Synergistic Inhibition of TNF α /TNFR and C5a/C5aR1 Pathways by two Progranulin Derivatives-Atsttrin and NC5-in Osteoarthritis Treatment

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Background Osteoarthritis (OA) is a chronic degenerative joint disease. Activation of the complement cascade produces anaphylatoxins such as C5a, which contribute to OA-associated inflammation. Our lab has long focused on progranulin (PGRN), a growth factor-like glycoprotein implicated in musculoskeletal diseases. Previous studies revealed that PGRN deficiency accelerates OA progression, and that PGRN and its engineered derivative Atsttrin bind to the cysteine-rich domains (CRDs) of TNFR1 and TNFR2, offering a potential therapeutic avenue (Tang, W., et al., *Science*, 2011). Since complement components also contain CRDs, we hypothesized that PGRN may modulate complement signaling. Thus, the objective of this study are 1) to determine whether PGRN and its derivative directly bind to C5a to antagonize its inflammatory effects, and 2) to explore whether dual inhibition of TNF α /TNFR and C5a/C5aR1 signaling via Atsttrin and NC5 produces a synergistic therapeutic effect in OA.

Methods All animal experiments were conducted in compliance with institutional guidelines and approved by the Institutional Animal Care and Use Committee of Yale University. ELISA and Co-IP assays were performed to investigate the interactions between PGRN or its derivatives and complement components (C1–C9). A surgery-induced osteoarthritis (DMM) mouse model (male, n=6 for each group) was established in C57/BL mice, followed by different treatment regimens. Disease progression was evaluated using clinical scoring and histological analysis of the joints.

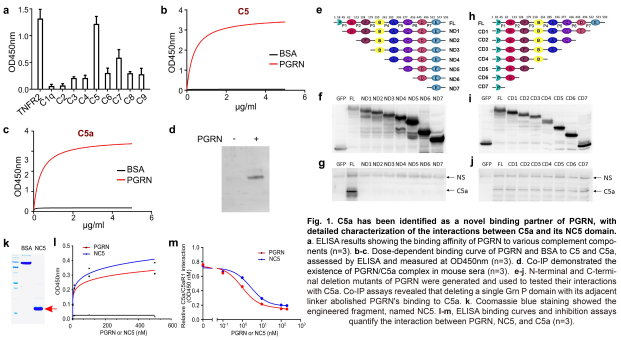

Fig. 1. C5a has been identified as a novel binding partner of PGRN, with detailed characterization of the interactions between C5a and its NC5 domain. **a**. ELISA results showing the binding affinity of PGRN to various complement components (n=3). **b-c**. Dose-dependent binding curve of PGRN and BSA to C5 and C5a, assessed by ELISA and measured at OD450nm (n=3). **d**. Co-IP demonstrated the existence of PGRN/C5a complex in mouse sera (n=3). **e-j**. N-terminal and C-terminal deletion mutants of PGRN were generated and used to tested their interactions with C5a. Co-IP assays revealed that deleting a single Grn P domain with its adjacent linker abolished PGRN's binding to C5a. **k**. Coomassie blue staining showed the engineered fragment, named NC5. **l-m**. ELISA binding curves and inhibition assays quantify the interaction between PGRN, NC5, and C5a (n=3).

Results **Terminal amino acid fragment (from aa 18-61) of PGRN, containing Grn P domain, is sufficient for binding to C5a.** To test whether PGRN could directly interact with complement components, we conducted solid-phase binding assays to screen the interactions between PGRN and central complement components. 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**). To determine which domains of PGRN are involved in binding to C5a, we generated N-terminal and C-terminal deletion mutants of PGRN and examined their interactions with C5a. Co-IP assays revealed that deleting a single Grn P domain with its adjacent linker abolished PGRN's binding to C5a (**Fig. 1e-j**), indicating the N-terminal 44 amino acids are crucial for this interaction. Further analysis with C-terminal deletions showed that they did not affect binding, and the N-terminal fragment, containing the Grn P domain, was sufficient for binding to C5a. We then generated an engineered fragment (named NC5 thereafter, aa 18-61), excluding the 17-aa signal peptide (**Fig. 1k**), and found that it bound to C5a with similar affinity as full-length PGRN in a dose-dependent manner (**Fig. 1l**). Notably, this fragment more effectively inhibited the C5a/C5aR1 interaction than PGRN, indicating that this derivative is a potent C5a antagonist (**Fig. 1m**).

