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Lubricin/Proteoglycan 4 Binding to CD44 Receptor: A Mechanism of Lubricin's suppression of *Pro*-inflammatory Cytokine Induced Synoviocyte Proliferation

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Abstract

Objective—To evaluate recombinant human proteoglycan 4 (rhPRG4) binding to CD44 receptor and its consequence on cytokine induced synoviocyte proliferation.

Methods—rhPRG4 binding to CD44 and competition with high molecular weight hyaluronic acid (HMW HA) was evaluated using a direct enzyme linked immunosorbent assay (ELISA) and surface plasmon resonance. Sialidase-A and O-glycosidase digestion of rhPRG4 was performed and CD44 binding was evaluated using ELISA. Rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) were stimulated with interleukin-1 beta (IL-1β) or tumor necrosis factor alpha (TNF-α) for 48 hours in the presence or absence of rhPRG4 or HMW HA at 20, 40 and 80μg/ml and cell proliferation was measured. CD44 contribution was assessed by co-incubation with a CD44 antibody (IM7). The anti-proliferative effect of rhPRG4 was investigated following treatment of Prg4−/− synoviocytes with IL-1β or TNF-α in the presence or absence of IM7.

Results—rhPRG4 binds CD44 and interferes with HMW HA CD44 binding. Removal of sialic acid and O-glycosylations significantly increased CD44 binding by rhPRG4 ($p<0.001$). rhPRG4 and HMW HA at 40 and 80μg/ml significantly suppressed IL-1β induced RA-FLS proliferation ($p<0.05$). rhPRG4 at 20, 40 and 80μg/ml significantly suppressed TNF-α induced RA-FLS proliferation ($p<0.05$). CD44 neutralization reversed the effect of rhPRG4 on IL-1β and TNF-α stimulated RA-FLS and the effect of HMW HA on IL-1β stimulated RA-FLS. rhPRG4 inhibited

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COI Statements:

Authors AA and MJ, LZ, and KL have nothing to declare.

Author GJ holds patents related to use of recombinant human proteoglycan-4 and holds equity in Lubris LLC, MA, USA..

Author TS hold patents related to use of recombinant human proteoglycan-4, is a paid consultant for Lubris LLC, MA, USA and holds equity in Lubris LLC, MA, USA.

Author KE is a co-author on pending patent application related to recombinant human proteoglycan-4.

cytokine-induced proliferation of Prg4−/− synoviocytes which could be prevented by blocking CD44.

Conclusion—Lubricin is a novel putative ligand for CD44 and may control synoviocyte overgrowth in inflammatory arthropathies via a CD44-mediated mechanism.

Keywords

Lubricin; proteoglycan-4; CD44; Fibroblast-like synoviocytes

Introduction

Lubricin/proteoglycan 4 (PRG4) is a mucinous glycoprotein secreted by synovial fibroblasts and the superficial zone chondrocytes [1–3]. Lubricin's protein core is 1,404 amino acid long with *N* and *C* termini and a central mucin domain. The central mucin domain is heavily glycosylated via O-linked (β1-3) Gal-GalNAc oligosaccharides, and is configured to form a nanofilm that exerts repulsive forces, and provides the basis for its anti-adhesive and lubricating properties [4, 5]. Lubricin is abundant in synovial fluid (SF) and has a multifaceted function in joint homeostasis including boundary lubrication, prevention of adhesion of apposed cartilage surfaces and prevention of synovial overgrowth [6–8]. In pre-clinical animal models of surgically-induced osteoarthritis (OA), lubricin gene expression is down-regulated in articular cartilage and lubricin intra-articular administration in this setting reduces the extent of cartilage degradation and exhibits a disease-modifying activity [9–13].

Another macromolecule present in high concentration in SF is hyaluronan (HA). Lubricin interacts with HA, regardless of the latter's molecular weight, and this interaction may underpin the observed synergy in providing enhanced wear protection, improved lubrication and reduction in flexor tendon gliding resistance [14–17]. High-molecular weight HA has an established anti-inflammatory role, mediated by its interaction with the cell surface receptor cluster determinant 44 (CD44) [18, 19]. Given that HA and lubricin are the most abundant macromolecules in SF, they may share common biological effects related to joint homeostasis. The *C*-terminus of lubricin contains a hemopexin-like domain, which in membrane-type 1 matrix metalloproteinase (MT-MMP1), binds CD44 resulting in subsequent shedding of the receptor [20].

In this work, we have investigated the binding of recombinant human PRG4 (rhPRG4) to recombinant CD44 using a direct enzyme-linked immunosorbent assay (ELISA) format and surface plasmon resonance and evaluated the ability of rhPRG4 and high-molecular weight HA to suppress interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) induced proliferation of rheumatoid arthritis fibroblast like synoviocytes and the extent to which this is mediated by CD44 interaction. We have also studied the effect of IL-1β and TNF-α on Prg4−/− and Prg4+/+ synoviocytes proliferation and the interaction of rhPRG4 with CD44 on Prg4−/− synoviocyte proliferation following IL-1β or TNF-α stimulation.

Methods

Binding of rhPRG4, high-molecular weight HA (HMW HA), medium-molecular weight HA (MMW HA) and vitronectin to CD44 using a direct ELISA

High-binding microtiter plates (Corning, Sigma Aldrich, USA) were used to coat rhPRG4 (M_r=240 KDa), HMW HA (M_r=1,500 KDa) (R & D System, USA), MMW HA (M_r=300KDa) (R & D System) and vitronectin (M_r=75 KDa) (Sigma Aldrich) at 400μg/ml in PBS buffer (100μl per well) overnight at 4°C. rhPRG4 is a full-length product produced by CHO-M cells (Lubris, Framingham, MA, USA) [21]. Following washing with PBS +0.1% tween 20, wells were blocked with 2% bovine serum albumin (BSA; 300μl per well) for 2 hours at room temperature. CD44-IgG_1Fc (R & D systems) or IgG_1Fc (R & D systems), each at 1μg/ml (100μl per well), were added to the plate and incubated for 60 min at room temperature. Following washing with PBS+0.1% tween 20, anti-IgG_1Fc-HRP (Sigma Aldrich) was added at 1:10,000 dilution (100μl per well) and incubated for 60 min at room temp. Following washing with PBS+0.1% tween 20, the assay was developed using 1-step Turbo TMB ELISA reagent (ThermoScientific, USA) and absorbance was measured at 450 nm. Data represents the average of 4 independent experiments, each with triplicate wells per group.

