

RESEARCH ARTICLE

Cytokine-induced interleukin-1 receptor antagonist protein expression in genetically engineered equine mesenchymal stem cells for osteoarthritis treatment

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Abstract

Background: A combination of tissue engineering methods employing mesenchymal stem cells (MSCs) together with gene transfer takes advantage of innovative strategies and highlights a new approach for targeting osteoarthritis (OA) and other cartilage defects. Furthermore, the development of systems allowing tunable transgene expression as regulated by natural disease-induced substances is highly desirable.

Methods: Bone marrow-derived equine MSCs were transduced with a lentiviral vector expressing interleukin-1 receptor antagonist (IL-1Ra) gene under the control of an inducible nuclear factor-kappa B-responsive promoter and IL-1Ra production upon pro-inflammatory cytokine stimulation [tumor necrosis factor (TNF) α , interleukin (IL)-1 β] was analysed. To assess the biological activity of the IL-1Ra protein that was produced and the therapeutic effect of IL-1Ra-expressing MSCs (MSC/IL-1Ra), cytokine-based two- and three-dimensional *in vitro* models of osteoarthritis using equine chondrocytes were established and quantitative real-time polymerase chain reaction (PCR) analysis was used to measure the gene expression of aggrecan, collagen IIA1, interleukin-1 β , interleukin-6, interleukin-8, matrix metalloproteinase-1 and matrix metalloproteinase-13.

Results: A dose-dependent increase in IL-1Ra expression was found in MSC/IL-1Ra cells upon TNF α administration, whereas stimulation using IL-1 β did not lead to IL-1Ra production above the basal level observed in nonstimulated cells as a result of the existing feedback loop. Repeated cycles of induction allowed on/off modulation of transgene expression. *In vitro* analyses revealed that IL-1Ra protein present in the conditioned medium from MSC/IL-1Ra cells blocks OA onset in cytokine-treated equine chondrocytes and co-cultivation of MSC/IL-1Ra cells with osteoarthritic spheroids alleviates the severity of the osteoarthritic changes.

Conclusions: Thus, pro-inflammatory cytokine induced IL-1Ra protein expression from genetically modified MSCs might represent a promising strategy for osteoarthritis treatment.

KEYWORDS

bone marrow-derived MSCs, cartilage defect, cell-based therapy, inducible promoter

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1 | INTRODUCTION

Osteoarthritis (OA) is a multifactorial condition of diverse aetiology that affects all tissues in synovial joints and culminates in structural and functional joint 'organ' failure, including loss and erosion of articular cartilage, subchondral bone remodelling, synovial inflammation and osteophyte formation.^{1,2} OA is the most common chronic articular disorder and affects most mammalian species, including humans, horses, dogs, cats and sheep, all of which exhibit a similar pathogenesis.³⁻⁷ The pathophysiological events start with the degradation of extracellular matrix components of the articular cartilage either as a result of accumulated repetitive micro-damage, a major traumatic event, or metabolic and biochemical factors, and are driven principally by an early innate immune response that progressively catalyses degenerative changes.^{1,8} The disease process is difficult to interrupt and results in a vicious circle of inflammation and degradation, leading to a gradual loss of joint function and pain.

A number of putative mediators, including proteases, pro-inflammatory cytokines, growth factors and radicals, are implicated in the process, although pro-inflammatory cytokines appear to play a dominant role in the initiation and progression of articular cartilage destruction by disturbing the dynamic articular equilibrium between anabolic and catabolic processes.⁹⁻¹⁴ Interleukin (IL)-1 β is one of the key arthritogenic triggers because it is much more potent than tumor necrosis factor (TNF) α with respect to inducing cartilage destruction, requiring only 0.1–1.0% of the TNF α dose to achieve the same proteoglycan synthesis inhibition in chondrocytes.¹⁵ In joints, IL-1 β is synthesized by chondrocytes, osteoblasts, synoviocytes and mononuclear cells and exerts its effect via binding to membrane receptor IL-1 receptor (IL-1R)1, whereas binding to IL-1R2 forms an inactive ligand-receptor complex that is unable to activate the intracellular signal.^{14,16-19} Both, IL-1R1 and IL-1R2 also bind IL-1 α and IL-1R antagonist (IL-1Ra). In OA, there are increased levels of IL-1 β in the synovial fluid, synovial membrane, cartilage and the subchondral bone layer and, furthermore, the expression of IL-1R1 on the surface of chondrocytes and synoviocytes is also increased.²⁰⁻²² IL-1 β , via activation of the transcription factors nuclear factor-kappa B (NF- κ B), p38MAPK and c-Jun N-terminal kinase and their downstream signalling cascade, decreases the synthesis of key extracellular matrix (ECM) proteins in chondrocytes, such as collagen II and proteoglycans (e.g. aggrecan) and stimulates the production of matrix degrading proteases, such as matrix metalloproteinase 1 (MMP-1), MMP-3, MMP-13 and ADAMTS-4, a disintegrin and metalloproteinase with thrombospondin motifs 4.²³⁻²⁹ Furthermore IL-1 β induces apoptosis in chondrocytes via upregulation of the Bcl-2 protein family members, mitochondrial depolarization, reactive oxygen species production and dysregulation of enzymatic antioxidant defenses in chondrocytes.³⁰⁻³²

Damaged articular cartilage has little or no healing capacity because it is hypocellular, avascular, aneural and alymphatic, has a dense ECM that limits the migration capacity of the sparse population of chondrocytes, and has low metabolic activity.^{3,33} Hence, once damage has occurred, cartilage lesions are likely to progress.

Current treatment strategies are only symptom modifying, leaving a large unmet need for efficacious disease-modifying therapies, which has resulted in a growing interest in regenerative medicine approaches

to OA. Based on our increasing understanding of the signalling pathways and key molecules interacting in the cascade of joint homeostasis and the pathophysiological processes involved in the development of OA, inflammatory mediators represent promising potential targets for therapeutic interventions designed to reduce both symptoms and structural joint damage in OA.³⁴⁻³⁸ Given the pivotal role of IL-1 as a pro-inflammatory cytokine in the development of OA, its natural opponent IL-1Ra has been investigated in OA treatment.³⁹⁻⁴² *In vitro* and animal models of IL-1 inhibition by IL-1Ra showed a reduction of cartilage destruction associated with this therapy.³³ Commercially available kits that produce autologous conditioned serum containing an upregulated higher amount of IL-1Ra compared to the concentration found in unprocessed blood samples are available for humans, horses and dogs. However, multiple joint injections are recommended to achieve clinically meaningful results.^{41,43} Therapeutic intervention to limit articular cartilage damage is likely most efficient early in disease progression when there is a possibility of preserving or re-establishing articular homeostasis and enabling anabolic activity to balance the catabolic pathways activated by inflammatory mediators.⁴⁴ However, even in established OA, modulation of the articular microenvironment could significantly contribute to the treatment success.

Gene therapy is able to deliver a therapeutic gene to the target tissue ensuring a more constant and longer lasting therapeutic effect without the need of frequently repeated invasive articular injections. Gene therapy applications to treat OA or rheumatoid arthritis have been published showing promising results.^{8,45-47} To interfere with joint homeostasis only when needed, an adjustable gene therapy is an attractive approach for fine-tuning any potential genetic treatment.⁴⁶ Inflammation inducible promoters have shown their great value in the context of tunable gene therapy. Such disease- (inflammation) responsive promoters will be 'switched on' only when pro-inflammatory cytokines are present, and will be silent again once the joint flair has been successfully treated.⁴⁸⁻⁵⁰ In these cases, stimulation of the regulatory elements to enhance transgene expression from the outside would not be needed as is the need with tetracycline responsive promoter systems.⁵¹ Indeed, the OA-affected joint would initiate the expression of the therapeutic gene and hence treatment when IL-1 β and TNF α are upregulated in the stage of inflammation.

