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Progranulin deficiency exaggerates, whereas progranulin-derived Atsttrin attenuates, severity of dermatitis in mice

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ABSTRACT

PGRN and its derived engineered protein, Atsttrin, were reported to antagonize TNFα and protect against inflammatory arthritis [Tang, W. et al. (2011) The growth factor progranulin binds to TNF receptors and is therapeutic against inflammatory arthritis in mice. Science 332 (6028) 478–484]. Here we found that PGRN level was also significantly elevated in skin inflammation. PGRN−/− mice exhibited more severe inflammation following induction of oxazolone (OXA). In contrast, recombinant Atsttrin protein effectively attenuated inflammation in mice dermatitis model. In addition, the protective role of PGRN and Atsttrin in dermatitis was probably due to their inhibition on NF-κB signaling. Collectively, PGRN, especially its derived engineered protein, Atsttrin, may represent a potential molecular target for prevention and treatment of inflammatory skin diseases.

1. Introduction

Contact dermatitis is a common inflammatory skin disorder characterized by pruritic skin lesions, immunodysregulation and disrupted epidermal barrier function. Moreover, it is a commonly used model system to evaluate potential therapies for psoriasis and atopic dermatitis [1,2]. It is known that TNF-α plays a detrimental role in various inflammatory diseases [3]. To date, anti-TNF therapy has been used in clinic for autoimmune inflammatory diseases including psoriasis, which has been proved to be effective in some of these patients [4,5].

PGRN is a multi-functional growth factor, and plays a critical role in various physiological and disease processes such as wound healing [6], tumorigenesis [7], chondrogenesis [8] and inflammation [9–13]. PGRN is expressed in epidermis, and is also involved in skin repair and some skin diseases [6,14]. Recently, we reported that PGRN antagonized TNF-α and exhibited anti-inflammatory function in inflammatory arthritis models [3,11,12,15]. Herein we examined the expression pattern of PGRN in skin of human and mice under physiological and inflammation conditions, and determined the potential therapeutic function of PGRN, specially its derived recombinant Atsttrin, in inflammatory skin diseases using oxazolone-induced (OXA) contact dermatitis models.

2. Materials and methods

2.1. Mice

All animal studies were performed in accordance with institutional guidelines and approval by the Institutional Animal Care and Use Committee of New York University. The generation and genotyping of C57BL/6 background PGRN−/− mice have been described previously [10]. 2-Month old WT and PGRN−/− mice were used for these experiments.

2.2. Preparation of Atsttrin protein

The generation and purification of Atsttrin has been reported previously [11,12]. Briefly, the appropriate plasmid pGEX-Atsttrin was transformed into *Escherichia coli* DE3 for expression of GST fusion Atsttrin. Fusion protein was affinity-purified on glutathione-agarose beads as described before [16] with slight modification. The engineered proteins were digested with Factor Xa and the supernatant was collected. Following the removal of Factor Xa by

Abbreviations: PGRN, progranulin; WT, wild type; TNF-α, tumor necrosis factor α; IL-1β, interleukin-1β; IL-6, interleukin-6; NF-κB, nuclear factor-KappaB; pIκB-α, phosphorylated IκB-α; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; OXA, oxazolone; VEH, vehicle

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using Factor Xa Removal Resin (QIAGEN), the purity of resulted proteins was determined by SDS–PAGE.

2.3. Chemical-induced dermatitis mice models

OXA induced contact dermatitis model were established as previously described with minor modifications [17]. Briefly, mice were sensitized by a single application of 50 μl of 1.5% OXA (Sigma, MO, USA) in ethanol on the dorsal skin 7 days before challenge. Then two experiments were performed as follows.

2.3.1. Experiment 1

To testify the role of PGRN deficiency in dermatitis development, the the inner and outer surfaces of right ear from WT and PGRN−/− mice were challenged with 20 μl of 1% OXA in a mixture of acetone and olive oil (4:1), while left ear with mere acetone and olive oil (4:1) as vehicle control (VEH) every other day for 7 days and ear thickness of each group was measured every day with a micrometer (Mitutoyo, Kanagawa, Japan). Thereafter, ear draining lymph nodes and ear lobes were collected from both WT and PGRN−/− groups for further analysis. 10 mice were used for each group of all this set of experiments.