Concentration-dependent binding of rhPRG4, HMW HA, MMW HA to CD44 and competition between rhPRG4 and HA on binding to CD44

Microtiter plates were coated with 400, 200, 100, 20, 4, 2 and 0.1μg/ml of rhPRG4, HMW HA and MMW HA. The assay was performed as described above. The absorbance values in the IgG_1Fc wells were subtracted from the absorbance values in the CD44 IgG_1Fc wells and the corrected CD44 IgG_1Fc absorbance values were normalized to those of the 400μg/ml rhPRG4 group and data was expressed as percentage binding to CD44. Data represents the average of 4 independent experiments, each with triplicate wells per group.

To evaluate the competition between rhPRG4 and either HMW HA or MMW HA on binding to CD44, microtiter plates were coated with either CD44 IgG_1Fc or IgG_1Fc at 1μg/ml (100μl per well). Subsequently, wells were washed with PBS+0.1% tween 20 and blocked using 2% BSA (300μl per well) for 2 hours at room temperature. Either rhPRG4 at 5μg/ml or a combination of rhPRG4 (5μg/ml) and HMW HA or MMW HA at 0.01, 0.05, 0.25, 1, 5 or 25μg/ml were added to the wells (100μl per well) and incubated at room temperature for 60 min. Following washing with PBS+0.1% tween 20, lubricin-specific monoclonal antibody (9G3, Millipore, CA, USA) was added at 1:1,000 (100μl per well) and incubated for 60 min at room temp. Following washing with PBS+0.1% tween 20, goat anti-mouse IgG-HRP (ThermoScientific) at 1:1,000 dilution was added (100μl per well) and incubated for 60 min at room temp. The assay was developed as described above. The absorbance values in the IgG_1Fc wells were subtracted from the absorbance values in the CD44-IgG_1Fc wells and the corrected absorbance values in the rhPRG4+HA groups were normalized to the absorbance values of the rhPRG4 group and data was expressed as percentage binding to CD44. Data represents the average of 4 independent experiments, each with triplicate wells per group.

Concentration-dependent binding of rhPRG4 to CD44 and competition between rhPRG4 and HMW HA using surface plasmon resonance

Binding of rhPRG4 to CD44-IgG1Fc was investigated using surface plasmon resonance (Biacore T100, GE Healthcare Lifesciences, NJ, USA). Series S chips were functionalized using the human antibody capture kit (GE Life Sciences) and either CD44-IgG_1Fc or IgG_1FC was allowed to bind to the surface of the functionalized chips in flow cell 1 (Fc_1) and flow cell 2 (Fc_2), *respectively*. rhPRG4 was injected at 30μl/min for 8 min at concentrations of 300, 250, 200, 150, 100 and 50μg/ml followed by a 10 min dissociation using 0.1M HEPES, 1.5M NaCl, 30mM EDTA, and 0.5% P20 (GE Life Sciences). The surface of the chip was regenerated at the end of each cycle with 1 min pulse of 3M $MgCl_2$. Each analyte concentration was injected in duplicate. The resulting curves were double referenced (i.e. Fc_1-Fc_2, followed by subtraction of the 0μg/ml curve). Binding kinetics and binding affinity were determined by BiaEvaluation software, using 1:1 binding/ conformational change model and by steady-state equilibrium, *respectively*. To study the competition between rhPRG4 and HMW HA in binding to CD44, rhPRG4 was injected at concentrations ranging between 0 and 300μg/ml as described above. Following the end of dissociation phase, HMW HA was injected at 50μg/ml (30 μl per min) for 1 min. The double-referenced binding signals of rhPRG4 (at various concentrations) to CD44 were then plotted against the binding signals generated by HMW HA binding to CD44 following rhPRG4 injections.

Impact of removal of mucin-domain glycosylations on binding of rhPRG4 to CD44

rhPRG4 was digested using sialidase A (Prozyme, USA), O-glycosidase (New England Biolabs, USA) or a combination of sialidase A and O-glycosidase for 16 hours at 37°C. In the sialidase A digestion, 12μl of the enzyme (1U/200μl) was added to rhPRG4 in a total reaction volume of 180μl and a rhPRG4 final concentration of 300μg/ml. In the O-glycosidase digestion, 4.8μl of the enzyme (40million units/ml) was added to rhPRG4 in a total reaction volume of 180μl and a rhPRG4 final concentration of 300μg/ml under non-denaturing conditions. In the sialidase-A and O-glycosidase digestion, the enzymes were used in volumes identical to the ones stated above and incubated with rhPRG4 in a total reaction volume and final rhPRG4 concentration as stated above. The effect of sialidase-A and O-glycosidase digestions on rhPRG4 apparent molecular weight was determined by SDS-PAGE using 4–12% Bis-Tris gel (NuPage, life technologies, USA). A total of 20μl of rhPRG4 or enzyme-digested rhPRG4 was run under reducing conditions (200 mV for 60 min) followed by staining using Gelcode Blue Stain (ThermoScientific, USA). Binding of enzymatically-digested rhPRG4 to CD44 was compared to undigested rhPRG4 using the direct ELISA approach described above and using a rhPRG4 coating concentration of 30μg/ml. Data represents the average of 4 independent experiments, each with triplicate wells per group.

Pro-inflammatory cytokine-induced rheumatoid arthritis fibroblast-like synoviocyte proliferation and impact of rhPRG4 or HMW HA treatment

Rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS; Cell Applications, USA) between 3rd and 6th passages were used. In sterile 96 well plates, RA-FLS (5,000 cells per

well) were cultured in DMEM supplemented with 1% FBS and 1mM pyruvate and stimulated with IL-1β (20 ng/ml; R & D systems) or TNF-α (5 ng/ml; R & D systems) for 48 hours at 37°C in the absence or presence of rhPRG4 or HMW HA at a final concentration of 20, 40 or 80μg/ml. The total volume in each well was 200μl. Cell proliferation was determined using the CellTiter 96 AQueous one solution cell proliferation assay (MTS; Promega, USA) and the 490 nm absorbance was determined. Data is presented as the number of fold change in 490 nm absorbance compared to untreated RA-FLS. Data represents the average of 3 independent experiments with at least triplicate wells per treatment. To evaluate the contribution of CD44 to the effect of rhPRG4 or HMW HA, RA-FLS were stimulated with IL-1β or TNF-α as described above. Cells were treated with rhPRG4 or HMW HA (80μg/ml) in the absence or presence of IM7 (1 mg/ml; Abcam, USA), a CD44 neutralizing antibody that recognizes a conserved epitope across all CD44 isoforms [22], at a final dilution of 1:200. The total volume in each well was 200μl. Cell proliferation across the experimental groups was determined as described above and data is presented as the number of fold change in 490 nm absorbance compared to untreated control RA-FLS. Data represents the average of 3 independent experiments with at least triplicate wells per treatment.

