Supporting Online Material for

The Growth Factor Progranulin Binds to TNF Receptors and Is Therapeutic Against Inflammatory Arthritis in Mice

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Materials and Methods

Mice. All animal studies were performed in accordance with institutional guidelines and with approval by the Institutional Animal Care and Use Committee of New York University. C57BL/6, BALB/c, DBA/1J, TNFR1 knockout (Tnfrsf1a<sup>−/−</sup>) and TNFR2 knockout (Tnfrsf1b<sup>−/−</sup>) mice were obtained from Jackson Laboratories. TNFα transgenic (hemizygous) mice were obtained from Taconic. The generation and characterization of PGRN knockout mice has been described previously (9).

Preparation of rhPGRN and Atsttrin protein. Generation of PGRN stable line and purification of recombinant PGRN has been described in our previous publication (3). For expression of GST fusion Atsttrin, the appropriate plasmid pGEX-Atsttrin was transformed into E. coli DE3. Fusion protein was affinity-purified on glutathione-agarose beads as previously described (S1) with slight modification. Briefly, glutathione-agarose beads bearing GST fused Atsttrin was digested with Factor Xa for six hours at room temperature and the supernatant was collected. Following the removal of Factor Xa by using Factor Xa Removal Resin (QIAGEN), the purity of resulted Atsttrin was determined by SDS-PAGE and reverse phase HPLC.

Yeast two-hybrid (Y2H) library screen. Plasmid pDBleu-PGRN (a.a.21-588) encoding PGRN lacking signal peptide was used as bait to screen a Y2H murine 10.5-day embryonic cDNA library (Life Technologies) according to a modified manufacturer’s protocol. Briefly, bait plasmid was introduced into a yeast MAV203 strain that contained three reporter genes, HIS<sup>+</sup>, URA<sup>+</sup>, and Lac Z, and the cDNA library in the vector pPC86 was then transformed into the resultant Leu<sup>+</sup> yeast strain and plated on medium lacking tryptophan, leucine, histidine, and uracil but containing 25 mM 3-amino-1,2,4-trizone. After incubation for 7–10 days at 30°C, colonies were screened for β-galactosidase by a filter lift assay.

Assay of protein-protein interactions using the Y2H system. Independent yeast colonies were analyzed for the interaction of two proteins, one of which was fused to the Gal4 DNA binding domain and the other, to the VP16 transactivation domain. Our published procedures (S1) were followed for 1) growing and transforming the yeast strain MAV203 with the selected plasmids; and 2) determining β-galactosidase activity and growth phenotypes on selective media lacking tryptophan, leucine, histidine, and uracil.

To quantify the interactions, yeast colonies were harvested, lysed and activity determined by a liquid assay for β-galactosidase. β-galactosidase activity was monitored at 420 nm, and 1 unit of β-galactosidase activity was defined as the amount capable of hydrolysing 1 µmol of o-nitrophenyl-β-D-galactopyranoside to o-nitrophenol and D-galactose per min per cell.

Co-immunoprecipitation (Co-IP). Approximately 500 µg of cellular extract prepared from isolated human chondrocytes was incubated with anti-PGRN, anti-TNFR1, anti-TNFR2 or control IgG (25 µg/ml) antibodies for 1 hr, followed by incubation with 30 µl of protein A-agarose at 4°C overnight. Bound protein was examined by Western blotting with anti-TNFR1, anti-TNFR2 or anti-PGRN antibodies.

Solid-phase binding. To determine whether PGRN directly binds to TNFR in vitro, microtiter plates were coated with 500 ng of PGRN in 100 µl of TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) overnight at 4°C. Wells were blocked using 1% bovine serum albumin (BSA) in TBS buffer for 3 h at 37°C. After washing with TBS and 0.05% Tween, various amounts of TNFR1 extracellular domain or
TNFR2 extracellular domain (R & D System) were added. Bound protein from the liquid phase was detected by anti-TNFR1 or anti-TNFR2 antibodies.

To examine the inhibition of the TNF/TNFR or LTα/TNFR interaction by PGRN or Atsttrin, plates were coated with 100 ng of TNFα (R & D System) or LTα (R & D System). 100 ng of TNFR1 extracellular domain or 100 ng of TNFR2 extracellular domain (R & D System) was then added in the presence of various amounts of PGRN or Atsttrin. Bound TNFR was detected as described above.

**Surface plasmon resonance analysis.** A COOH1 sensor chip was installed and thermally equilibrated 10 min. SensiQ Pioneer contains a three channel flow cell enabling simultaneous analysis of multiple ligands. Channel 1 was immobilized with TNFR2 and channel 3 was immobilized with TNFR1. Channel 2 was left unmodified to serve as a reference. Immobilization by random amine coupling was used where surface carboxyl groups were activated by injecting a mixture of 4mM EDC and 1mM NHS in deionized water for 3.5 min. Each receptor sample was diluted to 30 μg/mL in 10 mM acetate pH 4.7 and injected for 20 min over the respective channel. Remaining NHS esters were capped by injecting 1M ethanolamine pH 8.0 for 4 min.

