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Three TNFR-binding domains of PGRN act independently in inhibition of TNF-alpha binding and activity

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1. ABSTRACT

PGRN was previously reported to bind to TNF receptors (TNFR) and is therapeutic against inflammatory arthritis. Here we present further evidences demonstrating the PGRN inhibition of TNF-alpha binding and activity, and clarifying the distinct mechanisms underlying TNF-alpha inhibition between PGRN and classic TNF-alpha-binding inhibitors. In addition, we present evidences indicating that three TNFR binding domains of PGRN act independently in binding to TNFR. Furthermore, changing the order of three TNFR-binding domains in Atsttrin, a PGRN-derived molecule composed of these TNFR-binding domains, does not affect its anti-inflammatory and anti-TNF activities in both collagen-induced inflammatory arthritis and human TNF--alpha transgenic mouse model. Taken together, these findings provide the additional molecular basis underlying PGRN/TNFR interaction and PGRN-mediated anti-inflammatory activity in various inflammatory diseases and conditions.

2. INTRODUCTION

Progranulin (PGRN), also known as granulin–epithelin precursor (GEP), proepithelin (PEPI), and PC-cell derived growth factor (PCDGF), is a growth factor containing seven and half granulin units in the order of P–G–F–B–A–C–D–E(1). Each granulin unit is composed of 59 amino acid residues and 12 of them are cysteines, PGRN is thus a highly cysteine-rich glycoprotein. Progranulin has multiple biological functions including anti-inflammation and immune regulations (2). PGRN also acts as a neurotrophic growth factor and stimulates neurite growth (3). Mutations of the *GRN* gene are known to lead to the development of frontotemporal lobar degeneration (FTLD)(4; 5). PGRN insufficiency in some autism patients results in reduced neurotropic support together with cumulative damage in association with dysregulated inflammation (6). Loss of function mutations in the *GRN* gene are related to pro-inflammatory cytokine dysregulation in FTLD patients (7). PGRN-deficient mice

have increased susceptibility to neuroinflammation and neuron loss following toxin–induced injury(8), whereas mice overexpressing PGRN exhibit a neuro-protective role by decreased pro-inflammatory cytokines IL-1β, IL-6, and TNF-alpha, and increased anti-inflammatory cytokine IL-10 in glial cells(9). In addition, mutations in *GRN* are also associated with increased prevalence of specific and related autoimmune diseases, including inflammatory arthritis (10).

PGRN-mediated inhibition of TNF-alpha activity has been well established (11; 12; 13; 14; 15; 16; 17; 18; 19). For instance, PGRN diminishes TNF-alpha-trigged production of reactive oxygen species in neutrophils (11). PGRN protected lung inflammation and the PGRN-mediated protective effect depended on PGRN/TNFR2 interaction (12). PGRN ameliorated ischemia-reperfusion induced neuronal injury, and this ameliorative effect resulted from the inhibition of the TNF-alpha binding to the neutrophil, and in turn, the suppression of TNF-alpha-induced neutrophil chemotaxis(15). Additionally, PGRN also played a protective role in atherosclerosis through the suppression of TNF-alpha-induced expression of ICAM-1 and VCAM-1 in endothelial cells (16). Furthermore, PGRN was also reported to protect vascular endothelium against atherosclerotic inflammatory reaction via attenuating NF-κB pathways (17). Interestingly, it was also reported that PGRN abrogated TNF-alpha-triggered a dose-dependent loss of the primary cilia in mesenchymal stromal cells through inhibiting NF-κB signaling intermediates IκB kinase -alpha, β, and γ, as well as NF-κB p65 (18). The inhibition of TNF-alpha activity by PGRN was also supported by a very recent report that PGRN antibodies entertain a proinflammatory environment in a subgroup of patients with psoriatic arthritis(19). PGRN-antibodies-positive patients had more frequent enthesitis or dactylitis, and the protective effects of PGRN were inhibited by serum containing PGRN antibodies in TNF-alpha-induced cytotoxicity assays (19). Here we present further evidences demonstrating (1) Dosage-dependent inhibition of PGRN on some TNF-inducible genes; (2) Dependence on availability of TNFR in cells, but not only TNF-to-PGRN ratio, for revealing significant inhibition of TNF by PGRN; (3) Independent action in binding to TNF of three TNFR-binding domains of Atsttrin, and (4) Changing the order of three TNFR-binding domains of Atsttrin does not affect its anti-inflammatory and anti-TNF-alpha activities *in vitro* and *in vivo*.