Effect of rhPRG4 treatment on nuclear translocation of nuclear factor kappa b (NFκB) following TNF-α stimulation of RA-FLS

RA-FLS (400,000 cells/well) were cultured and stimulated with TNF-α (5ng/ml) and treated with rhPRG4 (200μg/ml) or a commercially available NFκB translocation inhibitor MG 132 (3μM; Tocris Bioscience) for 24 hours in serum free media. Cells were harvested and nuclear extraction was performed using a commercially available kit (Thermo scientific). Total protein was measured using a micro bicinchonic acid (BCA) kit (Thermo scientific) and 3μg of nuclear extract of each experimental group were used. The p50 subunit of NFκB was detected in the nuclear extract using a commercially available NFκB DNA binding assay kit (Abcam). Data is presented as the number of fold change in NFκB nuclear levels compared to untreated control. To evaluate whether inhibition of NFκB translocation by rhPRG4 is CD44 dependent, the above experiment was repeated in the presence or absence of IM7 (1:1,000 dilution). Data represents the average of 3 independent experiments with at least triplicate wells per treatment.

Isolation of Prg4−/− and Prg4+/+ synoviocytes and CD44 immunocytochemistry

Synovial tissue was harvested from Prg4−/− and Prg4+/+ male mice (8–10 weeks old; 5–8 animals per genotype) and digested with pronase enzyme (2 mg/ml; Sigma Aldrich) in sterile HBSS buffer for 30 min at 37°C with shaking. This was followed by digestion with type I collagenase (1 mg/ml; Sigma Aldrich) for 4 hours at 37°C with shaking. The enzymatic reaction was stopped using DMEM+10% FBS. Cells were grown in DMEM +10%FBS and Prg4−/− synoviocytes were used between 2nd and 4th passages while Prg4+/+ synoviocytes were used in their second passage.

Prg4−/− and Prg4+/+ synoviocytes were grown in chamber slides (Thermoscientific). Cells were fixed in 4% formaldehyde for 15 min and washed twice with PBS buffer. Cells were permeabilized with 0.2% Triton X-100 for 10 min and washed 3 times with PBS buffer.

Cells were blocked with 2% BSA for 30 min. Synoviocytes were incubated with IM7 antibody (1:200) at 4°C overnight. Following washing three times with PBS, synoviocytes were incubated with Alexa Fluor 488 goat anti-rat IgG (Life Technologies) at 1:400 dilution for 1 hour in the dark. All incubations were performed at room temperature unless otherwise specified. Following washing with PBS for 5 min, Vectashield mounting medium containing DAPI (Vector Labs, Burlingame, CA, USA) was added. Cells were imaged with the Nikon Eclipse 90i Fluorescence Microscope using NIS Elements imaging software.

Impact of IL-1β and TNF-α on proliferation of Prg4−/− and Prg4+/+ synoviocytes and the effect of rhPRG4 on cytokine-induced Prg4−/− synoviocyte proliferation

In sterile 96 well plates, Prg4−/− and Prg4+/+ synoviocytes (10,000 cells per well) were cultured in DMEM supplemented with 1% FBS and 1mM pyruvate and stimulated with IL-1β at 10 ng/ml or TNF-α at 5 ng/ml for 48 hours at 37°C. The total volume in each well was 200μl. Cell proliferation was measured as described above and data is presented as fold proliferation above untreated controls. Data represents the average of 3 independent experiments with four wells per treatment.

In a separate set of experiments, Prg4−/− synoviocytes were stimulated with IL-1β and TNF-α as described above in the presence or absence of rhPRG4 (100μg/ml) and IM7 (1:200 dilution). The total volume in each well was 200μl. Cell proliferation across the experimental groups was determined as described above and data is presented as the number of fold change in 490 nm absorbance compared to untreated controls. Data represents the average of 4 independent experiments with 4 wells per treatment.

Statistical Analysis

Variables were initially tested for normality and equal variances. Variables that satisfied both assumptions were tested for statistical significance using Student's *t*-test or analysis of variance (ANOVA) with Tukey's post-hoc test for two group and more than two group comparisons, *respectively*. Variables that did not satisfy the normality assumption were tested using Mann-Whitney U test or ANOVA on the ranks. The level of statistical significance was set at $\alpha=0.05$. Data is graphically represented as the average ± standard deviation.

Results

rhPRG4 Binding to CD44

Binding of rhPRG4, HMW HA, MMW HA and vitronectin to CD44-IgG_1Fc fusion protein and IgG_1Fc is presented in figure 1A. The 450 nm absorbance in the CD44-IgG_1Fc group was significantly higher than the absorbance in the IgG_1Fc group for rhPRG4, HMW HA and MMW HA-coated wells ($p<0.001$). In contrast, there was no significant difference between CD44-IgG_1Fc and IgG_1Fc in the vitronectin-coated wells. The concentration-dependent binding of rhPRG4, HMW HA and MMW HA to recombinant CD44 is depicted in figure 1B. The percentage of recombinant CD44 binding was significantly higher in the rhPRG4-coated wells compared to the HMW HA or MMW HA-coated wells for the 400, 100, 20, 4 and 2 μg/ml concentrations ($p<0.001$). Additionally, the percentage of

recombinant CD44 binding was significantly higher in the rhPRG4-coated wells compared to the MMW HA coated wells for the 200μg/ml concentration ($p<0.001$). There were no significant differences in percentage of CD44 binding between the rhPRG4, HMW HA and MMW HA-coated wells at the 0.1μg/ml concentration. The competition between rhPRG4 and HMW HA or MMW HA in binding to recombinant CD44 is presented in figure 1C. HMW HA or MMW HA, at 0.05, 0.25, 1, 5 and 25 μg/ml significantly reduced rhPRG4's binding to CD44 ($p<0.05$).

The binding of rhPRG4 to recombinant CD44 was confirmed using surface plasmon resonance. rhPRG4 displayed a concentration-dependent association with, and dissociation from immobilized CD44-IgG_1Fc (figure 2A), with an apparent K_d = 38 nM based on a predicted rhPRG4 molecular weight of 240 KDa. rhPRG4 interfered with binding of HMW HA to recombinant CD44 as shown by an inverse relationship between the HMW HA binding signal intensity (*x-axis*) and the rhPRG4 binding signal intensity (*y-axis*) (figure 2B).

The effect of sialidase-A and O-glycosidase digestions on rhPRG4 binding to CD44

Sialidase-A digestion resulted in a significant increase in the percentage binding of rhPRG4 to CD44 compared to untreated control ($p<0.001$) (figure 3A). Similarly, O-glycosidase digestion resulted in a significant increase in the percentage binding of rhPRG4 to CD44 compared to untreated control ($p=0.008$). There was no significant difference in percentage CD44 binding between the sialidase-A digested and the O-glycosidase digested rhPRG4 ($p=0.105$). The percentage binding to CD44 in the sialidase-A and O-glycosidase co-digested rhPRG4 was significantly higher than sialidase-A digested rhPRG4 ($p=0.007$), O-glycosidase digested rhPRG4 ($p<0.001$) and untreated control ($p<0.001$). Digestion of rhPRG4 with sialidase-A and O-glycosidase resulted in reducing the apparent molecular weight of rhPRG4 to approximately 200 KDa (figure 3B).

