ROS rather than inhibition of the NAPDH oxidase. APPA significantly decreased the formation of NETs ($p<0.05$), which was probably via inhibition of NADPH oxidase and decreased degranulation. TNFα-induced NF-κB signalling was inhibited by APPA (600 μM), as was GM-CSF and IL-6 signalling via ERK1/2 and STAT3, respectively. APPA decreased TNFα-activated expression of IL-8 and TNFα mRNA but upregulated NRF2, an anti-inflammatory regulator of antioxidant proteins. APPA was also an effective inhibitor of IL-6 and chemokine expression (CCL3, CCL4) induced by the TLR8 agonist and chromatin remodelling agent, R848 ($p<0.05$). This agonist triggers the expression of these genes via endogenous TNFα secretion and activation of neutrophils; APPA was as effective as the biologic therapy Infliximab in this inhibition.

Conclusions: APPA does not significantly impair host defence neutrophil functions and may have significant anti-inflammatory potential in diseases characterised by dysregulation of cytokine expression or oxidative stress. APPA is a potent ROS scavenger, in addition to affecting neutrophil degranulation. These data also describe the novel finding that APPA is as effective as biologic drugs in inhibiting the effects of endogenous TNFα on immune cell activation. We believe APPA may have therapeutic potential in ROS- and/or TNFα-driven inflammatory conditions.

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INDUCIBLE DICKKOPF 1-MEDIATED INHIBITION OF CANONICAL WNT SIGNALING AMELIORATES CARTILAGE DESTRUCTION DURING EXPERIMENTAL OSTEOARTHRITIS

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Purpose: Balanced canonical Wnt signaling has been shown to be crucial for the development and homeostasis of joint tissues. Previously, we described increased expression of various members of the Wnt signaling in the synovium in two experimental osteoarthritis (OA) mouse models. In addition, we found increased expression of the canonical Wnt signaling key downstream target WISP1 in both the synovium and cartilage during experimental and human OA. Together, this suggests increased activation of the canonical Wnt signaling pathway in the OA joint. We and others described that increased canonical Wnt signaling associates with more joint pathology. Here, we determined the potency of Dickkopf 1 (*Dkk1*) overexpression-mediated inhibition of canonical Wnt signaling to reduce pathology in the complex environment during experimental OA. Because a constitutively enhanced expression has profound effects on the development of target tissues such as the articular cartilage and bone which affects the development of OA, we here used inducible *Dkk1*-transgenic mice.

Methods: Inducible *Dkk1*-transgenic mice were generated by backcrossing *rtTA* and *tetO-Dkk1* transgenic mice. The mice used in this study were homozygous for *rtTA* and heterozygous for *Dkk1*, since a complete inhibition of β-catenin as key intracellular factor in canonical Wnt signaling, similar to the constitutive activation of β-catenin mimicking increased canonical Wnt signaling, has been shown to be detrimental for the articular cartilage. *Dkk1*-overexpression was induced by feeding mice food supplemented with doxycycline. Collagenase-induced osteoarthritis (CIOA) or destabilization of the medial meniscus (DMM) experimental animal models of OA were induced in mice with (on dox) or without (off dox) doxycycline supplementation. Gene expression was determined with qRT-PCR. Joint pathology was assessed using histology after hematoxylin & eosin or Safranin O & Fast Green staining. Protease activity was determined by immunohistochemical staining of the aggrecan neoepitope NITEGE.

Results: First, we ensured that the $rtTA^{+/+}/Dkk1^{+/-}$ had no basal phenotype in their cartilage and subchondral bone when compared with $rtTA^{+/+}/Dkk1^{-/-}$ mice at the age of induction of the OA models. Mice off dox showed comparable articular cartilage and a comparable ratio between the surface of Fast Green stained collagenous and total area in the subchondral bone. Moreover, we observed that addition of dox to the food resulted in a strongly increased *Dkk1* expression in both the cartilage and synovium of $rtTA^{+/+}/Dkk1^{+/-}$ as compared to off dox controls. Next, we tested whether doxycycline supplementation itself affected the severity of the experimental OA models as studied in $rtTA^{+/+}/Dkk1^{-/-}$ mice. However, we observed comparable cartilage degeneration between mice that were on dox and off dox after induction of CIOA and DMM, therewith excluding dox effects. Interestingly, $rtTA^{+/+}/Dkk1^{+/-}$ mice on dox, and thus overexpressing *Dkk1*, developed significantly less cartilage damage both after induction of CIOA and DMM, which was associated with decreased levels of the aggrecan neoepitope NITEGE, but showed comparable levels of synovial inflammation.