3. MATERIALS AND METHODS

3.1. Preparation of recombinant proteins

PGRN was purified from conditioned medium of HEK-EBNA cells, which were stably transfected with human PGRN with C-terminal His-tags as described previously (20). Atsttrin -alpha and Atsttrin β were expressed as GST fusion proteins in corresponding expression plasmids based on pGEX-3X vectors in E.coli. Fusion proteins were affinity-purified on glutathione-agarose as described previously (20). Factor Xa was used to release Atsttrin -alpha and Atsttrin β, respectively, from the fusion proteins. The protein purity was determined with SDS-PAGE, and activity was measured with a TNF-alpha blocking assay.

3.2. Real-time Quantitative PCR

BMDMs isolated from C57BL/6 mice or human THP-1 cells were stimulated with 20 ng/ml TNF-alpha in the presence or absence of various amounts of PGRN for 24 h. Total RNA was extracted from cells using the RNAeasy Kit. The following sequence-specific primers were used for the real-time qPCR: 5'-tgtgga gttcgagga accct-3' and 5'-tgccttggctggtgctg-3' for mouse CXCL9, 5′-ggatggctgtcctagctctg-3′ and 5'-tgagctagggaggacaagga-3′ for mouse CXCL10, 5'- gtggacccaactggaagctgtt-3' and 5'-aatacacggtgatggtagcggaag-3' for mouse ICAM-1, 5'-gaccatggagcctgtcagttttga-3' and 5'-gccactgaattgaatctctggatcc-3' for mouse VCAM-1, 5'-gcaaggaaccccagtagtgagaaag-3' and 5'-gaagggcttggggcaaattgt-3' for human CXCL9, 5'-atcaaactgccattctgatttgctg-3' and 5'-ggacaaaattggcttgcaggaata-3' for human CXCL10, 5'-ccggaaggtgtatgaactgagca-3' and 5'-agtccagtacacggtgaggaaggtt-3' for human ICAM-1, 5'-ttgggaacgaacactcttacctgtg-3' and 5'-ggccactcaaatgaatctctggatc-3' for human VCAM-1. The presence of a single specific PCR product was verified by melting curve analysis, and the experiments were repeated three times.

3.3. Assay for inhibiting TNF-alpha binding to cell surface by PGRN

This experiment was performed according to the protocol provided by the manufacturer (R&D System). RAW264.7. and THP-1 cells were suspended in 0.4% BSA PBS buffer. The cells were pre-treated with BSA or PGRN (5μg) for 15 min. 50ng of biotinylated TNF-alpha was added for 1 hour, then FITC-labeled streptavidin was added. The staining was measured by flow cytometry at the NYU core facility.

3.4. Solid phase binding assay

Microtiter plates were coated with 100 ng TNFR in 100 μl of TBS buffer (10mM Tris/HCl, 150mM NaCl, pH7.4.) containing 0.5.%BSA. After blocking, various amounts of several batches of Atsttrin -alpha or Atsttrin β, as indicated, was incubated for 1 h, 10 ng biotin-labelled TNF-alpha was then added and incubate for another 2 h, bound protein from the liquid phase was detected by streptavidin conjugated with horseradish peroxidase.

3.5. Yeast two-hybrid system

pDB-Atsttrin-alpha was used as templates to generate pDB-Atsttrin β and pDB-Atsttrin γ through fusion PCR technique. Extracellular domain of TNFR1 and TNFR2 were cloned into the pPC86 vector. The same strategy was used as the discovery of PGRN-TNFR interaction, referred to as a modified yeast-two-hybrid (Y2H) system in previous reports (14). Positive interactions between the bait and prey protein couples were displayed as blue dots on the membrane.

3.6. 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, PBS, Enbrel, or Atsttrin were administered intraperitoneally twice a week, starting on day 18 following primary immunization. To determine therapeutic effects, Atsttrin were also applied to mice with established moderate arthritis (clinical score ~ 7).

3.7. Human TNF-alpha transgenic model (TNF-Tg)

To determine the therapeutic anti-TNF effects of Atsttrin-β, TNF-Tg mice with moderate arthritis (clinical score ~9) were administered intraperitoneal PBS or Atsttrin-β (2.5. mg/kg body weight) twice a week for two weeks.

3.8. 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 destruction. Sections were also stained with 0.1% Safranin O for detection the loss of cartilage proteoglycans.

