Figure 1

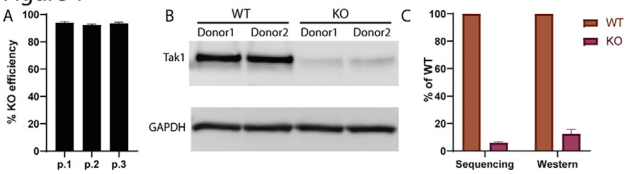
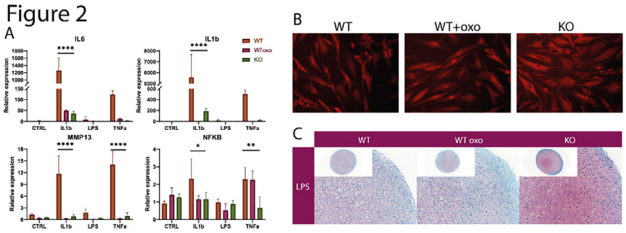


Figure 2



PRESENTATION NUMBER: 46
INJECTABLE HYDROGELS FUNCTIONALIZED WITH ANTIBODY FRAGMENTS AS INTRA-ARTICULAR CYTOKINE SINKS NEUTRALIZING PRO-CATABOLIC CYTOKINES

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Purpose: Intra-articular (IA) injection with biologics like cytokine neutralizing antibodies to treat osteoarthritis (OA) have been explored, but so far success is limited. One of the key obstacle for the efficacy of intra-articular therapies is the short retention time of these biologics in the joint space due to rapid clearing by the synovial membrane. To overcome these limitations, we developed a new concept that potentially can increase the therapeutic value of pro-inflammatory cytokine neutralizing antibody fragments in the management of OA. The concept is based on the use of injectable hydrogels functionalized with the variable domain of single chain heavy chain only antibodies (VHHs) for cytokine capture. VHHs can be easily isolated using phage-display technology, recombinantly engineered and produced in yeast. These hydrogels can either be pre-crosslinked in microgels before injection or injected as polymer-VHH conjugates that cross link in a hydrogel in situ. Here, hyaluronic acid was used as model polymer after substitution with tyramine and maleimide groups. The tyramine moiety is used in an enzymatic cross-linking reaction to yield stable macromolecular hydrogel networks. The maleimide group is used for thiol-maleimide chemistry coupling a recombinantly engineered VHH with a free Cysteine in its C-terminal tail to the polymer backbone. We hypothesized that this strategy to functionalize hydrogels with the VHH can retain biological activity of neutralizing VHH. To proof the concept of these so-called cytokine sinks we have selected an anti-TNFα VHH that effectively neutralizes TNFα, which plays an essential role in cartilage degradation in inflammatory arthritis.
Methods: To achieve directed conjugation of the VHH to the polymer, we have introduced an unpaired cysteine by using recombinant DNA technology in the C-terminus of the anti-TNFα VHH. Afterwards, the modified VHH was produced in yeast and purified. The conjugation of the VHH to the hyaluronic acid was performed by incubating the VHH with tyramine and maleimide functionalized hyaluronic acid. Characterization of binding affinity for both the non-conjugated VHH and conjugated VHH were measured by ELISA. hydrogels functionalized with the VHH was prepared by mixing the hyaluronic acid-VHH conjugates with enzyme peroxidase and H_2O_2. Biological activity of the conjugated VHH and hydrogels functionalized with the VHH was measured using an NFκb responsive luciferase reporter cell line after stimulation with TNFα. An equimolar concentration of non-conjugated VHH was used as a control.
Results: We show successful conjugation of the VHH to the polymer backbone. The VHH functionalized polymer could be used for making stable hydrogels after tyramine mediated cross linking. This modification did not affect the biological activity of the VHH. Using an NFκb responsive luciferase reporter cell line we demonstrated that hydrogels functionalized with the VHH efficiently inactivated TNFα and that this inhibition was comparable to inhibition by an equimolar concentration of non-conjugated VHH.
Conclusions: We successfully developed a biocompatible and efficient way to couple VHH to hyaluronic acid. These conjugates could be used for in situ generation of cytokine sinks capable of capturing different pro-catabolic cytokines in treatment of OA. Our Results demonstrate that cytokine sinks have great potential for neutralizing inflammatory pro-catabolic cytokines after intra-articular injection by increasing the retention time of neutralizing antibody fragments in the joint cavity.