Conclusions: Our study shows that inhibition of canonical Wnt signaling by DKK1 might prove an efficient way to limit OA-related cartilage degeneration and therefore might be an interesting option for clinical therapy.

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CHANGES IN SOX9 AND RUNX2 PROTEIN ACTIVITY CORRELATE TO THE HEALTH STATE OF HUMAN PRIMARY CHONDROCYTES

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Purpose: SOX9 and RUNX2 are the master transcription factors involved in cartilage and bone development respectively. Altered SOX9 and RUNX2 activity is implicated in osteoarthritis (OA) pathophysiology and hypertrophic differentiation of chondrocytes. We have previously shown that SOX9 and RUNX2 protein activity can be measured by Transcription Factor - Fluorescence Recovery After Photobleaching (TF-FRAP). To decipher the underlying signaling mechanism in OA pathophysiology, we aim to use TF-FRAP to correlate SOX9 and RUNX2 mobility to the health state of the chondrocytes. We hypothesize that the mobility of SOX9 increases with OA progression, while the mobility of RUNX2 decreases in chondrocyte hypertrophy. We measured the mobility and correlated protein activity of these transcription factors at the single cell level by TF-FRAP in healthy, preserved and OA chondrocytes.

Methods: Healthy human primary chondrocytes (hPCs) were purchased from Articular Engineering, USA. Human preserved and OA hPCs were isolated from patients undergoing total knee replacement therapy. hPCs were seeded on glass coverslips and transfected with either SOX9-mGFP or eGFP-RUNX2. TF-FRAP was performed in at least 40 cells per condition in two healthy donors and three donors for each preserved and OA chondrocytes. TF-FRAP measurements were done using a Nikon A1 confocal microscope (Japan). In TF-FRAP, a small circular region (Ø 25 pixels) of the nucleus was bleached using a high intensity laser and the recovery of the fluorescence in the bleach spot is recorded. For every TF-FRAP measurement, 25 pre-bleach images and 260 post-bleach images were acquired at 4 frames per second for 60 seconds, with a frame size of 256x256 pixels. Higher immobile fraction (i.e., the fraction of transcription factors bound to DNA) and higher half-time recovery (longer DNA interaction times) were associated with a higher transcriptional activity. For all donors, early passage ($\leq$3) cells were used for TF-FRAP and qPCR measurements. To correlate protein mobility and DNA binding with the protein activity, we quantified the expression levels of SOX9 target genes (*ACAN* and *COL2A*) and RUNX2 target genes (*MMP13* and *COL10*) by qPCR.

Results: In the healthy hPCs, the SOX9 immobile fraction and half-time recovery were 60% (SD ±6%) and 14.2 sec (SD ±3.2 sec) respectively. In preserved hPCs, the SOX9 immobile fraction and half-time to recover was significantly lower, ranging from 50% - 44% (SD ±7% - ±9.7%), and 14.2 - 13.3 sec (SD ±3.9 - ±4.5 sec) respectively. In OA hPCs, the SOX9 immobile fraction and half-time recovery was significantly lower than in the healthy and preserved hPCs, ranging from 48.6% - 42.8% (SD ±7.3% - ±8.4%), and half-time to recover ranged from 13.3 - 12.2 sec (SD ±3.9 - ±5.3 sec). RUNX2 immobile fraction and half-time to recover data spread was large in healthy, preserved and OA hPCs. In healthy hPCs, immobile fraction and half-time to recover were ranging from 33.6% - 47.9% (SD 19.4% - 20.7%) and 13.97 sec - 14.46 sec (SD 7.9 - 10.1 sec) respectively. In the preserved hPCs, the immobile fraction and half-time to recover were ranging from 38.8% - 47.0 % (SD 19.7% - 22.2%) and 11.7 sec - 14.8 sec (SD 6.8 - 8.2 sec) respectively. In OA hPCs, the immobile fraction and half-time to recover ranged from 33.0% - 41.02% (SD 20.3% - 25.7%) and 11.2 sec - 12.9 sec (SD 7.4 - 9.1 sec) respectively. We have an initial non-quantitative indication for a distinct cell population that, irrespective of the health state, shows decreased RUNX2 mobility, which could be indicative of a hypertrophic cell state. We will show the quantification and segregation. RT-qPCR measurements show decreased SOX9 and increased RUNX2 target gene expression during OA progression.