The samples of analytes (TNFα, rhPGRN and Atsttrin) were diluted to 400 nM in running buffer and each was injected using the FastStep™ injection type. Signal The FastStep™ injection incorporates a wide dilution range of a stock sample in a single injection. The dilution range is created from the stock solution within the Pioneer fluidics so additional vials are not required for a traditional dilution series. The FastStep™ injection used produced a doubling dilution series over 6 concentrations starting at 400 nM for each analyte. Following the analyte injection, dissociation of analyte-ligand complex was monitored for 8 min before the surface was regenerated by injecting 10 mM NaOH for 2 min. Each sample was injected in duplicate and a buffer blank was included for double referencing. The sample binding response over a blank surface (channel 2) and the response recorded for injection of a blank buffer are subtracted from the sample binding response over the receptor coated surface to accurately resolve specific binding in the presence of non-specific binding and systematic bias. The FastStep™ injection type allowed a full kinetic assay to be performed in a single injection and it used only one stock solution. All parameters of the analysis were fitted globally using 1:1 kinetic model.

**Flow cytometry assay.** Raw264.7 cells were resuspended in 10 mM PBS, and $1 \times 10^5$ cells were pretreated with different dose of rhPGRN or Atsttrin for 30min, after which the Biotinylated human TNFα (Bt-hTNFα, R&D Systems) was added. Cells were incubated for 30 min in 4°C. 10 μl of avidin-FITC was added and incubated for a further 30 min at 4°C in the dark. The cells were washed twice and re-suspended in 200 μl wash buffer for final flow cytometric analysis.

**Neutrophil activation assay.** Wild type or PGRN-deficient neutrophils resuspended in ice-cold Krebs-Ringer phosphate buffer with glucose (KRPG) were used for measurement of H₂O₂ production using the scolopetin assay (S2) with $1.5 \times 10^4$ neutrophils per well. Cells were treated with 100 ng/ml of TNFα (R&D systems) in the presence or absence of Atsttrin, and incubated at 37°C for 3 h. H₂O₂ production was measured in a fluorescence microplate reader.

**Nitrite production assay.** Bone marrow cells collected from the femurs of 5- to 8-wk-old wild type or PGRN-deficient mice were suspended in culture medium ($α$-MEM containing L-glutamine, penicillin, streptomycin and heat-inactivated 10% FBS) supplemented with 10 ng/ml M-CSF and cultured overnight. Non-adherent bone marrow derived macrophages (BMDMs) were cultured in 96-well flat-bottom plates ($1.25 \times 10^5$ cells/well) for 2 days in the presence of M-CSF (10 ng/ml). BMDMs were then
incubated for 24 h with 100 ng/ml TNF (R&D systems) in the presence or absence of various amounts of Atsttrin. Cell culture supernatants were harvested and tested for NO via the Griess reaction.

**In vitro suppression assays.** CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were purified from the peripheral blood of healthy human donors between ages of 16 and 75 years. Briefly, the whole blood was incubated (20 min, 22°C) with RosetteSep™ human CD4⁺ T cell enrichment cocktail (StemCell Technologies, Vancouver, BC, Canada). The remaining un-sedimented cells were loaded onto Ficoll-Paque Plus (Amersham Bioscience, Piscataway, NJ), isolated by density centrifugation, and washed with PBS. In second round of purification, CD4⁺ T cells were separated into CD25⁻ and CD25⁺ populations with magnetically coupled mAb against human CD25 (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4⁺CD25⁺ T cells were mixed at ratio 1:3 or 1.25 × 10⁵ : 5 × 10⁵ with CD4⁺CD25⁻ T cells at final concentration 2×10⁶/ml and treated with different doses of rhPGRN (10-500 ng/ml). Then, the cells were plated on anti-CD3 mAb (5µg/ml) pre-coated 24-well plates in the absence or presence of human TNFα (210-TA; R&D Systems Inc (Minneapolis, MN)) and cultured for 24-48 hr. Cytokine secretion was determined by ELISA by using Human IFNγ Cytoset™ (Biosource; Camarillo, CA).