2.3.2. Experiment 2

To verify the therapeutic role of Atsttrin for contact dermatitis, the right ear of WT mice were challenged with OXA as mentioned in experiment 1 every other day for 14 days, during which time PBS or Atsttrin (2.5 mg/kg body weight) were delivered through intraperitoneal injection every 3 days and ear thickness was measured every other day. Afterwards, ear lobes were collected from both treatment groups for further analysis. 7 mice were used for each group of all this set of experiments.

2.4. Real-time RT-PCR

Total RNA was extracted from ear tissue of each indicated experimental group using an RNeasy kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using a RT-for-PCR kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Reactions were performed in a 20-μl SYBR Green PCR volume in a 96-well optical reaction plate formatted in the 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Sequence-specific primers in the present study are synthesized as follows: 5′-AATCTCACAGCAGCACATCA-3′ and 5′-AAGGTGCTCATGTCCTCATC-3′ for IL-1β; 5′-CCTTCCTACCCCAATTTCCAAT-3′ and 5′-GCCACTCCTTCTGTGACTCCAG-3′ for IL-6; 5′-AAT GCT GAC TAT GGC TAC AAA A-3′ and 5′-AAA ACT GAT GCG TGA AGT GCT G-3′ for COX-2; 5′-ACA GGAGGGGTTAAAGCTGC-3′ and 5′-TTGTCTCCAAGGGACCAGG-3′ for iNOS; 5′-TGGTGGAGCAGCAAGAGCAA-3′ and 5′-CAGTGGACAGTAGACGGAGGAAA-3′ for PGRN; 5′ TACAAGCTGGCTGGTGGGGA-3′ and 5′-GTCGCGGGTCTCAGGACCTT-3′ for NF-κB2; 5′-AGAACATCATCCCTGCATCC-3′ and 5′-AGTTGCTGTTGAAGTCGC-3′ for GAPDH. The presence of a single specific PCR product was verified by melting curve analysis and for each gene, the experiments were repeated three times.

2.5. Western blotting

Total ear extracts from each experimental and control group were homogenized and proteins were collected. Proteins were resolved on a 10% SDS–polyacrylamide gel and electroblotted onto a nitrocellulose membrane. After blocking in 5% non-fat dry milk in Tris buffer-saline–Tween 20 (10 mM Tris–HCl, pH 8.0; 150 mM NaCl; and 0.5% Tween 20), blots were incubated with polyclonal anti-PGRN, anti-phosphorylated IκB-α (pIκB-α) or anti-iNOS (diluted 1:1000) antibody for 1 h. After washing, the secondary antibody (horseradish peroxidaseconjugated anti-rabbit immunoglobulin; 1:2000 dilution) was added, and bound antibody was visualized using an enhanced chemiluminescence system (Amersham Life Science, Arlington Heights, IL, USA).

2.6. Histology

The mice ear specimens were fixed in 10% Formalin, dehydrated, cleared with dimethylbenzene, and then embedded in olefin. At least 4 consecutive 6-μm sections were obtained from the sagital planes, and stained using hematoxylin and eosin (HE) for routine morphologic analysis. The epidermal thickess was examined using OsteoMeasure software (OsteoMetrics, Inc., Decatur, GA).

2.7. Immunohistochemistry

Seven skin samples were harvested from the legs of randomly selected psoriasis patients with plaque-stage psoriasis vulgaris, who received treatment at Department of Dermatology, Qilu Hospital, Shandong University from January 2011 to December 2011. Seven samples of normal skin tissues were collected from the same anatomic location of healthy volunteers from the Department of Plastic Surgery of Qilu Hospital. The study was approved by the Ethical Committee of Qilu Hospital of Shandong University and a written inform consent was obtained from each participant in this experiment. Special care was taken to minimize stress on the tissue when performing the biopsy. All samples were fixed for 48 h in 10% formalin and prepared as reported before [14]. Besides, ear tissues from indicated experimental groups were collected, and prepared as described previously [18,19]. The skin samples were then incubated with anti-PGRN or anti-p-IκB-α polyclonal antibody (1:100 dilution; Santa Cruz Biotechnology) at 4 °C overnight, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 60 min at room temperature. The signal was detected using the Vector Elite ABC Kit (Vectastain; Vector).

