Modic change bone marrow neutrophils are activated and activated neutrophils degrade cartilage endplates

I. Heggli[1,2]*, T. Mengis[1,2], N. Herger[1,2], M. Farshad[3], M. Habib[4], C.P.Ames[5], O. Distler[1,2], A.J. Fields[4], S. Dudli[1,2]

[1]Center of Experimental Rheumatology, Department of Rheumatology, University Hospital Zurich, University of Zurich, Zurich, Switzerland; [2]Department of Physical Medicine and Rheumatology, Balgrist University Hospital, Balgrist Campus, University of Zurich, Zurich, Switzerland; [3]Department of Orthopedics, Balgrist University Hospital, University of Zurich, Zurich, Switzerland; [4]Department of Orthopaedic Surgery, University of California San Francisco, San Francisco, CA, USA, [5]Department of Neurological Surgery, University of California San Francisco, San Francisco, CA, USA Disclosures: None.

INTRODUCTION: Inflammation is a hallmark of painful vertebral bone marrow lesions called Modic changes (MC). MC occur adjacent to damaged cartilage endplates (CEPs). Neutrophilic infiltrates and dysregulated granulopoiesis are indicative for neutrophil involvement in the MC pathomechanisms. Clarification of the role of neutrophils in inflammatory MC processes could help for the development of currently non-existing disease-modifying MC treatments. We hypothesized that i) activated blood neutrophils degrade CEPs, ii) MC bone marrow neutrophils are activated and have higher fractions of immature band neutrophils, and iii) that MC bone marrow neutrophil supernatant exhibits higher neutrophil elastase activity.

METHODS: To investigate the effect of activated blood neutrophil release on CEP composition, we first isolated neutrophils from fresh human peripheral blood of one healthy donor (obtained from STEMCELL) using the EasySep™ Human Neutrophil Isolation Kit (STEMCELL Technologies), and collected supernatants of stimulated (100 nM PMA for 3h at 37°C) and unstimulated neutrophils at 25 mio neutrophils / ml and 12.5 mio neutrophils / ml, respectively. We then collected circular 4 mm CEP biopsies from L4/5 and L5/S1 (n=6) from patients undergoing anterior lumbar interbody fusion surgery at the University of California San Francisco, USA. From each donor, 3 CEP biopsies were halved and exposed for 18h at 37°C to either 1A) 0.75 U/ml collagenase P (positive control) vs. 1B) Hanks balanced salt solution (HBSS); 2A) 25 mio neutrophils / ml stimulated vs. 2B) unstimulated supernatant; or 3A) 12.5 mio neutrophils / ml activated vs. 3B) unstimulated supernatant. Exposure supernatants and CEP tissues were collected and analyzed for sulfated glycosaminoglycans (sGAGs) using a dimethylmethylene assay. Relative sGAG release was tested against the null hypothesis (μ0=100%) using a one-sample t-test. P values<0.05 were considered statistically significant.

To assess MC bone marrow neutrophil activation, heterogeneity, and neutrophil elastase release, we isolated bone marrow neutrophils from low back pain patients with MC undergoing lumbar spondylodesis at the Balgrist University Hospital, Switzerland. From each patient, a bone marrow aspirate from a MC lesion and from an unaffected vertebral body (intra-patient control) was collected prior screw insertion. First, we isolated $CD45^+CD66b^+$ neutrophils by cell sorting and performed bulk RNA sequencing (n=7+7). To identify dysregulated biological processes and pathways, we performed bioinformatic overrepresentation analysis (ORA) with upregulated differentially expressed genes (DEGs) ($p < 0.01$). Terms were considered to be significant for false discovery rate (FDR) < 0.05. Second, we analyzed neutrophil activation ($CD66b^+$ intensity) (n=10+10) and proportions of mature neutrophils ($CD66b^+CD10^+CD11b^{high}$) and immature band neutrophils ($CD66b^+CD10^-CD11b^{high}$), myelocytes ($CD66b^+CD10^-CD11b^{int}$), and promyelocytes ($CD66b^+CD10^-CD11b^-$) (n=7+7) with flow cytometry. CD66b median fluorescence intensity (MFI) and fractions of neutrophil subsets were compared between MC and controls with paired t-tests and p-values < 0.05 were considered to be statistically significant. Third, we isolated bone marrow neutrophils as described for blood neutrophils and cultivated MC and control bone marrow neutrophils (25 mio neutrophils / ml) for 3h. Neutrophil elastase activity was measured with a fluorogenic neutrophil elastase substrate and the relative activity (MC vs. control) was tested against null hypothesis (μ0=100%) using a one sample t-test. P-values<0.05 were considered statistically significant.

