β, CTGF, and GDF5), and anti-catabolic factors (TIMPs) in degenerated IVD microenvironment[15] and in osteoarthritic cartilage and synovium.[19]

Regarding TGF-β, it is known to enhance proteoglycans and COL2 in NP cells 3D cultures,[42] and it is conventionally

used to induce MSCs differentiation into an NP-like phenotype.[15] This cytokine has a potent regulatory and inflammatory activity and, among others, regulates MSCs immune responses.[41] In a human MSCs/IVD fragments coculture model, an upregulation of *TGF-β1* by MSCs, and a decrease of *TNF-α*, accompanied by upregulation of *IL-1β* expression by IVD cells, were observed over time in culture.[29] This contrasts with our results, which show a decrease of TGF-β1 production, related with a decrease of inflammatory markers *IL-6*, *IL-8*, and *TNF-α* expression, by bovine IVD cells. We hypothesize that it may be due to the differences between study models (human *vs.* bovine) and/or the culture stimulation with IL-1β.

Concerning matrix production, *MMP1* and *MMP3* expression by IVD cells was upregulated 2 days after coculture with MSCs and no stimulatory effect of MSCs was observed at ECM level after 16 days. Although MSCs stimulation of proteoglycans and COL2A1 production in IVD were reported in different literature models, the inflammatory environment was not addressed in those models.[43–45] Moreover, *in vivo* observations showed increased ECM components only after 12[46] to 48 weeks.[43] In agreement with our results, van Buul *et al*[19] did not observe upregulation of ECM gene expression in human osteoarthritic cartilage explants cultured with MSC-conditioned medium; the expression of *IL-1RA* was upregulated, whereas a disintegrin and MMP with thrombospondin motifs (ADAMTS)*5* and *COL2A1* were downregulated.

IVD organ cultures have been developed to address specific questions, although with limitations such as lack of vascularization and immune response, and the inability to perform pain assessment.[47] Concerning vascularization, since mature native IVD lacks vasculature, this limitation is of minor relevance in disc organ culture models. Another point to take into consideration is the fact that although the cell density in human disc is very low,[48,49] there is a need to repopulate the matrix of a degenerated IVD. For cell transplantation, MSCs expansion is required. However, during MSCs *ex vivo* expansion, these cells might become senescent, and overexpress MMPs, catabolic factors, and proinflammatory cytokines.[50] Moreover, increased proliferation may lead to a decrease in differentiation capacity.[51]

Here, MSCs seemed to have an anti-inflammatory, but not an anticatabolic effect on IVD cells. This mode-of-action seems to work *via* a feedback loop, with increasing production of proinflammatory factors by MSCs. The literature suggests that MSCs have an immunomodulatory response when in an inflammatory environment,[20] and that they are mainly triggered to first counteract inflammation instead of stimulating matrix formation.[19] The regenerative therapies proposed overtime have been intended to act at early stages of disease development, and to pursue less invasive, long-term effective and safe approaches.[5,15,52] Ideally, a single MSCs injection, at an early degenerative stage, should be able to modulate the IVD inflammatory status toward a pro-regenerative environment for MSCs to differentiate into IVD-like cells and, together with the remaining native cells, produce healthy ECM, while improving patients pain and mobility. In the future, it would be of interest to explore MSCs effect in more complex models of IVD degeneration/inflammation and associated pain, for example, in the presence of immune cells. Ultimately, the establishment of an experimental setup of IVD/macrophages coculture, under proinflammatory/degenerative conditions could be used to better evaluate the MSCs immunomodulatory effect.

➢ Key Points

- ❑ The aim of this study was to evaluate the regenerative and immunomodulatory behavior of MSCs in the proinflammatory/degenerative IVD.
- ❑ MSCs produce higher amounts of the proinflammatory molecules IL-6, IL-8, and PGE2 in co-culture with IVD and in presence of IL-1β.
- ❑ MSCs have an immunomodulatory effect in degenerated IVD cells, possibly based on a cytokine feedback loop.

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