A combination of tissue engineering methods employing progenitor cells such as mesenchymal stem cells (MSCs) or induced pluripotent stem cells together with gene therapy highlights a novel approach for targeting OA and cartilage defects and the early data reveal promising results.^{45,52,53} Genetically manipulated MSCs employed in *in vitro* and *in vivo* studies have shown their capacity to improve the quality of the repair tissue that would normally fill articular cartilage defects.⁵⁴⁻⁵⁷ MSC-based gene therapy approaches not only allow the delivery of a therapeutic gene (either anti-inflammatory or anabolic) to treat OA, but also open the possibility of delivering genes that enhance chondrogenesis such as TGF- β and hence promote the regenerative capacity of MSCs to fill the cartilage defect with a hyaline-like cartilage providing mechanical properties close to the natural healthy articular cartilage.^{46,55}

Recently, we have shown that equine MSCs can be efficiently transduced with lentiviral vector in which the firefly luciferase reporter gene was driven by an inflammation inducible promoter.⁴⁹

The present study aimed to investigate tuneable IL-1Ra expression in equine MSCs using a composite, NF- κ B-responsive enhancer/promoter regulated by the pro-inflammatory cytokines TNF α and IL-1 β . Accordingly, a bicistronic lentiviral vector carrying the gene for equine IL-1Ra under the control of the inducible NF κ B/minimal cytomegalovirus immediately early (minCMV) CMV promoter region was constructed and the ability to produce IL-1Ra via transduced equine MSCs stimulated with TNF α and IL-1 β was determined. In addition, the biological activity and therapeutic effect of the IL-1Ra protein produced from transduced MSCs was analysed *in vitro*.

2 | MATERIALS AND METHODS

2.1 | Preparation and characterisation of IL-1Ra-expressing equine MSCs

Plasmid pAlli10-target1916 harbouring the coding sequence of the equine IL-1Ra gene (GenBank accession number U92482) under the control of synthetic NF- κ B-responsive promoter identical to the one used in our previous work⁴⁹ was custom-synthesized by Trenzyme (Konstanz, Germany). To obtain vector plasmid pSEWNfKbIL-1Ra, a 753-bp EcoRI-EcoRI fragment containing the NF- κ B-responsive elements, the minimal CMV promoter and the equine IL-1Ra gene was isolated from pAlli10-target1916 plasmid and inserted into the lentiviral vector plasmid pHR'SIN-SEW⁵⁸ linearized using EcoRI. Recombinant lentivirus particles were produced and the respective titre was estimated as described previously.⁴⁹

MSCs were isolated from bone marrow collected from the iliac crest of a 3-year old male horse euthanized for reasons not related to the present study. MSCs were cultured as described previously.⁴⁹ MSCs were collected in accordance with the 'Good Scientific Practice. Ethics in Science und Research' regulation implemented at the University of Veterinary Medicine Vienna. The animal owner's consent to collect and analyse the material and to publish resulting data was obtained in accordance with the standard procedures of the University of Veterinary Medicine Vienna.

To obtain SEWNfKbIL-1Ra-transduced MSCs (MSC/IL-1Ra), cells in passage 4 were transduced with virus vector supernatant at a multiplicity of infection (MOI) of 6 in the presence of 8 μ g/ml polybrene. Transduction efficiency was determined by counting the number of enhanced green fluorescent protein (EGFP) expressing cells using fluorescence-activated cell sorting (FACS) analysis. Additionally, the number of EGFP copies in the genomic DNA (corresponding to the number of provirus integration events) was quantified by quantitative polymerase chain reaction (PCR). Accordingly, DNA was isolated utilizing the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) in accordance with the recommended protocol for cultured cells. EGFP copy numbers were determined by quantitative PCR (qPCR) using a gene specific TaqMan probe assay as described previously.⁵⁹ Quantitative PCR was carried out on the AriaMx Realtime PCR System (Agilent Technologies Inc., Santa Clara, CA, USA) using 25- μ l reaction mixes, including 20 ng of DNA template, 0.2mM each dNTP, 3mM MgCl₂, 1 \times buffer B2 (Solis BioDyne, Tartu, Estonia), 300 nM each primer, 200 nM probe, 50 nM ROX reference dye (Biotium, Fremont, CA,

USA) and 1 unit of HOT FIREPol DNA polymerase (Solis BioDyne). After initial denaturation at 95°C for 10 min, 45 amplification cycles of 95°C for 15 s and 60°C for 1 min were performed. A standard dilution series made of EGFP-containing plasmid DNA was included to determine EGFP copy numbers, as described previously.⁵⁹ EGFP copy numbers per cell were calculated, assuming a DNA content of 6.6 pg per one mammalian cell.

2.2 | Analysis of IL-1Ra protein production in transduced equine MSCs

To test the inducibility of IL-1Ra production, MSC/IL-1Ra cells were seeded at densities of 3×10^3 and 6×10^3 cells/well of a 96-well plate. Two different cell densities were tested with regard to the planned short term (IL-1Ra induction, decline after cytokine withdrawal) and long-term (repeated induction) experiments (see below). Twenty-four hours later, cells were stimulated with different concentrations (0.1, 5 and 10 ng/ml) of recombinant human IL-1 β or recombinant human TNF α (both ImmunoTools, Friesoythe, Germany) for 48 h based on the results of previous work using a vector design similar to that employed in the present study.⁴⁹ The IL-1Ra protein concentration was measured in the cell supernatant using the RayBio Equine IL-1Ra enzyme-linked immunosorbent assay (ELISA) Kit (RayBiotech, Inc., Norcross, GA, USA) in accordance with the manufacturer's recommendations and the Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland). Relative IL-1Ra production was calculated as fold increase of stimulated cells compared to nonstimulated cells. To correlate the IL-1Ra protein production with the cell amount seeded, cells were counted using Trypan Blue Stain 0.4% (Molecular Probes, Carlsbad, CA, USA) in a COUNTESS Automated Cell Counter (Invitrogen, Carlsbad, CA, USA) at the time of supernatant harvesting and the IL-1Ra protein production from 10 000 cells within 48 h was calculated (normalized production).

For analysis of IL-Ra decline after cytokine withdrawal, 6×10^3 MSC/IL-1Ra cells were seeded per well into a 96-well plate. After 24 h, cells were stimulated with 10 ng/ml TNF α for 48 h and, subsequently, the medium was changed every day. After 2, 4 and 6 days, the IL-1Ra protein concentration was measured and relative IL-1Ra production was calculated as described above.

For repeated induction experiments, 3×10^3 MSC/IL-1Ra cells were seeded per well of a 96-well plate and stimulated after 24 h with 10 ng/ml TNF α : first stimulation for 48 h - 144 h off-cytokine (first drop-down) - second stimulation for 48 h - 144 h off-cytokine (second drop-down). At all indicated time points, the IL-1Ra protein concentration was measured and relative IL-1Ra production was calculated as described above. All experiments were performed in duplicate with at least two independent experimental replicates.

2.3 | Analysis of IL-1Ra gene expression in transduced equine MSCs

IL-1Ra gene expression was analysed using real time q-PCR (RT-qPCR). Therefore, the MSC/IL-1Ra cells were seeded at a density of 1.4×10^5 cells/well in the six-well plate. Twenty-four hours later, cells were stimulated with 10 ng/ml TNF α for 48 h and, subsequently, the medium was changed every day. At the indicated time points

(stimulation for 48 h followed by cytokine withdrawal for 24, 48, 72, 96, 120, 144 and 168 h), cells were harvested using 500 μ l of QIAzol lysis reagent (Qiagen) and stored at -80°C until further processing. RNA extraction was performed using the Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, CA, USA) in accordance with the recommended protocol, including the in-column DNase I treatment. Chondrocytes spheroids were mechanically homogenised on a MagNA Lyser instrument (Roche, Basel, Switzerland) using 1.4-mm ceramic beads at 6500 rpm for 25 s prior to RNA extraction. RNA concentrations were measured on the NanoDrop 2000c UV spectrophotometer (Thermo Scientific, Waltham, MA, USA). The RNA integrity of selected samples was assessed on the Agilent 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies Inc.). The determined RNA integrity numbers ranged from 9.1 to 10. For RT-qPCR, 1 μ g of total RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Scientific) in accordance with the manufacturer's instructions. Controls without RT-enzyme were included to monitor for PCR amplification of residual DNA. RT-qPCR primers were designed using the PrimerQuest assay design

tool (<http://eu.idtdna.com/PrimerQuest/Home/Index>; Integrated DNA Technologies, Coralville, IA, USA) or taken from the literature.^{60,61} Assay details are provided in Table 1. All assays were validated by the generation of standard curves for determining PCR reactions efficiencies (Table 1). RT-qPCR was performed in 20- μ l reaction volumes including 20 ng of cDNA, 200 nM each primer and 1 \times Kapa Sybr Fast qPCR Master Mix ROX Low (Kapa Biosystems, Wilmington, MA, USA). All samples were analysed in duplicate on the AriaMx Realtime PCR System (Agilent Technologies Inc.) using the temperatures: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 3 s and 60°C for 20 s, and a melting curve analysis over a temperature range of 65°C to 95°C . Four candidate reference genes (RGs) GAPDH, REEP5, SNRPD3 and VCP were included for normalization.⁶¹ The expression stability of all RGs was assessed using the BestKeeper analysis tool,⁶² identifying VCP as the most stable gene [SD (\pm Cq) VCP: 0.44, REEP5: 0.55, GAPDH: 0.57, SNRPD3: 0.66]. Target gene expression levels were normalized to those of VCP and relative expression changes were calculated using the comparative $2^{-\Delta\Delta\text{CT}}$ method.⁶³

