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Progress report: ISSLS Research Grant sponsored by Taisho Pharmaceutical



Dear ISSLS Research Grant Committee members,

We are grateful to ISSLS and Taisho Pharmaceutical for funding our study entitled **“Does complement deficiency influence intervertebral disc phenotype? Insights from C6 and CD59 deficient mice organ culture investigations”** in 2020. The grant has been providing us the opportunity to collect preliminary data as proof-of-concept whether disc degeneration is a complement-mediated disease.

The Progress Report with more detailed information on the results obtained so far, issues with evaluations of wild type mice and future perspectives is submitted together with this letter.

Please, do not hesitate in contacting me if further questions arise.

Once again, thank you very much for this great support to our research.

Sincerely,

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Content

1. Introduction	1
2. Results	2
3. Discussion and conclusions	4
4. Future perspectives.....	4

1. Introduction

We would like to acknowledge ISSLS and particularly Taisho Pharmaceutical for funding our study entitled **“Does complement deficiency influence intervertebral disc phenotype? Insights from C6 and CD59 deficient mice organ culture investigations”** in 2020. The grant has been providing us the opportunity to collect preliminary data as proof-of-concept whether disc degeneration is a complement-mediated disease.

In the intervertebral disc (IVD), several mechanisms are involved in the imbalance of anabolic and catabolic activities towards higher proteolytic activity during degeneration. Products of extracellular matrix breakdown, as well as crystal deposits are known to trigger inflammation. A wide number of inflammatory mediators, including prostaglandins, interleukins (IL-1, -6, -8) and tumor necrosis factor (TNF)- α , have been described in DD catabolic processes. The secretion of these mediators by nucleus (NP) and annulus (AF) cells, as well as infiltrated immune cells (e.g. macrophages, T-cells, neutrophils) can result in cell autophagy, senescence and apoptosis (Molinos et al., *J R Soc Interface*, 2015). Ultimately, structural damage of the outer AF provides an opportunity for blood vessels and nerves to invade the disc and cause pain (Adams et al., *Spine*, 2006). Deposition of terminal complement complex (TCC), a complement system activation product that contributes to cell lysis and inflammation, has been shown to play a role in articular cartilage disorders, such as osteoarthritis (Wang et al., *Nat Med*, 2011) and to increase its production with increase of the degree of disc degeneration (Teixeira et al., *Eur Spine J*, 2020). Hence, it is worthwhile to investigate the effect of complement activation in disc degeneration (DD). There is no knowledge if the disc inflammatory/degenerative environment plays a role in complement activation and if the TCC is an effective target for IVD therapeutics and suitable models for mechanistic investigations are required.

The first part of this project focused on investigating the influence of C6 deficiency (a complement component that or CD59 deletion has on the IVD phenotype. Based on the observations of Wang and colleagues (2011) in articular cartilage, we expect that C6 deficiency will decrease age-related changes in the IVD, whereas CD59 depletion will contribute to accelerated DD. Over the past year, we have focused our efforts in the investigations associated with work package 1 (WP1) on the phenotypic characterization of the intervertebral discs (IVDs) of C6 deficient (C6-def) and CD59-knockout (CD59-ko) mice regarding gene expression profile of IVD cells and IVD morphology was performed at three different ages: 13-, 32- and 52-weeks. To analyze mechanisms of complement activation during IVD degeneration, an *ex vivo* organ culture model of mouse lumbar spines is being developed (data not shown). The organ culture

model enables the evaluation of pro-inflammatory/degenerative factors and their effect on IVDs from the mice deficient for complement-related molecules. The ex-vivo model will clarify whether the IVD cells with complement deficiency retain their different complement effects and whether TCC formation can be activated using the established model.

2. Results

So far, the following results were obtained and will be presented at the 2021 ISSLS Virtual Meeting:

Gene expression analysis was performed on cells isolated from tail IVDs of C6-def and CD59-ko mice. Due to breeding problems, gene expression analysis of 32- and 52-weeks old wild type (WT) mice is still ongoing, but it is expected to be completed until end of the year. Animals were analyzed separately depending on their sex and age. Here, gene expression results for male animals were compared at three different ages. Comparisons between male and female were also performed (data not shown), but significant differences have only been observed for *COL1A1* expression between 13-weeks old female and male CD59-ko mice.

Markers of apoptosis (*BCL2*), cell metabolism (*C-FOS*), and inflammation (*IL-6*), as well as complement component *C6* were analyzed and are depicted in Figure 1. At 13-weeks, the relative mRNA expression of *BCL2*, *C-FOS* and *IL-6*, but not *C6*, was higher for 13-weeks old C6-def mice, when compared to WT and CD59-ko animals with the same age. For C6-def mice, *BCL2* was upregulated at 32-weeks, but then downregulated again at 52-week. For CD59-ko mice, it was upregulated at 32- and 52-weeks when compared to 13-weeks, but with no differences between the two older animal groups. Interestingly, C6-def animals did not present significant changes in *C-FOS*, *IL-6* or *C6* expression with ageing, whereas for CD59-ko mice it was increased and higher than in C6-def mice at 32- ad 52-weeks of age.