4. RESULTS

4.1. Inhibition of TNF-alpha-inducible genes by PGRN in both murine and human cells

Different from regular TNF-alpha-binding inhibitors, such as TNF antibodies or soluble TNFR, PGRN possesses multiple other actions in addition to its binding to TNFR and the inhibition of TNF-alpha/TNFR interaction, including activation of various signaling pathways and target genes expression, thus it is conceivable that PGRN will activate many downstream genes, instead of pure inhibition on TNF-alpha-activated signaling and downstream genes. To isolate the TNF-inducible genes that are also inhibited by PGRN or its derived Atsttrin -alpha, we performed whole genome array with primary mouse bone marrow-derived macrophages (BMDM) cells treated with TNF-alpha in the absences or presence of Atsttrin. Approximately 2000 TNF-inducible genes were found to be suppressed by Atsttrin -alpha. Four significant hits, including CXCL9, CXCL10, VCAM-1 and ICAM-1, were selected and their inhibition by PGRN was confirmed by Real-time qPCR. As shown in Figure 1, PGRN significantly inhibited the TNF-mediated inductions of these genes in BMDM (Panel A) and human THP-1 (Panel B) cells. In addition, PGRN inhibition of TNF-inducible ICAM-1 and VCAM-1 is in accordance with the recent findings in endothelial cells (15; 16).

4.2. PGRN inhibition of TNF binding largely depends on the numbers of cell surface TNFRs

We have reported that different amounts of PGRN was needed to achieve the clear inhibition of TNF-alpha binding to various cells (14; 20), indicating that variable number of TNFRs are expressed in different cell types. To further support the concept that PGRN inhibition of TNF-alpha highly depends on the number of TNFRs, we examined the PGRN inhibition of TNF-alpha binding to cell surfaces with various numbers of murine Raw264.7 cells (Figure 2A) and human THP-1 cells (Figure 2B) at fixed PGRN/TNF-alpha ratio. PGRN demonstrated clear inhibition of TNF-alpha binding in total cell number of 1×10^4 cells, the inhibition became weaker when cell number was increased to 1×10^5. It is conceivable that PGRN will not demonstrate significant inhibition on TNF-alpha binding at even higher cell density. Same tendency was also observed with THP-1 cells. In addition, alteration in inhibition was also noticed at the same cell number between two cell lines. Collectively, these set of data clearly indicate that PGRN inhibition of TNF-alpha binding highly depends on the availability of TNFR on the cell surface.

4.3. Three TNFR-binding domains of PGRN act independently in TNFR binding

Atsttrin is composed of three TNFR-binding domains of PGRN (20). To determine whether changing the order/sequence of these TNFR-binding domains affect the binding to TNFR, we generated two additional constructs expressing recombinant proteins Atsttrin β & γ (Original Atsttrin was renamed as Atsttrin -alpha) in different order (Figure 3A). Similar to Atsttrin -alpha, both Atsttrin β and Atsttrin γ interacted with TNFR1 and TNFR2 in a yeast-2-hybrid assay (Figure 3B). These findings indicate that each domain of Atsttrin acts independently and thus change of the order does not abolish the binding activity to TNFR. Intriguingly, it seems that Atsttrin β and Atsttrin γ show stronger binding affinity to TNFR compared to Atsttrin -alpha, at least in yeast, suggesting that alteration of fragment order may lead to variation in protein folding and thus enhance the binding affinity. The finding that Atsttrin β exhibited stronger binding to TNFR promoted us to determine whether Atsttrin β also affected the TNF-alpha/TNFR interaction. As shown in Figure 3C, Atsttrin β demonstrated a dose-dependent inhibition of the interaction between TNF-alpha and TNFR1/TNFR2.

4.4. Atsttrin β inhibits inflammation in mice with collagen-induced arthritis (CIA) and in TNF transgenic mice, as does Atsttrin -alpha

In vitro findings above led us to determine whether Atsttrin β also has anti-inflammatory activity *in vivo*, we tested its potential effect in CIA model first. Briefly, 18 days after immunization, the DBA1 mice were divided into 4 groups with 9 mice in each group: PBS, Enbrel/eternacept (serving as positive control), Atsttrin -alpha, and Atsttrin β. Mice were injected at a dose of 2.5.mg/kg body weight twice a week. As is shown in Figure 4A, Atsttrin β significantly prevented CIA development, similar to Atsttrin -alpha. To examine the therapeutic effect of Atsttrin β in the established CIA, DBA1 mice with the average arthritic score of around 7 were treated as indicated in Figure 4B. Similar to Atsttrin -alpha, Atsttrin β also effectively inhibited, or even

