Abschlussbericht / Final report

zur Forschungsförderung der Deutschen Wirbelsäulenstiftung zum Bewilligungs-Bescheid vom 11.12.2020

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2. Projektname / Project Name:

Komplement-Modulation durch zellfreies MSC-Sekretom als neue Therapieoption bei Bandscheibendegeneration? Untersuchungen an murinen Wirbelsäulen-Organkulturen

Englischer Titel:
Complement modulation by cell-free MSC secretome as a new therapeutic option for intervertebral disc degeneration? Studies on murine spinal organ cultures)

Da wir aktuell an der finalen Version einer Publikation zu unserer von der DWS geförderten Studie arbeiten, erlauben wir es uns den Bericht, der Auszüge aus diesem Manuskript enthält, in englischer Sprache zu verfassen.

3. Projekt-Code Verwaltung: Verwendungszweck T84-S2: **D.6568**

4. Datum Zuwendungsbescheid / Date of grant decision: 11.12.2020

5. Einleitung / Introduction

The healthy intervertebral disc (IVD) consists of a central gelatinous nucleus pulposus (NP), which is surrounded by the concentric lamellae of anulus fibrosus (AF) to allow spinal flexibility, shock absorption and pressure distribution.[1] The causes and pathomechanisms of IVD degeneration (IVDD) are not completely understood and there is no generally accepted disease model that fully incorporates the intricate interplay of all biological and biomechanical aspects leading to the histomorphological, radiographic and functional changes of degenerated discs while also addressing their relevance in relation to the clinic of LBP.[2] IVDD is a multifactorial process correlated with increased cell apoptosis and senescence, biomechanical stress factors, degeneration of the water-binding extracellular matrix (ECM) and tissue inflammation responses, among others.[3] In addition, it has been shown that the formation of terminal complement complex (TCC), an activation product of the complement system that acts as an inflammatory trigger and induces cell lysis,[4] correlates positively with the degree of disc degeneration.[5] It has already been demonstrated that C6 deficiency, which is essential for the formation of the TCC complex, protects osteoarthritic mice from collagen degradation;[2] however, the underlying pathomechanisms are still poorly understood.

Over the past decades, cell-based regenerative approaches, particularly utilizing adult mesenchymal stem cells (MSC),[6] have shown promising potential in ameliorating musculoskeletal pathologies, including IVDD.[7, 8] However, the unique and harsh microenvironment of degenerated discs has remained a considerable challenge by limiting long-term survival of transplanted cells and several obstacles regarding bench to bedside translation are yet to overcome.[9] The therapeutic efficacy of MSC has been shown to be mainly mediated by paracrine signaling factors including the entire secretome and extracellular vesicles (EV).[10] EV present a distinct fraction among the plethora of biologically active components of the entire secretome and are nowadays considered vital mediators of intercellular communication through transfer of proteins, lipids, and nucleic acids within their lipid membrane.[11]

In this study, we hypothesized that MSC-derived EV may ameliorate the inflammatory response and modulate the degenerative disc cell phenotype *in vitro*. Importantly, we expected IL-1β primed EV to display improved potential as a therapeutic tool for IVDD compared to non-primed EV, as already explored in an AF organ culture experiment for IL-1β primed MSC secretome.[12] The pleiotropic effects observed in secretome experiments[13] may be primarily mediated through transfer of content from EV to recipient IVD cells. Hence, identification of factors possibly involved in the mechanism of action, as well as detailed comparison of the effects of whole secretome *versus* EV treatment were main focuses of this work.

6. Zielsetzung / Objective

The following objectives were investigated in the present study in two work packages (WP1-2):

i) development of an immunomodulatory and regenerative therapeutic strategy based on preconditioned MSC-secreted EV
ii) investigation of the therapeutic potential of EV with respect to complement modulation and disc regeneration in an ex vivo model using novel complement-deficient and -sufficient organ cultures of the mouse spine

Overall, this study aimed to contribute to a better understanding of the interplay between TCC formation and disc degeneration.