**CD4⁺ T cells differentiation in vitro.** Naïve CD4⁺ T cells were enriched by CD4 T cell negative selection kit (STEM CELL) combined with addition of Bio-anti CD-25 and Bio-anti-CD44 for depleting CD25⁺ regulatory T cells and CD44⁺ activated cells. Cells were activated by coated anti-CD3 and soluble anti-CD28 for Th1 (anti-IL-4, 10µg/ml; IL-12, 10ng/ml),Th2 (anti-IFNγ, 10µg/ml; anti-IL-12, 10µg/ml; IL-4, 20ng/ml), Th17 (anti-IFNγ, 10µg/ml; anti-IL-12, 10µg/ml; anti-IL-4, 10µg/ml; IL-6, 20ng/ml; TGFβ, 1ng/ml), and Treg (anti-IFNγ, 10µg/ml; anti-IL-12, 10µg/ml; anti-IL-4, 10µg/ml; IL-2, 2ng/ml; TGFβ, 2.5ng/ml) in the absence (Ctrl) or presence (rhPGRN) of 100ng/ml of rhPGRN polarization for 5 days. After re-stimulation with PMA and ionomycin in the presence of Golgi plug for 4 hours, cell surface staining was performed with anti-CD4 followed by intracellular cytokine staining for IL-4, IFNγ, IL-17 and FoxP3.

**Osteoclast formation and activity.** BMDMs were obtained as described above and the resultant preosteoclasts were cultured in medium supplemented with 10 ng/ml M-CSF and 100 ng/ml TNF for 4 days. The cells were fixed with formalin and stained for tartrate-resistant acid phosphatase (TRAP) with a TRAP solution containing 100 mM sodium acetate buffer (pH 5.0), 50 mM sodium tartrate, 0.1 mg/ml sodium naphthol AS-MX phosphate, 0.6 mg/ml Fast Violet LB, and 0.1% Triton X-100. TRAP-positive cells appeared dark red and TRAP-positive multinucleated cells (TRAP⁺-MNCs) containing more than three nuclei were counted using light microscopy. Osteoclast activity was assessed with pit formation assays by using the BD BioCoat Osteologic bone cell culture system (BD Biosciences).

**Cytotoxicity assay.** Rhabdomyosarcoma A673/6 cells were seeded in a 96-well plate at 3×10⁴ cells/well and incubated for 16 h. Serial dilutions of PGRN, Atsttrin, or BSA were prepared in medium containing 80 pg/ml TNFα and 2 µg/ml actinomycin D. Following incubation at 39°C for 18-24 hr, each well was rinsed once with PBS. The adherent cells were then fixed by adding 10% formalin in PBS for 15 min at room temperature, and stained with 0.5% naphthol blue black in 9% acetic acid and 0.1M sodium acetate. After 30 min at room temperature, the cells were rinsed with distilled water, and the bound dye was eluted with 50mM NaOH. Absorbance of the eluted dye was determined at a wavelength of 630 nm.
Cartilage explants. Articular cartilage collected from wild type or PGRN deficient mice was dispensed into 96-well plates and incubated overnight in control, serum-free Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) containing 25 mM HEPES, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin. Fresh control medium (0.2 ml) containing TNFα (5 ng/ml) was then added (day 0). On day 2, supernatants were collected and the COMP degradation determined by immunoblotting with anti-COMP antibodies.

Collagen antibody induced arthritis (CAIA) model. 5 to 6-week-old female BALB/c mice were injected intravenously with a 2 mg cocktail containing four antibodies against type II collagen (Chondrex, LLC, Seattle, WA) (day 0). On day 3, an intraperitoneal injection of lipopolysaccharide (LPS) (Chondrex, LLC, Seattle, WA) was given to enhance the incidence and severity of disease (50 μg/mouse). CAIA mice were given an intraperitoneal injection of PBS, 10 mg/kg rhPGRN, or 10 mg/kg Atsttrin every other day, starting on day 1. Hind paw thickness was measured every other day, using a digital caliper.

Collagen induced arthritis (CIA) model. 8-week-old male DBA1/J mice were immunized via a 0.1 ml intradermal injection at the base of the tail with 100 μg chicken type II collagen (Chondrex, LLC, Seattle, WA) emulsified with an equal volume of complete Freund’s adjuvant (CFA) containing 4 mg/ml heat-denatured mycobacterium (Chondrex, LLC, Seattle, WA) (day 0). To determine preventative effects, various amounts of rhPGRN or Atsttrin were administered intraperitoneally every other day or once a week, starting on day 19 following primary immunization. To determine therapeutic effects, various amounts of Atsttrin were also applied to mice with established severe arthritis (clinical score ≥ 10).

8-week-old male PGRN-deficient mice and their wild type littermates (C57BL/6 background) were primarily immunized with chicken type II collagen (Chondrex, LLC, Seattle, WA) (as described in the preceding paragraph), and a booster injection of 100 μg chicken type II collagen emulsified with an equal volume of complete Freund adjuvant (Chondrex, LLC, Seattle, WA) was administered on day 21. To determine whether PGRN could reverse the inflammation seen in challenged PGRN-deficient mice, recombinant PGRN (10 mg/kg body weight) was injected intraperitoneally every other day beginning at 4 weeks following the initial primary immunization.