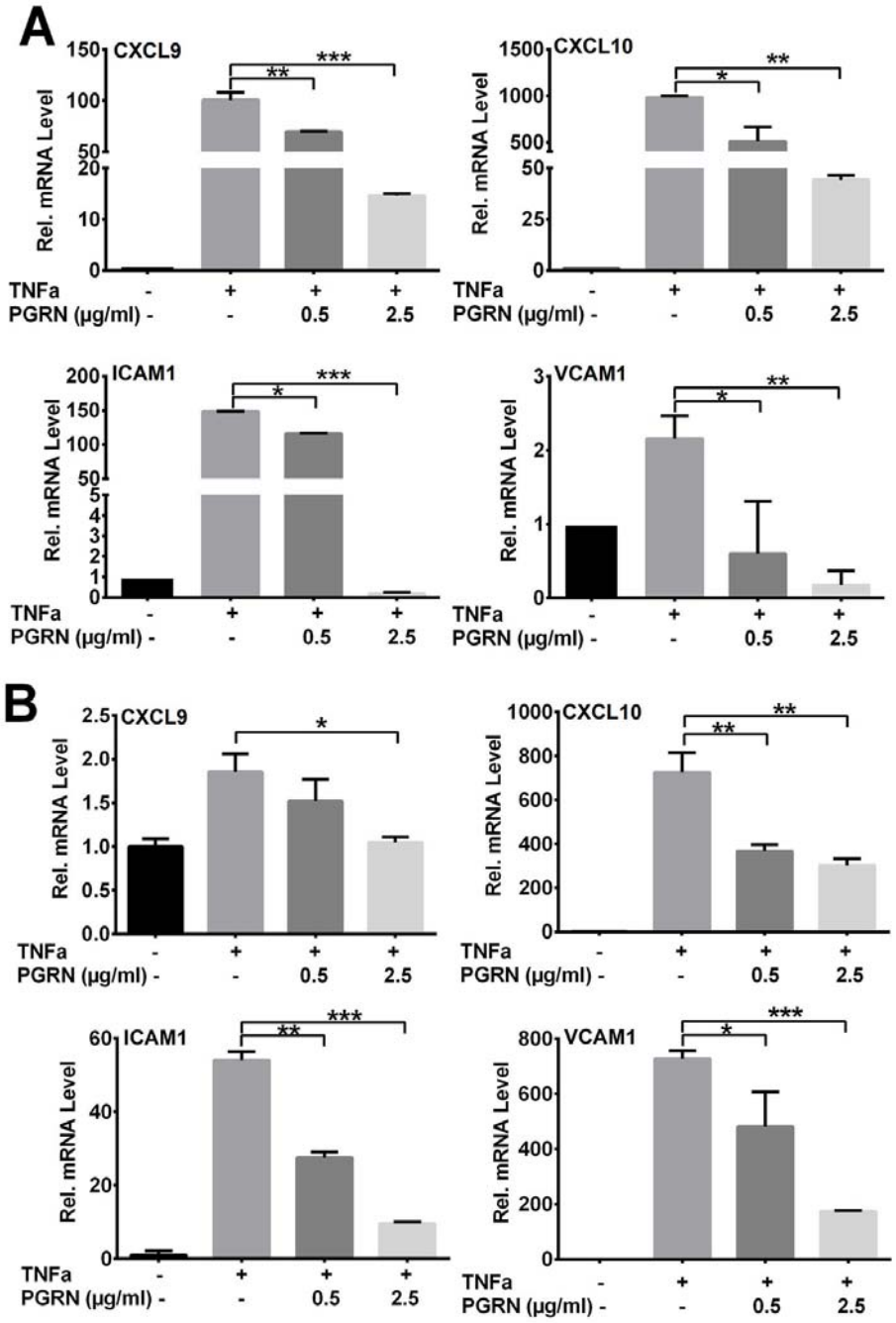

Figure 1. (A & B) Dosage-dependent inhibition of TNF-alpha-activated expressions of CXCL9, CXCL10, ICAM-1 and VCAM-1 by PGRN. BMDMs (A) or THP-1 (B) cells were stimulated with 20ng/ml TNF--alpha in the presences of various amounts of PGRN, as indicated, for 24hrs. The data shown are representative of 3 independent experiments. *p< 0.0.5, **p< 0.0.1, ***p< 0.0.01.

reversed, the disease progression. Intriguingly, the disease relapsed approximately five days later following the halt of treatment. In addition, histological analysis and Safranin O staining revealed that Atsttrin β markedly inhibited tissue destruction, bone erosion, and loss of proteoglycan (Figs. 4C, 4D).