Impact of rhPRG4 and HMW HA treatments on cytokine induced proliferation of RA-FLS

IL-1β and TNF-α induced RA-FLS proliferation over a 48 hour period (figure 4A). Treatment with 40 and 80μg/ml rhPRG4 or HMW HA significantly suppressed RA-FLS proliferation with IL-1β stimulation ($p<0.05$). Treatment with 20, 40 and 80 μg/ml rhPRG4 significantly suppressed RA-FLS proliferation with TNF-α stimulation ($p<0.05$). Treatment with HMW HA did not result in suppressing RA-FLS proliferation with TNF-α. Co-treatment with IM7 reversed the effect of rhPRG4 and HMW HA on IL-1β stimulated RA-FLS as shown by the lack of significant difference in fold change of absorbance between the IL-1β stimulated RA-FLS treated with rhPRG4+IM7 or HMW HA+IM7 and IL-1β stimulated RA-FLS (figure 4B). Similarly, Co-treatment with IM7 reversed the effect of rhPRG4 on TNF-α induced RA-FLS proliferation.

Effect of rhPRG4 treatment on TNF-α induced NFκB nuclear translocation in RA-FLS

TNF-α treatment resulted in significant NFκB nuclear translocation compared to untreated controls ($p<0.001$) (figure 4C). Treatment with rhPRG4 or MG132 significantly reduced NFκB nuclear translocation compared to TNF-α-treated RA-FLS ($p<0.001$). NFκB nuclear translocation in the TNF-α+rhPRG4+IM7 group was significantly higher than NFκB

translocation in the TNF-α+rhPRG4 group ($p<0.001$) and was not significantly different from the TNF-α group.

CD44 localization in Prg4−/− and Prg4+/+ synoviocytes and the CD44-mediated anti-proliferative effects of rhPRG4

CD44 immunocytochemistry of Prg4−/− and Prg4+/+ synoviocytes is shown in figure 5A. Intense green fluorescence, indicative of CD44 localization, was observed for Prg4−/− synoviocytes. Alternatively, absent or faint green fluorescence was observed for Prg4+/+ synoviocytes. IL-1β and TNF-α treatment resulted in a significant increase in Prg4−/− synoviocytes proliferation compared to untreated Prg4−/− synoviocytes ($p<0.001$) (figure 5B). In contrast, only IL-1β stimulation resulted in a significant increase in Prg4+/+ synoviocyte proliferation compared to untreated Prg4+/+ synoviocytes ($p<0.001$). Additionally, IL-1β treatment resulted in significant increase in proliferation of Prg4−/− synoviocytes compared to TNF-α treatment ($p=0.002$).

Treatment with rhPRG4 significantly inhibited IL-1β and TNF-α induced proliferation of Prg4−/− synoviocytes ($p<0.001$) (figure 5C). Co-treatment with IM7 reversed the effect of rhPRG4. This is illustrated by the significant increase ($p<0.001$) in Prg4−/− synoviocyte proliferation in the IL-1β+rhPRG4+IM7 and TNF-α+rhPRG4+IM7 groups compared to IL-1β+rhPRG4 and TNF-α+rhPRG4 groups, respectively. There was no significant difference in Prg4−/− synoviocyte proliferation between TNF-α+rhPRG4+IM7 and TNF-α groups. In contrast, Prg4−/− synoviocyte proliferation was significantly higher in the IL-1β group than the IL-1β+rhPRG4+IM7 group ($p<0.001$).

Discussion

CD44 is a major cell surface receptor, with various isoforms generated by alternative splicing and glycosylation that plays a major role in inflammation [23]. A well-established ligand for CD44 is HMW HA, where HMW HA binds to an extracellular motif in CD44 with homology to other HA-binding proteins resulting in subsequent intracellular uptake of HMW HA [24–26]. In experimental OA models, chondrocyte CD44 expression is increased with disease progression and cartilage CD44 expression may correlate with disease severity in humans [27, 28]. Other CD44 ligands include extracellular matrix components *e.g.* collagens, fibronectin and laminin [29, 30]. HMW HA suppresses matrix metalloproteinase-13 (MMP 13) and aggrecanase-1 expression in OA and RA chondrocytes and synoviocytes mainly via a CD44-mediated interaction [31–37]. Additionally, HMW HA, via CD44 interaction, binds to OA osteoblasts and osteoclasts and suppresses MMP-13 production and expression of receptor activated NFκB ligand (RANKL), *respectively* [38, 39]. Using ELISA, we have demonstrated that rhPRG4, HMW HA and MMW HA specifically bind to chimeric CD44 with extremely low non-specific binding. In contrast, vitronectin that shares significant sequence homology with lubricin [2] does not show any binding specificity towards CD44. Furthermore, using a combination of ELISA and surface plasmon resonance, we demonstrate that rhPRG4 binds to CD44 in a concentration-dependent manner with comparable affinity to HMW HA. Furthermore, rhPRG4 competes with HMW HA in binding to CD44. The presence of an excess of HMW or MMW HA only

reduced rhPRG4 binding to CD44 by approximately 50%. Additionally, the presence of rhPRG4 bound to CD44 prevented HMW HA from binding to CD44 in a concentration-dependent manner and may indicate that rhPRG4 and HMW HA share a common binding site on the receptor. In the joint environment where HA SF concentration is roughly 10 times higher than that of lubricin, and based on our competitive binding data, it is expected that lubricin will be able to bind to CD44 on surface of synoviocytes and chondrocytes and exert a CD44-mediated biological function in the presence of HA.

Lubricin's boundary lubricating ability is mediated by the O-linked (β1-3)Gal-GalNAc oligosaccharides [5]. A combination of neuraminidase and beta 1,3, 6 galactosidase digestions reduced lubricin's boundary lubricating ability by 50% [5]. Lubricin isolated from RA SF samples contains increased core 1 glycosylation structures and displays the sulfated epitope that is proposed to be part of the L-selectin ligand [40]. Additionally, lubricin from RA SF binds L-selectin in a glycosylation-dependent manner and coats polymorphonuclear granulocytes recruited to inflamed synovia and SF of patients with RA, pointing to a potential role for lubricin in inflammation [41]. In our work, silaidase-A and O-glycosidase treatments have individually resulted in enhancing rhPRG4's binding to CD44 receptor. Cumulative sialidase-A and O-glycosidase digestions have resulted in even more significant binding to CD44 by rhPRG4 compared to individual enzyme digestions. Sialidase-A cleaves branched and unbranched terminal sialic acid residues from glycoproteins, while O-glycosidase catalyzes the removal of cores 1 and 2 from glycoproteins. The enhancement in CD44 binding indicates that neither the core 1 glycosylation nor the sialic acid terminal residues are required in rhPRG4 binding to CD44. In contrast, removal of these residues may lead to a conformational change in the rhPRG4 semi-rigid rod shaped structure that results in enhanced interaction with CD44.