2.8. Flow cytometry

Single-cell suspensions from the cervical draining lymph nodes were prepared through isolating LN cells by crushing the nodes and filtering through a 70-μm strainer (BD Biosciences). Antibody PerCP-Cy5-conjugated anti-CD4 was used for surface staining. For intracellular staining of Foxp3, cells were fixed and permeabilized with a commercial Foxp3 staining kit (eBiosciences) and the cells were stained with Alexa Fluor 647-conjugated anti-Foxp3 (BD Biosciences).

2.9. Statistical analysis

The data were expressed as mean ± standard deviation (S.D.). The Statistical Package for Social Sciences version 17.0 (SPSS Inc, Chicago, IL) was used for standard statistical analysis including one-way ANOVA and Student's *t*-test. Statistical significance was achieved when a value of $P < 0.05$.

3. Results

3.1. Progranulin (PGRN) expression was upregulated in inflammatory skin

To examine the expression pattern of PGRN in skin, immunohistochemistry was performed in skin samples from normal control and psoriasis patients. Fig. 1A showed that PGRN was detectable in normal skin and its level was elevated in epidermis of psoriasis

patients. Moreover, high-resolution analysis indicated that PGRN was particularly expressed in extracellular matrix of skin in psoriasis (Fig. 1A, panel d). To examine the expression profiling of PGRN in skin inflammation of mice, OXA-induced dermatitis model was established in WT mice as described in experiment 1, and immunohistochemistry was performed. Fig. 1B revealed that PGRN expression was dramatically enhanced in skin after induction of dermatitis. To further verify this alteration of PGRN expression, we performed real-time PCR and Western blotting for VEH and OXA induced WT ears ($n = 3$, respectively), and both RNA (Fig. 1C) and protein levels of PGRN (Fig. 1D) were dramatically elevated in skin of dermatitis.

3.2. Deficiency of PGRN led to exaggerated skin inflammation in mice contact dermatitis model

To investigate the role of PGRN in skin inflammation, OXA-induced dermatitis model was established in WT and PGRN−/− mice as described in experiment 1. Both genotypes exhibited remarkable increase of ear thickness following stimulation of OXA, but the ear thickness in PGRN−/− group was significantly larger than WT littermates in dermatitis (Fig. 2A). The ear samples were also assessed via histology and the result revealed that while there was no obvious difference between the two genotypes in VEH groups, PGRN−/− mice exhibited thicker epithelium and increased infiltration of inflammatory cells than WT mice in OXA-induced mice models (Fig. 2B). Real-time PCR assay of ears indicated there was no significant difference in the levels of inflammatory parameters, including IL-1β, IL-6, COX-2 and iNOS between WT and PGRN−/− mice in VEH control groups, while deficiency of PGRN resulted in significantly higher levels of all those molecules in OXA-induced dermatitis model (Fig. 2C–F). iNOS is reported to mediate inflammatory reaction in OXA-induced dermatitis models [20]. To further determine the effect of PGRN deficiency on the induction of iNOS in skin inflammation, Western blotting assay for iNOS was then performed. Fig. 2G indicated that iNOS was undetectable in ear homogenates of both genotypes without OXA challenge ($n = 1$, respectively), while PGRN−/− ear expressed dramatically higher level of iNOS than WT mice following OXA induction ($n = 3$, respectively). In addition, cervical lymph node cells from dermatitis models were harvested, and flow cytometry was performed to compare regulatory T cell (Treg) development in both genotypes. The result demonstrated that Treg/total T cell ratio was dramatically lower in PGRN−/− group (Fig. 2H), impling the defect of Treg development in skin inflammation.