Conclusions: Our data show that the SOX9 and RUNX2 transcriptional activity is differentially regulated depending on the health state of hPCs. If we assume a correlation between mobility and transcription factor

activity, then we can say that SOX9 protein activity decreases with increasing OA progression. RUNX2 mobility data showed large spread especially in the preserved and OA donors possibly due to cells were in the different health state in the OA joint. Our findings at the single cell level provide novel insights into SOX9 and RUNX2 protein activity with respect to OA progression.

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INHIBITION OF NADPH OXIDASES ABROGATES FIBRONECTIN FRAGMENT INDUCED INTEGRIN SIGNALING AND MATRIX METALLOPROTEINASE 13 RELEASE IN HUMAN CHONDROCYTES

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Purpose: OA is characterized by the degradation of cartilage extracellular matrix (ECM), including the production of ECM protein fragments. One of these, fibronectin fragments (FN-f), found in OA cartilage and synovial fluid, promotes cartilage matrix destruction through activating the α5β1 integrin and increasing MMP production. Reactive oxygen species (ROS) are necessary second messengers in the chondrocyte integrin signaling pathways that mediate MMP-13 production in response to FN-f stimulation. NADPH oxidases (Noxes) generate ROS in response to a variety of extracellular stimuli. The purpose of this study was to investigate the role of Noxes as mediators of ROS production and α5β1 signaling in human chondrocytes in response to FN-f.
Methods: Primary human chondrocytes were isolated from normal donor tissue or OA tissue removed at the time of knee arthroplasty. Prior to FN-f stimulation, media was changed to serum-free media. Chondrocytes were treated with 10 μM VAS2870 (pan-Nox inhibitor) or 20 μM GKT 137831(dual Nox1/4 inhibitor) for 1 h before the treatment of 1 μM FN-f overnight (to study MMP release) or 30 min (to study the activation of cell signaling). Oxygen is the substrate of Nox reactions. To assess the effect of oxygen tension on FN-f induced MMP release, chondrocyte monolayers from same donors were cultured in normoxic (20%) or low oxygen (3%) conditions. Cell lysates or conditioned media were collected and analyzed by immunoblotting to assess MMP-13 production and the activation of MAP-kinases (JNK, ERK, and P38) following FN-f stimulus. To assess Nox isoform expression, seven described Nox isoforms and subunits of Nox2 and Nox4 p22phox, p67phox, p47phox and p40phox were analyzed by Real-Time PCR. Protein levels of Nox2 and Nox4 in response to FN-f were analyzed by immunoblotting.
Results: FN-f induced MAP-kinases (JNK, ERK, and p38) signaling and MMP-13 production by normal chondrocytes were inhibited by the pan Nox inhibitor VAS2870 but not by the dual Nox1/Nox4 inhibitor GKT 137831 (Fig 1) suggesting Noxes other than Nox1/4 were required for FN-f activity. Similar findings were noted with OA chondrocytes. Low

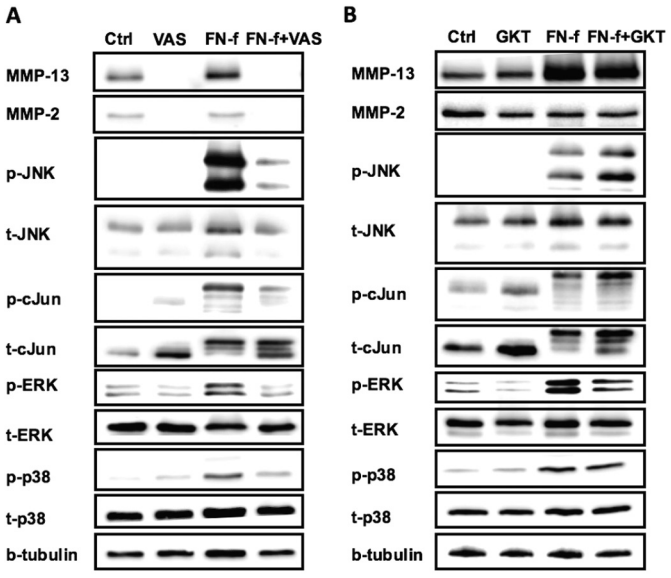

Figure 1 Effect of Nox inhibitors on FN-f induced MMP production and MAP kinase activation in normal chondrocytes. (A) VAS2870 blocked FN-f induced MMP and MAP kinase activation. (B) GKT137831 did not block FN-f induced MMP or MAP kinase activation. Data are representative from n = 3 donors.

