Inhibitor N5-1 reduces cartilage damage and promotes anti-inflammatory macrophage polarization *in vivo*

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INTRODUCTION: The transcription factor interferon regulatory factor 5 (IRF5) plays key roles in regulating innate and adaptive immune responses and has been implicated in several autoimmune diseases. IRF5 promotes inflammatory M1 macrophage polarization, making it a therapeutic target of recent interest for osteoarthritis (OA) treatment. Studies have reported elevated IRF5 expression in both synovial macrophages and chondrocytes of OA patients, but mechanisms to inhibit IRF5 are limited. N5-1 is a novel peptide inhibitor of IRF5 activation designed by the Barnes Lab, and preliminary data with *Irf5*$^{-/-}$ mice and N5-1-treated rats support the potential of IRF5 inhibition to decrease joint inflammation and degradation and increase cartilage remodeling. The purpose of this study is to investigate the effects of N5-1 injections on knee joint damage and inflammation in an OA rat model. We hypothesized that N5-1 treatment would increase proteoglycan and other anabolic marker levels, decrease pro-inflammatory marker expression, and shift macrophage polarization to an anti-inflammatory M2 phenotype.

METHODS: The animal model and protocols were approved by the IACUC of the Feinstein Institutes. An OA rat model was used in which 20 rats underwent anterior cruciate ligament (ACL) transection and a surgical lesion to the front corner of the medial meniscus. Control and N5-1 treatment injections were administered into the right knee synovial cavity twice a week for four weeks. For 14 rats, injections began immediately after surgery, with seven rats receiving 50 µL PBS (Group 1) while the other seven received 100 µM N5-1 in 50 µL PBS (Group 2). For the remaining six rats, injections began after a four-week waiting period post-operation, with three rats receiving 50 µL PBS (Group 3) while the other three received 100 µM N5-1 in 50 µL PBS (Group 4). After four weeks of injections, the knee joints were dissected, fixed in 10% formalin, and decalcified with formic acid decalcification solution. The joints were cut evenly in the sagittal plane and embedded in paraffin, and the medial samples were sectioned (7-8 µm in thickness) and mounted. Safranin-O/Fast Green staining was performed to identify proteoglycan content, and immunostaining was performed to analyze the expression of type II collagen, MMP-3, CD68, and CD163. Comparisons between groups were analyzed with Mann-Whitney U tests to determine statistical significance.

RESULTS: Group 2 had significantly higher OARSI scores compared to Group 1 (p<0.05), indicating that N5-1 treatment increased proteoglycan content and reduced OA severity. COL2 expression was increased in the N5-1 group but did not reach statistical significance, and there were no significant differences in MMP-3 expression; further study with a greater sample size may help identify any changes in expression for these markers. There were no significant differences in total CD68 expression, but total CD163 expression and the ratio of CD163:CD68 were significantly higher in Group 2 compared to Group 1 (p<0.05, p<0.05, respectively). Further analysis of CD68 in the growth plate region showed significantly higher CD68 expression in Group 2 compared to Group 1 (p<0.05). Additionally, Group 2 had significantly lower CD68 expression (p<0.01) and significantly higher CD163 expression (p<0.05) compared to Group 1, along with a significantly higher CD163:CD68 ratio (p<0.01). In all analyses, there were no significant differences observed between Groups 3 and 4, likely due to a low sample size.

DISCUSSION: These data demonstrate that N5-1 treatment provides some protection of articular cartilage in the knee joint and shifts macrophage polarization toward an anti-inflammatory phenotype, particularly in synovial macrophages. Significantly greater Safranin-O staining and a trend toward greater COL2 expression demonstrate a less severe disease state following N5-1 treatment, and this may be the result of a greater M2 macrophage presence as shown by significantly higher CD163 expression. Significantly higher CD68 expression in the growth plate region suggests greater osteoclast activity, which may indicate greater regeneration potential, but further study is needed to explain this elevated expression. To our knowledge, this study is the first to report on the effects of N5-1 treatment *in vivo* in an osteoarthritis model. Our study provides information on the chondroprotective effects of N5-1 treatment and presents evidence supporting the mechanism of altered macrophage polarization to decrease cartilage degradation and OA progression.