Human TNF-alpha transgenic (TNF-Tg) mice develop an inflammatory arthritis phenotype spontaneously (21). We next took advantage of this animal model to test the anti-TNF-alpha activity of Atsttrin β *in vivo*. Briefly, TNF-Tg mice with the established arthritis (arthritic score ~9) were administered intraperitoneal PBS (n=6) or Atsttrin

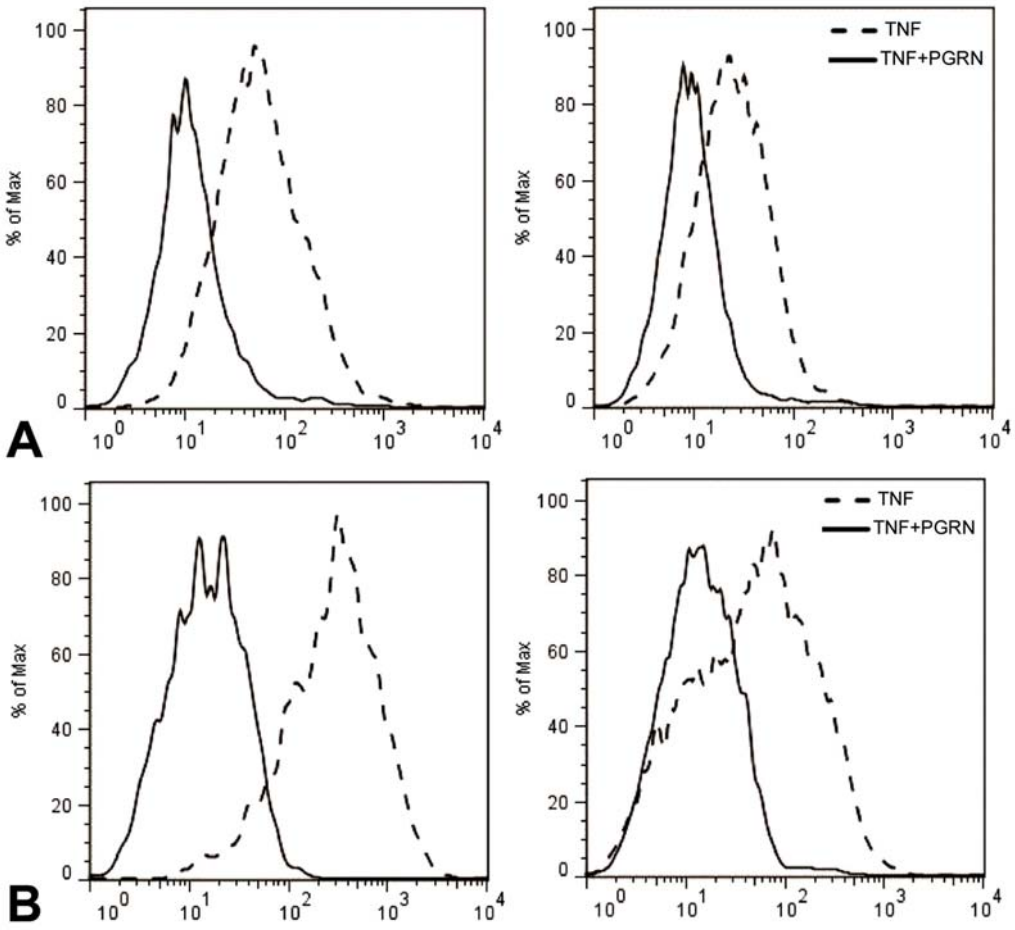

Figure 2. (A & B) Dependences on the cell number used of PGRN inhibition of TNF-alpha binding to cell surface. 1×10^4 (left panel) or 1×10^5 (right panel) RAW264.7. (A) or THP-1 (B) cells were incubated with 50ng biotinylated-TNF-alpha in the absence (dotted line) or presence (solid line) of 5μg PGRN. The binding of biotinylated-TNF-alpha to cells were examined with FACS.

β (n=6) at a dose of 2.5.mg/kg body weight twice per week for 2 weeks. As shown in Figure 4E, Atsttrin β also displayed significant anti-inflammatory/anti-TNF-alpha effect in this model.

5. DISCUSSION

Although the inhibition of TNF-alpha activity by PGRN has been reported independently by several laboratories(11; 12; 13; 14; 15; 16; 17; 18; 19), recent short report claimed that PGRN inhibition of TNF-alpha was not observed in their experiments (22). One possibility for explaining their inability is the use of improperly folded proteins. PGRN is a highly cysteine-rich glycoprotein, and contains numerous internal disulfide bonds, which are critical for maintaining the proper folding and conformation of this protein (23). Indeed, proper folding of PGRN is critical for its binding to TNFR, as DTT treatment, which is known to disturb the formation of disulfide bonds and in turn affecting protein folding, completely abolished binding of PGRN to TNFR, but did not inhibit the binding of PGRN to Sortilin(14). Posttranslational modifications of PGRN could be another important factor affecting its activity. Recombinant PGRN produced from a HEK-EBAN stable line and PGRN purchased from R&D Systems exhibited a slight difference in gel mobility assay, indicating that they may not contain the similar modifications and in turn conformation and activity accordingly. In addition, variations in inhibiting TNF-alpha binding were also observed among different batches purified from the same stable clone or purchased from the same vendor (14). Variations in inhibiting TNF-alpha activities among purification batches of PGRNs from various sources are quite similar to the discrepancy between different purifications of perlecan, a PGRN-binding glycoprotein (24). Perlecans have been shown to have significant variations in glycosylation and function, and can vary dramatically in cell-based assays or in growth factor binding assays (25; 26; 27). Atsttrin, an engineered molecule composed of three TNFR-binding domains and