RESULTS: Effect of activated blood neutrophils on CEP composition: Exposure of CEPs to neutrophil supernatants caused significant release of sGAG from the CEP tissues in a dose-dependent manner (25 mio neutrophils/ml: 380.1% ± 177, p = 0.01; 12.5 mio / ml: 123.7% ± 22.3, p = 0.05, Tukey Post Hoc test: 25 mio/ml vs. 12.5 mio/ml: p = 0.02, positive control: 545.0% ± 302.8, p = 0.02) (**Figure 1a**), showing that activated blood neutrophils damage CEPs.

MC bone marrow neutrophil characterization: Transcriptomic analysis of MC bone marrow neutrophils revealed upregulated pathways associated with inflammation and neutrophil activation (**Figure 1b**). Furthermore, among the top five upregulated biological processes in MC bone marrow neutrophils were calcium associated processes "calcium ion transmembrane transport" (FDR<0.001) and "positive regulation of cytosolic calcium ion concentration" (FDR<0.001) (not shown), indicating increased neutrophil degranulation, a feature of activated neutrophils. This underscored that MC bone marrow neutrophils have an activated pro-inflammatory transcriptome. Flow cytometric analysis revealed significantly higher CD66b MFI on MC bone marrow neutrophils (ΔMC-control = 1475 MFI ± 1801, p=0.03), further supporting neutrophil activation in MC. Moreover, in six out of seven patients, we found increased proportions of immature band neutrophils (ΔMC-control = 6.69 % ± 8.00%, p=0.07) (**Figure 1c**). Immature band neutrophils expand under inflammatory conditions. This indicates an increased demand for neutrophils in the MC bone marrow and suggests a role in inflammatory MC pathomechanisms. Neutrophils also expand in joints of patients with rheumatoid arthritis and promote articular cartilage damage[1]. Since cartilage damage was found to be mediated by neutrophil elastase, we analyzed neutrophil elastase activity in unstimulated MC and control neutrophil supernatants. In four out of five patients, we found higher neutrophil elastase activity in MC (212.9%±148.0, p=0.16) (**Figure 1d**), indicating increased production of an enzyme that potentially (further) damages CEPs in MC.

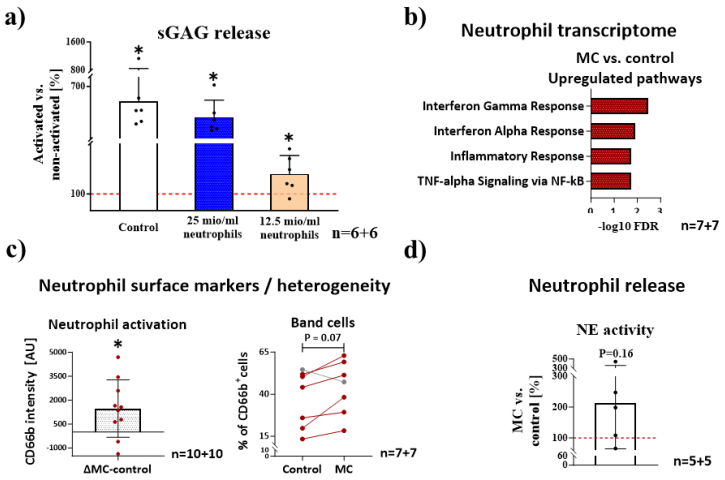

Figure 1a) Relative sGAG released from CEP tissues exposed to collagenase P (white bar) or activated blood neutrophil supernatants (blue/yellow bar). **b)** Upregulated pathways in MC BM neutrophils identified by bulk RNA sequencing. **c)** CD66b intensity as a measurment of neutrophil activation (left) and fractions of immature band cells in MC BM identfied by flow cytometry. **d)** Neutrophil elastase activity measured with a fluoregenic NE substrate. *P-value<0.05.

DISCUSSION: Here we show that activated blood neutrophils degrade CEPs and that MC bone marrow neutrophils are activated release more neutrophil elastase. Therefore, it is plausible that inflammatory MC pathomechanisms include a vicious cycle of neutrophil activation and CEP damaging. Whether MC neutrophil supernatant leads to increased sGAG release in vitro remains to be elucidated.

RELEVANCE: CEP damage promotes a pro-inflammatory disc/bone marrow crosstalk in MC and coincides with increased nerve fiber density. Hence, these findings could have implications for treatment strategies to mitigate inflammation and CEP damage in MC.

REFERENCES: [1]Carmona-Rivera C et al.JCI Insight. 2020 Jul 9;5(13):e139388.