3.3. Atsttrin attenuated skin inflammation in mice contact dermatitis model

PGRN and its derived engineered protein, Atsttrin, protected against inflammatory arthritis in mice models [12]. To assess whether Atsttrin also has therapeutic effect in inflammatory skin disease, OXA-induced dermatitis model was established in WT mice and treated with PBS or Atsttrin as described in experiment 2. Ear thickness was measured and Atsttrin treatment group displayed significantly less increase of ear thickness than PBS group (Fig. 3A). Histological assessment of ear samples showed that thickening of epithelium and infiltration of inflammatory cells were also inhibited in Atsttrin treatment group (Fig. 3B and C). Moreover, RNA was collected from ear tissue of both treatment

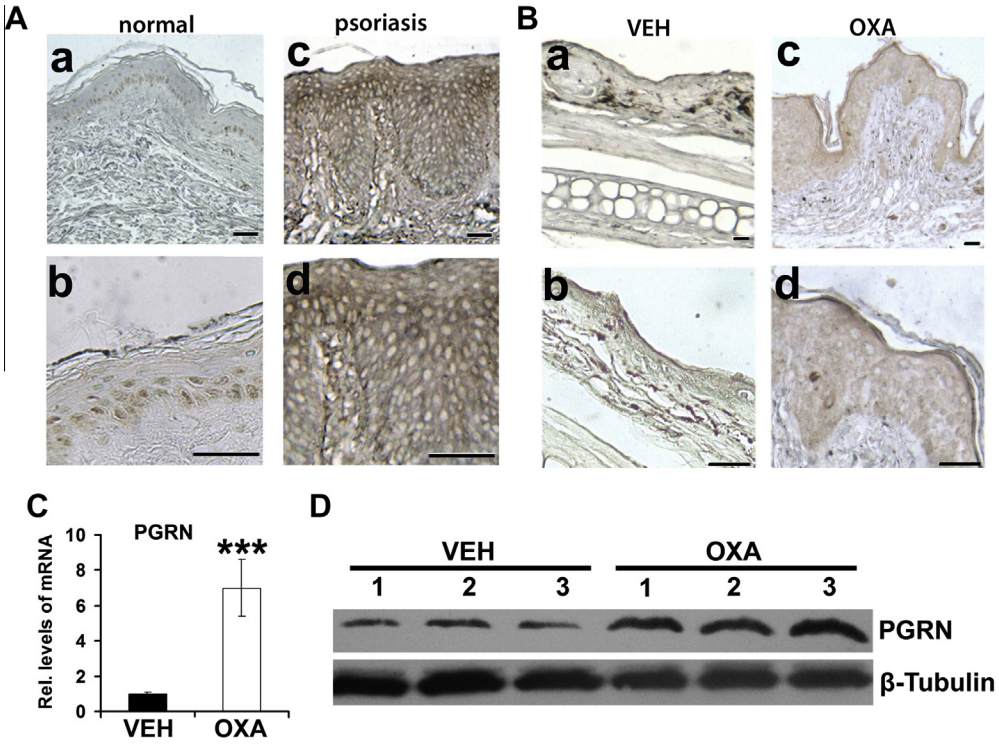

Fig. 1. The expression pattern of PGRN in skin of human and mice. (A) PGRN was expressed inside cells of normal human skin (panels a and b). Its expression level was elevated in skin of psoriasis patients (panel c) and high-resolution analysis showed that PGRN was particularly detectable in extracellular matrix (panel d). Skin samples from normal control ($n = 7$) and psoriasis patients ($n = 7$) were collected and were stained with anti-PGRN antibody (brown), then counterstained with methyl green (green), and representative pictures are shown. (B) PGRN expression in WT mice skin induced by vehicle (VEH) (panels a and b), assayed by immunohistochemistry. PGRN level was elevated in oxazolone-induced dermatitis model (OXA) (panel c) and high-resolution analysis showed that PGRN was particularly distributed in extracellular matrix (panel d). (C) Relative level of PGRN in ear tissue of WT mice stimulated with vehicle or oxazolone, assayed by real-time PCR. $^{***}P < 0.005$. (D) Expression level of PGRN in ear tissue of WT mice with challenge of vehicle or oxazolone ($n = 3$, respectively), assayed by Western blotting. Bars = 40 μm.