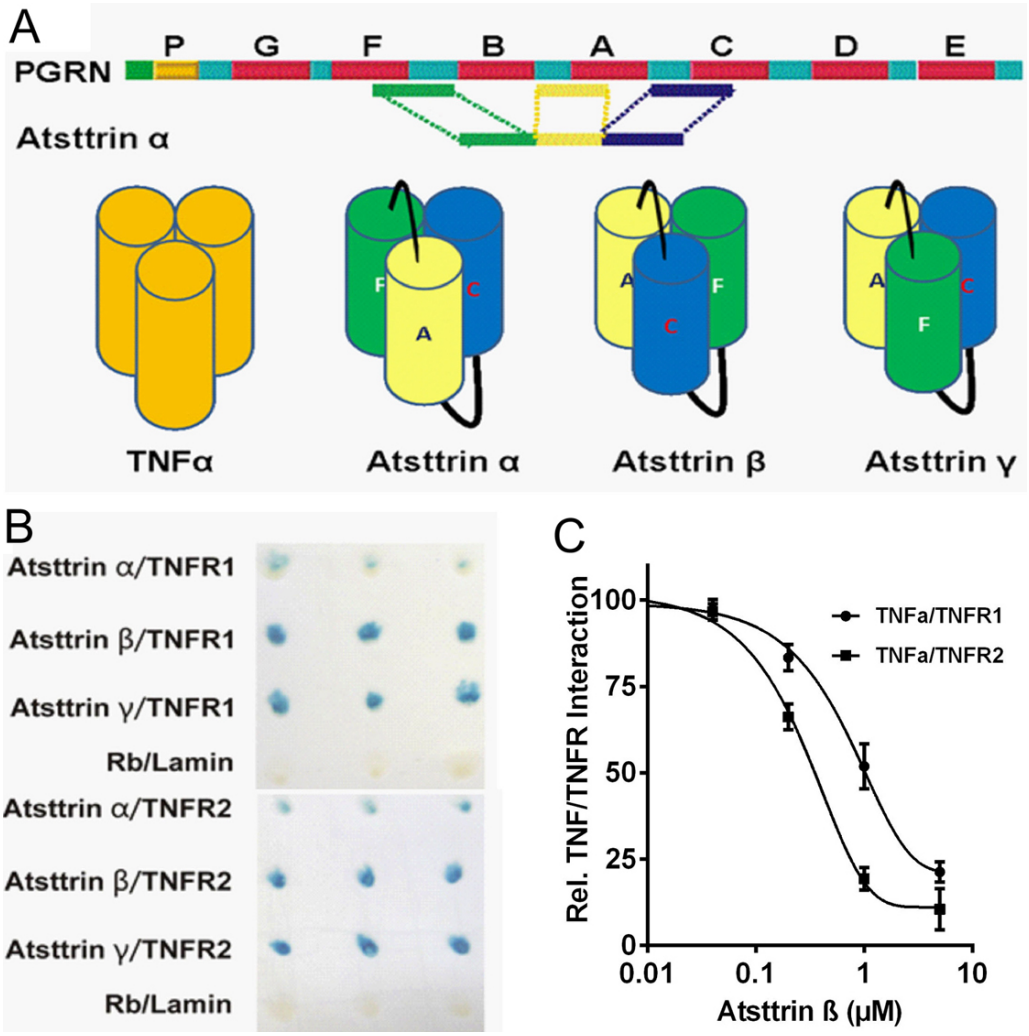

Figure 3. Each of three TNFR-binding domains of PGRN acts independently in TNFR binding. (A) Diagrams showing the structures of PGRN and its derived Atsttrin, and proposed models for explaining the independent action of three domains which comprise Atsttrin -alpha , Atsttrin β and Atsttrin γ. (B) Atsttrin β and Atsttrin γ bind to TNFR, as does Atsttrin -alpha (Yeast-two hybrid Assay). Rb/Lamin pair serves as a negative control. (C) Atsttrin β inhibits the binding of TNF-alpha to TNFR1 and TNFR2 (Solid-phase binding assay). Microtiter plate coated with TNFR1 or TNFR2 was incubated with biotin-labeled TNF-alpha in the presence of various amounts of Atsttrin β, as indicated, and the bound TNF-alpha was detected by streptavidin conjugated with horseradish peroxidase. Values are mean ± s.d.

known to inhibit TNF-alpha as well, also demonstrated high variation in the folding among purification batches from the same expression clone in reverse phase HPLC assay (data not shown). Accordingly, variability in inhibiting TNF-alpha binding and activity by Atsttrin was observed as well. The variation and instability in Atsttrin folding is probably due to presence of 17 cysteine residues within Atsttrin, which is composed of 154 residues (14).

