Toll-like receptor 2 signaling of cartilage endplate cells amplifies inflammation in Modic type 1 changes

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INTRODUCTION: Vertebral bone marrow lesions, known as Modic changes (MCs), strongly associate with cartilage endplate (CEP) damage and disc degeneration. Discs adjacent to MCs release higher amounts of inflammatory cytokines and degenerate faster. Yet, the causal link between MCs, CEP damage, and disc degeneration is unknown. It is assumed that the inflammatory factors from the degenerating disc drain through the damaged cartilage endplate into the bone marrow where they activate bone marrow cells. Inflammatory factors may include cytokines and extracellular matrix (ECM)-derived danger associated molecular patterns (DAMPs) that are generated during disc degeneration and cause inflammation by binding to toll-like receptor 2 or 4 (TLR2, TLR4). TLR2 and TLR4 signaling in disc cells has been causally linked with disc degeneration. Although cell density in the hyaline cartilage of CEPs is about 4-times higher than in the fibrocartilage of the disc, it is unknown if CEPC express TLRs and if they have a pathologic role. TLR signaling in CEPs might be relevant in MCs to amplify and propel the inflammatory signal from the disc to the bone marrow. The aims of this study were (i) to identify the presence of TLRs and their effect on downstream genes in cartilage endplate cells (CEPC), and (ii) to compare the expression of TLRs and downstream activated genes on CEPC from Modic type 1 changes (MC1), MC2 or degenerated non-Modic change (nonMC) cartilage endplates.

METHODS: CEPCs from degenerated discs (6 nonMC, 4 MC1, 4 MC2) were isolated from fresh cartilage endplate tissue of spinal fusion surgery patients that signed informed consent for further use of surgically removed biological material. CEPC were enzymatically isolated overnight with 0.05% collagenase P (Roche) in Dulbecco's modified eagles medium supplemented with 10% fetal calf serum, 5% penicillin streptomycin, 5% HEPES and expanded to passage 1-2. To assess response of CEPCs to inflammatory stimuli, CEPS were treated for 24h or 48h with (i) tumor necrosis factor α (TNF-α) to simulate the inflammatory milieu provided by the degenerating disc, with (ii) TLR2/6 and TLR2/1 specific ligands Pam2CysSerLys4 (Pam2csk4), Pam3CysSerLys4 (Pam3csk4), respectively, to investigate the signaling mechanism, with (iii) ultrapure E. coli lipopolysaccharide (LPS) as a TLR4 ligand, or with (iv) the 30kDa N-terminal fibronectin fragment (FNf30), a known ECM-derived DAMP from the disc. To assess the specificity of Pam2csk4 signaling, CEPS were pre-treated for 2 h with the TLR2 inhibitor TL2-C29 before adding Pam2csk4. In all conditions gene expression of all TLRs as well as of inflammatory genes (IL-6, IL-8) and matrix proteases (MMP1, MMP2, MMP3, MMP9 and MMP13) were measured with quantitative real-time polymerase chain reaction (qPCR). The -ΔΔCt method was used for analysis with Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as the reference gene. Statistical analysis was done by fitting a mixed-effects model, followed by multiple comparisons on $\log_2$ fold changes. In addition, surface expression levels of TLR2 were measured with flow cytometry on untreated, Pam2csk4, and Pam3csk4 treated cells and analyzed with multiple paired t-tests on mean fluorescence intensity. Statistical analysis was performed with GraphPad Prism v10.0.2.