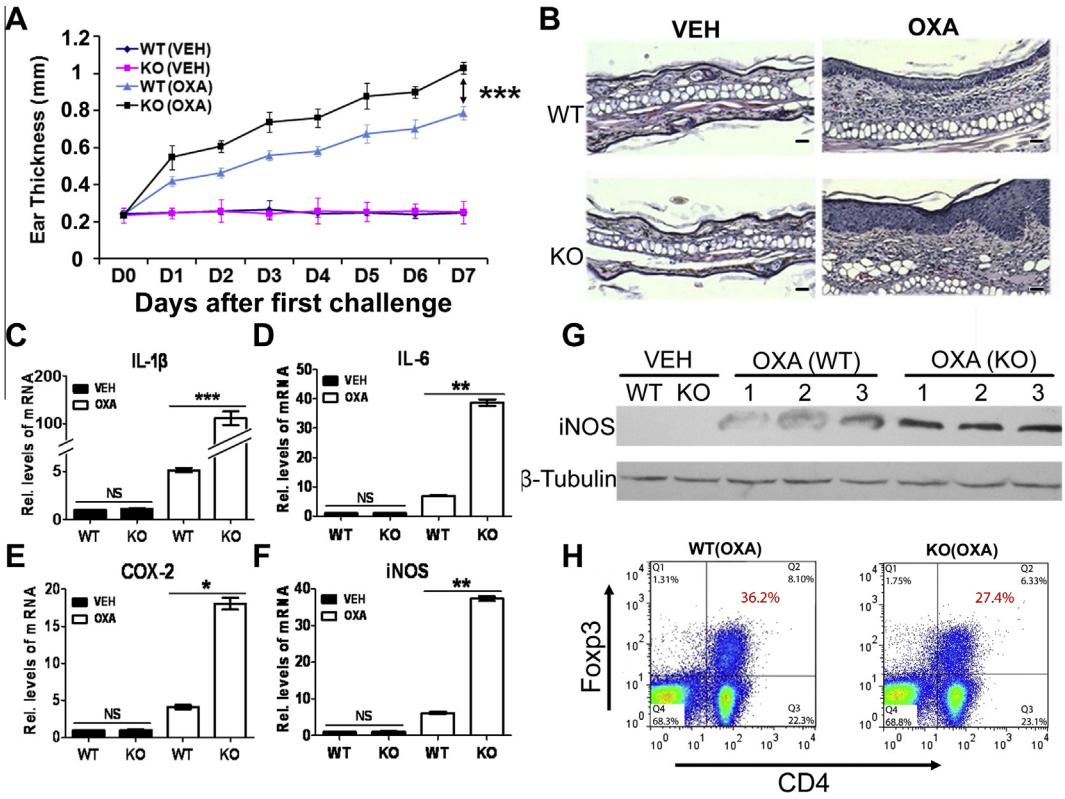

Fig. 2. Loss of PGRN resulted in more severe inflammation in oxazolone-induced dermatitis. (A) Ear thickness was dramatically increased in WT and PGRN−/− mice after induction of oxazolone compared with vehicle (VEH) groups, while deficiency of PGRN led to significantly increased ear thickness ($n = 10$ for each group). $^{***}P < 0.005$ vs. WT dermatitis group. (B) PGRN−/− mice exhibited more severe thickening of epidermis and infiltration of inflammatory cells than WT mice, assayed by HE staining. Scale bar, 50 μm. (C–F) RNA levels of inflammatory parameters including IL-1β, IL-6, COX-2 and iNOS were significantly higher in ear tissue of PGRN−/− mice than WT mice following oxazolone induction, as measured by real-time PCR. The values are the mean ± S.D. of 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$ vs. WT group. (G) Expression of iNOS in ear extracts of WT and PGRN−/− mice with challenge of oxazolone ($n = 3$, respectively) or vehicle ($n = 1$, respectively), assayed by Western blotting. (H) Treg development in cervical lymph node of WT and PGRN−/− mice in dermatitis model, detected by flow cytometry. Numbers in red in the upper right quadrant represent the Foxp3+ population as a percentage of CD4+ cells. Results are representative of 3 independent experiments.

groups, followed by real-time PCR. The results demonstrated that levels of inflammatory parameters IL-1β, IL-6, COX-2 and iNOS, were significantly lower in Atsttrin treatment group (Fig. 3D–G). Total protein was also collected from ears of both PBS and Atsttrin treatment group ($n = 3$, respectively), and Western blotting was performed for iNOS level. As shown in Fig. 3H, Atsttrin treatment markedly reduced iNOS protein level in dermatitis.