The synovia of patients with RA contain considerable amounts of various CD44 isoforms and are generally present to a higher degree compared to OA or normal synovia [42, 43]. RA-FLS play an important role in the invasiveness of the synovia of patients with RA. The expression of a unique CD44 variant (CD44v7/8) contributes to the proliferation of RA-FLS *in vitro* [44] and pharmacological agents that bind cell surface CD44 with subsequent receptor shedding have shown efficacy in experimental arthritis models [45]. IL-1β and TNF-α induced RA-FLS proliferation with higher cell proliferation observed with TNF-α stimulation, which is in agreement with other published reports [46]. rhPRG4 inhibited the IL-1β and TNF-α induced RA-FLS proliferation in a mechanism that involves CD44 binding. The downstream effect of rhPRG4 and CD44 interaction is the inhibition of nuclear translocation of NFκB. In our cell proliferation assay, HMW HA inhibited IL-1β induced proliferation of RA-FLS but did not inhibit TNF-α induced proliferation. As with rhPRG4 treatment, the effect of HMW HA was reversed with a CD44 antibody, indicating the role of CD44 in mediating this effect.

Prg4−/− mice exhibits early signs of cartilage degeneration, demonstrated by surface fibrillations and increased joint coefficient of friction compared to Prg4+/− and Prg4+/+ mice [47]. Additionally, Prg4−/− mice exhibit increased activated caspase-3 chondrocyte staining in articular cartilage compared to age-matched Prg4+/+ cartilage [48] and synovial hyperplasia and overgrowth is evident in Prg4 −/− mice, with no obvious synovial

hyperplasia in Prg4 +/− or Prg4 +/+ mice [8]. Prg4−/− synoviocytes display enhanced CD44 staining compared to Prg4+/+ synoviocytes. Additionally, pro-inflammatory cytokines induced significant proliferation of Prg4−/− synoviocytes with no appreciable effect on Prg4+/+ synoviocytes. Combined, these observations indicate an ongoing inflammation in Prg4−/− joints with a proliferative synoviocyte phenotype resembling that of RA-FLS. rhPRG4 inhibited cytokine-induced Prg4−/− synoviocyte proliferation and this effect was mediated by rhPRG4-CD44 interaction. Neutralizing CD44 completely reversed the anti-proliferative effect of rhPRG4 following TNF-α stimulation and partially reversed the anti-proliferative effect of rhPRG4 following IL-1β stimulation. This difference related to rhPRG4-CD44 interaction in the setting of TNF-α and IL-1β stimulation of Prg4−/− synoviocytes may potentially be due to rhPRG4's ability to modulate other signaling pathways independent of its ability to interact with CD44.

In summary, we presented data suggesting that rhPRG4 binds to recombinant CD44 and competes with HMW HA in binding to the receptor. The level of sialylation and core 1 glycosylations on rhPRG4 protein core are not essential to the latter's ability to bind CD44. rhPRG4 exerts an anti-proliferative effect on RA-FLS subsequent to IL-1β or TNF-α stimulation. Interestingly, rhPRG4 concentrations that demonstrate this anti-proliferative effect are significantly lower than the rhPRG4 concentration required to provide boundary lubrication. This anti-proliferative effect of rhPRG4 is mediated by its interaction with CD44, resulting in a downstream inhibition of NFκB nuclear translocation. Furthermore, rhPRG4 inhibiting IL-1β and TNF-α induced proliferation of Prg4−/− synoviocytes mechanistically involves CD44. Lubricin-CD44 interaction is a novel mechanism that contributes to lubricin's joint homeostatic role [49] and provides insights into the function of this glycoprotein in the joint that complements its boundary lubricating properties

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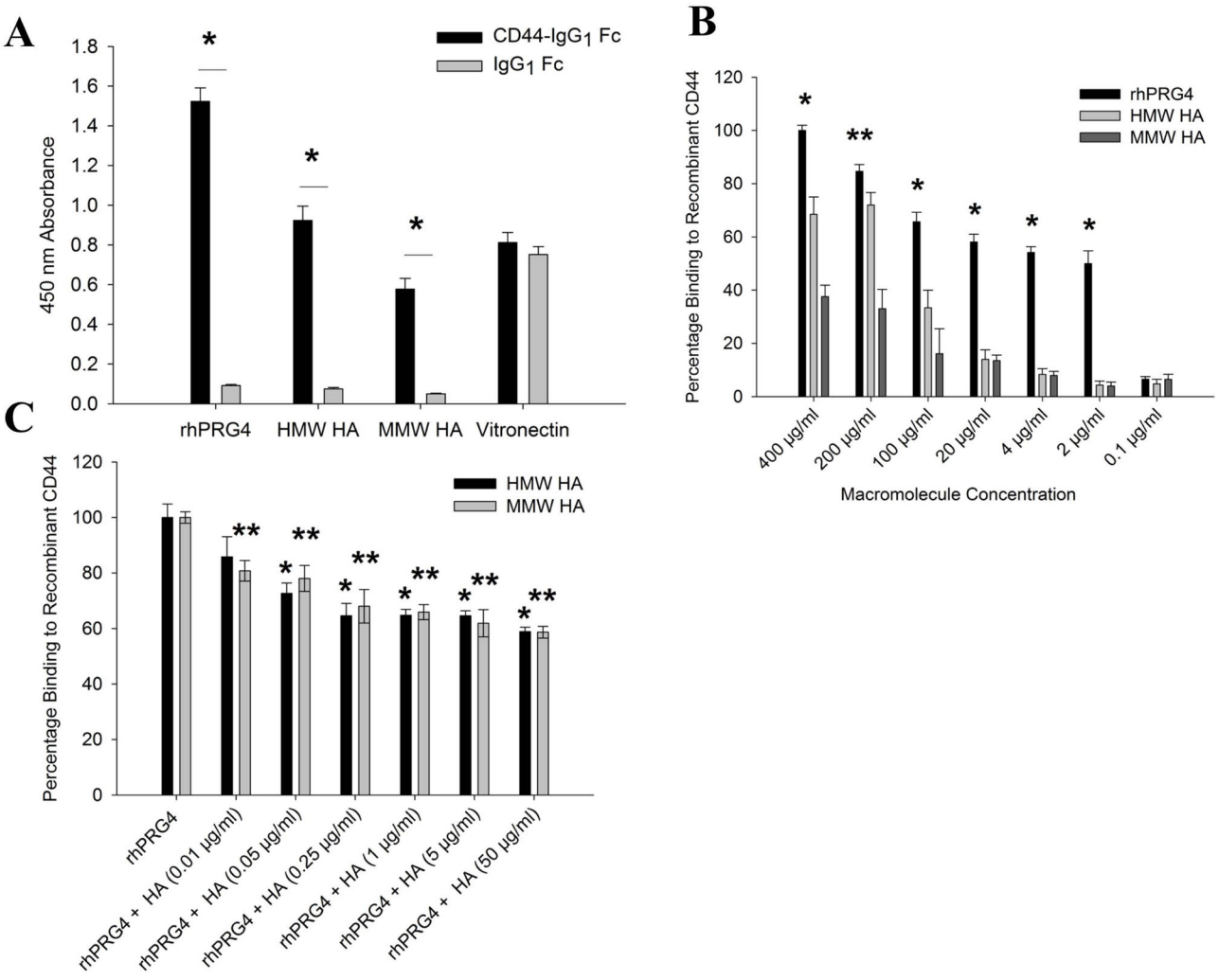