To determine the TNFR dependence of Atsttrin’s therapeutic effects, 8-week-old male Tnfrsf1a−/−, Tnfrsf1b−/− and wild type C57BL/6 mice were immunized as described above. And the mice with established mild arthritis (clinical score 4-6) were injected intraperitoneally with various amounts of Atsttrin once every week.

Human TNFα transgenic model (TNF-Tg): TNF-Tg/Grn+/− mice were generated by crossing TNF-Tg mice with Grn−/− mice, and subsequent TNF-Tg/Grn+/− mice were obtained by crossing TNF-Tg/Grn−/− with Grn−/− mice. The spontaneous development of inflammatory arthritis in TNF-Tg, TNF-Tg/Grn+−, and TNF-Tg/Grn−/− mice was recorded, and paws were scored for disease severity. To evaluate the possible therapeutic effects of PGRN, TNF-Tg mice with established arthritis (clinical score 4-6) were administered intraperitoneal PBS or rhPGRN (10 mg/kg body weight) twice per week. At 4 weeks, the treatment groups were then switched. To determine the possible therapeutic effects of Atsttrin, TNF-Tg mice with mild (clinical score 4-6) or severe (clinical score ≥ 10) arthritis were administered intraperitoneal PBS or Atsttrin (2.5 mg/kg body weight) twice per week.

Evaluation for clinical arthritis. Clinical signs of arthritis in CAIA, CIA, and TNF-Tg mouse models were evaluated to determine arthritis incidence. Each paw was evaluated and scored individually using a
0 to 4 scoring system. The paw scores were summed to yield individual mouse scores, with a maximum score of 16 for each animal (S3). The scores, as follows: a paw score of 0, no signs; 1, mild swelling confined to the tarsal bones or ankle joint; 2, mild swelling extending from the ankle to the tarsal bones; 3, moderate swelling extending from the ankle to the metatarsal joints; and 4, severe swelling encompassing the ankle, foot and digits, or ankylosis of the limb.

**Histopathological examination of joints.** Following routine fixation, decalcification, and paraffin embedding, tissue sections were prepared and stained with hematoxylin and eosin. All slides were coded and submitted for evaluation by investigators blinded to the experimental conditions. The extent of synovitis, pannus formation, and bone/cartilage destruction was determined using a graded scale, as follows: grade 0, no signs of inflammation; grade 1, mild inflammation with hyperplasia of the synovial lining without cartilage destruction; grades 2 through 4, increasing degrees of inflammatory cell infiltrate and cartilage/bone destruction37. Sections were also stained with 0.1% Safranin O or TRAP for detection of cartilage proteoglycans or osteoclast activity, respectively.

**TNF intracellular signaling assays.** BMDMs were stimulated with 10 ng/ml TNFα in the presence or absence of 2.5nM rhPGRN or Atsttrin. At the indicated times, total cell lysate or cytoplasmic and nuclear extracts were prepared following our published protocol (S4). Cell extracts (~30 μg) were resolved on a 10% SDS-PAGE gel, and probed using specific antibodies against total and phosphorylated IKKα, IKKβ, IkBa, NF-κB p65, Erk1/2, p38, and JNK.

**Immunohistochemistry.** The tarsal joints of CIA mice treated with PBS, rhPGRN, or Atsttrin were sectioned, deparaffinized, rehydrated, and incubated in Tris buffer (10 Mm Tris-HCl (pH 8.0), 150mM NaCl). The samples were then incubated with rabbit anti-mouse phosphorylated IkBa (1:100) at 4°C overnight. Then, the sections were incubated for 30 min with biotinylated anti-rabbit IgG (Vector, Burlingame, CA) and subsequently stained using a biotin-streptavidin-peroxidase protocol (Vector). Horseradish peroxidase (HRP) activity was detected using 3,3’-diaminobenzidine and H2O2. Slides were counterstained with 0.5% Methyl green.

**Chromatin Immunoprecipitation (ChIP).** Our previous protocol was followed (S4). Briefly, BMDMs were stimulated with TNFα (10 ng/ml) for 1h, in the presence or absence of rhPGRN/Atsttrin (2.5nM). The cells were then treated with formaldehyde, and the sonicated supernatant fraction was incubated with antibodies against NF-κB p65 (Santa Cruz Biotechnology) or control IgG at 4°C overnight. 10% of the chromatin-immunoprecipitated DNA was used as template to amplify a 230 bp-segment of the IkBa gene promoter bearing a NF-κB binding site using forward primer 5’-TGGCGAGGTCTGACTGTTGG-3’, and reverse primer 5’-GCTCATCAAAAAAGTTCCTGTCG-3’.

