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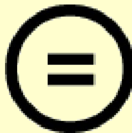

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의학박사 학위논문

알레르기성 기도 염증에서 대식세포 유래 progranulin 의 역할

Macrophage-derived progranulin produced in the early sensitization period plays an important role in the development of allergen induced T_H2 dominant airway inflammation

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이 논문을 의학박사 학위 논문으로 제출함

2018 년 2 월

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Abstract

Heterogeneity of asthma in its pathophysiology and clinical phenotypes has led to the development of new treatment strategies in which a biologic agent targets a key pathway of a certain inflammatory cascade. Progranulin, as a new biomarker in asthma was suggested based on a series of studies that demonstrated its anti-inflammatory role in animal models of acute lung injury and emphysema, also on a clinical study of asthma that showed its association with airway obstruction and blood neutrophilia. The specific function of progranulin and its mechanism of action in asthma is unknown, therefore, we aimed to investigate its immunologic role in asthma. To evaluate the effect of allergen exposure to progranulin in the airway, house dust mite (HDM) extract was administered intranasally in a mouse model and we found increased progranulin levels along with type 1 and 2 cytokines in bronchoalveolar lavage (BAL) fluid. The source of progranulin was alveolar macrophages. The effect of macrophage-derived progranulin on airway was evaluated with intranasal treatment of recombinant progranulin in mice and the results showed increased alveolar macrophages, neutrophils and type 2 cytokines, and the cytokines were produced from natural killer T (NKT) cells and lung epithelial cells. In the model of macrophage-derived progranulin deficient mice, HDM exposure did not induce innate and adaptive immune responses, and these responses were restored by progranulin supplement in the sensitization period. Also, the deficiency in macrophage-derived progranulin resulted in markedly decreased inflammation in histologic analysis of lung tissue after HDM exposure. Thus, the presence of macrophage-derived

progranulin in the airway was shown to be important for the generation of T helper cell type 2 (T_H2) immune response to HDM allergen exposure.

Keywords: progranulin, macrophage, asthma

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1. Introduction

1.1. Asthma, a heterogeneous disease of the airway

Asthma is a common chronic disease of the airway, and traditionally, it is characterized by inflammatory, functional and structural changes leading to airway hyper-responsiveness and airflow limitation which is usually reversible.[1] Asthma affects more than 300 million individuals of all age, and its prevalence is continuously increasing worldwide.[2] The clinical features of asthma include, shortness of breath, wheezing and cough and it is diagnosed clinically based on history of variable respiratory symptoms together with variable expiratory airflow limitation from pulmonary function tests.[3]

Until early 2000s, the importance of nonspecific anti-inflammatory and bronchodilator medications – combination of inhaled corticosteroids (ICS) and β_2-adrenergic receptor agonists – was emphasized and the majority of patients using such medications as standard therapies were well controlled. However, in approximately 5-10% of the asthma population, the disease remained symptomatic and inadequately controlled despite the stepwise treatment according to the Global Initiative for Asthma (GINA) guidelines.[4] The proportion of uncontrolled asthma is small, but they carry a high risk of serious morbidity and mortality, and use the largest share of economic resources and health-care services,[5] therefore, additional therapeutic breakthrough was required for those who were poorly controlled.

Meanwhile, the body of evidence grew suggesting that responses of people with asthma to nonspecific anti-inflammatory drugs were dependent on the presence and type of airway inflammation.[6,7] It was then demonstrated that a specific antibody to the allergy-related factor immunoglobulin E (IgE) showed efficacy in reducing exacerbations in the targeted 'allergic' asthma population.[8] Thus, the term 'asthma' evolved from one describing a single disease entity to a heterogeneous disease group encompassing multiple phenotypes or endotypes, each defined by distinct clinical, functional and pathological patterns.[1]

Many researchers now believe that heterogeneity of asthma roots from the different types of airway inflammation each with its unique underlying pathobiology linked to its associated clinical phenotype.[1] However, inflammation has not been measured frequently in practice to diagnose asthma, and even when it was measured, it was seldom used for personalizing treatment for the heterogeneous groups of patients. The most commonly used phenotypic classification has been; 1) allergic to non-allergic forms based on history of rhinitis or eczema, onset of disease and skin prick tests or laboratory measurement of total IgE and specific IgEs to common aeroallergens[9] and 2) eosinophilic to non-eosinophilic forms based on cellular analysis of sputum or blood samples.[10] The data show that about 50-80% of patients with severe asthma have allergic asthma[11], and based on the current knowledge that patients with persistent eosinophilic airway inflammation tend to have more severe disease and frequent

exacerbations[12], the research on pathobiology of inflammation in these patients have been briskly carried out as the two phenotypes overlap immunologically into a broader category of T helper cell type 2 (T_H2)-associated asthma.