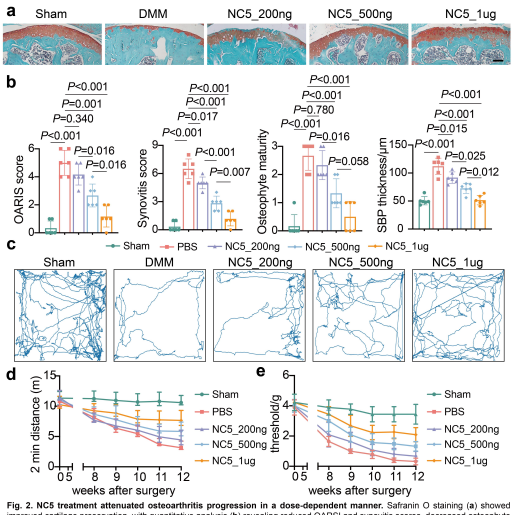

Fig. 2. NC5 treatment attenuated osteoarthritis progression in a dose-dependent manner. Safranin O staining (a) showed improved cartilage preservation, with quantitative analysis (b) revealing reduced OARSI and synovitis scores, decreased osteophyte maturity, and thinner subchondral bone plate thickness in NC5-treated groups (male, n=6), scale bar=100μm. Locomotor tracking (c,d) demonstrated enhanced activity, while pain assessments (e) indicated increased walking distance and higher pain thresholds compared with DMM controls (male, n=6).

Dose-dependent protective effects of NC5 in OA. Safranin O staining (**Fig. 2a, b**) showed that NC5 preserved cartilage structure, reduced synovitis and reduced matrix loss in a dose-dependent manner. Open field tracking and von fery test demonstrated improved mobility (**Fig. 2c,d**) and relieved pain (**Fig. 2e**) in NC5-treated mice.

The combination of Atsttrin and NC5 demonstrates promising synergistic effects in protecting against the progression of OA compared to single-agent treatments, even at lower doses. The combination of Atsttrin and NC5 synergistically attenuated disease severity, yielding therapeutic benefits that surpassed those of Atsttrin or NC5 alone. In Safranin O staining and quantitative analysis (**Fig. 3a,b**), treatment with Atsttrin or NC5 alone partially preserved cartilage, while the combination markedly enhanced cartilage protection. Open field tracking (**Fig. 3c,d**) and Von fery pain assessments (**Fig. 3e**) revealed that co-administration improved mobility and pain thresholds more effectively than either agent alone. Furthermore, IHC (**Fig. 3f**) demonstrated stronger Col2 expression and reduced Mmp13 levels in the combined treatment, highlighting superior protection of cartilage matrix integrity and suppression of matrix degradation. Collectively, these findings suggest that Atsttrin and NC5 act in a complementary and synergistic manner to more effectively slow OA progression, promote cartilage repair, and improve functional outcomes.

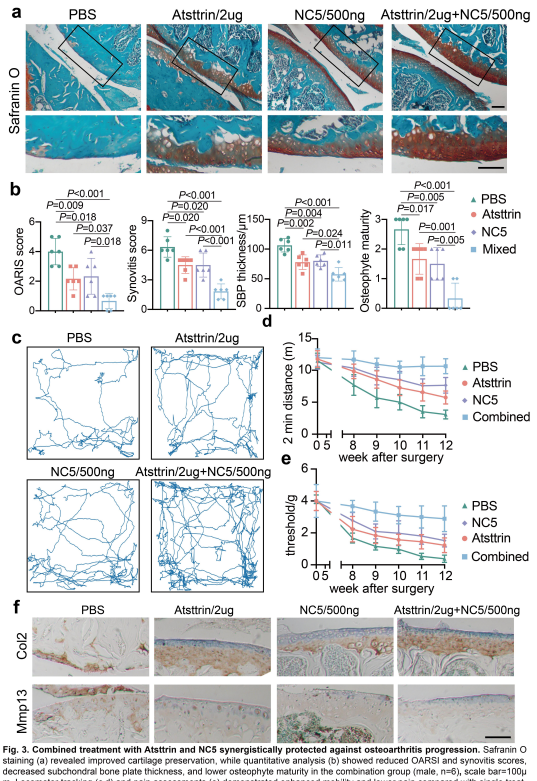

Fig. 3. Combined treatment with Atsttrin and NC5 synergistically protected against osteoarthritis progression. Safranin O staining (a) revealed improved cartilage preservation, while quantitative analysis (b) showed reduced OARSI and synovitis scores, decreased subchondral bone plate thickness, and lower osteophyte maturity in the combination group (male, n=6), scale bar=100μm. Locomotor tracking (c,d) and pain assessments (e) demonstrated enhanced mobility and lower pain compared with single treatments. IHC (f) further confirmed stronger Col2 expression and reduced Mmp13 expression, indicating superior cartilage matrix protection with combined therapy. (male, n=6), scale bar=100μm.

Discussion The combination of NC5 with Atsttrin exerted significant synergistic effects in treating OA, through simultaneously inhibiting C5aR1 and TNFR1 inflammatory signaling and activating TNFR2 protective pathway, while avoiding the tumorigenic activity associated with full-length PGRN.

Conclusion The synergistic inhibition of TNF α /TNFR and C5a/C5aR1 signaling by the progranulin derivatives-Atsttrin and NC5-enhances treatment efficacy in OA. Further clinical investigations are warranted to validate these findings and explore the potential of translating this approach into OA treatment strategies, potentially offering patients a more effective and safer option for disease management.