Metabolic Regulation Of Synovial Macrophage Activation In A Novel Proteoglycan 4 Conditional Knockout Mouse

Khaled A. Elsaid[1], Ling Zhang[2], Thomas Zhao[2], Gregory D. Jay[2]
[1]Chapman University, Orange, CA, [2]Rhode Island Hospital, Providence, RI
elsaid@chapman.edu

DISCLOSURES: Khaled A. Elsaid (N), Ling Zhang (N), Thomas Zhao (N), Gregory D. Jay (4, Lubris LLC)

INTRODUCTION: Proteoglycan 4 (PRG4) is a mucinous glycoprotein that fulfills a homeostatic role in the joint. In joints lacking *Prg4* expression, synovial hyperplasia, fibrosis, and accumulation of pro-inflammatory macrophages progressed with age and these pathological changes were partially reversed with *Prg4* re-expression. PRG4 regulates macrophage activation via binding to its cognate receptor, CD44 and activation of protein phosphatase 2A (PP2A) and downstream inhibition of xanthine oxidase (XO) expression. In this context, XO-derived reactive oxygen species (ROS) was shown to drive NLRP3-dependent IL-1β secretion by macrophages. We aimed to characterize early metabolic changes in the synovium with respect to XO modulation and downstream synovial macrophage immune activation in a novel conditional knockout mouse model, where animals are born Prg4 competent and *Prg4* expression can be inactivated with Cre recombination. We hypothesized that XO upregulation in setting of Prg4 deficiency drives pro-inflammatory phenotypic switching of synovial macrophages. **METHODS:** *Prg4*$^{Frt/GT}$;*R26*$^{FlpoER/rTTA}$;Tg:Tet-on-Cre are transgenic mice, where the *Prg4*^{Frt} allele normally expresses the Prg4 protein and was designed to flank the first two exons of Prg4 with a flippase recognition target and "LOXP" sites. Inducing the flippase activity with tamoxifen (TAM) inactivates the Frt allele and thus creates a knockout state. TAM (0.1mg/gram) or corn oil (Veh) (100µl) administration occurred in 4 weeks-old animals for 10 days and histological analyses and synovial tissue collection for RNA and synovial macrophage (SM) isolations were performed 6 weeks later. Histological analyses included Prg4 and XO immunostainings by Mab S6.79 and anti-XO antibody (Abcam) (1:100 dilutions for both antibodies), respectively followed by DAB staining as well as hematoxylin and eosin (H&E). In TAM and Veh administered animals, RNA was isolated from synovial tissues of both knee joints and pooled together to represent one independent biological sample and multiplexed gene expressions were performed using pre-validated panels for murine immune activation status and cell metabolism using the nCounter technology (NanoString). In addition, Synovial tissues from 2-3 animals were pooled together and subjected to SM isolation. Isolated SMs (500,000 cells per well) were activated using LPS (100ng/ml) and IFNγ (20ng/ml) and intracellular ROS levels were quantified using the DCFDA/H2DCFDA kit (Abcam). Alternatively, glycolytic activation of SMs was monitored in real time using a Seahorse Analyzer displaying proton efflux rate (PER), a marker of pro-inflammatory SM activation. Pharmacological treatments included febuxostat (Feb; XO inhibitor; 25µM) (Cayman Chemicals) and echinomycin (HIF-1α inhibitor; 50nM). Statistical analyses included Student's *t*-test and ANOVA followed by post-hoc Tukey's test. **RESULTS:** Abolishment of *Prg4* expression was evident in TAM mice, and this was associated with enhanced XO staining and synovial hyperplasia (**Fig.1A&1B**). In TAM synovia (n=3), glycolysis, hypoxia and oxidative stress pathways were upregulated compared to Veh synovia (n=3) (**Fig.2A**) and upregulated individual genes of interest included *Xdh* (gene symbol for XO; *p=0.01*) and *Hif1a* (*p=0.008*) (**Fig.2B**). ROS levels were higher in TAM SMs at baseline and following LPS+IFN activation (**Fig.3A**). Feb treatment reduced ROS level in TAM SMs (**Fig.3B**; *p<0.001*). TAM SMs more readily switched to glycolysis (**Fig.3C&3D**) and this switch was inhibited by Feb (**Fig.3E**; *p<0.01*) and HIF-1α inhibitor (**Fig.3F**; *p<0.0001*) treatments. **DISCUSSION:** *Prg4* inactivation induced synovial hyperplasia and XO upregulation. Immune activation, glycolysis and hypoxia pathways were also activated in the synovium due to *Prg4* inactivation. SMs from Prg4 deficient animals had a higher ROS burden likely due to XO upregulation and XO and HIF-1α contributed to exaggerated inflammatory activation of these macrophages. **SIGNIFICANCE:** PRG4 is a significant modulator of SM phenotypic switching and restoring PPRG4-mediated synovial homeostasis is potentially therapeutic in chronic synovitis. **ACKNOWLEDGEMENT:** Drs. Yajun Cui and Matt Warman and Mara Coyan for generating the conditional knockout allele.