3.4. Deficiency of PGRN elevated, whereas recombinant Atsttrin inhibited, the expression and activity of NF-κB signaling

It is known that PGRN and Atsttrin inhibited activation of NF-κB signaling pathway in inflammatory arthritis models [12], together with the reports that NF-κB signaling played a critical role in skin inflammatory diseases [21], promoted us to determine whether PGRN deficiency or treatment of Atsttrin affected NF-κB signaling in OXA-induced dermatitis model. To investigate the potential effect of PGRN deficiency on NF-κB signaling in dermatitis, RNA extracts were collected from ears of both genotypes with or without challenge of OXA in experiment 1, and the NF-κB2 level was measured using real-time PCR. As revealed in Fig. 4A, while there was no significant difference between two genotypes in control uninduced ears, NF-κB2 level was significantly higher in ear tissue of PGRN−/− mice than WT group in OXA induced dermatitis. Furthermore, total proteins were collected from ears of experiment 1, and a Western blotting was performed for examining the phosphorylated IκB (pIκB-α), which indicates the activity of NF-κB signaling pathway. Fig. 4B showed that pIκB-α was faint in VEH challenged ear of both genotypes, while PGRN−/− group exhibited dramatically increased pIκB-α than WT littermates in dermatitis. To examine the regulation of NF-κB signaling by Atsttrin, RNA extracts were harvested from ear tissues of experiment 2, followed by real-time PCR. Result indicated that Atsttrin significantly reduced NF-κB2 level compared with PBS treatment group in OXA-induced dermatitis model (Fig. 4C). Moreover, immunohistochemistry for pIκB-α was performed on ear sections of both treatment groups in experiment 2. PBS group demonstrated strong signal of pIκB-α in cytoplasm of cells in epidermis of dermatitis model, while this was dramatically attenuated in Atsttrin group (Fig. 4D).

4. Discussion

PGRN and Atsttrin were reported to be therapeutic in inflammatory arthritis in mice [12]. In addition, loss of PGRN increased the susceptibility to collagen-induced inflammatory arthritis [12]. In this study we used OXA-induced contact dermatitis model and found that deficiency of PGRN also led to exaggerated skin inflammation. Interestingly, Atsttrin, an engineered protein that is comprised of the three fragments of PGRN responsible for binding to TNF receptors [12], attenuated the severity of skin inflammation in contact dermatitis.

PGRN is reported to be secreted by epithelial cells and inflammatory cells of skin, and its level in skin is elevated in some pathological condition and skin trauma [14,22,23]. Here we found that