TABLE 1 Primer used for RT-qPCR

Accession number*	Gene symbol	Gene name	Oligo	Sequence (5' to 3')	Amplicon size (bp)	PCR efficiency (%)	Reference
XM_014733894.1, XM_005602799.1	ACAN	<i>Equus caballus</i> aggrecan	Forward Reverse	CCTGACTCCAGTGGTCTTATC GTCGTGGACCACCTAATTCTATC	99	0.952	–
NM_001081764.1, XM_005611082.2	COL2A1	<i>Equus caballus</i> collagen type II alpha 1 chain	Forward Reverse	GCCCGTCTGCTTCTTGTAATA CGTGACTGGGATTGGAAAGT	91	0.932	–
NM_001163856.1	GAPDH	<i>Equus caballus</i> glyceraldehyde-3-phosphate dehydrogenase	Forward Reverse	GGCAAGTTCATGGCACAGT CACACATATTCAGCACCAGCAT	129	0.904	⁶¹
NM_001317261.1, XM_001495926.4	IL1B	<i>Equus caballus</i> interleukin 1 beta	Forward Reverse	GGCCTCAAGGAAAAGAACCT TGGGTACATTGCAGACTCA	175	0.924	⁶⁰
XM_014730883.1, XM_005599766.2	IL1RN	<i>Equus caballus</i> interleukin 1 receptor antagonist	Forward Reverse	GACACCTAATCTCTCTCTCTCT GATTCTGAAGGCTTGATCTTTG	95	0.997	–
NM_001082496.2	IL6	<i>Equus caballus</i> interleukin 6	Forward Reverse	CAAGCACCGTCACTCCAGTTGC CATCTTCTCCCAGGGTAGTGGG	90	0.944	⁶⁰
NM_001083951.2	IL8	<i>Equus caballus</i> C-X-C motif chemokine ligand 8	Forward Reverse	TGCTTTCTGCAGCTCTGTGT GCTCCGTTGACGAGCTTTAC	181	0.949	⁶⁰
NM_001081847.2	MMP1	<i>Equus caballus</i> matrix metalloproteinase 1	Forward Reverse	CCGAAGGGAACCTCGGTGG TGGCTGGTCCACATCTGCTC	93	0.919	⁶⁰
NM_001082495.2	MMP3	<i>Equus caballus</i> matrix metalloproteinase 3	Forward Reverse	GGCAACGTAGAGCTGAGTAAAGCC CAACGGATAGGCTGAGCACGC	91	0.914	⁶⁰
NM_001081804.1	MMP13	<i>Equus caballus</i> matrix metalloproteinase 13	Forward Reverse	GCCTCAAAGTTTGGTCTGATG GGTAGAAGTACCATGCTCTTT	109	0.914	–
NM_001081935.1, XM_014735868.1, XM_014735869.1 XM_005599722.1	PTGES REEP5	<i>Equus caballus</i> prostaglandin E synthase <i>Equus caballus</i> receptor accessory protein 5	Forward Reverse Forward Reverse	CGCTCCTGAAGGCTGTAAAT CAGATACTGAAGGAGCCAGTAAAG CCTGAAGCACGAGTCCCAG CCCAGTAAATTCACGGCAGC	83 114	0.932 0.925	– ⁶¹
XM_001489060.4	SNRPD3	<i>Equus caballus</i> small nuclear ribonucleoprotein D3 polypeptide	Forward Reverse	ACGCACCTATGTTAAAGAGCATG CACGTCCCATTCCACGTC	120	0.995	⁶¹
NM_001081819.2, XM_005603490.1	TNF	<i>Equus caballus</i> tumor necrosis factor	Forward Reverse	TTCTCGAACCCCAAGTGACAAG GCTGCCCTCGGCTT	65	0.927	⁶⁰
XM_005605574.1	VCP	<i>Equus caballus</i> valosin containing protein	Forward Reverse	GAGTGAGATCAGGCGAGAACG CCTCTTCCACCTCCATGGC	56	0.925	⁶¹

*National Center for Biotechnology Information (NCBI), Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>).

2.4 | Cytokine-induction of OA-like processes in equine chondrocytes *in vitro*

To isolate equine chondrocytes, articular cartilage of a 4-year-old horse euthanized for reason unrelated to the presented research topic was obtained by dissection, cut into small pieces and processed as described previously.⁶⁴ Isolated chondrocytes were cultivated in cell culture medium consisting of Dulbecco's modified Eagle's medium low glucose/Ham's F12 (1:1), supplemented with 10% fetal bovine serum, antibiotic/antimycotic mix solution (100 000 units penicillin, 100 mg of streptomycin and 250 µg of amphotericin B per litre medium) and an additional 1.25 µg/ml amphotericin B (chondrocyte medium; all chemicals purchased from Sigma, St Louis, MO, USA). Cells were frozen after first passage and used in the experiments no later than in passage 3.

To set up the two-dimensional (2D) OA *in vitro* model, 2×10^5 equine chondrocytes per well were seeded in a six-well plate and grew in a monolayer. Forty-eight hours later, culture medium was changed to a fresh one containing either IL-1β or TNFα at a concentration of 5, 10 or 50 ng/ml. After 24, 48 and 72 h, cells were harvested using QIAzol lysis reagent and subjected to RT-qPCR as described above. The expression pattern of the following OA-related genes was analysed: aggrecan (ACAN), collagen IIA1 (COL2A1), IL-1β, interleukin-6 (IL-6), interleukin-8 (IL-8), MMP1, MMP3, MMP13, TNFα, prostaglandin-E2 synthase (PTGES) (Table 1). Expression patterns (target genes normalised to VCP) of cytokine-treated cells were compared with mRNA expression in nontreated cells set to 1.

To set up the three-dimensional (3D) OA *in vitro* model, 5×10^5 equine chondrocytes per Eppendorf Tube were centrifuged (200 × g for 1 min at room temperature) and allowed to form spheroids within 24 h of cultivation. Afterwards, four to six spheroids were transferred per well of a six-well plate and incubated in fresh medium containing either IL-1β, TNFα or a combination of both (10 ng/ml). After 24, 48 and 72 h, the expression pattern of OA-related genes (IL-6, IL-8, MMP1, MMP13, ACAN, COL2A1; normalised to VCP) was analysed using RT-qPCR as described above and cytokine-treated spheroids were compared with mRNA expression of nontreated spheroids arbitrarily set to 1.

2.5 | Application of conditioned medium produced from MSC/IL-1Ra cells

To evaluate the biological activity of the produced IL-1Ra protein, conditioned medium from MSC/IL1Ra cells was tested in the 2D OA *in vitro* model. To produce conditioned medium, MSC/IL-1Ra cells were seeded at a concentration of 1.5×10^6 cells per T75 flask and stimulated with 10 ng/ml TNFα 24 h later or left unstimulated. After 48 h of stimulation, medium was changed for a fresh one without cytokine. Twenty-four hours later, conditioned medium was harvested and centrifuged (600 × g for 5 min at room temperature) to avoid cell carry-over. To exclude the possibility that effects are related to other factors produced from MSCs, conditioned medium of stimulated and unstimulated nontransduced MSCs was produced accordingly. Conditioned media were applied to chondrocytes (2×10^5 cells/well of the six-well plate seeded 48 h earlier) concomitantly with 10 ng/ml IL-1β.

In a second approach, chondrocytes were pre-treated with conditioned medium for 24 h and subsequently treated with 10 ng/ml IL-1β. After 24 h, RT-qPCR analysis of ACAN, COL2A1, IL-6, IL-8, MMP1 and MMP13 was performed as described above. Chondrocytes cultured in MSC medium containing IL-1β for OA induction were used as controls and mRNA levels obtained in these cells were arbitrarily set to 1.