**Reporter gene.** To examine whether PGRN and Atsttrin inhibit TNF-mediated transactivation of NF-κB-dependent reporter genes, BMDMs grown to ~50% confluence were transfected with 1 μg of the p6XNF-κB-Luc reporter plasmid and 1 μg of the pSVGal plasmid (internal control), using FuGene HD (Roche Applied Science, Basel, Switzerland). 48 hours following transfection, the cells were starved overnight and then stimulated for 6 h with 10 ng/ml TNFα in the presence or absence of 0.1, 0.5, or 2.5nM rhPGRN or Atsttrin. Luciferase and β-Galactosidase activities were then measured.
**Real-time PCR.** Total RNA was isolated from BMDMs stimulated with TNFα (10ng/ml) in the presence or absence of rhPGRN or Atstatrin (2.5nM) for 3h, and then reverse-transcribed to cDNA. Real time PCR was performed with the following sequence-specific primers: 5’-TACAAGCTGGCTGGTGAGGA-3’ and 5’-GTCGCGGGTCTCAGGACCTT-3’ for NFκB2; 5’-AATCTCACAGCAGCAGCACA-3’ and 5’-AAGGCTCCTGTCCTGCTC-3’ for IL-1β; 5’-CCTTCCTACCCCAATTCCCAAT-3’ and 5’-GCCACTCCTTCTGTGACTCCAG-3’ for IL-6; 5’-CTTCACCACCAGGAGGC-3’ and 5’-GACGGCACCATGGGGTAG-3’ for GAPDH.

