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CONTROLLED RELEASE OF CD200 INHIBITS INFLAMMATORY MACROPHAGES AND CHONDROCYTE CATABOLISM

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Purpose: In post-traumatic osteoarthritis (PTOA), inflammation plays a central role in which macrophages in the synovium are classically activated to a M1-like state and secrete pro-inflammatory cytokines. The ensuing chronic loop of self-perpetuating tissue damage and macrophage activation ultimately exacerbates cartilage degradation and joint degeneration. When macrophages are polarized to an anti-inflammatory M2-like phenotype, they can trigger anti-inflammatory and immunosuppressive response within the joint. Therefore, we aim to polarize the macrophages in OA joints from a M1-like to a M2-like state using CD200, an endogenous immunomodulatory protein. Given the specific expression of the CD200 receptor (CD200R) on myeloid cells, with the highest expression in macrophages, CD200 engagement of CD200R results selectively in the polarization of M1 macrophages towards an M2-like state. The underlying hypothesis of this work is that the presentation of CD200 to macrophages in the inflamed synovium will shift their cytokine profile from pro-inflammatory to pro-regenerative, thereby protecting joint tissues from progression of degeneration. The goal of this study was to engineer a controlled release system for CD200, based on poly (lactic-co-glycolic acid) (PLGA) microparticles (MPs), to provide immunomodulation of macrophages and chondroprotection to chondrocytes *in vitro*.

Methods: The soluble form of the extracellular domain of the CD200 protein was produced recombinantly using CHO cells. CD200-loaded PLGA MPs and blank MPs were prepared using a water-in-oil-in-water double emulsion-solvent evaporation method. The microparticles' diameters were analyzed using ImageJ and images taken on an inverted optical microscope. Encapsulation efficiency was determined by dissolving the MPs in 0.5N sodium hydroxide and quantifying total protein using a μBCA assay. To determine release, PLGA MPs encapsulating Alexa488-tagged CD200 were incubated in release buffer at 37°C and the fluorescent signal from released CD200 was measured on a spectrophotometer over a 50-day period. The functional activity of CD200 following encapsulation was determined by incubating the MPs with primary mouse bone-marrow derived macrophages (BMDMs) 24 hours prior to classical activation and compared with an equivalent dose of free CD200. Following 12 hours of LPS and IFNγ stimulation, the secretion of pro-inflammatory cytokines (TNFα and IL6) and nitric oxide (NO) was measured using ELISAs and the Griess Assay respectively. The gene expression of primary mouse articular chondrocytes exposed for 48 hours to (1) conditioned media from M1 stimulated BMDMs and (2) conditioned media from M1 stimulated BMDMs treated with CD200 MPs was also determined. Statistical analysis was conducted on GraphPad PRISM 7.01 whereby error bars indicate standard deviations. Statistical comparison of means was conducted in GraphPad *via* a 1-way ANOVA with Dunnett's test for the protein secretion and *via* a Kruskall-Wallace test with Dunn's multiple comparison tests for the gene expression.

Results: CD200-loaded PLGA microparticles with a median diameter of 17μm and an encapsulation efficiency of 85.5% ± 9.8% were formulated. Over 50 days, 65.6% ± 4.6% of the protein cargo was released (Fig. 1) following first order kinetics. Following the 24-hour incubation of the CD200 loaded MPs with the BMDMs, the cells were classically stimulated for 12 hours with ultrapure (UP) LPS and IFNγ leading to increases in TNFα, IL6 and NO stimulation in control conditions (Fig. 2). Free CD200 as well as CD200-loaded MPs decreased this stimulation to comparable levels, suggesting the released CD200 is indeed biologically active. The blank MPs induced increases

in TNFα and IL6 compared to control, suggesting additional pro-inflammatory signaling in classically stimulated BMDMs. Chondrocytes, exposed to conditioned media from the M1+CD200 group of BMDMs, expressed catabolic mediators such as MMP13, MMP3, ADAMTS 4 and 5 at lower levels than chondrocytes incubated with media from the M1 group (Fig. 3).

Conclusions: Following encapsulation using the water-in-oil-in-water double emulsion-solvent evaporation method, the soluble form of the extracellular domain of CD200 was successfully incorporated into PLGA MPs for controlled release over 50 days, without compromising bioactivity. This will be ultimately be critical when used in *in vivo* applications, due to the rapid clearance of exogenously delivered proteins in the joint. While the CD200 MPs demonstrated the ability to decrease pro-inflammatory cytokine production, further studies will be needed to characterize the extent of M2 polarization. M1 macrophages that were treated with the CD200 MPs showed potential chondroprotective effects on chondrocytes *via* reduction in expression of catabolic enzymes. Future studies will test the MP system in a PTOA animal model wherein impact of CD200 on the immune profile of the joint can be evaluated. This study marks the first time the delivery of CD200 to alter the inflammatory cascade in a PTOA joint has been investigated. Given the promising results from the *in vitro* work, there is significant potential for the CD200-CD200R inhibitory signaling pathway to be leveraged as an intra-articular treatment of PTOA.