2.6 | Co-cultivation of MSC/IL-1Ra cells with osteoarthritic chondrocytes

To model a therapeutic approach, MSC/IL-Ra cells were co-cultured with osteoarthritic chondrocyte spheroids (3D OA model). Therefore, spheroids were prepared as described above, transferred to a six-well plate, and OA was induced by adding either IL-1β or TNFα, or a combination of both (10 ng/ml). After 24 h of OA induction, transwell inserts containing MSC/IL-Ra cells [seeded separately 24 h earlier at the concentration of 1×10^5 cells/insert (0.4 µm pore size, polycarbonate membrane)] were added to the chondrocytes. Without a change of medium (i.e. in the presence of the cytokines mentioned above), chondrocyte spheroids were co-cultivated with MSC/IL-RA cells for an additional 24, 48 or 72 h when RT-qPCR analysis of ACAN, COL2A1, IL-6, IL-8, MMP1 and MMP13 was performed as described above. Chondrocyte spheroids grown in the absence of MSC/IL-1Ra cells but treated with the respective cytokines for OA induction were used as controls and their mRNA levels were arbitrarily set to 1.

2.7 | Statistical analysis

For statistical analysis of the IL-1RA protein concentration measured via ELISA, as well as mRNA expression of OA-related genes assessed by RT-qPCR, the results of the respective control groups were arbitrary set to 1 and a relative increase or decrease was computed (as the fold change). Mean values and 95% confidence intervals were calculated from the technical replicates. Lower confidence limits greater than 1 (i.e. increase) and upper confidence limits less than 1 (i.e. decrease) were assumed to be significant.

3 | RESULTS

3.1 | Preparation and characterisation of IL-1Ra-expressing equine MSCs

Upon successful isolation from bone marrow, MSCs were transduced with the lentivirus vector SEWNFKBIL-1Ra. In this vector, IL-1Ra expression in transduced cells is driven by the inducible NF-κB/minCMV promoter, whereas EGFP expression is ensured from the constitutively active SFFV promoter. A single round of transfection using VSV-G (i.e. vesicular stomatitis virus G-protein) pseudotyped SEWNFKBIL-1Ra vector particles at a MOI of 6 resulted in more than 90% transduced cells (MSC/IL-1Ra), as determined by EGFP-specific FACS analysis. The qPCR revealed 1.06 ± 0.07 EGFP copies per cell, resulting statistically in one integrated provirus molecule per transduced MSC.

3.2 | Induction of IL-1Ra expression in transduced equine MSCs

Inducibility of the IL-1Ra protein production was measured in the supernatant of MSC/IL-1Ra cells stimulated for 48 h with different concentrations of IL-1 β or TNF α using species-specific ELISA. In nonstimulated MSC/IL-1Ra cells, basal IL-1Ra protein levels (7.3 and 13.9 ng/ml/48 h for 3×10^3 and 6×10^3 cells seeded) were detected as a result of the presence of minimal CMV promoter sequences in the synthetic NF- κ B-inducible promoter. As expected, TNF α induced IL-1Ra protein expression in a dose-dependent manner (Figure 1A). An up to five-fold increase in IL-1Ra production was observed in cells stimulated with 10 ng/ml TNF α . Interestingly, no changes in IL-1Ra protein production were observed upon IL-1 β stimulation (Figure 1A). Therefore, the subsequent induction experiments were performed using TNF α only. The cell seeding density influenced the absolute IL-1Ra protein production over the definite period of time; however, the relative changes (fold-changes) remained constant irrespective of the cell amount seeded (Figure 1A). In non-infected, parental MSCs, no IL-1Ra protein was detected. The IL-1Ra protein production per 10^4 cells was similar irrespective of the initial seeding density (Figure 1B). In 48 h, nonstimulated cells produced approximately 1 ng/ml IL-1Ra protein, and protein concentration was increased to approximately 4 ng/ml in cells stimulated with 10 ng/ml TNF α (Figure 1B).

In the next step, we analysed the repeated inducibility of IL-1Ra expression from MSC/IL-1Ra cells. However, use of the same experimental set-up as that employed in our previous work (48 h of induction followed by 48 h of off-stimulation)⁴⁹ was not sufficient to bring the IL-1Ra protein levels back to those observed in nonstimulated MSC/IL-1Ra cells. Accordingly, we analysed the decline of the IL-1Ra expression over time in stimulated MSC/IL-1Ra cells both at mRNA and protein levels. Upon stimulation with 10 ng/ml TNF α for 48 h, IL-1Ra mRNA levels in stimulated cells were significantly higher (approximately 9.5-fold) compared to nonstimulated cells (Figure 2A). IL-1Ra mRNA expression continuously decreased over the following days, reaching the basal level between days 4 and 7 after TNF α withdrawal (Figure 2A). Similarly, IL-1Ra protein production in MSC/IL-1Ra cells stimulated with 10 ng/ml TNF α declined in the absence of TNF α stimulus over time (Figure 2B).

Protein levels returned to baseline at day 6 after cytokine withdrawal (Figure 2B). Therefore, 6 days without cytokine stimulation was considered sufficient for the repeated stimulation experiment.

Finally, MSC/IL-1Ra cells were repeatedly stimulated with 10 ng/ml TNF α (stimulation for 48 h – 144 h withdrawal of cytokine – second stimulation for 48 h – second 144 h withdrawal of cytokine). After the first stimulation, the IL-1Ra protein levels were increased six- to seven-fold followed by regress to IL-1Ra protein basal levels after 6 days of cultivation without TNF α (Figure 2C). Repeated stimulation also yielded a statistically significant three-fold increase in IL-1Ra protein production. Another 6 days without the cytokine stimulus resulted in IL-1Ra protein basal levels again (Figure 2C).

3.3 | 2D osteoarthritis *in vitro* model

In the 2D OA *in vitro* model, the expression pattern of ten OA-related genes (IL-1 β , IL-6, IL-8, TNF α , MMP1, MMP3, MMP13, COL2A1, ACAN, PTGES) of equine chondrocytes was analysed after IL-1 β or TNF α stimulation (Figure 3; see also Supporting information, Figure S1). Compared to nontreated cells, mRNA expression of ACAN and COL2A1 was statistically significant decreased after both IL-1 β or TNF α stimulation (Figure 3; see also Supporting information, Figure S1). Based on these observations, analysis of ACAN, COL2A1, IL-6, IL-8, MMP1 and MMP13 mRNA levels and treatment with the respective cytokine(s) (10 ng/ml) was chosen for further experiments to monitor osteoarthritic changes in further experiments using OA *in vitro* models.

3.4 | Application of conditioned medium produced from MSC/IL-1Ra cells

To evaluate the biological activity of the IL-1Ra protein produced by the genetically modified MSCs, conditioned medium from these cells was

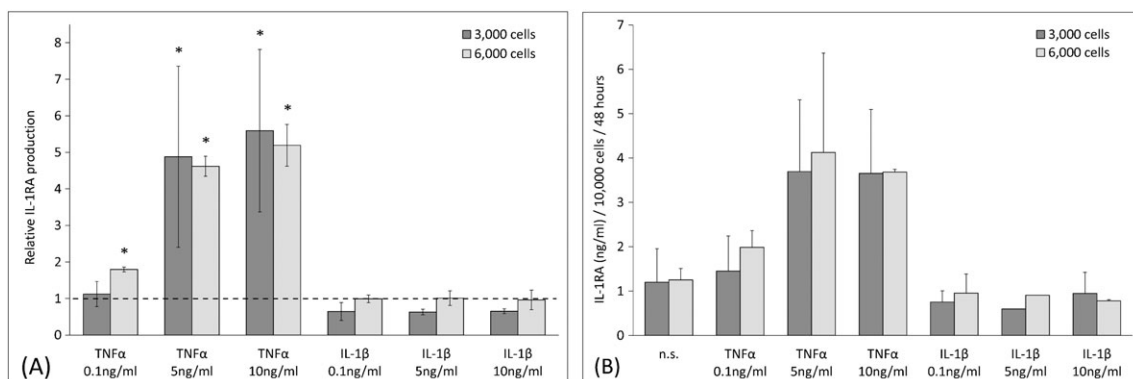


FIGURE 1 Inducibility of IL-1Ra protein production in MSC/IL-1Ra cells upon cytokine stimulation. Relative increase of IL-1Ra protein amount (A) in the supernatant of transduced MSCs compared to the respective nonstimulated cells arbitrary set to 1. Absolute IL-1Ra protein production (B) in 48 h calculated per 10 000 cells. Cells were seeded in a concentration of 3×10^3 or 6×10^3 cells/well of a 96-well plate and stimulated with different concentrations of TNF α and IL-1 β for 48 h. The results (*) are considered significant with a lower confidence limit set greater than 1. Data show mean values; whiskers represent 95% confidence intervals