**Statistical analysis.** For comparison of treatment groups, we performed unpaired t-tests (Mann-Whitney), paired t-tests, and one-way or two-way ANOVA (where appropriate). For ANOVA, we used Bonferroni post hoc analysis to compare treatment groups. All statistical analysis was performed using GraphPad Prism Software (version 4.01). Statistical significance was achieved when p<0.05.
Fig. S1 (A) PGRN interacts with TNFR1 in chondrocytes (Co-IP assay). The cell lysates of human chondrocytes were incubated with anti-PGRN, anti-TNFR1, or control IgG antibodies, and bound protein was examined by Western blotting with the corresponding antibodies, as indicated.  
(B) PGRN directly binds to TNFR1 and TNFR2 (Solid phase binding). Microtiter plates were coated with 500 ng of rhPGRN in 100 µl of TBS buffer (50mM Tris/HCl, 150mM NaCl, pH7.4). After blocking, various amounts (0-500ng) of extracellular domain of TNFR1 (left) or extracellular domain of TNFR2 (right) were added to each well, and bound protein from the liquid phase was detected by antibody against TNFR1 or TNFR2, followed by a secondary antibody conjugated with horseradish peroxidase.
Fig. S2 PGRN deletion enhances TNF-induced COMP degradation. Cartilage explants isolated from wildtype (WT) and PGRN deficient (KO) mice were cultured in the presence of 5 ng/ml of TNF-α for 2 days. The media were separated on non-reduced SDS-PAGE gels and COMP was detected using an anti-COMP antibody. Intact COMP and COMP fragments are indicated by arrows and arrowheads, respectively.
Fig. S3 (A) **PGRN overcomes TNFα downregulation of Treg suppressive function.** Freshly purified human CD4^+CD25^+ T cells (Treg) were pre-treated with indicated concentrations of PGRN before adding TNFα (50 ng/ml), mixed with CD4^+CD25^- cells (Teff) at ratio 1:3, and plated on immobilized CD3 monoclonal antibody (mAb). Cytokine secretion was determined by ELISA by using Human IFNγ Cytoset™. Data represent the average of three independent experiments. Values are mean ± s.d. (B) **PGRN prevents TNFα-induced down-regulation of Foxp3 expression in Treg cells.** Freshly purified Treg cells were pre-treated with PGRN (250 ng/ml) for 30 min before adding TNFα (50 ng/ml). Foxp3 expression was determined by FACS after 18 hours. Representative experiment of two is shown.
**Fig. S4  PGRN promotes Treg differentiation.** Naïve CD4⁺ T cells were enriched by CD4 T cell negative selection kit combined with addition of Bio-anti CD-25 and Bio-anti-CD44 for depleting CD25⁺ regulatory T cells and CD44hi activated cells. Cells were activated by coated anti-CD3 and soluble anti-CD28 for Th1 (anti-IL-4, 10µg/ml; IL-12, 10ng/ml), Th2 (anti-IFNγ, 10µg/ml; anti-IL-12, 10µg/ml; IL-4, 20ng/ml), Th17 (anti-IFNγ, 10µg/ml; anti-IL-12, 10µg/ml; anti-IL-4, 10µg/ml; IL-6, 20ng/ml; TGFβ, 1ng/ml), and Treg (anti-IFNγ, 10µg/ml; anti-IL-12, 10µg/ml; anti-IL-4, 10µg/ml; IL-2, 2ng/ml; TGFβ, 2.5ng/ml) in the absence (Ctrl) or presence (rhPGRN) of 100ng/ml of rhPGRN polarization for 5 days. After re-stimulation with PMA and ionomycin in the presence of Golgi plug for 4 hours, cell surface staining was performed with anti-CD4 followed by intracellular cytokine staining for IL-4, IFNγ, IL-17 and FoxP3.
Fig. S5 PGRN inhibits, whereas TNFα stimulates, IFNγ production in Teff cells in a TNFR-dependent manner. Teff cells were purified from the peripheral blood of human donors and pre-treated cells with 2 µg/ml of either anti-TNFR1 or anti-TNFR2 antibodies for 1 hour before adding TNFα (50 ng/ml) or rhPGN (250 ng/ml). Then, the cells were plated on anti-CD3 mAb (5 mg/ml) and cultured for 24 hr. Cytokine secretion was determined by ELISA by using Human IFNγCytoset™. Average of two different experiments is shown.
Fig. S6 PGRN-deficiency increases, whereas recombinant PGRN prevents, loss of cartilage matrix, tissue destruction, and osteoclast activity in collagen-induced arthritis (CIA) model. (A) Safranin O stained sections of tarsal joints from each experimental group. Arrows indicate loss of matrix staining. Scale bar, 200μm. (B) TRAP stained sections of tarsal bones in WT and KO mice with CIA. TRAP+ osteoclasts are indicated by arrows. Scale bar, 200μm. (C) H&E stained sections and histological evaluation of ankle joints in KO mice with CIA treated with PBS or rhPGRN. Scale bar, 200μm. Values are mean ± s.d. ***P<0.001 versus the control PBS group. (D) Safranin O stained sections of tarsal joints from PGRN deficient CIA mice treated with PBS or rhPGRN. Arrows indicate loss of matrix staining. Scale bar, 200μm. (E) TRAP stained sections of tarsal bones in PGRN deficient CIA mice treated with PBS or rhPGRN. TRAP+ osteoclasts are indicated by arrows. Scale bar, 200μm.
Fig. S7 Deletion of PGRN increases joint deformation, tissue destruction, loss of cartilage matrix and calvarial osteoclast activity in TNF transgenic mice. (A) Photographs of frontal paws of 12-week-old TNF-Tg, TNF-Tg/Grn<sup>+/−</sup>, and TNF-Tg/Grn<sup>−/−</sup> mice. (B) H&E stained sections and histological evaluation of wrist joints in TNF-Tg, TNF-Tg/Grn<sup>+/−</sup>, and TNF-Tg/Grn<sup>−/−</sup> mice. Scale bar, 200μm. Values are mean ± s.d. ***P<0.001 versus the control TNF-Tg group. (C) Safranin O stained sections of wrist joints in 12 week old mice from each experimental group. Arrows indicate loss of matrix staining. Scale bar, 50μm. (D) TRAP stained sections of calvaria from 12 week old TNF-Tg, TNF-Tg/Grn<sup>+/−</sup>, and TNF-Tg/Grn<sup>−/−</sup> mice. TRAP<sup>+</sup> osteoclasts are indicated by arrows. Scale bar, 200μm.