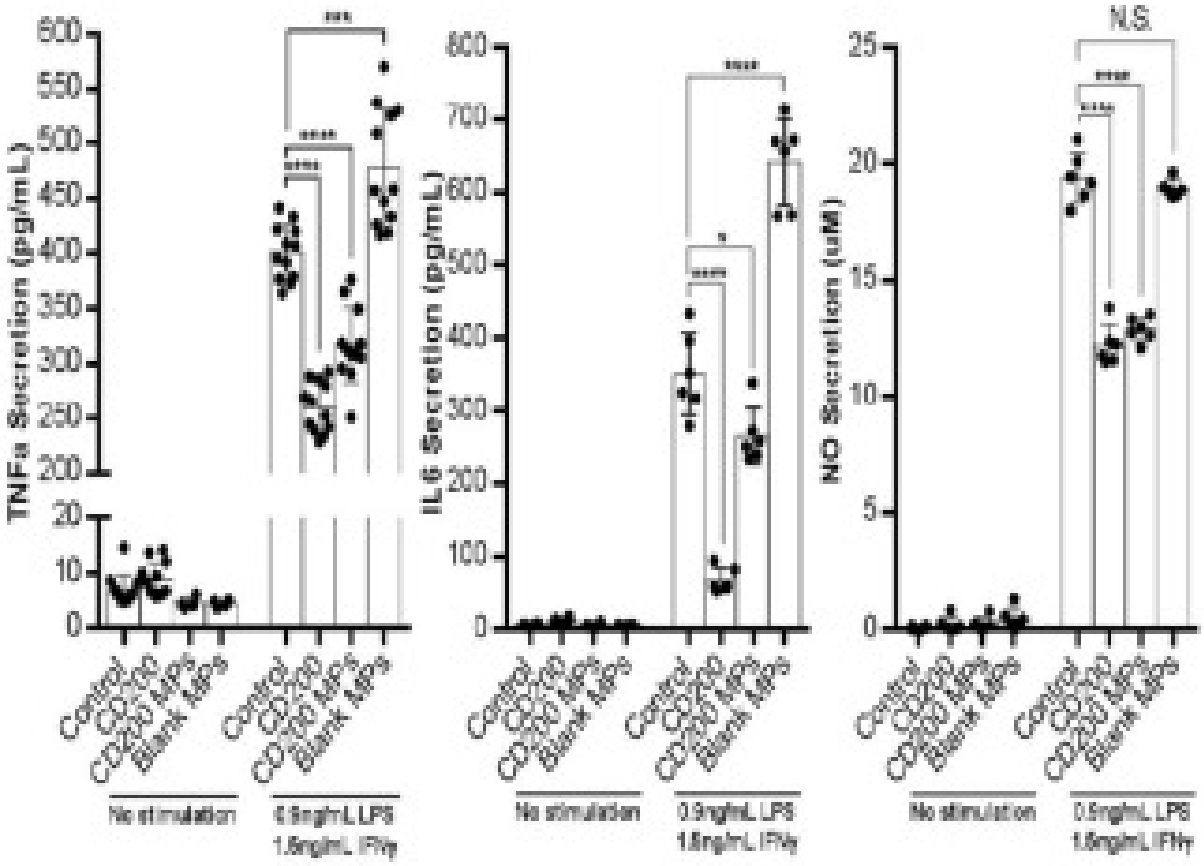
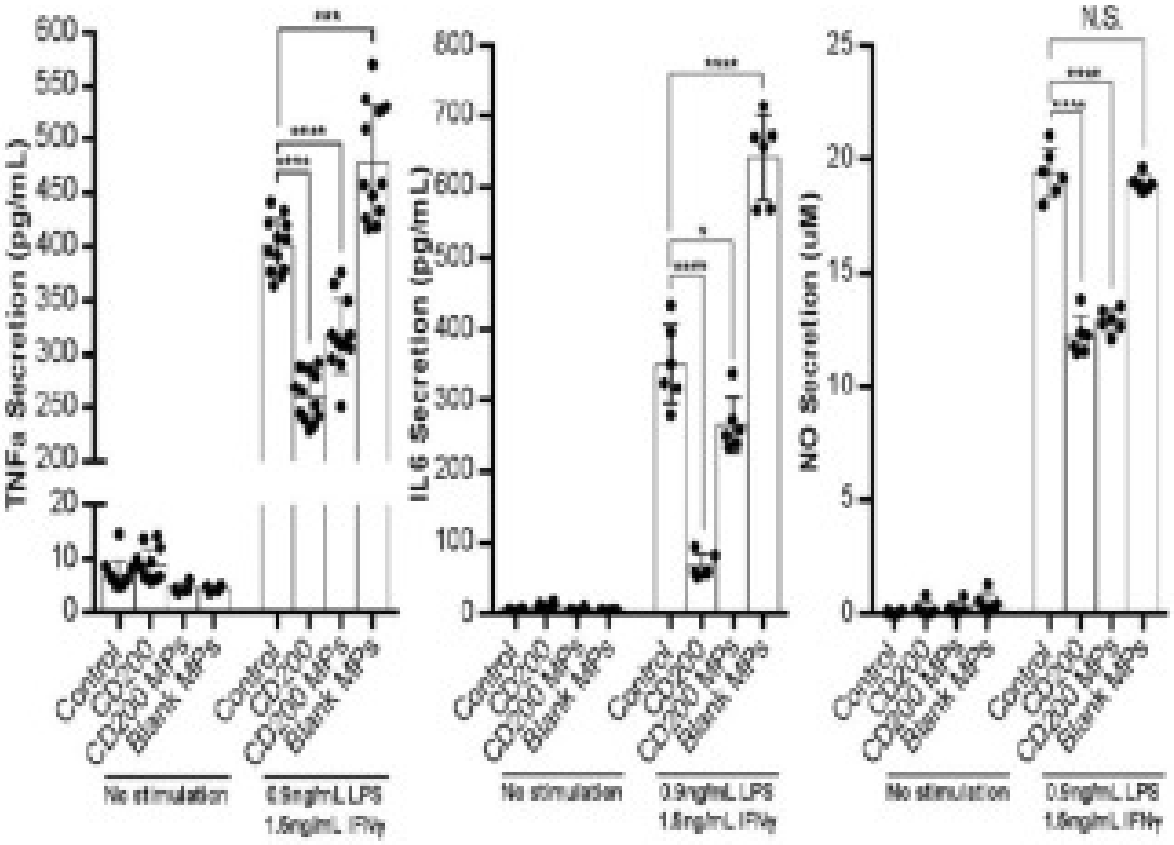