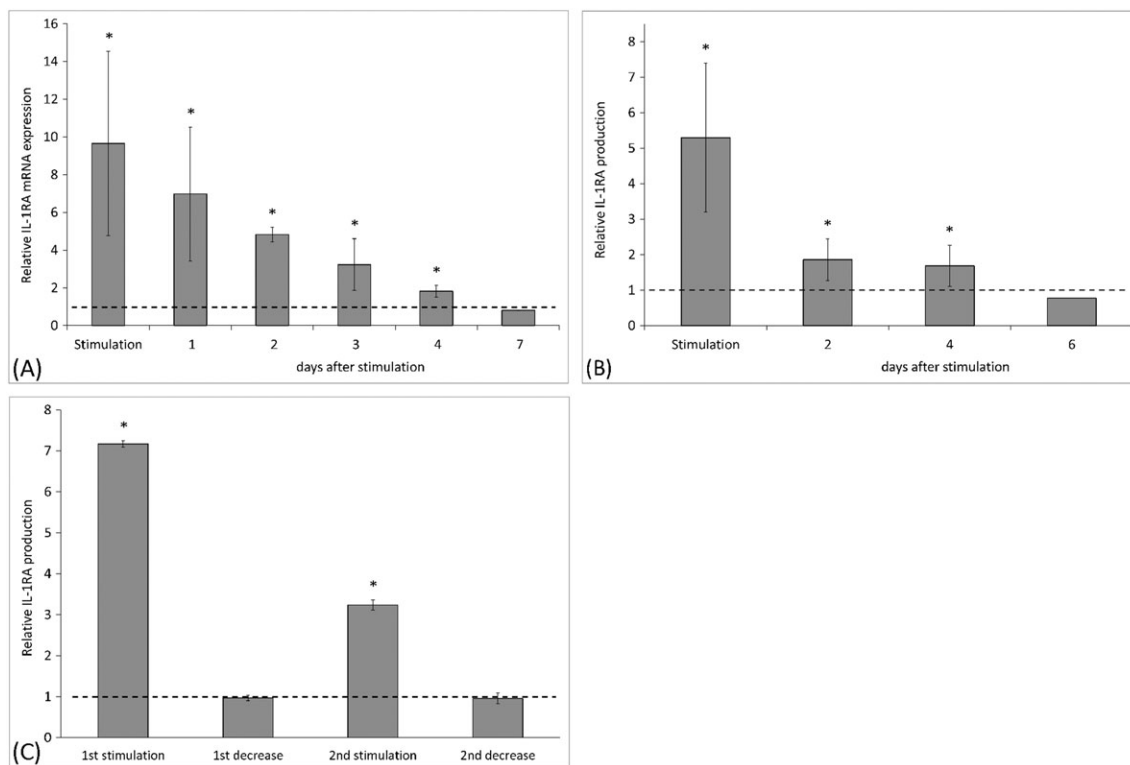


FIGURE 2 Decline of IL-1Ra expression after stimulus withdrawal (A, B) as a prerequisite for repeated inducibility of IL-1Ra production in MSC/IL-1Ra cells (C). To analyse the decrement in transgene expression, cells were stimulated with 10 ng/ml TNF α for 48 h and subsequently cultivated in the absence of cytokine. Relative changes in IL-1Ra mRNA expression (A) and IL-1Ra protein levels (B) in MSC/IL-1Ra cells are shown compared to the basal expression of nonstimulated MSC/IL-1Ra cells arbitrary set to 1. To analyse repeated transgene inducibility, cells were stimulated with 10 ng/ml TNF α for 48 h (first increase) and subsequently kept for another 144 h in standard medium (first decrease). The same stimulation regime was repeated afterwards (second increase and second decrease). The results (*) are considered significant with a lower confidence limit set greater than 1. Data show mean values; whiskers represent 95% confidence intervals

tested in the 2D OA *in vitro* model and mRNA levels of ACAN, COL2A1, IL-6, IL-8, MMP1 and MMP13 were analysed (Figure 4; see also Supporting information, Figure S2). To exclude the possibility that the effects are related to other factors produced from MSCs, conditioned medium of nontransduced MSCs was used as a control. Application of 24-h conditioned medium of stimulated and nonstimulated MSC/IL-1Ra cells together with 10 ng/ml IL-1 β resulted in a 2.5-fold increase of ACAN mRNA levels, whereas only minor changes were observed in COL2A1 expression levels (Figure 4). Furthermore, chondrocytes treated with conditioned medium from stimulated and nonstimulated MSC/IL-1Ra cells revealed significantly decreased mRNA levels of IL-6, IL-8, MMP1 and MMP13. Conditioned medium from stimulated and nonstimulated, nontransduced MSCs resulted in unchanged expression levels of ACAN but revealed up-regulation of IL-6, IL-8 and MMP1 compared to control chondrocytes with OA phenotype. Only marginal differences were observed between conditioned medium from stimulated and nonstimulated naïve MSCs as well as MSC/IL-1Ra cells. Similar changes in gene expression for ACAN, COL2A1, MMP1 and MMP13 were obtained when chondrocytes were pre-treated with the conditioned medium and the OA-changes were induced 24 h later; however, up-regulation of IL-6 and IL-8 was not seen (Supporting information, Figure S2).

3.5 | 3D osteoarthritis *in vitro* model

To better mimic the natural situation, chondrocytes were grown in spheroids. For this 3D OA *in vitro* model, compact spheroids were

formed within 24 h after a single centrifugation step and cultivation of cells on a non-adherent surface. Pre-formed spheroids were subjected to cytokine treatment and mRNA expression of ACAN, COL2A1, IL-6, IL-8, MMP1 and MMP13 genes was analysed (Figure 5). Generally, changes in mRNA expression were stronger in the 3D OA model compared to the 2D OA model. Stronger effects were produced by combined treatment (IL-1 β + TNF α) and longer stimulation (24 h versus 72 h). As expected, matrix related gene expression was reduced by approximately 60% (ACAN) and 90% (COL2A1) in cytokine-treated spheroids, whereas expression of pro-inflammatory cytokines (IL-6, IL-8) and matrix-degrading proteases (MMP1, MMP13) was increased by up to 400-fold (Figure 5). Based on these results, 24 h of cytokine treatment was considered to be sufficient for OA induction in the 3D *in vitro* model.

3.6 | Co-cultivation of MSC/IL-1Ra cells with osteoarthritic chondrocytes

To model a therapeutic approach, OA-induced chondrocyte spheroids were co-cultured with MSC/IL-1Ra cells in the presence of pro-inflammatory cytokines (IL-1 β , TNF α or a combination of both). Expression levels of ACAN, COL2A1, IL-6, IL-8, MMP1 and MMP13 were compared with those of osteoarthritic chondrocytes grown in the absence of MSC/IL-1Ra cells (Figure 6). The mRNA levels of ACAN and COL2A1 genes were significantly increased. A significant