Selection of the dosages of PGRN only based on the ratio of PGRN to TNF-alpha may be insufficient for demonstrating the clear inhibition of TNF-alpha by PGRN, since PGRN inhibition of TNF-alpha binding also closely depends on the numbers and availability of TNFR on cell surface (Figure 2A), which is clearly different from the regular TNF-alpha-binding inhibitors, such as anti-TNF-alpha antibodies. Even if the ratio of PGRN-TNF-alpha is high, as long as the unoccupied TNFRs in cells are still available, TNF-alpha will be able to bind to receptors and activates its signaling and target genes. We propose a competitive receptor binding of TNF-alpha and PGRN to TNFR, in which TNFR is a limited factor. In case of TNFR abundance, TNF-alpha to PGRN ratio become irrelevant, as both TNF-alpha and PGRN will bind to TNFR and will lead to partial or insignificant inhibition. To attain significant inhibition PGRN must saturate available TNFR binding sites and compete out TNF-alpha binding to TNFR. Additionally, it is well established that expressions of TNFRs vary greatly among different cell types or even the same cell type from different donors. Furthermore,

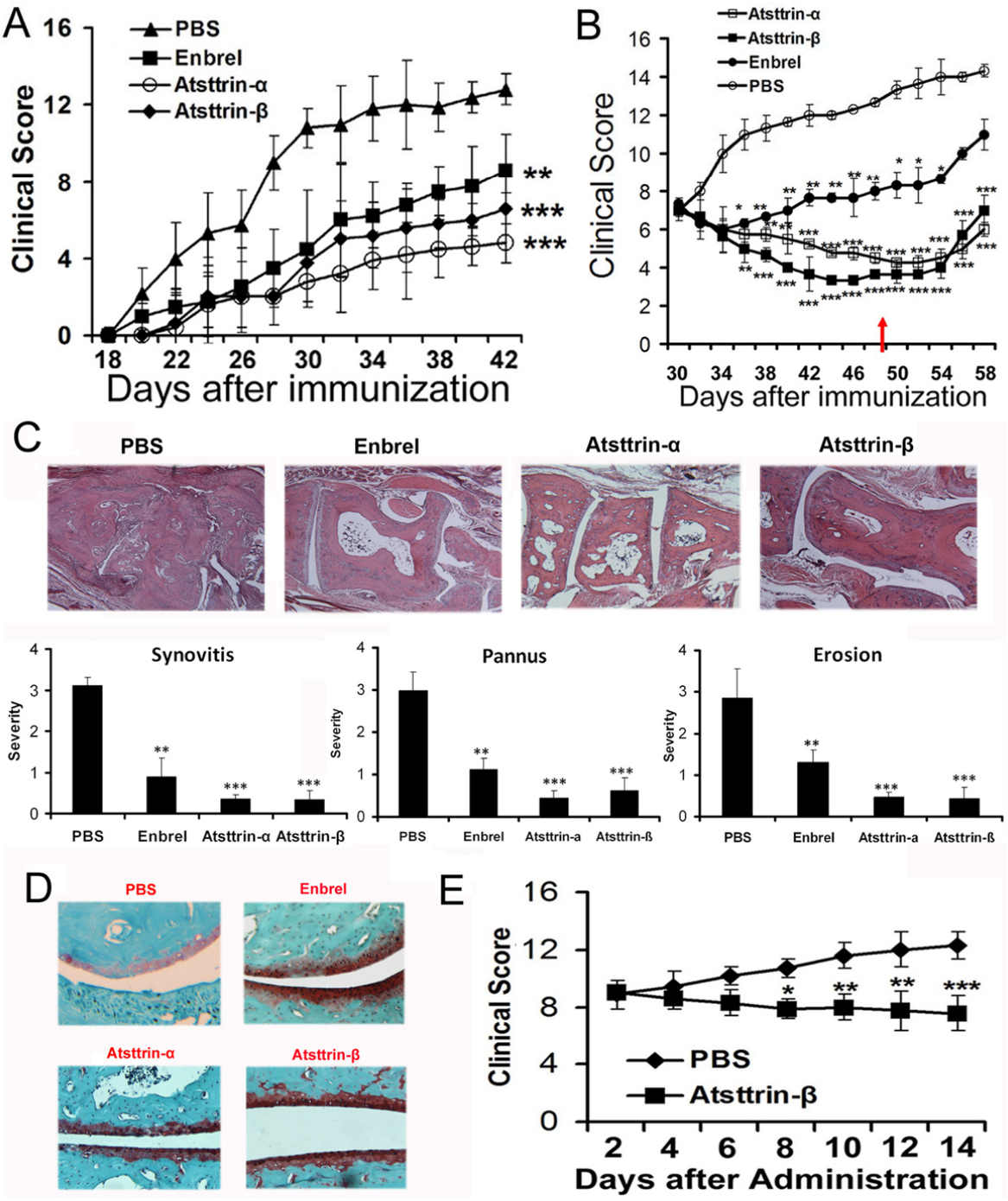

Figure 4. Effects of Atsttrin -alpha and Atsttrin β in CIA and in TNF-Tg mice. (A) Clinical arthritis scores in PBS, Enbrel (serving as positive control), Atsttrin -alpha, or Atsttrin β treated CIA mice. (*n*=9/group). (B) Therapeutic effects of Atsttrin in established CIA mice receiving intraperitoneal injections of indicated treatment (*n*=9/group). Arrow indicates the time point when treatment was ceased. (C) H&E stained sections and evaluation of synovitis, pannus formation, and erosion of tarsal joints in CIA mice sacrificed at day 42 following primary immunization and treatment (starting day 18) with PBS, or indicated antagonists. (D) Safranin O stained sections of CIA tarsal joints on day 42 following primary immunization and treatment with PBS or indicated antagonists. (E) Clinical arthritis scores in TNF-Tg mice treated with PBS or Atsttrin β. Human TNF-Tg mice with moderate arthritis (n=6/group) treated with Atsttrin β significantly ameliorated the severity of inflammation. p< 0.0.5, **p< 0.0.1, ***p< 0.0.01 versus the control PBS group.

demonstrating the interaction of PGRN/TNFR is highly dependent on the target tissue/cell type, for instance, no interaction between PGRN and TNFR1 was detected in human regulatory T cells (not shown), which are known to express only TNFR2.