7. Methodik / Methods

7.1. WP1: Isolation and characterization of human and mouse EV secreted by MSC

To investigate hypothesis 1, bone marrow MSC from human and mouse were expanded up to passage 6 and then primed as previously described.[12] Briefly, the MSC were cultured in 6-well plates at a cell density of $1x10^6$ MSC/well with 5 mL MSC-qualified medium with or without 1 ng/mL recombinant human or mouse interleukin (IL)-1β, and kept under 6% O_2 for 2 days. MSC were analyzed for metabolic activity and proliferation by resazurin assay. The expression of pro-apoptotic gene *BAX*, pro-inflammatory markers *IL6* and *IL1β*, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and prostaglandin E_2 (PGE_2) production. EV were purified from the entire secretome by ultracentrifugation. EV morphology, particle diameter and polydispersity were assessed by transmission electron microscopy and dynamic light scattering. Western blotting of CD63 and calnexin was performed. Secretome and EVs produced by non-primed (Control-Sec and Control-EV, respectively) or IL-1β-primed MSCs (IL-1β-Sec and IL-1β-EV, respectively) were investigated by proteomics (n=4) or stored at -80°C for application in WP2.

7.2. WP2: Effect of EV on IVD cells and tissues

WP2a: Effect of EV on human AF cells

Human AF cells isolated from IVD biopsies of disc degeneration patients were cultured without or with recombinant human IL-1β (1 ng/mL) for 2 days (n=4-8). Subgroups were treated with i) Control-Sec, ii) IL-1β-Sec, iii) Control-EV, and iv) IL-1β-EV. Unstimulated AF cells were used as control. AF cells were analyzed for mitochondrial metabolic activity by resazurin assay, expression of pro-apoptotic gene *BAX*, matrix metalloproteinases *MMP1* and *MMP3*, collagen type I (*COL1A1*), tissue inhibitor of MMPs *TIMP1* and pro-inflammatory cytokines *IL1β*, *IL6* and *IL8*, and *GAPDH*. PGE_2 production was quantified in the AF cell culture supernatants.

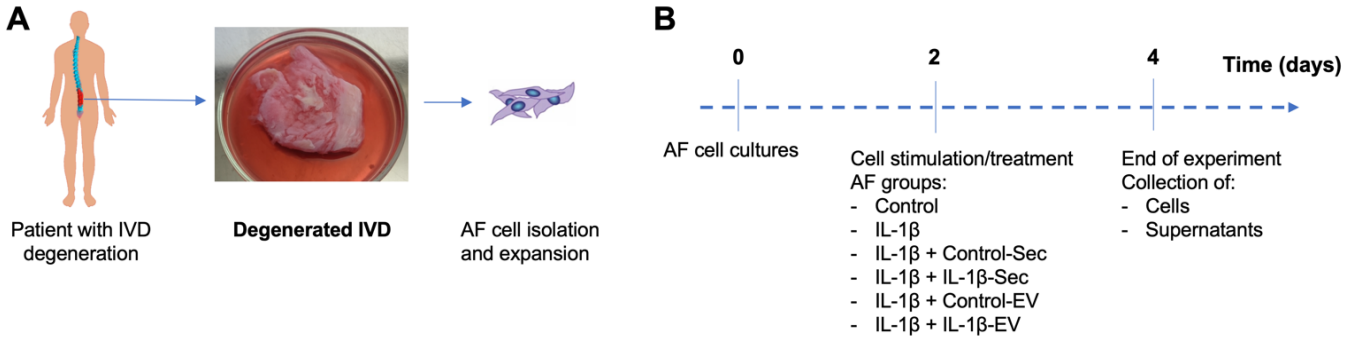


Fig. 1: A) Annulus fibrosus (AF) cell isolation from human intervertebral disc (IVD) tissues. **B)** Experimental timeline.

WP2b: Effect of EV on mouse spine organ cultures

The complex molecular relationship between disc degeneration and complement system activation was investigated in WP2b with a particular focus on TCC formation/inhibition. For that, organ cultures of wild type (WT), C6-deficient and CD59-deficient mouse lumbar spines were established under

proinflammatory/degenerative conditions. In addition, the therapeutic potential of EV produced on complement modulation and disc regeneration was also investigated. Due to breeding limitations, only spines from WT and C6-deficient mice were treated with mouse EV generated by primed WT mouse MSC (Fig. 2A). Briefly, lumbar spines (L1-L6) of 13-week-old male mice (n=4-6) were cultured for 6 days after dissection with standard ("basal") disc medium, reduced oxygen atmosphere (37ºC, 6% O_2 and 8.5% CO_2) and saturated humidity, as previously described for bovine discs.[14] Degenerative conditions were simulated by medium supplementation with 10 ng/mL IL-1β.