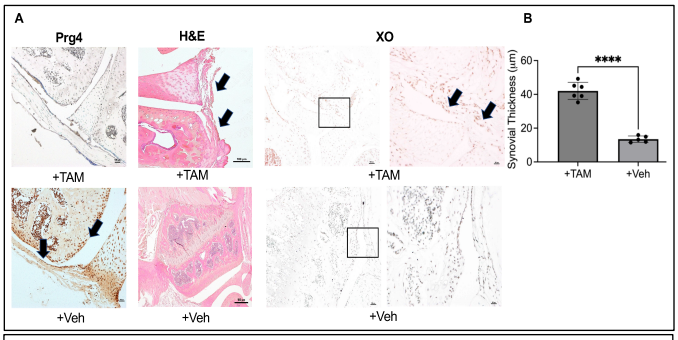

Figure 1 (above) Conditional inactivation of *Prg4* was associated with synovial hyperplasia and enhanced xanthine oxidase (XO) staining in synovial tissues. Mice were injected with tamoxifen (0.1mg/gram) (TAM) or corn oil (Veh) intraperitoneally for 10 days starting at 4 weeks old and knee joints were harvested 6 weeks later. Immunohistochemical staining included Prg4, XO in addition to hematoxylin and eosin (H&E). ****$p<0.0001$. **A)** Representative images of TAM and Veh-administered mice showing abolishment of Prg4, enhancement of XO staining, and synovial hyperplasia in a TAM-administered mouse, shown by arrows. **B)** Mean thickness of synovial membranes of TAM-administered mice (n=6; 3 males and 3 females) was higher than corresponding thickness in Veh-administered mice (n=5; 3 males and 2 females).

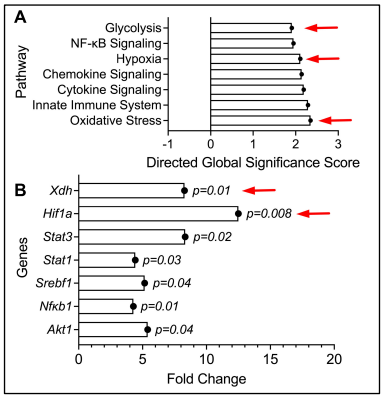

Figure 2 (left) Multiplexed gene expressions of synovial tissues from TAM or Veh-administered *Prg4* conditional knockout mice. Data is presented as significance scores (pathways) or fold change (individual genes) of TAM vs. Veh (n=3 in each group). **A)** Upregulated pathways in synovia from *Prg4* null animals included glycolysis, hypoxia, and oxidative stress (indicated by arrows). **B)** Xanthine oxidase (*Xdh*) and hypoxia inducible factor one alpha (*Hif1a*) genes were upregulated in *Prg4* null synovial tissues.

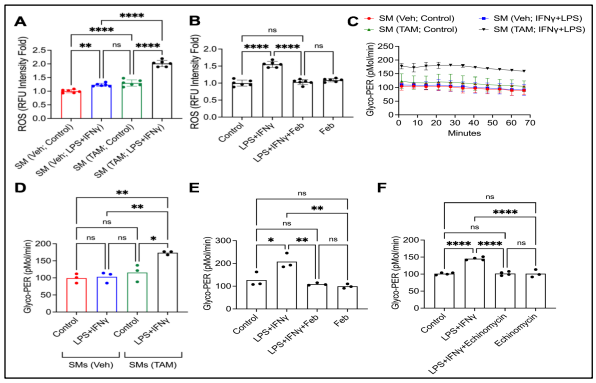

Figure 3 (left) Reactive oxygen species (ROS) and real-time monitoring of glycolysis of synovial macrophages (SMs) isolated from tamoxifen (TAM)-treated or vehicle (Veh)-treated *Prg4* conditional knockout mice using proton efflux rate (PER). (n=3-6 in each group). NS: non-significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$. **A)** TAM SMs had higher ROS at baseline and in response to LPS+IFNγ. **B)** Febuxostat (Feb; xanthine oxidase inhibitor; 25µM) reduced ROS in TAM SMs. **C)** TAM SMs had higher PER compared to Veh SMs in response to LPS+IFNγ. **D)** Mean PER of stimulated TAM SMs was higher than Veh SMs. **E)** Feb reduced PER of stimulated TAM SMs. **F)** Echinomycin (hypoxia inducible factor alpha inhibitor;50nM) reduced PER of stimulated TAM SMs.