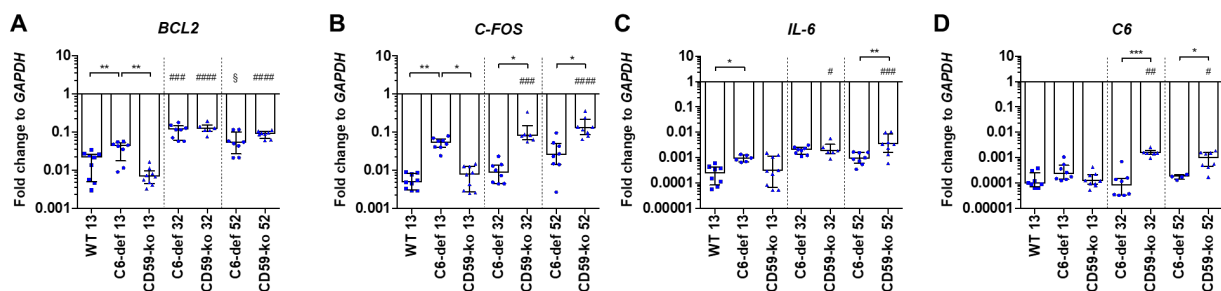


Figure 1. Gene expression analysis of male mouse IVD cells isolated from tail IVDs of 13-, 32- and 52-weeks old mice. Relative mRNA expression of **A)** apoptosis (*BCL2*), **B)** cell metabolism (*C-FOS*), **C)** inflammation (*IL-6*) and **D)** complement activation (*C6*) markers. Results were normalized to mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). n = 6-9, #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001 (significant differences to 13-weeks old animals with the same deficiency); §p < 0.05 (significant differences to 32-weeks old animals with the same deficiency); *p < 0.05, **p < 0.01, ***p < 0.001 (significant differences between animals with the same age).

The gene expression of cellular complement regulation molecules (*CD46* and *CD55*) was also determined (Figure 2). Relative mRNA expression of *CD46* was higher at 32- and 52-weeks old compared to 13-weeks old animals in both phenotypes, and it was significantly higher in 52-weeks old CD59-ko animals than in C6-def mice. A similar trend was observed for *CD55*.

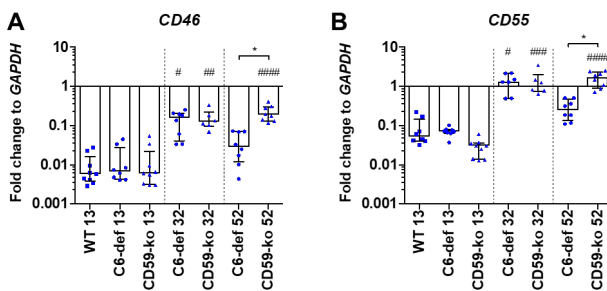


Figure 2. Gene expression analysis of male mouse IVD cells isolated from tail IVDs of 13-, 32- and 52-weeks old mice. Relative mRNA expression of cellular complement regulators **A)** *CD46* and **B)** *CD55*. Results were normalized to mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). $n = 6-9$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.0001$ (significant differences to 13-weeks old animals with the same deficiency); * $p < 0.05$ (significant differences between animals with the same age).

Matrix metalloproteinase *MMP-3* and ECM components collagens type I and II (*COL1A1* and *COL2A1*, respectively) were also analyzed (Figure 3). The relative mRNA expression of the matrix-degrading enzyme (*MMP3*) increased with age of the animals in both phenotypes. Significantly higher *MMP-3* expression was observed for 13-weeks old C6-def mice *versus* WT, whereas for 52-weeks *MMP-3* expression was downregulated in IVDs of C6-def compared to CD59-ko mice. The relative expression of *COL1A1* was significantly higher in 13-weeks old C6-def mice than in WT and CD59-ko animals of the same age. Furthermore, it seemed to decrease with age for C6-def mice (significant decrease at 52-weeks), while it increased for CD59-ko animals. The expression of *COL2A1* followed a similar profile as *COL1A1*.

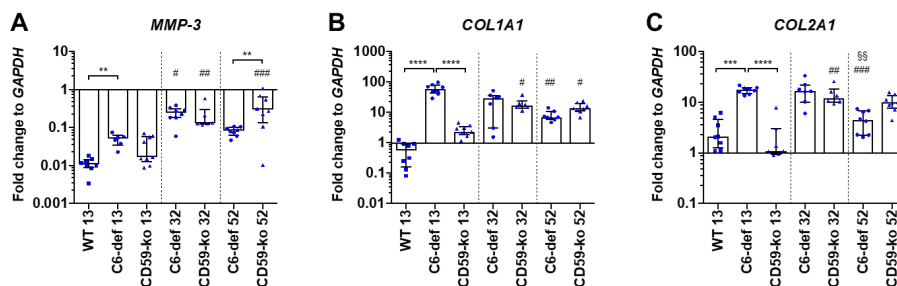


Figure 3. Gene expression analysis of male mouse IVD cells isolated from tail IVDs of 13-, 32- and 52-weeks old mice. Relative mRNA expression of **A)** matrix metalloproteinase *MMP-3* and ECM components **B)** *COL1A1* and **C)** *COL2A1*. Results were normalized to mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). $n = 6-9$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.0001$ (significant differences to 13-weeks old animals with the same deficiency); * $p < 0.05$ (significant differences between animals with the same age).