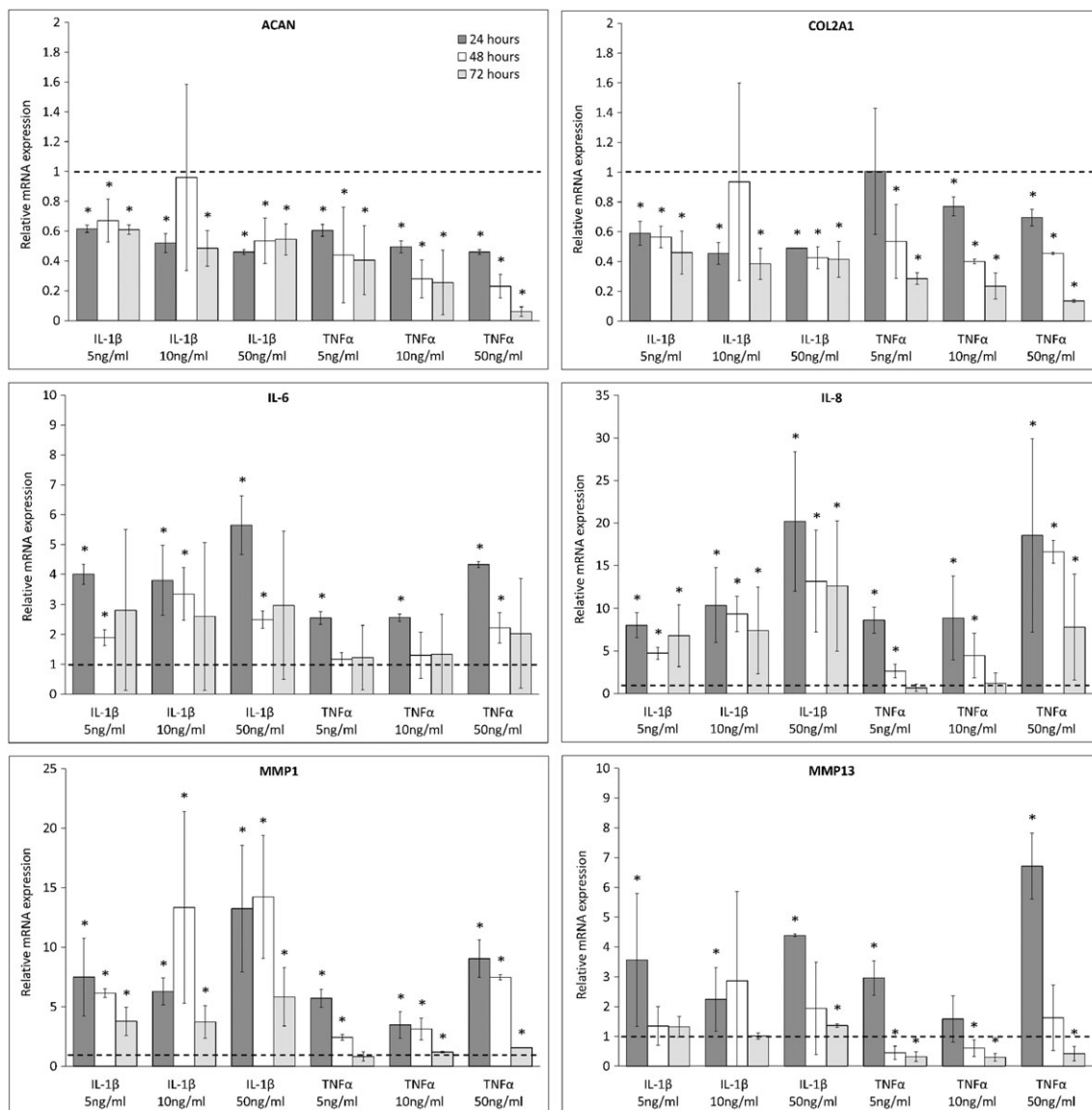


FIGURE 3 Changes in OA-related gene expression in the 2D osteoarthritis *in vitro* model. Equine chondrocytes grown in a monolayer were treated with different concentrations of IL-1 β or TNF α for 24, 48 and 72 hours. The mRNA levels of aggrecan, collagen IIA1, interleukin-6, interleukin-8, matrix metalloproteinase-1 and matrix metalloproteinase-13 were measured using RT-qPCR. Relative changes in mRNA levels of cytokine-treated chondrocytes are shown compared to nontreated chondrocytes arbitrary set to 1. Lower confidence limits greater than 1 and upper confidence limits less than 1 were assumed as statistically significant (*). Data show mean values; whiskers represent 95% confidence intervals

decrease in IL-6, MMP1 and MMP13 expression levels was observed in co-cultured chondrocytes, whereas the changes in IL-8 mRNA levels were less obvious and inconsistent. These effects were most distinct after OA induction with IL-1 β and less prominent after treatment with TNF α and the combination of IL-1 β and TNF α .

4 | DISCUSSION

As a result of the limited self-renewal capacity of adult articular cartilage, cartilage defects and osteoarthritis lead to intense investigation on clinically applicable cartilage regenerating techniques.⁶⁵ MSCs represent an interesting cell source for cartilage repair because of their multilevel action (mostly paracrine activity, organelle and molecule transfer via tunnelling nanotubes, exosome and microvesicle transfer

of molecules, as well as marginally also differentiation into replacement cell type)⁶⁶⁻⁶⁸ and several preclinical and clinical trials have provided initial evidence of their efficacy and safety in the treatment of OA.^{69,70} However, MSCs have a tendency to undergo hypertrophic differentiation and, when exposed to an inflammatory environment, they show a reduced chondrogenic differentiation capacity.⁷¹⁻⁷⁴ Hence intra-articular application of MSCs has not yet achieved full restoration of hyaline cartilage. Employing the gene therapy concept to increase cartilage regeneration by expression of specific genes (e.g. anti-inflammatory cytokines, growth and transcription factors) represents another promising therapeutic strategy.⁷⁵ Combining cellular therapy using MSCs with gene transfer can combine the advantages of both innovating strategies and might help to overcome challenges that arise from using naïve MSCs. However, when using gene transfer techniques resulting in host genome

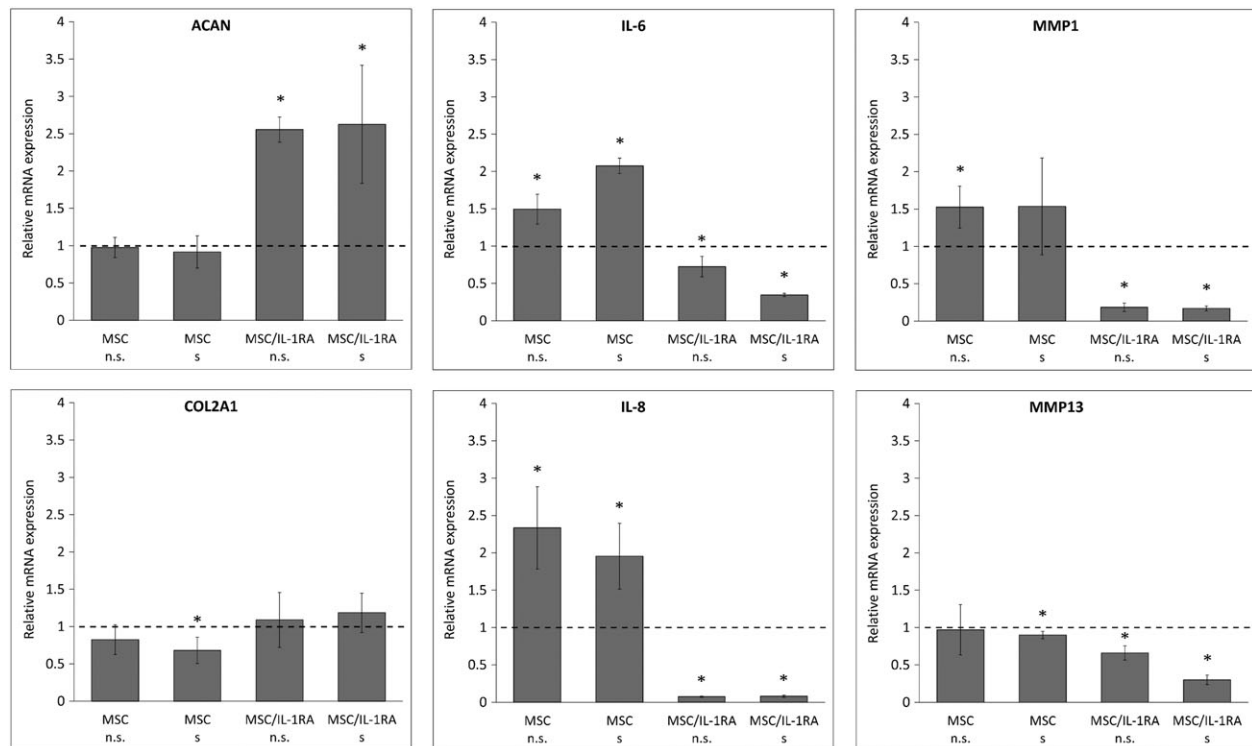


FIGURE 4 Conditioned medium of MSC/IL-1Ra cells blocks OA onset in cytokine treated equine chondrocytes. Cytokine-free conditioned medium from TNF α -stimulated (s) or nonstimulated (n.s.) MSCs or MSC/IL-1Ra cells was transferred onto equine chondrocytes grown in a monolayer and OA changes were induced by concomitant application of 10 ng/ml IL-1 β . After 24 h, mRNA levels of aggrecan, collagen IIA1, interleukin-6, interleukin-8, matrix metalloproteinase-1 and matrix metalloproteinase-13 were measured using RT-qPCR. Chondrocytes cultured in normal medium containing IL-1 β for OA induction were used as controls and mRNA levels obtained in these cells were arbitrary set to 1. Relative changes in gene expression in conditioned medium-treated chondrocytes are shown. Lower confidence limits greater than 1 and upper confidence limits less than 1 were assumed as statistically significant (*). Data show mean values; whiskers represent 95% confidence intervals