RESULTS SECTION: Gene expressions in untreated CEPC of all TLRs except TLR8 and TLR9 could be identified (data not shown). Yet only TLR2 expression was significantly increased after stimulation with TNF-α, Pam2csk4, Pam3csk4, LPS and FNf30 (Figure 1A), suggesting a role of TLR2 in CEPC under inflammatory conditions. Pam2csk4 and Pam3csk4 also upregulated MMP1, MMP9 and MMP13 expression (Figure 1A), indicating that TLR2 signaling on CEPC can trigger degenerative changes. The addition of TL2-C29 inhibited in a concentration-dependent manner the upregulation of all TLR2 responsive genes except MMP9 (Figure 1B), proving that these effects were indeed TLR2-mediated. Based on these results TLR2 protein levels were measured with flow cytometry, which showed a significant increase in TLR2 on the cell surface upon TLR2/6 heterodimer stimulation by Pam2csk4 ($p = 0.006$) (Figure 1C), but no increase upon TLR2/1 heterodimer stimulation with Pam3csk4 (Figure 1D). This indicates that the increase in gene expression is mirrored at the protein level for the TLR2/6 heterodimer, unlike the TLR2/1 heterodimer. This implies a potentially more important role for TLR2/6 signaling.
Next, the CEPC were stratified into the groups nonM, MC1 and MC2 CEPC ($n = 6 + 4 + 4$, respectively) to identify potential differences in TLR expression levels or the effect of TLR stimulation on the gene expression. Untreated MC1 CEPC had a significantly higher TLR2 expression level ($p = 0.029$, predicted (least square (LS)) mean difference = 1.80 ± 0.69) and a slightly higher expression level of TLR6 ($p = 0.070$, predicted (LS) difference = 1.33 ± 0.72) than nonMC CEPC (Figure 2A). Stimulation with Pam2csk4 upregulated TLR2 expression in MC1 almost double as strong as in nonMC CEPC ($p = 0.076$, fold change = 3.92 ± 0.84) (Figure 2B).

DISCUSSION: These are the first experiments to show that CEPCs express TLRs, that CEPCs can induce TLR2-mediated inflammation, and that an inflammatory environment enhances TLR2 and TLR6 expression in CEPCs, making them even more responsive to TLR2/6 ligands. This could play a substantial role in amplification of the inflammatory environment in degenerated discs. We also found that MC1 CEPC have a higher expression of TLR2, which is further increased through TLR2/6 stimulation in a positive feedback loop. This suggests that TLR2/6 signaling is an engaged mechanism in MC1 and that MC1 CEPCs are more sensitive to TLR2/6 ligands. This may add to CEP inflammation and enhance CEP resorption. Consequently, MC1 CEPC can extend the inflammatory milieu from the disc to the bone marrow, making TLR2 highly relevant in the pathogenesis of MC1.

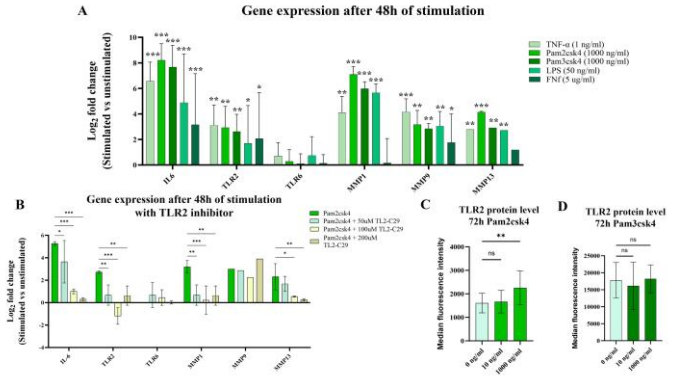

Figure 1 (A) Gene expression of CEPC treated with TNF-α, Pam2csk4, Pam3csk4, LPS and FNf30 shown as $\log_2$ fold change to unstimulated. **(B)** Inhibition of gene expression upregulation by Pam2csk4 through TLR2 inhibitor. **(C/D)** Flow cytometry measurement of TLR2 on CEPC after 72h incubation with **(C)** Pam2csk4 or **(D)** Pam3csk4 shown as median fluorescence intensity.

SIGNIFICANCE/CLINICAL RELEVANCE: Increased TLR2 expression and signaling in MC1 CEPC may be an important contributor to the development of MC1. TLR2 inhibition may offer a novel approach to hinder the development of MC1 adjacent to degenerated discs.

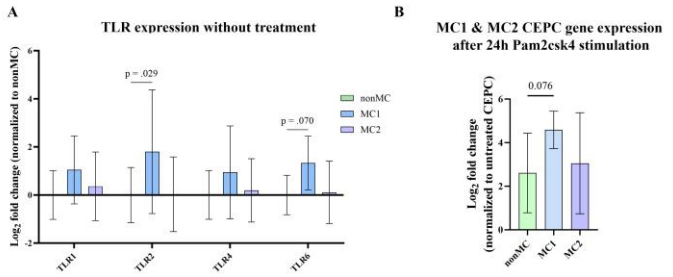

Figure 2. (A) Comparison of MC1 and MC2 CEPC TLR expression to nonMC CEPC without prior stimulation. **(B)** TLR2 gene expression compared between nonMC, MC1 and MC2 CEPC after 24h Pam2csk4 stimulation and normalized to unstimulated.