SIGNIFICANCE: There is currently no cure for OA, and this study supports the therapeutic potential of the novel IRF5 inhibitor N5-1 to reduce inflammation, improve joint microenvironment conditions, promote bone regeneration, and prevent OA progression. Further, the varying inflammatory profiles observed in different parts of the knee joint suggest that knee OA should be considered a systemic pathology to improve the design of therapeutic strategies for OA treatment.

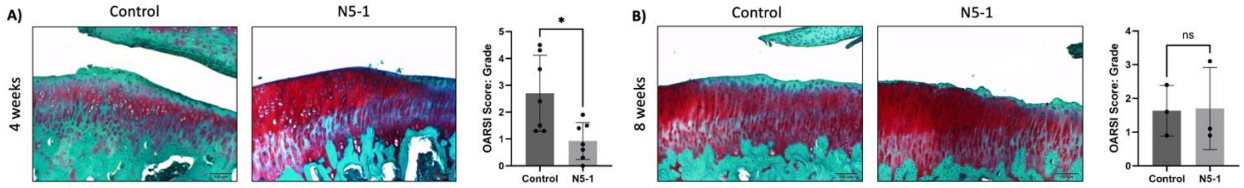

Figure 1. Safranin-O staining and OARSI scoring for control and N5-1 treated groups that received injections starting A) immediately post-op (4 weeks, n=7), and B) four weeks post-op (8 weeks, n=3). * p<0.05.

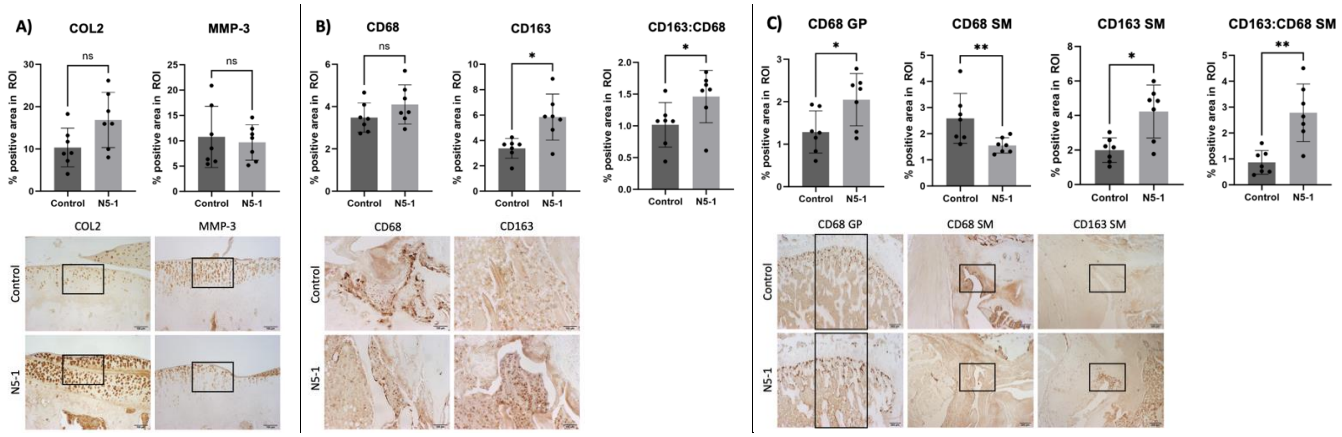

Figure 2. Quantification and representative images of A) COL2 and MMP-3 expression, B) total CD68 and CD163 expression, and C) CD68 and CD163 expression in the growth plate region (GP) and synovial membrane (SM) for control and N5-1 treated groups that received injections starting immediately post-op (n=7). * p<0.05, ** p<0.01.