integration of the therapeutic cargo, which is usually necessary for persisting gene transfer, concerns associated with genotoxicity and mutagenic effects have to be considered. To minimize this risk, we have used the third generation lentivirus vector system. These vectors, which are well characterized with the existing regulatory framework to achieve its successful translation into the clinical stage, were recently used for gene addition to haematopoietic stem cells and have yielded clinical benefits without vector-related complications.^{76,77}

Controllable and tunable transgene expression is highly desirable to maintain joint homeostasis. We have previously shown that equine MSCs can be efficiently transduced with a lentiviral vector harbouring a reporter gene under the control of an inflammation inducible promoter and the transgene expression can be induced by pro-inflammatory cytokines IL-1 β or TNF α .⁴⁹ To further elaborate this strategy in the present study, we have analysed the feasibility of a biological active MSC-based gene therapy in an *in vitro* model of OA. The gene encoding the IL-1Ra protein was chosen as therapeutic gene because it was shown that administration of IL-1Ra (either recombinant or present in autologous conditioned serum) is efficient in the treatment of cartilage degeneration in OA.⁷⁸⁻⁸⁰ Furthermore, gene therapy approaches utilizing virus-mediated transfer of the IL-1Ra gene into chondrocytes *in vitro* and *in vivo* revealed therapeutic potential.⁸¹⁻⁸³

IL-1Ra expression was increased by up to five-fold in MSC/IL-1Ra cells stimulated with TNF α , which is in line with our data based on

transduced MSCs with NF- κ B-inducible luciferase expression (MSC/Luc) using the same NF- κ B-promoter reported previously.⁴⁹ Upon IL-1 β stimulation, no changes in IL-1Ra protein production were observed compared to baseline levels. Because, in our previous study, both cytokines induced luciferase expression in MSC/Luc cells in a dose-dependent manner, we presume that baseline IL-1Ra protein production from MSC/IL-1Ra cells occupies the IL-1 β receptor, blocks IL-1 β binding and prevents promoter induction. Therefore, the IL-1Ra protein expression levels following IL-1 β stimulation remain similar to those measured in nonstimulated MSC/IL-1Ra cells. This finding represents the first proof of biological activity of the expressed transgene.

Transgene expression was also detected in nonstimulated MSC/IL-1Ra cells as a result of the presence of the minimal CMV promoter sequence within the inducible promoter, which is in agreement with our previous observations,⁴⁹ as well as with the observations of other studies.⁸⁴ The concentration of IL-1Ra protein produced from nonstimulated MSC/IL-1Ra cells (1.2 ng/ml/10 000 cells/48 h) is within the range of IL-1Ra levels in autologous conditioned serum.^{39,85}

The use of tightly regulated inducible promoters such as those described recently by Mohamed *et al.*⁵⁰ might increase the specificity of expression.

Surprisingly, the decrease in IL-1Ra expression in MSC/IL-1Ra cells after TNF α withdrawal was three times slower (both on mRNA and protein levels) than the drop in luciferase expression previously observed in MSC/Luc cells,⁴⁹ probably as a result of the short half-life

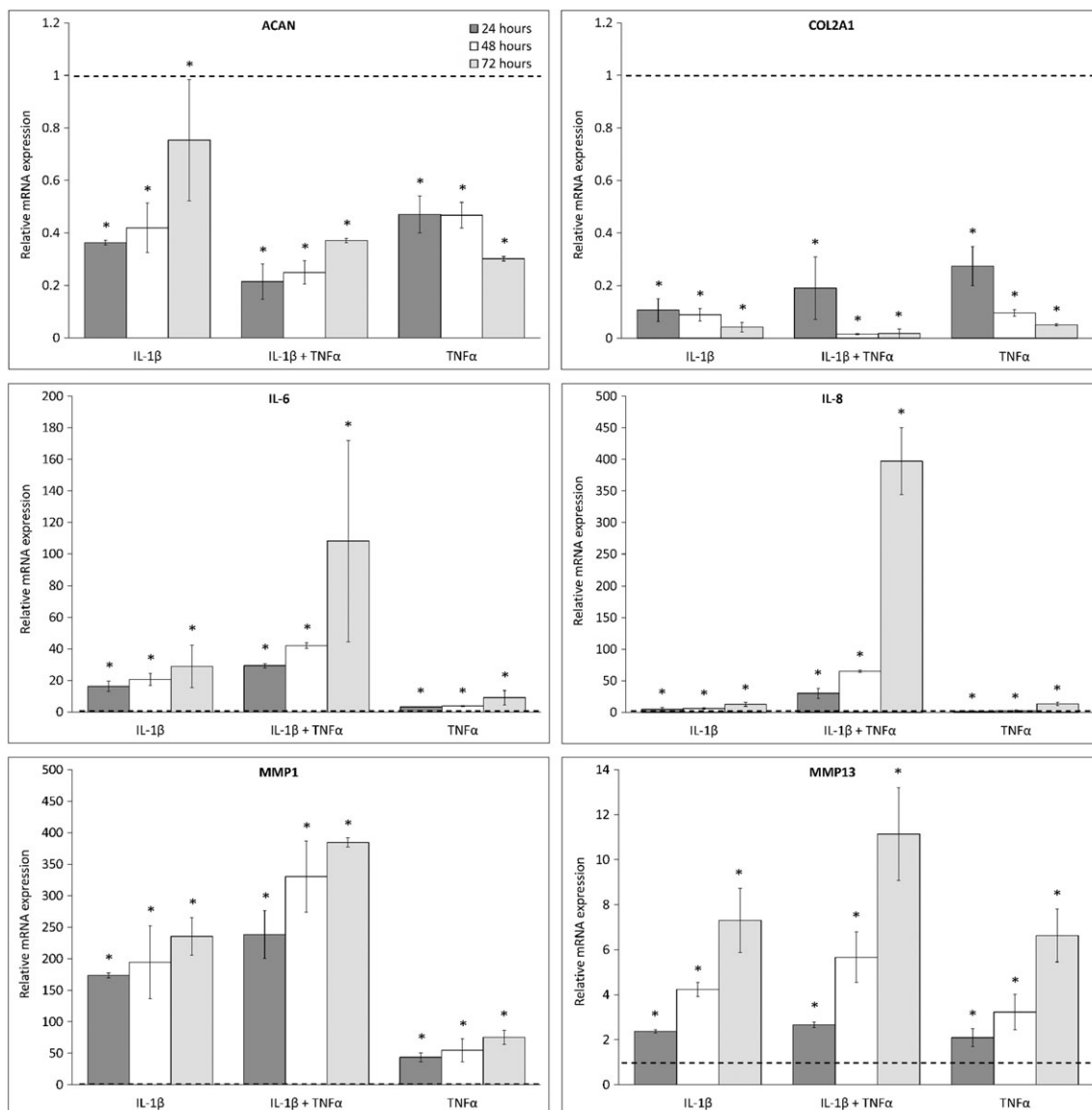


FIGURE 5 Changes in OA-related gene expression in the 3D osteoarthritis *in vitro* model. Equine chondrocytes grown in spheroids were treated with 10 ng/ml IL-1 β or TNF α , or a combination of both, for 24, 48 and 72 h. The mRNA levels of aggrecan, collagen IIA1, interleukin-6, interleukin-8, matrix metalloproteinase-1 and matrix metalloproteinase-13 were measured using RT-qPCR. Relative changes in mRNA levels of cytokine-treated chondrocytes are shown compared to nontreated chondrocytes arbitrary set to 1. Lower confidence limits greater than 1 and upper confidence limits less than 1 were assumed as statistically significant (*). Data show mean values; whiskers represent 95% confidence intervals

of luciferase of less than 4 h.⁸⁶ Repeated stimulation of IL-1Ra production was possible, although IL-1Ra levels after the second stimulation were reduced to 50% compared to the first one. This is in accordance with our observations using luciferase expressing MSCs.⁴⁹