Figure 2: TNF-α, IL6 and NO secretion by non-stimulated (M0) and classically activated (M1) primary mouse BMDMs treated with soluble CD200, CD200 MPs and blank PLGA MPs. N.S. = non-significant, *$p \leq 0.05$, ***$p \leq 0.001$, ****$p \leq 0.0001$ between indicated groups.



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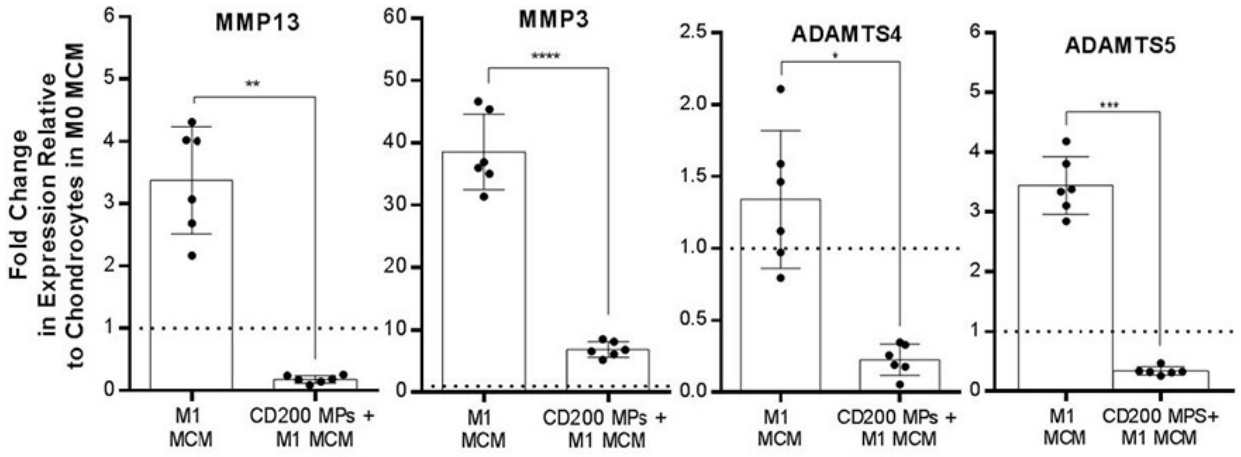

Figure 3: Gene expression of catabolic mediators by chondrocytes incubated in conditioned media from classically activated BMDMs that were untreated (M1 MCM) or treated with CD200 MPs (CD200 MPs + M1 MCM) *p≤0.05, **p≤0.01, ***p≤0.001,****p≤0.0001 between indicated groups.