Fig. S8 No single granulin unit (A, B, C, D, E, F, or G) (A) or linker region (P1, P2, P3, P4, P5, P6 or P7) (B) is able to bind to TNFR2. (Left) Schematic diagram of PGRN constructs used to map those of its fragments that bind to TNFR2. (Right) β-Galactosidase assays.
Fig. S9 Granulin F-P3 (A), P4-granulin A and P5-granulin C (B) exhibit a weak interaction with TNFR2. (Left) Schematic diagram of PGRN constructs used to map those of its fragments that bind to TNFR2. (Right) β-Galactosidase assays.
Fig. S10 (A) Identification of Atstrrin (Y2H assay). (Left) Schematic diagram of PGRN constructs used to map those of its fragments that bind to TNFR2. (Right) β-Galactosidase assays. (B) Schematic of Atstrrin composition. Three fragments derived from PGRN are indicated with three different colors. (C) Amino acid sequence of Atstrrin.
Fig. S11 (A) Quantification of protein–protein interactions between PGRN and TNFR subfamily were determined using the β-galactosidase (β-gal) liquid assay. The plasmid encoding one of the members in TNFR subfamily (as indicated) linked to VP16AD, and the plasmid encoding PGRN linked to Gal4DBD were co-transformed into yeast strain MAV203. The β-gal activity represents the average of three transformants from three independent experiments. The β-gal activity produced by the association of PGRN with TNFR1 was set to 1. Tie2 receptor was used as a negative control. Values are mean ± s.d. (B) Co-IP assay. The cell lysates of RAW264.7 cells were immunoprecipitated (IP) with control IgG or anti-PGRN antibodies, and bound protein was examined by Western blotting (WB) with the corresponding antibodies, as indicated. The interaction of PGRN/TNFR1 is employed as a positive control. (C) Atsttrin specifically binds to TNFR1 and TNFR2. The plasmid encoding one of the members in TNFR subfamily (as indicated) linked to VP16AD, and the plasmid encoding Atsttrin linked to Gal4DBD were co-transformed into yeast strain MAV203. Yeast transformants were examined for β-galactosidase activity.
Fig. S12 Characterization of recombinant Atsttrin. **(A)** SDS-PAGE analysis. GST-Atsttrin and Atsttrin are indicated by arrows and arrowheads, respectively. **(B)** Reverse HPLC analysis. The reverse-phase HPLC was performed at 25°C using a Waters 2695 HPLC Module equipped with Waters 2487 dual absorbance detector. An Analytical C18 HPLC column was employed. 35μl of sample at 10μM concentration was injected. Sample was separated over a gradient of 0-60% acetonitrile in the presence of 0.08% TFA at a flow rate of 0.8 ml/min.
Fig. S13 Atsttrin inhibits the binding of LTα to TNFR1 and TNFR2 (solid phase binding). Microtiter plate coated with lymphotoxin-α (LTα) was incubated with TNFR1 or TNFR2 in the presence of various amounts of Atsttrin, as indicated, and the bound TNFR to LTα was detected by corresponding antibodies. The interaction between LTα and TNFR in the absence of Atsttrin was set as 1. Values are mean ± s.d.
Fig. S14 (A) Atstrin inhibits TNFα-induced H₂O₂ production in neutrophils. Neutrophils were treated with TNFα in the presence of various amounts of Atstrin, as indicated, and H₂O₂ production was measured. n=4. Values are mean ± s.d. **P<0.01, ***P<0.001. (B) Atstrin inhibits TNFα-induced nitrite production in bone marrow derived macrophages. M-CSF pretreated BMDMs were incubated with TNFα in the presence of various amounts of Atstrin, as indicated, and the supernatants were tested for NO production. n=4. Values are mean ± s.d. **P<0.01, ***P<0.001. (C) Atstrin does not exhibit cytotoxic effects in rhabdomyosarcoma A673/6 cells. Actinomycin D-treated A673/6 cells were incubated with varying concentrations of Atstrin for 24 hours and stained with naphthol blue black. Absorbances were determined at 630 nm. Cells without treatment and cells treated with 80 pg/ml TNFα served as negative and positive controls, respectively. Values represent mean ± s.d. (D) Atstrin and PGRN neutralize TNFα-induced cytotoxicity in rhabdomyosarcoma A673/6 cells. The data are presented relative to the amount of killing by 80 pg/ml TNFα in the absence of inhibitors (as 100%). Values are mean ± s.d. (E) Left panel: representative TRAP staining of TNFα stimulated culture in the presence or absence of Atstrin. TRAP⁺ staining indicates osteoclast formation. Scale bar, 50µm. Right panel: mean number of TRAP-positive multinucleated cells (MNCs). Values are mean ± s.d. ***P<0.001 versus the control (TNFα + M-CSF) treatment group. (F) Left panel: representative photomicrographs of resorption pits. Scale bar, 50µm. Middle and Right panel: mean number of pits and areas of osteoclast-mediated bone resorption. Values are mean ± s.d. ***P<0.001.