On day 6, the medium was replaced with supplementation according to the following groups: i) 5% mouse serum (MS) alone collected from the respective animal, ii) 10 ng/mL recombinant mouse IL-1β as proinflammatory/degenerative stimulus, or also treated with iii) mouse IL-1β-EV (Fig. 2B). After 2 days of stimulation (day 8 of organ culture), the IVDs were isolated from each segment were investigated by gene expression analysis for anti-apoptotic marker *Bcl2*, complement regulators *Cd46*, *Cd55* and *Cd59*, inflammation- and matrix catabolism-associated markers *Il6* and *Mmp3*, *Col1a1* and *Col2a1*, and *Gapdh*. After 14 days (day 21 of organ culture), the spine segments were analyzed by µCT according to Mödinger et al.[15] The disc height index (DHI) was calculated as described by Choi and colleagues.[16] To assess the degree of degeneration, histological grading was performed on safranin O/fast green-stained mid-coronal sections of the lumbar intervertebral discs by at least two independent observers. Degenerative changes in NP and AF were graded according to the modified Thompson scale as described by Choi et al.[16] Investigations at day 21, including immunohistochemical investigations of the distribution of IL-6, TCC, MMP-3, COL1A1 and COL2A1, are ongoing and could not be completed within the funding period of this project.

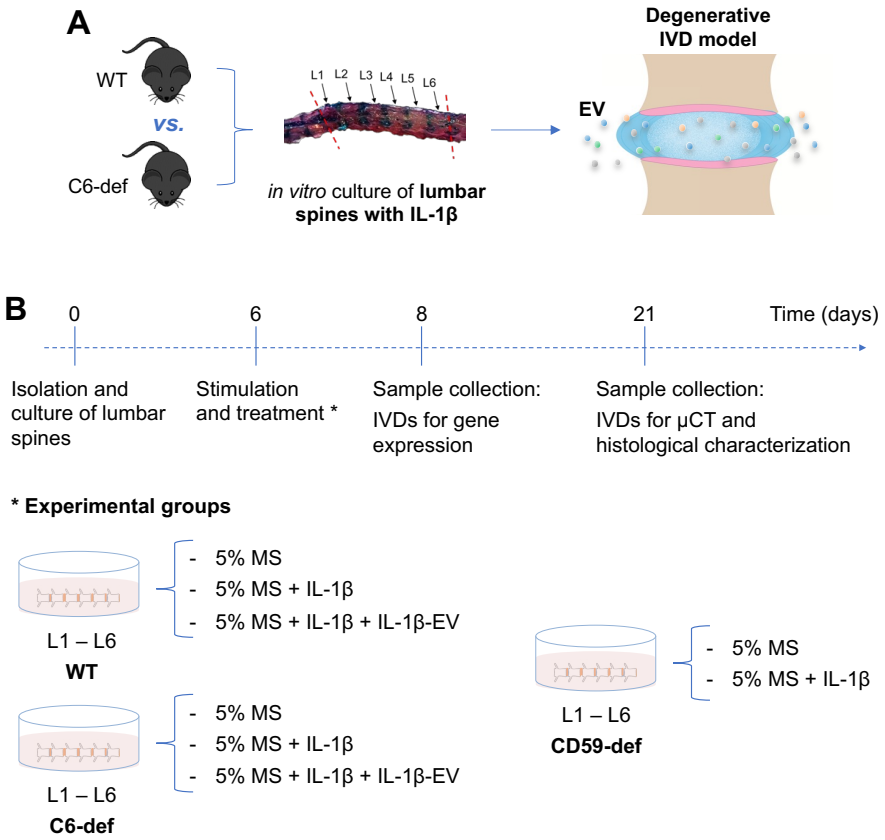


Fig. 2: A) Schematic diagram of the work package 2b. **B)** Experimental timeline and groups.
def, deficient; EV, extracellular vesicles; IL-1β, interleukin-1β; MS, mouse serum; WT, wild type.

8. Ergebnisse / Results

8.1. WP1: Isolation and characterization of human and mouse EV secreted by MSC

The aim of this subproject is to characterize EV produced by primed human and mouse MSC in comparison to the whole MSC secretome. To overcome initial difficulties in the isolation of MSC from mouse blood marrow, the protocol for the isolation of EV was first established using human MSC. The established protocol was successfully translated to mouse cells. Representative data for human MSC, secretome and EV is presented. Similar results were observed for mouse MSC-derived secretome and EV.

IL-1β-primed human MSC proliferated more and were more active than human MSC cultured under control conditions ($p<0.05$, Fig. 3). A significant downregulation of *BAX*, upregulation of *IL6* and *IL1β*, as well as higher PGE_2 production were observed in the primed group versus control MSC ($p<0.01$, Fig. 4), indicating a pro-inflammatory cell phenotype.

