

Hyper-activation of Complement System in Osteoarthritis and Spinal Cord Injury: Exploring the Interplay between Progranulin and C5a/C5aR1 Signaling

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Disclosure: None

Introduction: The complement system, composed of more than 30 component proteins, regulators and receptors, has been linked to a number of immunological and inflammatory conditions. Activation of the complement cascade generates anaphylatoxins particularly C5a. It has been reported that C5 deficiency attenuated arthritis in mouse osteoarthritis (OA) model, and signaling through the C5a-C5aR axis was implicated in inflammatory response following spinal cord injury (SCI). Our lab has long standing interests in investigating the role of progranulin (PGRN, encoded by *Grn* gene), a growth-factor like glycoprotein with multiple functions, in musculoskeletal disorders. In our previous research, we found PGRN deficiency exaggerated OA progression and impaired neurological recovery and accelerated inflammatory response following SCI. This impact is, at least partially, attributed to PGRN modulation of macrophage polarization. In addition, we previously reported that PGRN binds to the cysteine-rich domains (CRD) of TNFR2 (Tang, W., et al, Science, 2011), together with the fact that complement proteins also contain CRDs, led us to determine whether PGRN physically interacted with and functionally interplayed with the complement system. Thus, the objective of this study is 1) to determine whether PGRN directly binds to C5a, thereby antagonizes the effect of C5a in macrophage polarization, and 2) to define the importance of PGRN/C5a-C5aR1 interplay in the course of OA and SCI. By probing the intricate relationship between PGRN and C5a/C5aR1 signaling, we aim to unlock novel insights into the interplay of these pivotal elements and their impact on the onset and progression of OA and SCI.

Methods: All animal studies were performed in accordance with institutional guidelines and with approval by the Institutional Animal Care and Use Committee of Yale University. Solid phase binding and FACS were used to examine the interactions between PGRN and complement components. The binding affinity between PGRN and C5a was determined using Analytical Surface Plasmon Resonance (SPR) by Essai Sciences, LLC (Stillwater, OK). Destabilization of the medial meniscus (DMM) OA model and SIC models were established on WT, *Grn*^{-/-} and *Grn*^{-/-};*C5ar1*^{-/-} mice.

Results: C5a is a novel binding partner of PGRN, and PGRN inhibits the binding of C5a to C5aR1 and to the cell surface. To test whether PGRN could directly interact with complement components, we thus conducted solid-phase binding assays to screen the interactions between PGRN and central complement components, including C1-C9, C5a and C3a. Our findings revealed a robust binding of PGRN to C5 and C5a, with a dose-dependent interaction. Notably, the binding affinity exhibited by PGRN towards C5a was comparable to that of the positive control TNFR2 (Fig. 1a-c). In addition, the existence of PGRN/C5a complex in mouse sera was demonstrated by Co-IP (Fig. 1d, e), and PGRN binds to C5a with a high affinity of KD 7.18±0.07nM measured by Analytical Surface Plasmon Resonance. Further functional assays demonstrated that PGRN inhibits C5a binding to C5aR1 and effectively blocks C5a binding to cell surfaces (Fig. 1f, g), indicating that PGRN acts as a natural antagonist of C5a signaling.

PGRN antagonizes C5a's effect on macrophage polarization. In line with the published data showing that C5a regulates macrophage polarization, we found that C5a enhanced M1 macrophage polarization while inhibited M2 macrophage polarization. Interestingly, PGRN played opposite role on regulating macrophage polarization, and more importantly it antagonized C5a's effect on macrophage polarization.

C5aR1 deficiency reverses the exacerbation of OA progression seen in *Grn*^{-/-} mice. ELISA analysis showed a significant increase in C5a levels in the synovial fluid of OA patients than healthy controls, indicating hyperactivation of the complement cascade in OA synovial fluid. PGRN levels were also significantly higher in the synovial fluid of OA patients compared to healthy controls (Fig. 2a). Furthermore, a positive correlation was found between the levels of PGRN and C5a in OA patients ($r = 0.5475$, $P < 0.0001$) (Fig. 2b, 2c). Thus, we set to investigate the interplay of PGRN/C5a in vivo using various genetically deficient mice with surgically induced DMM model. The results showed that deletion of C5aR1 partially reversed the exaggerated OA phenotype, including severe cartilage loss and increased OA-associated pain observed in *Grn*^{-/-} OA mice (Fig. 2d). Currently, we are in the process of evaluating the intricate interplay between PGRN and C5a/C5aR1, specifically in their role in regulating macrophage polarization in the course of OA.