An *in vitro* model of osteoarthritis was used to study the therapeutic potential of MSC/IL-1Ra cells further. Cytokine addition to the culture medium has been shown to produce OA-like changes in chondrocytes (altered OA-relevant gene expression) and therefore represents a well-established *in vitro* model and evaluation tool in experimental OA therapy research.⁸⁷⁻⁸⁹ Because isolated chondrocytes are prone to dedifferentiation in 2D culture and redifferentiate in 3D culture,^{90,91} we performed all experiments with cells in low passage number (< passage 3) and carried out experiments in 2D and 3D culture to determine the chondrogenic phenotype of the chondrocytes. Because the data in the literature differ with respect to cytokine

concentration and the duration of treatment, we first analysed the effect of cytokine concentrations and combination of cytokines over 72 h on target gene expression in chondrocytes cultured in 2D and 3D. For further experiments, a cytokine concentration of 10 ng/ml was chosen because it produced distinct alteration in gene expression and is pathophysiologically relevant.^{92,93} Inflammation-induced osteoarthritic changes were most apparent after 24 h of cytokine treatment, which is in agreement with the results reported by Rai *et al.*⁸⁷ Furthermore, changes in gene expression were more pronounced in the 3D OA model than those measured in the 2D OA model.

Biological activity of the produced recombinant molecule was further demonstrated by the protective effect of conditioned medium from MSC/IL-1Ra cells blocking the OA onset in cytokine-treated chondrocytes. Basal IL-1Ra protein levels obtained in nonstimulated MSC/IL-1Ra cells were sufficient to exert this effect. Conditioned medium from nontransduced MSC had no protective effect and,

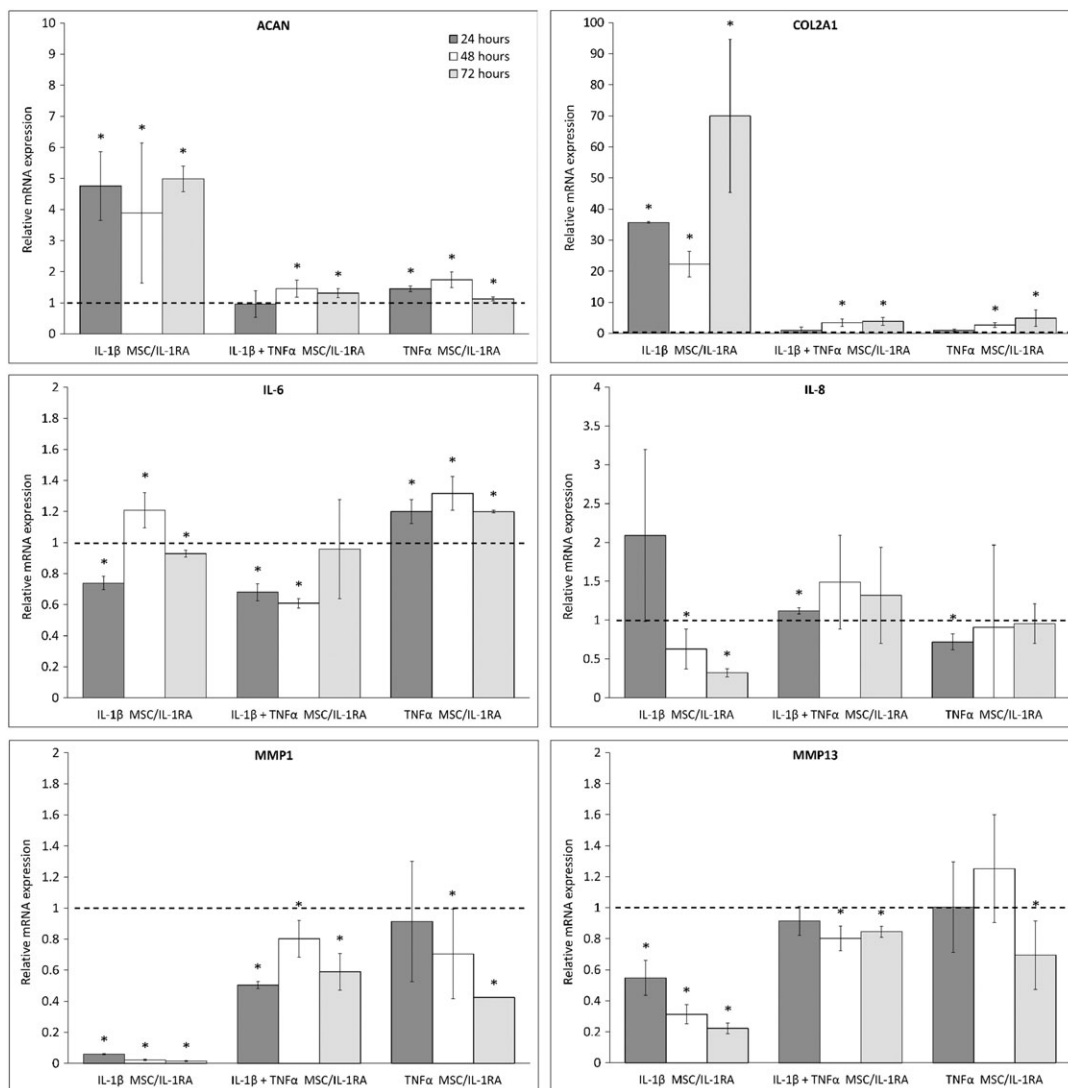


FIGURE 6 Co-cultivation of MSC/IL-1Ra cells with osteoarthritic spheroids alleviates the severity of the osteoarthritic changes. Using a transwell system, OA-induced chondrocyte spheroids were co-cultured with MSC/IL-1Ra cells in the presence of 10 ng/ml IL-1 β or TNF α , or a combination of both, for 24, 48 and 72 hours. The mRNA levels of aggrecan, collagen IIA1, interleukin-6, interleukin-8, matrix metalloproteinase-1 and matrix metalloproteinase-13 were measured using RT-qPCR. Relative changes in mRNA levels of cytokine-treated, co-cultured chondrocytes are shown compared to cytokine-treated chondrocyte spheroids grown in the absence of MSC/IL-1Ra cells, arbitrary set to 1. Lower confidence limits greater than 1 and upper confidence limits less than 1 were assumed as statistically significant (*). Data show mean values; whiskers represent 95% confidence intervals

instead, the contrary effect was observed, because chondrocytes treated with conditioned medium from stimulated and nonstimulated, nontransduced MSC revealed up-regulation of IL-6, IL-8 and MMP1, as well as down-regulation of COL2A1 genes. These observations were fairly unexpected because it is known that MSCs secrete factors inhibiting inflammatory processes⁹⁴ and a protective effect of MSC co-cultivated with IL-1 β -treated chondrocytes in a rat osteoarthritic model was reported recently.⁹⁵ We are aware that these expression changes are influenced by several parameters that are closely related to the experimental set-up (e.g. co-cultivation versus conditioned medium, timing and duration of cytokine treatment, cell cultivation and harvesting time for RNA analysis), which might differ among the research groups and publications. Therefore, any direct comparison might be difficult. Co-cultivation of MSC/IL-1Ra cells with osteoarthritic spheroids alleviated the severity of osteoarthritic changes and this effect was most distinct after OA induction with IL-1 β . This is

not surprising because IL-1 β is one of the key arthritogenic triggers and is much more potent than TNF α with respect to inducing cartilage destruction.¹⁵

In conclusion, we could repeatedly induce transgene expression via cytokine stimulation of MSC/IL-1Ra cells. The IL-1Ra that was produced was biological active. Furthermore, we were able to demonstrate the protective ability of the IL-1Ra protein in an *in vitro* OA model analysing the genes responsible for extracellular matrix proteins, the enzymes for matrix degradation and pro-inflammatory cytokines. Future studies are planned that focus on an improvement of tight regulation of transgene expression and an assessment of the therapeutic potential of the described system in a relevant *in vivo* model.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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