Although little is understood about asthma patients with neutrophil dominant inflammation, this subset has been identified in both stable and severe asthma.[13,14] The nomenclature for this subset varies by different study groups, which include non-T_H2 asthma, neutrophilic asthma, T_H1 and/or T_H17 dominant asthma. In fact, IL-17A and IL-17F – proinflammatory cytokines that are released by T_H17 cells and crucially involved in neutrophilic inflammation – were found upregulated in bronchial biopsy samples obtained from severe asthma patients.[15] *Moore et al* showed that sputum neutrophilia was greatest in individuals who had generally adult-onset and severely obstructed (and incompletely reversible) asthma and highest-intensity healthcare usage and systemic corticosteroid use.[16] However, there does not exist a representative biomarker that can assemble this subgroup of asthma and no consensus exists as to what level of neutrophilia should define the subtype.

1.2. Pathobiology of asthma

To overcome the unmet need in the treatment of uncontrolled asthma with heterogeneous inflammation, there have been remarkable advances in the discovery of molecular pathogenesis of asthma.

Asthma originates from complex interactions between genetic factors and environmental agents such as aeroallergens, respiratory viruses and airborne pollutants.[17] In particular, allergens can be taken up by dendritic cells in the airway lumen, which process antigenic molecules and present them to naïve T helper (T_H0) cells. The cytokine milieu determines the type of antigen presentation-dependent differentiation of T lymphocytes. For example, IL-12 produced by dendritic cells promote T_H1 polarization, whereas commitment towards the T_H2 lineage is driven by IL-4, which is probably released from mast cells, T cells, eosinophils and basophils.[18] Moreover, the innate cytokine thymic stromal lymphopoietin (TSLP) and IL-33 are secreted in large amounts by bronchial epithelial cells and mast cells of patients with asthma[19], thus eliciting the development of T_H2-adaptive responses[20] and inducing dendritic cells to release the chemokines CC motif chemokine 17 (CCL17) and CCL2, which recruit T_H2 cells upon binding to their receptor: CC chemokine receptor 4 (CCR4).[21] As a consequence, T_H2 cells that express CCR4 synthesize cytokines, IL-3, IL-4, IL-5, IL-6, IL-9, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF)[22]. These cytokines in turn, stimulate the maturation and recruitment of eosinophils and mast cells. IL-5 promotes eosinophil maturation and survival, which is synergized by eotaxin and CCL5[23,24]. The type 2 innate lymphoid cells (ILC2) are also involved when the T_H2 polarizing cytokines (TSLP and IL-33) are released by epithelial cells by viruses or allergens, which is another important source of type 2

cytokines that orchestrate allergic immune response[25]. IL-4 and IL-13 promote B cell-operated synthesis of IgE antibodies[26,27]. In addition to T_H2 cells, IL-9 releasing T_H9 cells become activated, leading to the growth and recruitment of mast cells, which release both preformed and newly synthesized mediators[28].

Other subsets of T lymphocytes contribute to asthma pathobiology, which include T_H17 cells. They produce IL-17A and IL-17F, which stimulate bronchial epithelial cells and subepithelial fibroblasts to secrete neutrophil chemoattractants such as chemokine CXC motif ligand 1 (CXCL1) and IL-8[21]. T_H17 cells were also suggested to contribute to the pathogenesis of allergic asthma, thus worsening its severity[29]. Tumor necrosis factor-α (TNF-α) was also shown to exert pleiotropic effects on inflammatory and structural cells of the airway in asthma[30,31]. Furthermore, exacerbations can be caused by respiratory viruses, and in some cases it can be facilitated by deficient epithelial synthesis of antiviral cytokines such as interferon-β (IFN-β) and IFN-γ released by T_H1 cells[32].