Figure 1. Binding of recombinant human proteoglycan 4 (rhPRG4), high-molecular weight hyaluronic acid (HMW HA), and medium molecular weight hyaluronic acid (MMW HA) to recombinant human CD44 receptor. Data represents the average of 4 independent experiments with triplicate wells per group

1A Binding of rhPRG4, HMW HA, MMW HA and vitronectin to CD44-IgG_1Fc and IgG_1 Fc.

*450 nm absorbance in the CD44-IgG_1 Fc wells were significantly higher than the IgG_1 Fc wells for rhPRG4, HMW HA and MMW HA ($p<0.001$).

1B Concentration-dependent CD44 binding of rhPRG4, HMW HA and MMW HA.

*CD44 binding to rhPRG4 was significantly higher than to HMW HA or MMW HA ($p<0.001$).

**CD44 binding to rhPRG4 was significantly higher than to MMW HA ($p<0.001$).

1C Competition between rhPRG4 (5µg/ml) and either HMW HA or MMW HA (0.01µg/ml to 50µg/ml) on binding to CD44 coated on 96-well ELISA plates.

*Percentage CD44 binding in HMW HA+rhPRG4 wells was significantly lower than rhPRG4 wells ($p<0.05$).

**Percentage CD44 binding in MMW HA+rhPRG4 wells was significantly lower than rhPRG4 wells ($p<0.05$).

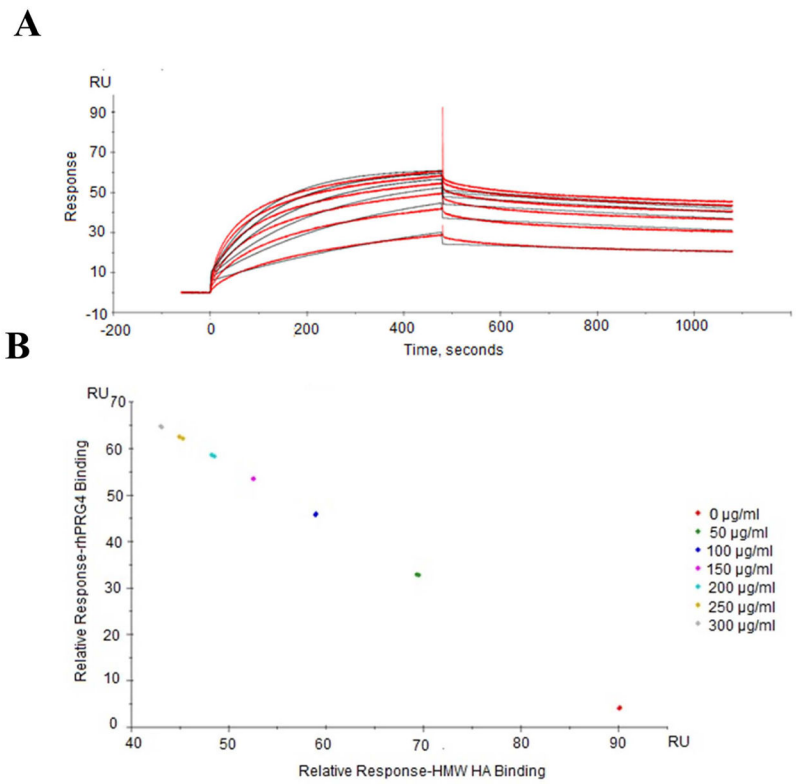

Figure 2. Binding of recombinant human proteoglycan 4 (rhPRG4) to recombinant CD44 and competition between rhPRG4 and high molecular weight hyaluronic acid (HMW HA) on CD44 binding using surface plasmon resonance

2A Sensogram depicting the concentration-dependent association and dissociation of rhPRG4 (300µg/ml to 50µg/ml) to immobilized CD44-IgG_1Fc. Red curves represent the binding curves of rhPRG4 to CD44 chimeric protein and the black lines represent the fitted 1:1 binding model.

2B Competition between rhPRG4 and HMW HA on binding to immobilized CD44-IgG_1Fc. rhPRG4 was injected at 300, 250, 200, 150, 100 and 50 µg/ml. Following dissociation of rhPRG4, HMW HA was injected at 50 µg/ml. As the concentration of rhPRG4 increased, subsequent HMW HA binding to CD44 decreased.