**Fig. S15 Effects of PGRN and Atstrrin in experimental model of CAIA.** (A) Arthritis severity and (B) paw thickness measurements in CAIA mice injected with PBS, rhPGRN or Atstrrin at 10 mg/kg body weight every other day (n=8/group). Values are mean ± s.e.m. ***P<0.001 versus the control PBS group. (C) H&E (top) and Safranin O (bottom) stained sections of CAIA ankle joints on day 14 following collagen antibody challenge and treatment with PBS, rhPGRN or Atstrrin. Arrows indicate loss of matrix staining. Scale bar, 100μm.
Fig. S16 Effects of PGRN and Atsttrin in experimental model of CIA. (A) Safranin O stained sections of CIA tarsal joints on day 41 following primary immunization and treatment with PBS or indicated antagonists. Arrows indicate loss of matrix staining. Scale bar, 100μm. (B) Three-dimensional microstructural analysis of ankle joints in CIA mice treated with PBS, rhPGRN or Atsttrin. Specimens were scanned using micro-computed tomography. Scale bar, 1mm. (C) TRAP stained sections of digital bones from each experimental group. TRAP^+ osteoclasts are indicated by arrows. Scale bar, 100μm. (D, E) Serum levels of IL-1β, IL-6, IL-10 and IL-13, and COMP, as measured by ELISA on day 41 in each experimental group. Values are mean ± s.d. *P<0.05, **P<0.01, ***P<0.001 versus the control PBS group.
Fig. S17 (A) Characterization of affinity-purified polyclonal antibodies against Atsttrin. Bacterial DE3 strain bearing the pET9d-Atsttrin’ was cultured for 3 hours, followed by the induction with 0.5mM IPTG for 2 more hours, and the cell extracts were separated by SDS-PAGE, followed by the detection with affinity-purified anti-Atsttrin antibodies. (B) Standard curves for indirect ELISA assays for PGRN (a) and Atsttrin (b). (C) Pharmacokinetic profiles of PGRN and Atsttrin in mice. PGRN-deficient mice received a single i.p. injection of 2.4 mg/kg rhPGRN or 1.2 mg/kg Atsttrin. At various time points, blood was collected and serum levels of PGRN (a) or Atsttrin (b) were determined by indirect ELISA. Values represent mean ± s.d. PGRN and Atsttrin were found to have half-lives of ~40 hours and ~120 hours, respectively. (D) Pharmacokinetic profile of Atsttrin in mice. PGRN deficient mice that received a single i.p. injection of Atsttrin (1.2mg/kg) were bled at the designated time points (3 mice per time point). The serum concentration of Atsttrin was determined by an indirect ELISA using anti-Atsttrin antibody. Values are mean ± s.d.
Fig. S18 (A) Dose dependency assay of Attrlin in CIA mice. Attrlin was administered at 0.02, 0.1, 0.5, or 2.5 mg per kg body once a week; n=8/group. Values are mean ± s.e.m. *P<0.05, ***P<0.001 versus the control PBS group. (B) A single dose of Attrlin (10 mg/kg) could effectively delay the onset of inflammation for approximately three weeks in CIA mice. Attrlin was administered at day 19 after collagen II challenge; n=8/group. Values are mean ± s.e.m. *P<0.05, **P<0.01, ***P<0.001 versus the control PBS group.
Fig. S19 Therapeutic efficacy of PGRN and Atstrrin in TNF-Tg mice. (A) Dose dependency assay of PGRN’s therapeutic effect in TNF-Tg mice. rhPGRN was administered at 0.1, 0.5, 2.5, or 10 mg per kg body twice a week; n=8/group. Values are mean ± s.e.m. **P<0.01, ***P<0.001 versus the control PBS (0mg/kg) group. (B) Administration of Atstrrin (2.5mg/kg body weight) twice every week led to the reversal of inflammation in TNF-Tg mice with mild arthritis (n=8; arthritic score ~5). In contrast, inflammatory arthritis was relapsed and gradually progressed following the discontinuation of Atstrrin. Arrow indicates the time point when Atstrrin treatment was ceased. (C) Atstrrin treatment of TNF-Tg mice with severe arthritis. TNF-Tg mice with severe arthritis (n=8; arthritic score ~12) treated with Atstrrin at the same dose and time interval as in (B) ameliorated the severity of inflammation. Data presented as the mean clinical score ±s.e.m.
## Table S1 Pharmacokinetic parameters of Atsttrin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tbody>
<tr>
<td>(C_{\text{max}}) ((\mu\text{g/ml}))(^1)</td>
<td>19.3</td>
</tr>
<tr>
<td>(T_{\text{max}}) (hour)(^2)</td>
<td>12</td>
</tr>
<tr>
<td>Terminal half-life (day)</td>
<td>5.0</td>
</tr>
<tr>
<td>Availability (%)(^3)</td>
<td>85.5</td>
</tr>
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\(^1\)The maximum concentration.
\(^2\)The time of reaching maximum concentration.
\(^3\)Percentage of i.p. dose that reaches blood sampling compartment.
References:

Author Contributions
C. J. Liu designed and supervised this study, analyzed data, and wrote and edited the manuscript. W. Tang, Y. Lu, and Q.Y. Tian designed and performed experiments, collected and analyzed data, and co-wrote the paper. Y. Zhang performed experiments in initial stages of the project, particularly in screening the yeast-two hybrid library. F.J. Guo, G.Y. Liu, N.M. Syed, Y.J. Lai, E.A. Lin, and L. Kong performed experiments, and collected and analyzed data. J. Su contributed to the analysis of Analytical Surface Plasmon Resonance with SensíQ Pioneer and performed reverse-phase HPLC. F. Yin and A.H. Ding provided PGRN null mouse lines and assisted in genotyping. A. Zanin-Zhorov and M.L. Dustin performed in vitro assays with human Treg and Teff cells. J. Tao, Z.N. Yin and J. Craft performed in vitro T cell differentiation assay. A.H. Ding, M.L. Dustin, S.B. Abramson, J. Q. Feng, and X.P. Yu assisted in analyzing the data and editing the manuscript.