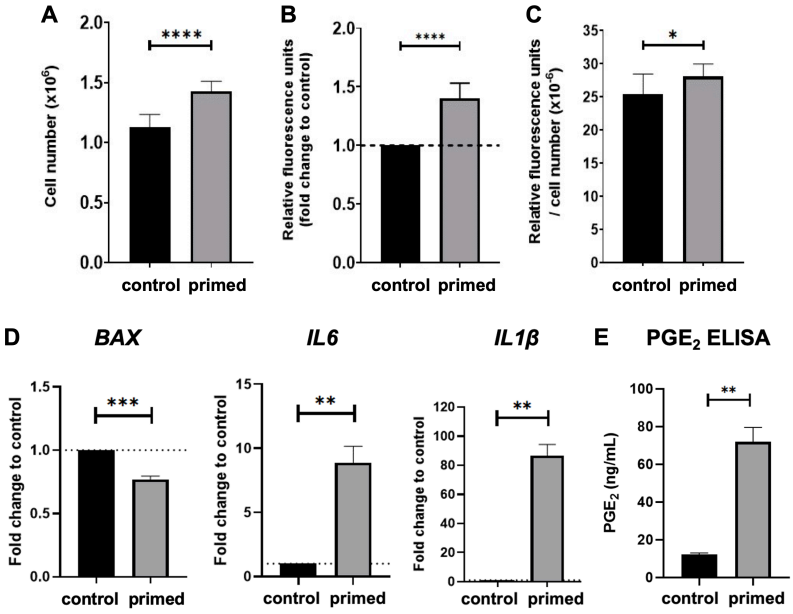
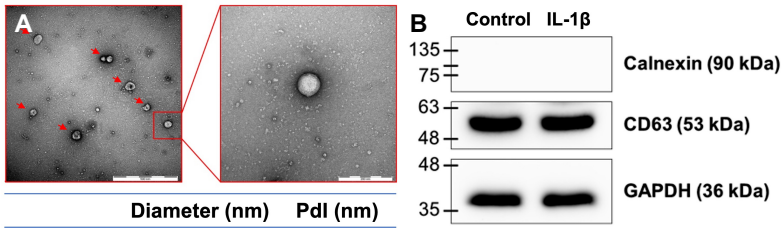


Fig. 3: MSC characterization 2 days after IL-1β priming. **A)** Number of MSC ($x10^6$) per well (of 6-well plate). **B)** Mitochondrial metabolic activity in relative fluorescence units normalized to the non-stimulated control group. **C)** Metabolic activity normalized to cell number. **D)** Gene expression of *BAX*, *IL6* and *IL1β*. **E)** PGE_2 production. n=4; Mann-Whitney test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The characterization of EV isolated from entire human MSC secretome showed that both Control- and IL-1β-EVs displayed typical size distribution (Fig. 4A), expressed the positive EV marker (CD63) and did not express the negative EV marker (calnexin) (Fig. 4B).



	Diameter (nm)	PdI (nm)
Control EV	105.9 ± 52.91	0.315
IL-1β EV	121.3 ± 56.47	0.340

Fig. 4: EV characterization. **A)** Transmission electron microscopy images of EV (scale bar, 200 nm; red arrows, EV). EV size distribution determined by dynamic light scattering. **B)** Representative western blots for the detection of Calnexin and CD63 in EV lysates (n=3).

In the proteomic analysis, several proteins were differentially regulated when comparing the proteome content of secretome (Fig. 5A) or EVs (Fig. 5B) produced by MSCs under IL-1β versus control conditions content of secretome or EV (Fig. 6A, B). Interestingly, only 6 proteins were common to IL-1β-Sec and IL-

1β-EV groups (Fig. 5C). Gene ontology analysis revealed that IL-6 was associated with both the regulation of body fluid levels and the extracellular matrix.