The development of both T_H2 cell and T_H17 cell of the T cell-mediated adaptive immunity is facilitated by an impairment of specific immunomodulatory cells, T regulatory (Treg) cells that produce IL-10 and transforming growth factor-β (TGF-β). Defective functioning of Treg cells can occur in all forms of asthma[33,34], and probably in severe subtypes[35].

Finally, many mediators, cytokines and growth factors produced by several

different cells involved in chronic asthmatic inflammation may also affect the functions and proliferation rates of airway structural cells, including epithelial cells, fibroblasts, smooth muscle cells and endothelial vascular cells[36].

Figure 1, adopted from the review article by *Pelaia et al*, well delineates the complex interactions between host and external factors and their subsequent molecular pathogenic cascade described in previous paragraphs[36]. All of the cytokines described can be potential targets of biological treatments, especially needed for patients who do not respond fully to high doses of ICSs. A better understanding of the pathogenic role of such cytokines can encourage the development and application of anti-cytokine treatment. Such therapies would help to achieve personalized treatments tailored for patients who express high levels of specific cytokines implicated in the pathobiology of different subtypes of severe asthma.

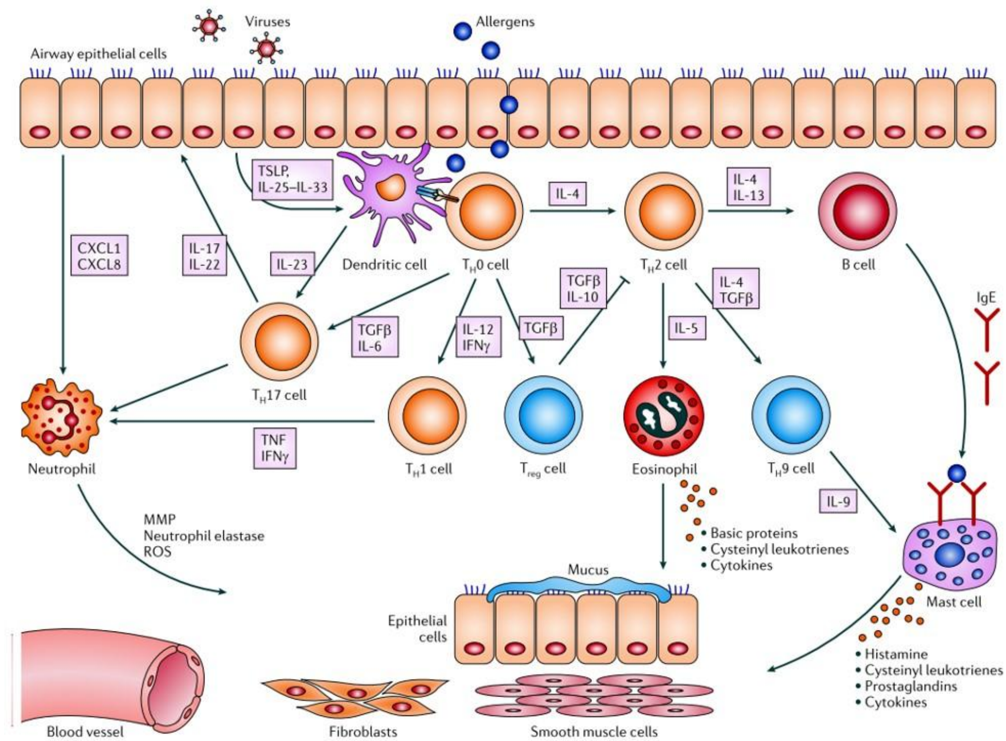


Figure 1. Pathobiology of asthma

Immune processes in the asthmatic airway. The pathway begins with the development of T_H2 cells and their production of the cytokines IL-4, IL-5 and IL-13. These cytokines stimulate allergic and eosinophilic inflammation as well as epithelial and smooth muscle changes that contribute to asthma pathobiology. Other contributing T lymphocyte are T_H17 cells, which produce IL-17A and IL-17F, which induce neutrophil recruitment and expansion.