C5aR1 deficiency reverses the exacerbation of inflammation seen in PGRN deficient mice with SCI. We also determined whether genetic deficiency in C5aR could reverse the exacerbation of inflammation observed in *Grn*^{-/-} mice with SCI. BMS locomotor scoring was performed for 4weeks after injury. In accordance with the published data, starting from 2weeks after surgery, C5aR1 deficient mice displayed significantly more hindlimb motor function than WT mice, and PGRN deficient mice exhibited impaired functional recovery as evidenced by significantly lower BMS scores than WT mice. Notably, C5aR deficiency substantially improve PGRN induced impaired locomotion function (Fig. 3a-c). Accordingly, Nissl staining of spinal cord tissue revealed significantly more severe neuronal injury in the PGRN-deficient mice group than in the WT mice group, while C5aR1-deficient mice exhibited markedly milder neuronal injury (Fig. 3d). And neuronal injury in double deficient mice is undistinguishable from that in WT mice (Fig. 3d). Immunofluorescence staining complemented these findings, clearly indicating reduced M1 macrophage presence and increased M2 macrophage infiltration in the spinal cord of C5aR1-deficient mice compared to WT mice. Intriguingly, PGRN-deficient mice showed an opposing effect on macrophage recruitment, which was effectively reversed by C5aR1 deficiency, effectively rescuing the altered macrophage polarization phenotype caused by PGRN deficiency.

Discussion: Protein-protein interaction screen led to the identification of C5a as a novel binding partner of PGRN. Additionally, PGRN inhibited the binding of C5a to its receptor C5aR1 and to the cell surface. Further, PGRN antagonized C5a's effect on macrophage polarization and C5aR1 deficiency neutralized the exacerbated joint degeneration and inflammation seen in PGRN deficiency mice in the context of OA and SCI, respectively. In summary, PGRN acts as a naturally occurring antagonist of C5a and interplays with C5a/C5aR1 signaling in mediating musculoskeletal disorders.

Significance: Identification of PGRN as a novel antagonist of the prominent C5a/C5aR1 pathway uncovers a new strategy for inhibiting this cardinal pathway involved in various diseases/conditions associated with hyper-activation of complement system, particularly OA and SCI.

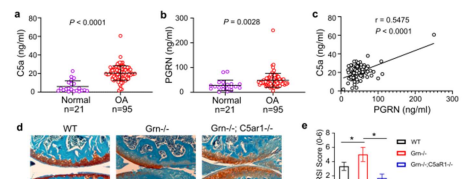
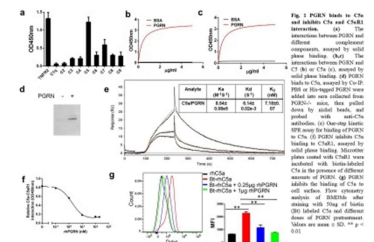


Fig. 2 C5aR1 deficiency reverses the exacerbation of OA progression in PGRN-deficient mice. a, b, Levels of C5a (a) and PGRN (b) in synovial fluids measured by ELISA. c, Association of C5a and PGRN levels in synovial fluids of OA patients. d, Representative Safranin O stained knee joint sections of DMM operated mice with indicated genetic background. Scale bar = 50µm. e, Quantitation of OARSI score. * $P < 0.05$.

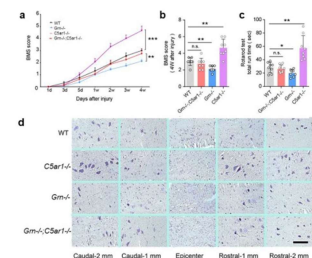


Fig. 3 C5aR1 deficient improves the impaired neuronal recovery in *Grn*^{-/-} mice after SCI. (a) BMS locomotor scoring in the SCI mice with different genetic background. (b) Pooled BMS scores for individual mice at 4weeks after injury. (c) Nissl rod test at 4weeks after injury. (d) Nissl staining of spinal cord. Scale bar: 100µm.