Recently, Chen and colleagues reported that PGRN stimulates the suppressive function of mouse regulatory T cells (Treg) through enhancing TNF-alpha-induced Treg proliferation (28). The effect of TNF-alpha on regulation of Tregs purified from mice and humans remains to be highly controversial. The data from Chen lab suggest that TNF-alpha promotes murine Treg activity *in vitro* (28), whereas in humans, TNF-alpha inhibits the suppressive function of Tregs through negative regulation of Foxp3 expression (29; 30; 31; 32). Our report that PGRN protects human Tregs from negative regulation by TNF-alpha (20) also supported the concept that TNF-alpha inhibits the suppressive function of Tregs. PGRN significantly protects Treg function from a negative regulation by TNFα, assayed by IFNγ secretion, and also prevented the downregulation of Foxp3 by TNF-alpha in Tregs (20). In Discussion section of Chen paper, they speculated that the effect of PGRN on Treg suppression in our study is due to the inhibition of IFNγ secretion from Teffs directly (28). This is wrong because PGRN was washed out after treatment of Tregs and PGRN was no present during the co-culture with Teffs (20). Interestingly, PGRN was found to be effective on human Tregs at doses between 50-250 ng/ml, where its effects on mouse Tregs occurred only at low nanomolar doses (28). Thus, it appears PGRN stimulates the suppressive function of both human and mouse Tregs, although the exact molecular mechanism remains to be further delineated.

It is well established that TNF family ligands bind to receptors in a heterohexameric 3:3 complex (33) . The three fragments of Atsttrin act independently for interacting with TNFR, as changing the order of these fragments does not affect their ability to binding to TNFR (Figure 3B). These data suggest that each TNFR-binding domain may function as a single TNF-alpha molecule, and the intact Atsttrin might resemble a TNF trimer through internal folding at their linker regions (Figure 3A). In addition, structural analysis revealed that each TNFR-binding domain contains both acidic and basic amino acids at the corresponding positions to TNF-alpha which are known to play critical roles in mediating TNF/TNFR interaction (34). Importantly, recombinant Atsttrin β demonstrate potent therapeutic effect in both inflammatory CIA and TNF Tg animal model (Figure 4). In addition, the therapeutic effect of Atsttrin β in TNF tg mice indicate that Atsttrin β effectively inhibits TNFR1-mediated TNF-alpha activity, as inflammation in this model is believed to be mediated primarily through TNF Receptor 1 (21; 35). Collectively, the finding that each TNFR-binding domain acts independently provides new insight into the mechanism underlying the association of PGRN and TNFR, and also provides us with additional interventions for treating inflammatory conditions clinically.

6. ACKNOWLEDGMENTS

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