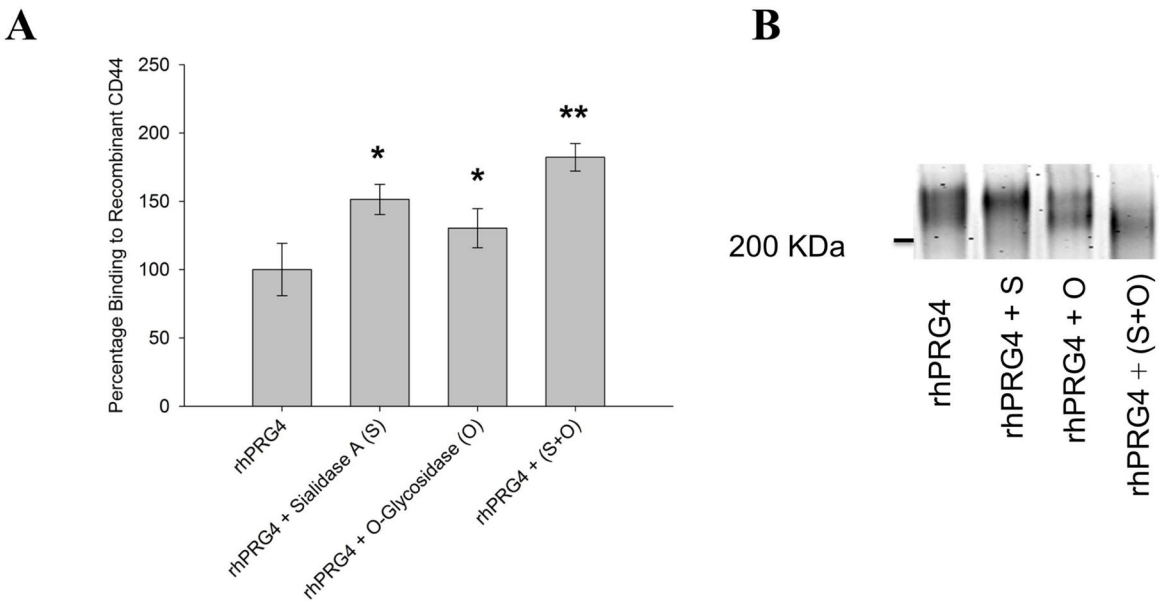

Figure 3. Impact of sialidase-A and O-glycosidase digestion of recombinant human proteoglycan 4 (rhPRG4) on binding of rhPRG4 to CD44. Data represents the average of 4 independent experiments with triplicate wells per group

3A Binding of rhPRG4, sialidase-A digested rhPRG4, O-glycosidase digested rhPRG4 and sialidase-A + O-glycosidase digested rhPRG4 to CD44. The 450 nm absorbance values across different groups were normalized to the absorbance values in the undigested rhPRG4 group.

*CD44 binding in the sialidase A-digested and O-glycosidase-digested rhPRG4 was significantly higher than undigested rhPRG4 ($p<0.01$).

**CD44 binding in the Sialidase-A + O-Glycosidase digested rhPRG4 was significantly higher than sialidase-A digested, O-glycosidase-digested and undigested rhPRG4 ($p<0.01$).

3B SDS-PAGE of rhPRG4, sialidase-A digested rhPRG4, O-glycosidase digested rhPRG4 and a combination of sialidase-A and O-glycosidase digested rhPRG4. The gel was stained overnight with Commassiee Blue. Digestion with sialidase-A and O-glycosidase resulted in reducing the apparent molecular weight of rhPRG4.

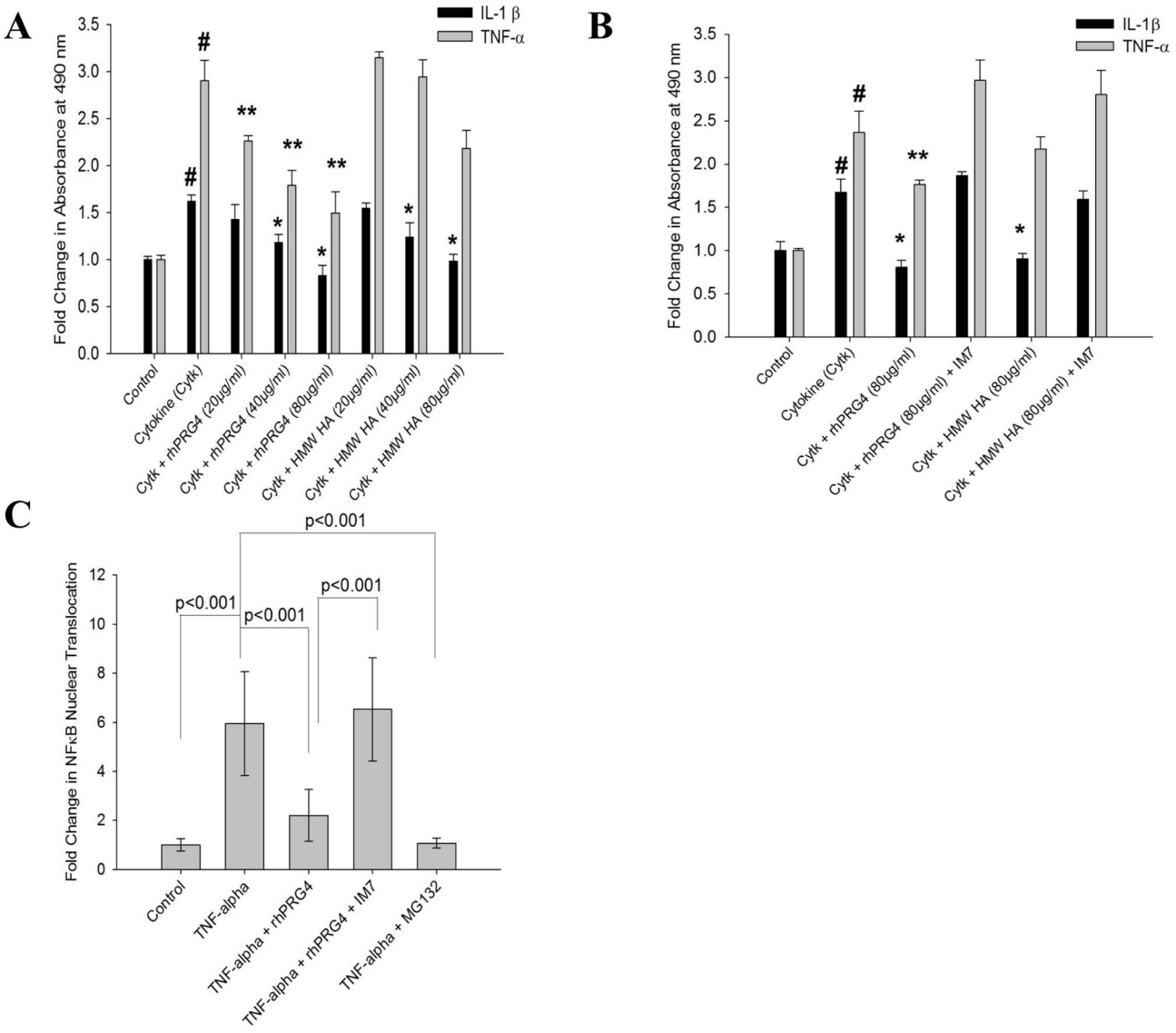

Figure 4. Impact of recombinant human proteoglycan 4 (rhPRG4) and high molecular weight hyaluronic acid (HMW HA) treatment on cytokine induced rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) proliferation. Data represents the average of 3 independent experiments with triplicate wells per treatment

4A Inhibition of cytokine induced RA-FLS proliferation by rhPRG4 and HMW HA.

#Cytokine stimulated RA-FLS had a significantly higher absorbance than untreated cells ($p<0.001$).