PRESENTATION NUMBER: 47
TNF-ALPHA INDUCES FGF1 IRES MEDIATED MESSENGER RNA TRANSLATION IN CHONDROCYTES

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Purpose: Ribosomes are exclusively required for the continuous translation of all proteins in cells. In addition to the major pathway of cap-mediated translation, Internal Ribosomal Entry Site (IRES)-mediated translation is a distinct level of translational regulation that is at a cell's disposal under stress conditions. It was demonstrated that thousands of human mRNAs contain IRESs. We hypothesized that environmental conditions relevant to osteoarthritis (OA), alter ribosome function in chondrocytes, which in turn contribute to OA pathophysiology. We used a candidate approach in a chondrocytic cell line to identify IRESs that differentially regulate mRNA translation in OA-like conditions.
Methods: Eleven eukaryotic IRESs known to mediate cap independent translation were cloned into a modified psiCHECKv2 backbone to generate bicistronic reporter plasmids (pRenilla_Firefly, pR_F). The SW1353 chondrocytic cell was cultured in DMEM/F12 supplemented with 10% FCS, 1% NEAA and antibiotics. Cells were stimulated for up to three days with two different pleiotrophic cytokines (10 ng/ml IL1-beta, 10 ng/ml TNF-alpha). SW1353 were transfected with bicistronic IRES reporter constructs or an empty control plasmid using Fugene. Dual luciferase reagents were used in combination with a luminescence detection injection system to measure IRES (Firefly) and cap-mediated (Renilla) translation. The IRES activity ratio was calculated by dividing Firefly/Renilla signal and is presented as a fold change. RT-qPCR was used to determine *FGF1* splice variant and total *FGF1* mRNA expression. ELISA was used to measure FGF1 secretion levels. Statistical analyses were performed with a student t-test.
Results: Our initial IRES activity screening revealed that 10/11 IRESs were 2-7 times more active in comparison with the empty control plasmid under normal culture conditions demonstrating basic activity of the IRES. Stimulation with TNF-alpha for 3 days led to increased FGF1 IRES activity (Fig. 1A; 59%). The empty control plasmid also produced ~18% more signal in the presence of TNF-alpha. Next, we determined the kinetic regulation of the FGF1 IRES activation by TNF-alpha. After overnight transfection of the reporter, TNF-alpha stimulation did not result in FGF1 IRES activation after 8 hours, but significant induction at 16 (42%) and 24 hours (52%) post-stimulation (Fig. 1B; TNF-alpha). Non-treated cells were taken along as a control and did not show any FGF1 IRES activation alterations (Fig. 1B; control). To establish whether the IRES activation was accompanied by induction of mRNA expression, we evaluated gene expression of all *FGF1* isoforms and the endogenous IRES containing *FGF1A* isoform. None of the FGF1 transcript variants was induced by TNF-alpha treatment after 1 or 3 days (Fig. 1C; *FGF1a* expression and data not shown). Gene expression was even significantly decreased at day 1 (2 fold) and day 3 (8 fold) compared to day 0. At day 3, gene expression of the IRES-containing transcript was even significantly reduced by 35% in the presence of TNF-alpha in comparison with control stimulation. Finally, we measured FGF1 protein levels in the medium to test if the observed IRES activation and decreased mRNA expression led to an increase in FGF1 protein synthesis. We found an induction of FGF1 protein levels in the medium at day 1 (1.7 fold) and day 3 (3.4 fold; Fig. 1D).
Conclusions: For the first time we demonstrate that a cytokine, TNF-alpha, can induce IRES-mediated mRNA translation of FGF1 in a chondrocytic cell-line. This was accompanied by an 8-fold reduction in *FGF1* mRNA levels, but a 3.4 fold accumulation of FGF1 protein levels in the culture medium. Our time-series experiments indicate that the IRES