1.3. New biologic agents for asthma

New medications have been developed based on molecular pathogenesis of asthma, in which a biologic agent that blocks a certain molecule in a targeted pathway gets selected and tested from animal to human studies. New biologic agents currently in market include, omalizumab targeting IgE and mepolizumab and reslizumab targeting IL-5 of the T_H2 immune pathway.[37-39] Also, benralizumab, dupilumab, and lebrikizumab, monoclonal antibodies against IL-5 receptor, IL-4 receptor and IL-13, respectively, have undergone phase 3 clinical trials and are expected to be in market in the near future.[40-42] Other candidate molecules associated with T_H2 dominant inflammation of asthma, such as blockers of IL-33 and TSLP are currently under development or early phase clinical trials.[43,44] Such advances are highly meaningful in that we know that there is a breakthrough for severe refractory asthma, and that we have now opened a new era of precision medicine for the treatment of asthma. However, they do not yet complete the puzzle of heterogeneity of asthma, not only because the effectiveness of these new medications need to be further assessed with long term clinical experience, and more importantly, biomarker studies in patients with inflammatory profiles other than T_H2-dominant subtype have not been satisfactory so far. It is partly due to the absence of a useful biomarker for these patterns of inflammation, which makes pre-identification of subjects who might respond to a certain biologics difficult. Brodalumab, a monoclonal antibody targeting IL-17

receptor had been tested in a phase 2 clinical trial but the results showed no treatment effect in subjects with asthma.[45]

Indeed, a wide range of cells and inflammatory molecules interact in a complex manner to lead to development of asthma in various severities and profiles of inflammation, which determine different clinical phenotypes of the disease[36]. In order to overcome the unmet need in the treatment of uncontrolled asthmatics, further studies are mandatory to identify the undiscovered pathogenic inflammatory pathway, especially in T_H1 and/or T_H17 high subtype of asthma.

1.4. Progranulin

Progranulin, also referred to as granulin-epithelin precursor[46], proepithelin[47,48], acrogranin[49], and PC cell-derived growth factor[50], is a 88-kDa glycoprotein composed of seven and a half cysteine-rich tandem repeats,[51] and it was originally isolated as an autocrine growth factor from the culture medium of the highly tumorigenic mouse teratoma-derived cell line PC[50,52]. Progranulin is digested into 6-kDa granulin peptides by many proteinases, including matrix metalloproteinase (MMP) 9, 12, and 14, elastase, and proteinase 3.[53-57]

1.4.1. The functions of progranulin

Progranulin is a multifunctional protein expressed in a wide range of tissues and

cell types.[58] Progranulin is highly expressed in epithelial cells, macrophages, fibroblasts and immune cells, including T cells and dendritic cells.[59-61] Progranulin has multiple physiological functions and is involved in many types of disease processes, including autoimmune disorders, cancer, and neurodegenerative diseases. Progranulin, as a growth factor, promotes cell proliferation and is crucial to the development and generation of fast-growing epithelial, endothelial, and cancer cells.[62] Progranulin levels are elevated in many types of cancer, including breast cancer, ovarian cancer, and cholangiocarcinoma.[63-65] Macrophage-derived progranulin is a key regulatory factor in the processes of inflammation and would healing[66,67] and its expression has been known to increase in response to tissue injury and coordinates physiological events for epithelial tissue maintenance and repair.[68] The importance of progranulin has also been emerging with respect to the immune system. Recently, progranulin has been reported to promote CD4+ T cells to differentiate into Treg cells.[69] Progranulin has also been found to be highly expressed in a subpopulation of neutrophils that can promote antibody diversity in B cells.[70]

Interestingly, there are many distinctive and contrasting functions executed by progranulin and granulin. For instance, progranulin, as opposed to granulin, blocks the TNF-α induced respiratory burst in neutrophils[54], whereas granulin B, but not progranulin, stimulates IL-8 expression in epithelial cells.[54] It is not clear, however, by which mechanism the single 6-kDa granulins mediate its biological function, as most

progranulin-binding membrane receptors need more than one granulin domain.[71]

1.4.2. Association between progranulin and respiratory diseases, including asthma

Studies in murine models of acute lung injury have identified progranulin to be a potent anti-inflammatory molecule, whose anti-inflammatory action was mediated by inhibition of neutrophil degranulation.[72,73] Another recent study on human sputum samples of chronic obstructive lung disease (COPD) has identified a higher progranulin level as an indicator of less severe neutrophilic inflammation.[74] *Lee et al* reported that progranulin expression was induced in the cigarette smoke extract exposed mouse lungs and that cellular apoptosis was augmented in the absence of progranulin.[75]