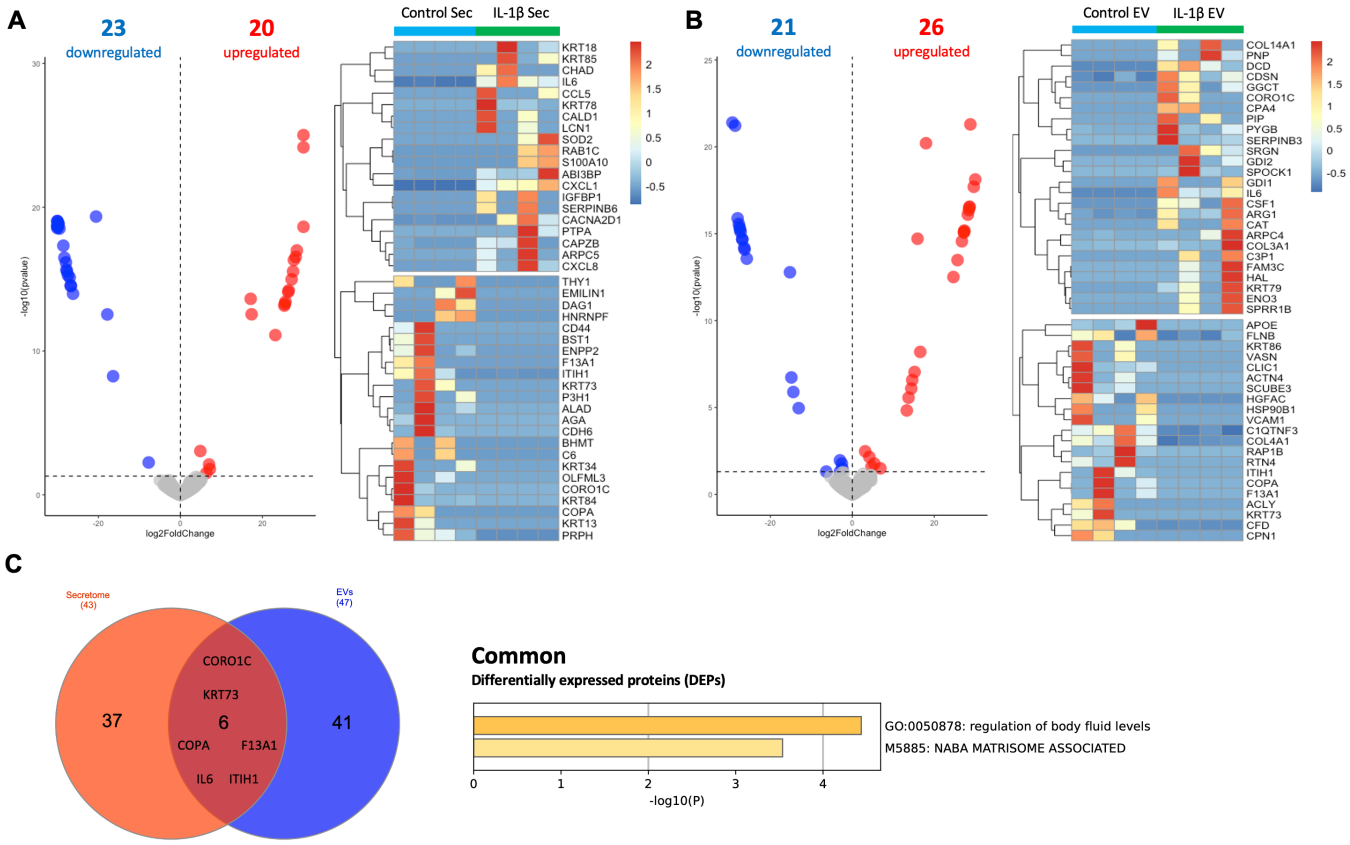


Fig. 5: Proteomic analysis of the secretome and EV produced by non-primed (Control-Sec and Control-EV, respectively) or IL-1β-primed MSC (IL-1β-Sec and IL-1β-EV, respectively). Volcano plot and heatmap of the differentially expressed proteins (DEPs), in which red represents upregulated DEPs and blue represents downregulated DEPs in **A)** IL-1β-Sec *vs.* Control-Sec and **B)** IL-1β-EV *vs.* Control-EV. **C)** Unique and common DEPs between IL-1β-EV and IL-1β-Sec. n=4, p<0.05.

8.2. WP2: Effect of EV on IVD cells and tissues

WP2a: Effect of EV on human AF cells

IL-1β stimulation downregulated the expression of *BAX* and increased the metabolic activity of AF cells (p<0.05, Fig. 6); however, no further effects of secretome or EV treatments were observed.

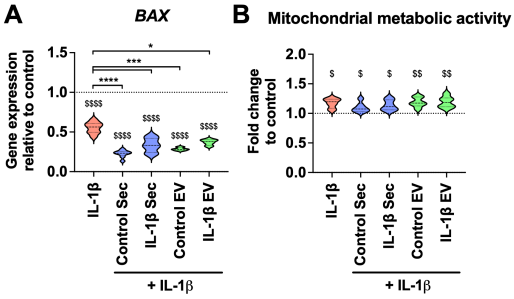


Fig. 6: A) Relative mRNA expression of human apoptosis marker *BAX* by AF cells. Results were normalized to expression level of *GAPDH* and unstimulated AF cells (control group). **B)** Mitochondrial metabolic activity of stimulated and treated AF cells, normalized to the control group. n=4-8; one-way ANOVA; $comparison to control, $p<0.05, $$p<0.01, $$$$p<0.0001; *comparison to IL-1β-stimulated group, *p<0.05, ***p<0.001, ****p<0.0001.