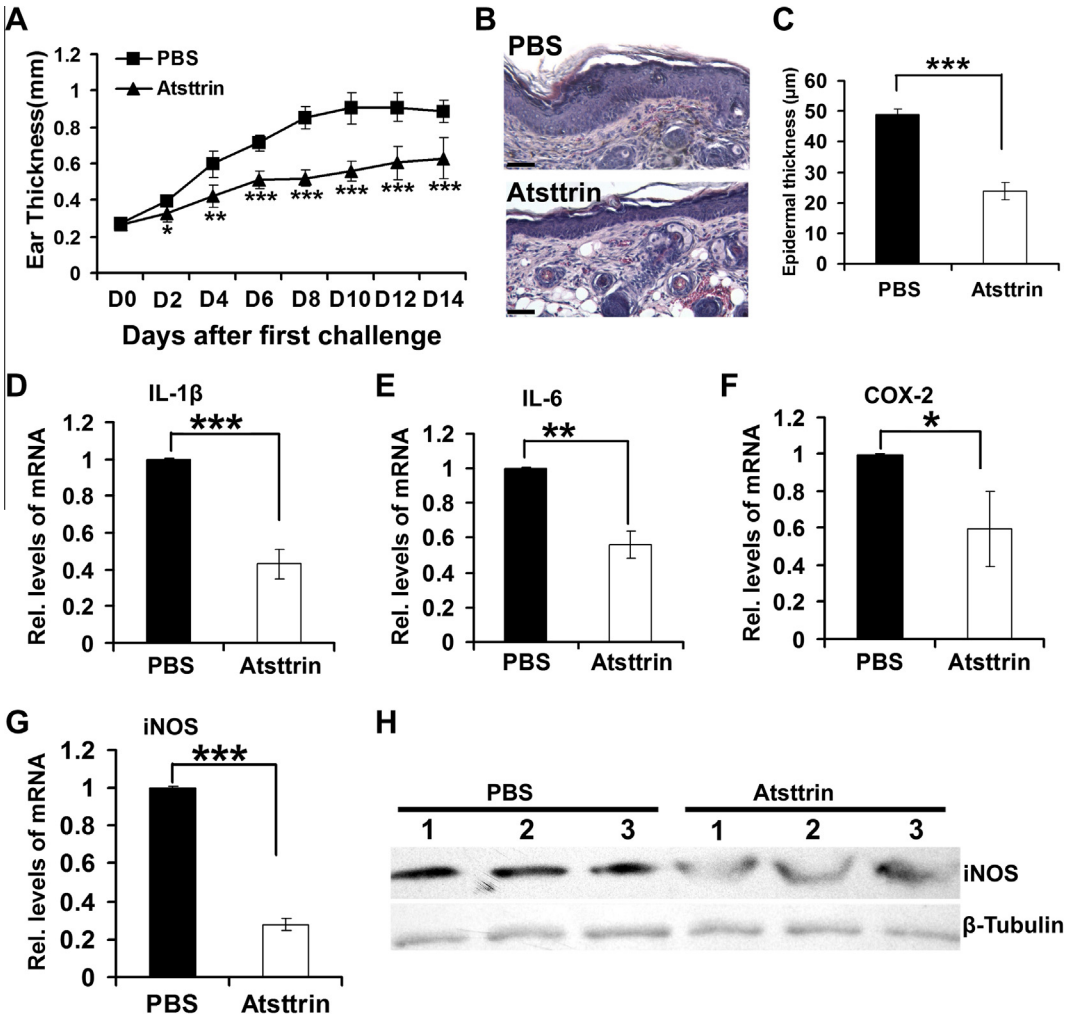

Fig. 3. PGRN-derived engineered protein Atsttrin attenuated skin inflammation in oxazolone induced dermatitis model. (A) Atsttrin treatment inhibited increase of ear thickness in oxazolone-induced dermatitis. Ear thickness was measured every other day for 14 days and compared between PBS and Atsttrin treatment groups ($n = 7$, respectively). (B and C) Atsttrin treatment attenuated thickening of epithelium and infiltration of inflammatory cells in dermatitis model, detected by HE staining. Scale bar, 50 μm. (D–G) Relative levels of IL-1β, IL-6, COX-2 and iNOS in PBS and Atsttrin treatment groups in dermatitis model, assayed by real-time RT-PCR. (H) Expression of iNOS in ear extracts of PBS and Atsttrin treated mice in dermatitis model, assayed by Western blotting. Each experiment was repeated in triplicate, and the values are the mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$ vs. PBS treatment group.

PGRN level was elevated in the skin of both psoriasis patients and mice dermatitis model, particularly in the extracellular matrix, compared with normal controls. This is also consistent with the previous report that PGRN level was dramatically elevated in the patients with osteoarthritis or rheumatoid arthritis, and played a protective role in the pathogenesis of arthritis [18]. Recently it was reported that PGRN level was also significantly elevated in the patients with systemic lupus erythematosus [24]. It is speculated that the elevation of PGRN expression in skin was secondary to the increased inflammatory cytokines in particular TNF-α which accelerated skin inflammation process, but the elevated level of PGRN was still not sufficient to neutralize the activities of such inflammatory cytokines.