*rhPRG4 or HMW HA (40 and 80μg/ml) treatment significantly reduced cell proliferation compared to untreated IL-1β stimulated cells ($p<0.05$).

**rhPRG4 (20, 40 and 80μg/ml) treatment significantly reduced cell proliferation compared to untreated TNF-α stimulated cells ($p<0.05$).

4B Inhibition of cytokine induced RA-FLS proliferation by rhPRG4 and HMW HA in the presence and absence of IM7.

[#]Cytokine stimulated RA-FLS had a significantly higher absorbance than untreated cells ($p<0.001$).

*rhPRG4 or HMW HA treatment had a significantly lower cell proliferation compared to untreated IL-1β or (rhPRG4 or HMW HA)+IM7 treatment ($p<0.05$).

**rhPRG4 treatment had a significantly lower cell proliferation compared to untreated TNF-α or rhPRG4+IM7 treatment ($p<0.05$).

4C rhPRG4 inhibition of TNF-α induced NFκB nuclear translocation in RA-FLS. Nuclear translocation of NFκB in the TNF-α+rhPRG4 group was significantly lower than TNF-α alone or TNF-α+rhPRG4+IM7 groups ($p<0.001$).

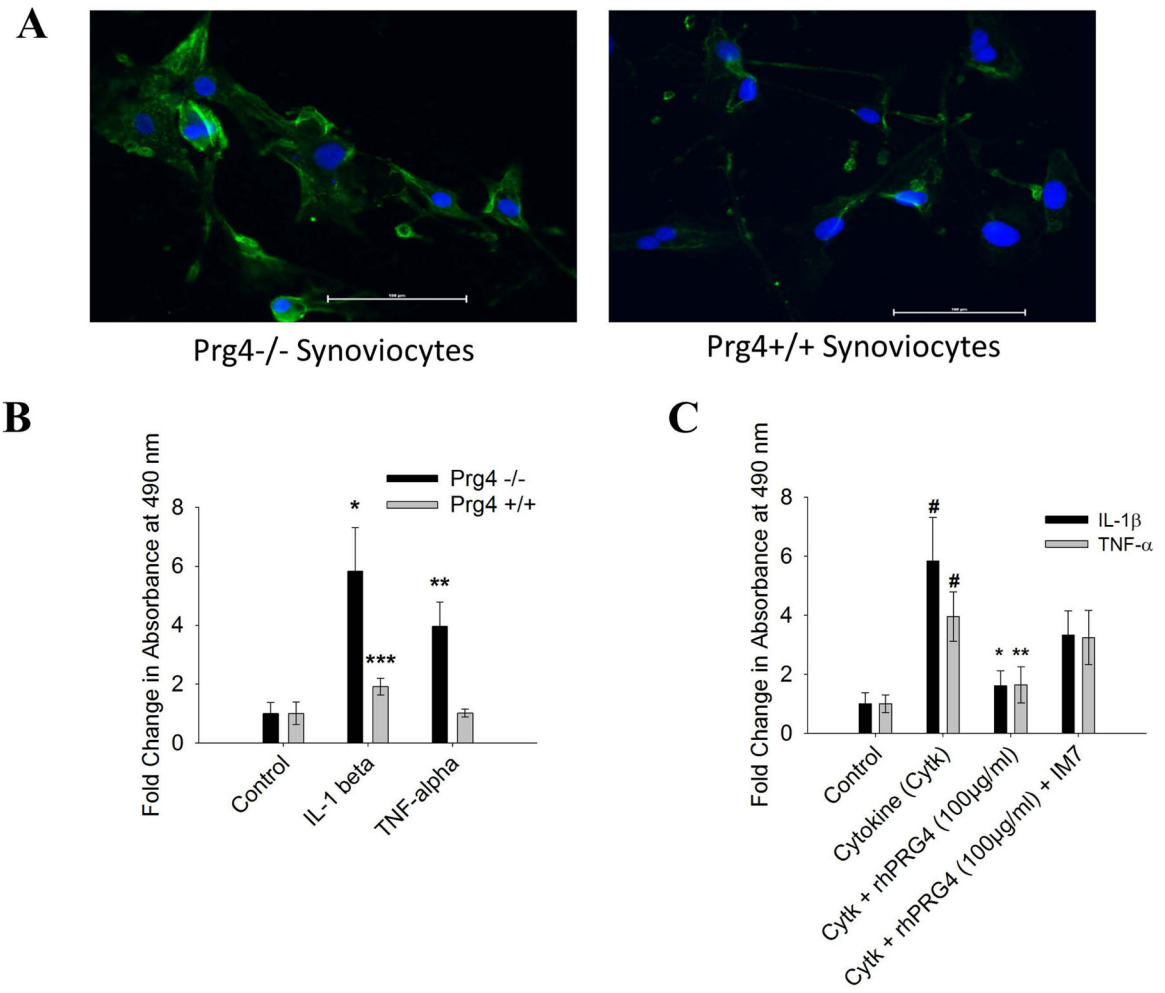

Figure 5. Impact of pro-inflammatory cytokines on Prg4−/− and Prg4+/+ synoviocyte proliferation and effect of recombinant human proteoglycan 4 (rhPRG4). The data represents the average of 4 independent experiments with 4 wells per treatment

5A Prg4−/− and Prg4+/+ synoviocytes immunocytostaining using anti-CD44 antibody (IM7) (Green) and DAPI (blue). Enhanced green fluorescence in Prg4−/− synoviocytes indicates increased CD44 localization compared to Prg4+/+ synoviocytes. Scale = 100 µm.

5B Cytokine induced proliferation of Prg4−/− and Prg4+/+ synoviocytes.

* IL-1β treated Prg4−/− synoviocytes had significantly higher proliferation than untreated Prg4−/− synoviocytes ($p<0.001$) or TNF-α treated Prg4−/− synoviocytes ($p=0.002$).

**TNF-α treated Prg4−/− synoviocytes had significantly higher proliferation than untreated Prg4−/− synoviocytes ($p<0.001$).

***IL-1β treated Prg4+/+ synoviocytes had significantly higher proliferation than untreated Prg4+/+ synoviocytes ($p<0.001$).

5C Impact of rhPRG4 treatment on cytokine induced Prg4−/− synoviocyte proliferation in the presence and absence of IM7.

#Cytokine stimulated Prg4−/− synoviocytes had a significantly higher absorbance compared to untreated cells ($p<0.001$).

*rhPRG4 treatment of IL-1β stimulated Prg4−/− synoviocytes significantly reduced cell proliferation compared to untreated IL-1β or rhPRG4+IM7 treatment ($p<0.001$).
**rhPRG4 treatment of TNF-α stimulated Prg4−/− synoviocytes significantly reduced cell proliferation compared to untreated TNF-α or rhPRG4+IM7 treatment ($p<0.001$).