AF cells were further characterized by gene expression and quantification of PGE_2 production. IL-1β stimulation of AF cells upregulated *MMP1*, *MMP3*, *TIMP1*, *IL1β*, *IL6*, *IL8* and increased the production of PGE_2 with respect to the control (p<0.05), whereas expression of *COL1A1* was downregulated (p<0.0001,

Fig 7), in line with previous observed in a different model.[17] Interestingly, similarly to IL-1β-Sec group, IL-1β-EV upregulated *TIMP1* and *COL1A1* expression in comparison to Control-EV (p<0.0001). Considering the proteomic findings (Fig. 5) we hypothesize that this effect may occur through modulation of IL-6 signaling.

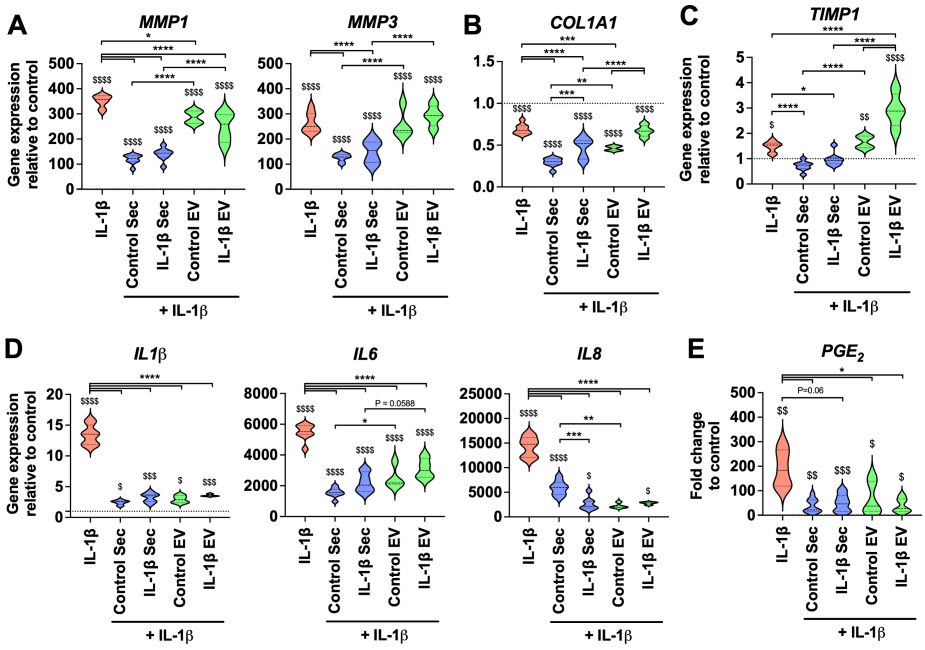


Fig. 7: Relative mRNA expression of human **A)** matrix metalloproteinases *MMP1* and *MMP3*, **B)** collagen type I (*COL1A1*), **C)** tissue inhibitor of MMPs *TIMP1* and **D)** pro-inflammatory cytokines *IL1β*, *IL6* and *IL8*. Results were normalized to expression level of *GAPDH* and unstimulated AF cells. n=4-8; one-way ANOVA; $comparison to control, $p<0.05, $$p<0.01, $$$p<0.001, $$$$p<0.0001; *comparison to IL-1β-stimulated group, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

WP2b: Effect of EV on mouse spine organ cultures

IL-1β stimulation of WT mouse lumbar spine segments downregulated the expression of *Bcl2* and upregulated the expression of *Il6* and *Mmp3* (p<0.05, Fig. 8), as previously shown for AF cells in 2D culture (Fig. 7), also in line with previous findings. [17] In addition to the IL-1β stimulation, IL-1β-EV supplementation reverted the effect of IL-1β alone on *Bcl2*, and upregulated the expression of complement regulators *Cd55* and *Cd59*, responsible for modulation TCC formation, in comparison to the control group (p<0.05). Additionally, Col1a1 was signiffiantly upregulated in the group treated with IL-1β-EV when compared to control spines cultured only with 5% MS (p<0.01).