Safranin-O/fast green stained IVD sections of C6-def, and CD59-ko mice were examined using the modified Thompson score. In general, 13-weeks old mice presented a low level of degeneration in both NP and AF as depicted in representative safranin-O/fast green images in Figure 4A. In about 8% of IVD samples, a score of 1 was attributed to the NP of 13-weeks old CD59-ko mice, whereas all C6-def animals present score 0 (Figure 8B). In comparison, a score of 1 was attributed to the AF of about 20% of both C6-def and CD59-ko mice, indicating lightly higher degeneration in the AF than in NP (Figure 8C). In the 32-weeks old mice, the C6-def mice show similar degeneration in NP and AF: 10% of NP and 16% of AF have were attributed a score of 1. All 32-weeks old CD59-ko mice presented a score of 0 in the NP and 1 in the AF. Although without statistical significance, a higher Thompson Score was found particularly in the NP and AF of 52-weeks old animals, especially in CD59-ko mice in comparison with C6-def, indicating slight stronger degeneration/age-related changes in the IVDs of the animals.

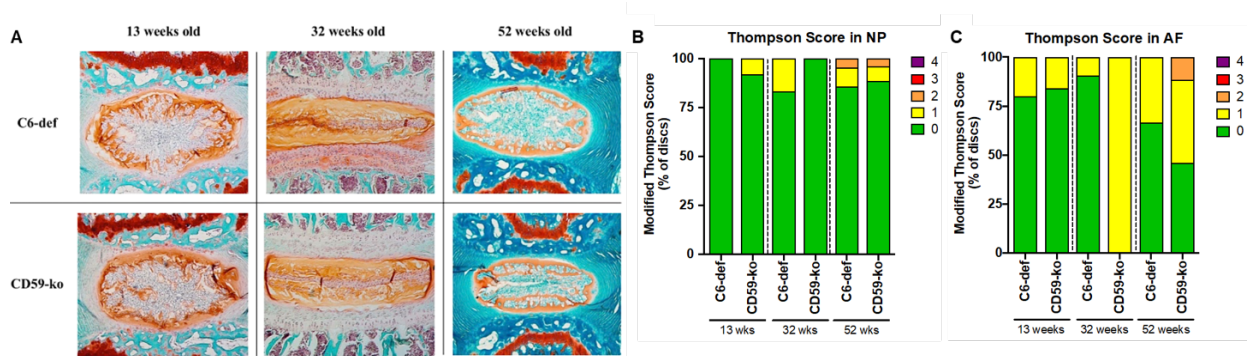


Figure 4. A) Safranin O/fast green stained samples from 13-, 32- and 52-weeks old C6-def and CD59-ko mice. Thompson Score in B) nucleus pulposus (NP) and C) annulus fibrosus (AF). IVDs of 13-, 32- and 52-weeks old C6-def and CD59-ko animals were analyzed (n=6-9).

3. Discussion and conclusions

Overall, IVDs from C6-def mice seem to present a different phenotype from CD59-ko mice. Particularly 52-weeks old aCD59-ko mice, present upregulated expression of *C-FOS*, *IL-6*, *C6*, *CD46*, *CD55*, and *MMP-3*, as well as slightly higher Thompson Score in the annulus fibrosus (AF) region, which indicates that the IVDs of CD59-ko animals display a slightly accelerated degeneration compared to C6-def rodents with ageing, which is in agreement with previous results observed for osteoarthritis (Wang et al., *Nat Med*, 2011) and our hypothesis. A manuscript is in preparation.

WP2 is focusing on *ex vivo* investigations of thoracolumbar spines from 13-weeks old WT, C6-def and CD59-ko mice and TCC formation. The focus is understanding the impact of different molecules that play a role in DD ($IL-1\beta$, cathepsin D) and how they may affect TCC formation. Moreover, screening of the therapeutic effect of TCC inhibitors (e.g. eculizumab) in the organ culture environment will be investigated in the second year of the project.

4. Future perspectives

Since we overcame the issues that postponed investigations of WT mice, we are confident to finish the data collection and the preparation of a manuscript with the results from WP1 by the end of 2021. Again, we are grateful to ISSLS and Taisho Pharmaceutical for funding the research. This ISSLS grant provides new relevant data to understand the role of TCC (and its inhibition) in disc degeneration, a prerequisite for targeting it in a potential therapeutic approach. This is the first step of a larger research program on this topic. The data generated in this proposal will build the foundation for a larger collaborative study with experts from different fields.

Ulm, 03.03.2021

Place, date

Graciosa Teixeira

Name of the issuer of this report

C. Weidling

Name of the project leader or responsible division manager (should not be the same as the issuer of the report)