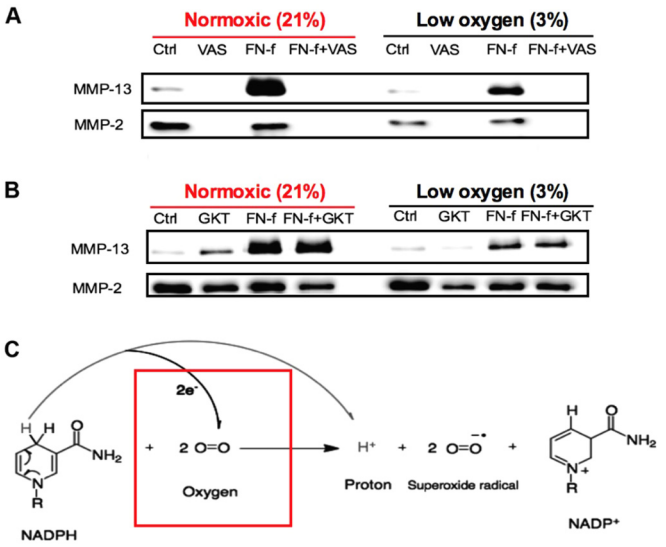

Figure 2 Low oxygen conditions partially decreased FN-f induced MMP-13 release but did not alter effects of Nox inhibition. Data are representative from n = 3 donors.

oxygen conditions partially decreased FN-f induced MMP-13 release as compared to normoxic conditions but Nox inhibition with VAS2870 was still effective (Fig.2). Moreover, we found Nox2 and Nox4 were highly expressed within human chondrocytes. FN-f stimulated the mRNA expression of Nox subunits, including p22phox and p67phox. FN-f stimulation did not affect Nox2 protein levels but increased Nox4 at early time points (30 min) and gradually led to Nox4 downregulation.
Conclusions: Collectively, these results indicate that NADPH oxidase activity is required for ROS production in response to FN-f that leads to MAP kinase activation and MMP-13 production. Inhibitor studies suggested Nox2 but not Nox1 or Nox4 was required. Future studies will investigate the role of Noxes in the development of OA *in vivo*.

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TRANSFORMING GROWTH FACTOR-β DAMPENS INTERLEUKIN-6 SIGNALING IN CHONDROCYTES BY DECREASING THE INTERLEUKIN-6 RECEPTOR

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Purpose: Interleukin-6 (IL-6) is a pro-inflammatory cytokine present in increased levels in serum and synovial fluid from patients with osteoarthritis (OA) and is even considered to be predictive for disease progression. Previous studies implicate IL-6 in articular cartilage destruction, which is the main hallmark of OA. In contrast, transforming growth factor-β (TGF-β) is an important homeostatic regulator of cartilage that can decrease the catabolic effects of pro-inflammatory cytokines in chondrocytes. Multiple mechanisms of cross-talk between TGF-β and IL-6 are reported in other tissues. In this study, we investigated whether TGF-β is able to control IL-6 signaling in chondrocytes.
Methods: Human primary chondrocytes and the human G6 chondrocyte cell line were stimulated with rhTGF-β1 (1.0 ng/ml). We determined mRNA expression levels of IL-6 and IL-6 receptor (IL-6R) using qPCR, measured IL-6 protein release using Luminex multianalyte technology and IL-6R protein levels by Western Blot. rhIL-1β was used as a positive control for IL-6 induction. Induction of signal transducer and activator of transcription (STAT)3 phosphorylation by TGF-β was determined using Western Blot, in presence of the IL-6R inhibitor Tocilizumab to study IL-6R dependency. Induction of suppressor of cytokine signaling (SOCS)3 gene expression was used as a read-out for IL-6/STAT3 mediated transcriptional activity.
Results: TGF-β increased both IL-6 mRNA expression and protein release in G6 and primary chondrocytes. Moreover, we observed activation of the main IL-6R downstream signaling protein STAT3 after TGF-β exposure, which could be completely prevented by blockade of the IL-6R, suggesting IL-6 dependency. We further explored whether TGF-β mediated IL-6/p-STAT3 signaling resulted in activation of target genes,