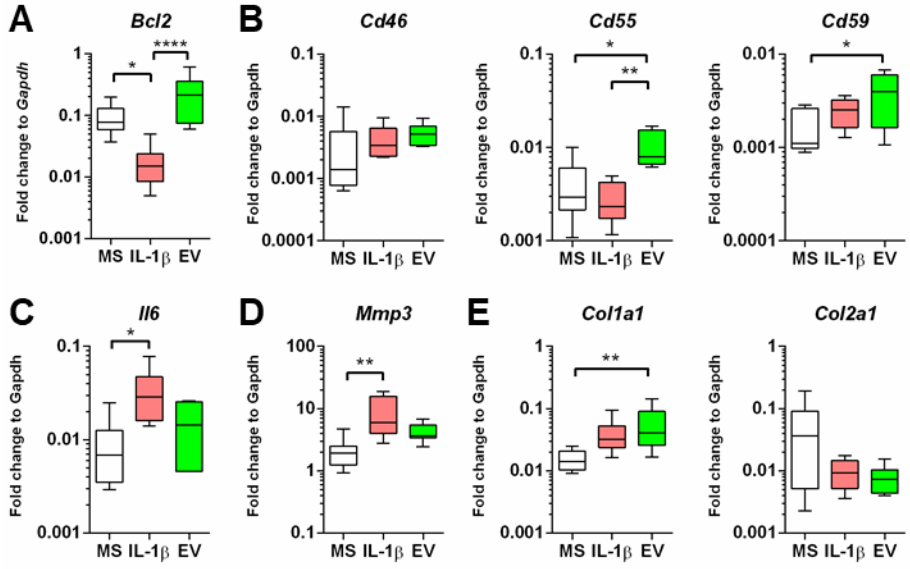


Fig. 8: Relative mRNA expression of mouse **A)** anti-apoptotic marker *Bcl2*, **B)** complement regulators *Cd46*, *Cd55* and *Cd59*, **C)** *Il6*, **D)** *Mmp3,* **E)** *Col1a1* and *Col2a1*. Results were normalized to expression level of *Gapdh* and unstimulated AF cells. n=6-8; Kruskal-Wallis test; *p<0.05, **p<0.01, ****p<0.0001.

Additionally, the effect of IL-1β-EV on organ cultures of lumbar spines from C6-def animals. The EV seemed to have an effect on *Bcl2* upregulation ($p<0.05$, Fig. 9). *Cd59* was upregulated in the EV group versus MS control. Interestingly, the Il6 and Mmp3 upregulation with IL-1β stimulation also observed in WT spines, was here reversed by the IL-1β-EV treatment ($p<0.05$). Additionally, the IL-1β-EV promoted an upregulation of *Col1a1* in comparison to IL-1β stimulation alone ($p<0.05$), but a downregulation of *Col2a1* versus MS samples was also observed ($p<0.05$). This may indicate a reparative effect via formation of fibrous tissue. To confirm this hypothesis, samples from day 21 will be investigated at protein level.

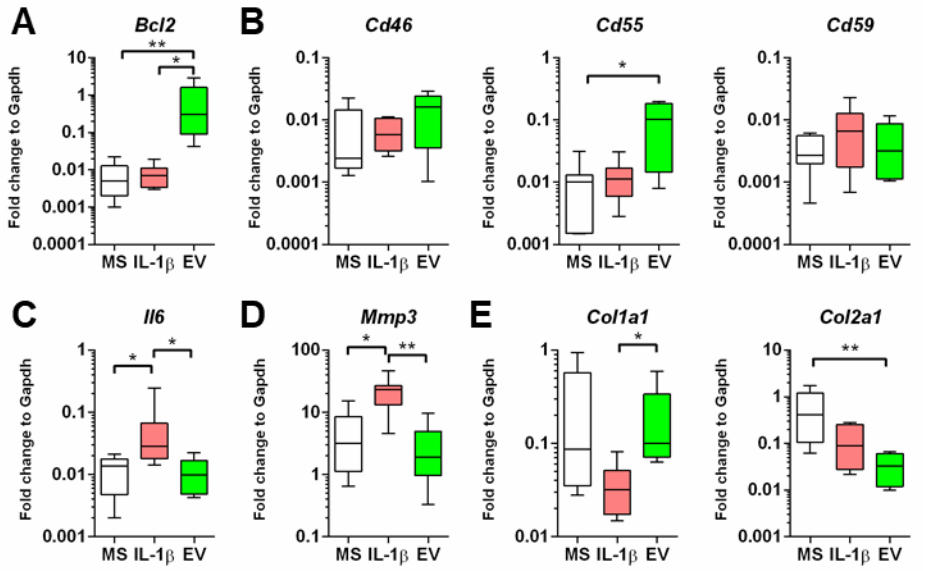


Fig. 9: Relative mRNA expression of mouse **A)** anti-apoptotic marker *Bcl2*, **B)** complement regulators *Cd46*, *Cd55* and *Cd59*, **C)** *Il6*, **D)** *Mmp3,* **E)** *Col1a1* and *Col2a1*. Results were normalized to expression level of *Gapdh* and unstimulated AF cells. n=6-8; Kruskal-Wallis test; *p<0.05, **p<0.01.