It is well-established that TNF-α acts as one of the dominant factors in various skin inflammatory diseases [4,25]. Anti-TNF agents have been used for treatment of psoriasis [26]. NF-κB signaling is a key pathway for TNF-α mediated inflammatory function and plays a critical role in progression of skin inflammation [27–29]. IL-1β, IL-6, COX-2 and iNOS are important downstream molecules of NF-κB signaling pathway [30] and are assayed as inflammatory markers in dermatitis [31,32].We reported that PGRN inhibited TNF-α mediated activation of NF-κB signaling in inflammatory arthritis in mice [12]. In this study, we found deficiency of PGRN enhanced, while treatment of Atsttrin effectively inhibited the expression and activity of NF-κB signaling pathway, and affected the levels of its downstream molecules in dermatitis models. This finding suggests the protective role of PGRN and Atsttrin maybe through repressing activation of NF-κB signaling pathway. Treg is known to fight against dermatitis in various models [33]. We found that deficiency of PGRN led to fewer Foxp3-positive Treg compared with WT littermates following OXA induction. In addition, PGRN was reported to selectively induce the differentiation of Treg from naïve T cells in vitro [12]. These findings suggest that the exaggerated inflammation seen in dermatitis of PGRN−/− mice might be also due to the defect in Tregs differentiation in PGRN-deficient dermatitis model.

Collectively, PGRN plays a protective role in the development of dermatitis, and its derived engineered protein, Atsttrin, may represent a promising therapeutic agent in treatment of inflammatory skin diseases, including contact dermatitis and psoriasis.

Conflict of interest

C.J. Liu is the co-founder and consultant in Atreaon, Inc.

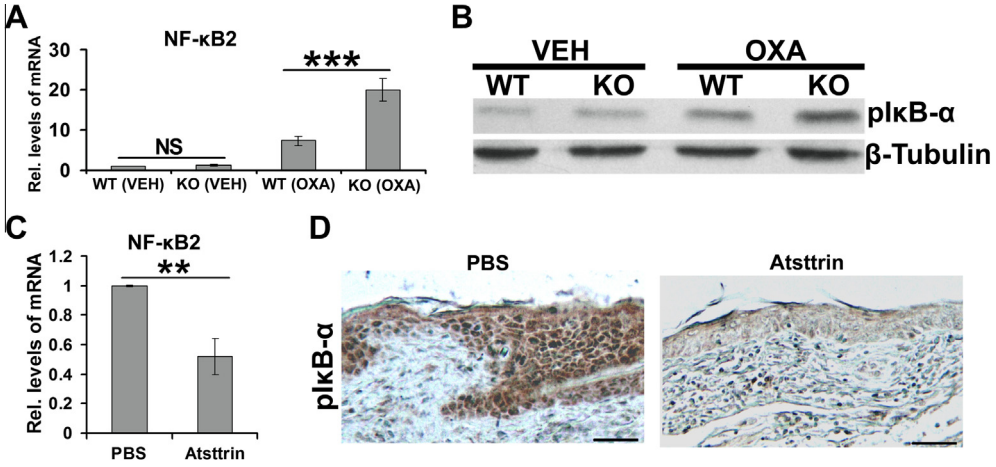

Fig. 4. PGRN and Atsttrin inhibited augmentation of NF-κB signaling in skin following oxazolone challenge. (A) Deficiency of PGRN significantly enhanced NF-κB2 level in oxazolone-induced dermatitis model, assayed by real-time PCR. RNA extract was harvested from ear tissue of mice in experiment 1 and real-time PCR was performed. (B) Expression of phosphorylated IκB-α (pIκB-α) was dramatically higher in ear extracts of PGRN−/− mice than WT mice in dermatitis, assayed by Western blotting. (C) Atsttrin treatment significantly reduced level of NF-κB2 in dermatitis of WT mice, assayed by real-time RT-PCR. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$ vs. PBS treatment control group. (D) Atsttrin treatment inhibited expression of pIκB-α in contact dermatitis of WT mice, assayed by immunohistochemistry. Scale bar, 50 μm. Each experiment was repeated in triplicate, and the values are the mean ± S.D.

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