9. Diskussion / Discussion

Our data have shown an anti-inflammatory effect of the entire secretome and EV on human AF cells. However, EV from primed MSC may have a stronger effect on AF matrix metabolism. EV enrichment with IL-6 may play a role in the modulation of IVD matrix metabolism, in line with the data from the AF cell culture investigations. IL-6 acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine and has been proposed as a serum biomarker of IVD degeneration.[27] Additionally, EV-encapsulated proteins may be longer protected from degradation than in the entire secretome, being beneficial for therapeutics. In first preclinical trials, treatment of degenerated human disc cells with MSC-derived EV promoted cell proliferation and viability, reduced apoptosis and induced early chondrogenesis.[18-20] EV treatment also promoted matrix anabolism by an upregulation of matrix genes such as aggrecan and COL2 and a downregulation of matrix degrading genes MMP-1 and MMP-3 in disc cells.[21] Moreover, EV demonstrated anti-oxidant and anti-inflammatory effects, possibly linked to a reduced gene expression of Caspase-1 and IL-1β, modulation of the inflammasome and repair of damaged mitochondria.[22] Moreover, first studies analyzing the therapeutic potential of EV in animal organ culture models have been published.[18, 22, 23] However, mechanisms mediating the documented effects are yet to be fully understood and there persists a lack of studies comparing the therapeutic potential of secretome and EV. Furthermore, secretome and/or EV content and effect on recipient cells highly depend on the functional state and microenvironment of the parental cell.[24] This understanding not only emphasizes the importance of a reproducible experimental setting,[25] but also highlights a still mostly unexplored potential of cell priming to enhance secretion and increase therapeutic effects.[10, 26]

The mouse lumbar spine model seems to simulate pro-inflammatory/degenerative conditions in vitro both in segments from WT and C6-def animals, as previously established for bovine.[14] Interestingly, no differences in the expression of complement regulators were observed between models, indicating that IL-1β may not be involved in TCC formation. Nevertheless, treatment with IL-1β-EV only significantly downregulated Il6 and Mmp3, markers of inflammation and catabolism, in the C6-def model, indicating that the EV may have a stronger effect on AF matrix metabolism via TCC modulation, representing a promising therapeutic approach. Nevertheless, further mechanistic investigation will be important to confirm the hypotheses raised from the data collected in the context of this project. A DFG proposal in which these results will be used as preliminary data is in preparation.

10. Angaben wo und wann die Ergebnisse publiziert wurden / Publications

Podium presentations (with reference to DWS funding):

- Annual meeting of the International Society for the Study of the Lumbar Spine, May 1-5 2023, Melbourne, Australia (to be presented)
- Annual congress of the German Spine Society (DWG), December 7-9 2022, Berlin, Germany (Best-of-Show Session)
- eCM20: Cartilage and Disc Repair and Regeneration, June 15-18 2022, Davos, Switzerland

Poster presentations (with reference to DWS funding):

- ORS PSRS Symposium, November 6-10 2022, Skytop, PA, USA
- Eurospine meeting, October 19-21 2022, Milan, Italy

The preparation of a manuscript with the results from WP1 including data from human cell experiments is planned to be finished by May 2023 and a second manuscript comprising the mouse EV characterization and organ culture experiments from WP2 by June 2023.

Two medical doctoral students were contributing to the experimental work. The data will be included in their medical doctoral theses currently in preparation.

We are grateful to the *Deutschen Wirbelsäulenstiftung* for funding this research. This grant has provided us the opportunity to collect preliminary data on whether MSC-priming with specific proinflammatory and degenerative cues can improve the therapeutic potential of the secreted EVs in the context of IVDD.

Ulm, 24.03.2023	Graciosa Teixeira	C. Neidlinger-Wilke
Place, date	Dr. Graciosa Teixeira, Co-PI	Dr. Cornelia Neidlinger-Wilke, PI

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