Based on the results of previous studies, we had assumed that progranulin would also have a role in the pathogenesis of asthma, presumably in neutrophilic asthma in which inflammation is dominant through T_H1 and/or T_H17 pathway. We had formerly carried out a study with serum samples and clinical data of asthma patients and showed that serum progranulin levels were lower in asthma compared with healthy controls. Its levels were lower in patients with worse pulmonary function and showed a negative correlation with blood neutrophil counts, therefore, we had suggested that serum progranulin may be an indicator of severe asthma with airflow limitation, particularly in neutrophilic asthma.[76]

1.5. Possible role of progranulin in allergic airway inflammation

The association was not strong between progranulin and neutrophil in asthma, and the exact position of progranulin in the pathomechanism of asthma is not clear. We could not rule out the possibility of progranulin effect on the balance of T_H1/T_H2 immune response or on the differentiation of naïve T cells to Treg cells. Furthermore, the role of progranulin limited to allergic asthma had not been studied so far.

Hence, we further aimed to evaluate the specific function of progranulin in the asthmatic airway and its mechanism of action, in the setting of house dust mite (HDM) induced inflammation. The biology of progranulin in asthma has not been studied to date, and our study is the first to explore.

2. Materials and methods

2.1. Mice

Wild type (C57BL6) and Lyzm (expressing Cre-recombinase in myeloid cell lineage, B6 background) mice were purchased from Jackson laboratory (Bar harbor, ME, USA). GRN mice (lox-P inserted in GRN gene, B6 background) were kindly donated from Professor Min Seon Kim in Asan Medical Center. To generate the macrophage-derived progranulin deficient mice, we mated Lyzm with GRN mice. The deletion of progranulin was confirmed by PCR genotyping of each gene specific primer in tail tissue of their offspring. Mating and maintenance of mice were performed in the facility at specific pathogen-free room, and all animal experiments were approved by the Institutional Animal Care and Use Committee of Asan Medical Center.

2.2. Materials for stimulation of cell line or mice

To induce the immune responses in mice or the production of inflammatory cytokines from cell lines, we purchased and stimulated with HDM extract (*Dermatophagoides pteronyssinus* (Der p) from Korean National Arthropods of Medical Importance Resource Bank, Seoul, Korea) and recombinant mouse progranulin (R&D systems, Minneapolis, MN, USA). In order to establish the conventional alum model, we purchased ovalbumin (grade V) from Sigma-Aldrich (St. Louis, MO, USA) and aluminum hydroxide (Imject Alum, Thermo Scientific, Rockford, IL, USA).

2.3. Culture and stimulation of mouse alveolar macrophage, epithelial cell and NKT cell

Alveolar macrophage cell line (MH-s, ATCC), epithelial cell line (MLE12, ATCC) and NKT cell line (DN32.D3) were cultured in RPMI, DMEM/F12 (1:1) or DMEM (Welgene, Gyeongsan-si, Korea), respectively, with 10 % fetal bovine serum (Biowest, MO, USA) and 1x antibiotics (Welgene). All cells were seeded as 1.0 x 10^6/ml concentration with 500 μl media in 24-well cell culture plate (Corning, NY, USA), and we stimulated each cell with HDM extract, progranulin or aluminum hydroxide, respectively, after overnight stabilization. We harvested culture supernatant at each time-point and evaluated cytokine production with sandwich ELISA system.

2.4. Establishment of airway inflammation in animal model

The overall picture of establishment of airway inflammation in the animal models is delineated in figure 2. To evaluate the cytokine expression in the sensitization period of HDM extract exposure, we administered a single dose of 30 μg HDM allergen to wild type mice intranasally, and evaluated immunological parameters at baseline, 1, 2, 4, 8, 16, 32, and 48 h.

The role of progranulin produced by HDM extract exposure in the sensitization period was studied in wild type mice with administrations of 1, 10 and 100 ng recombinant mouse progranulin, and the evaluations were done 16 h after administration.

To evaluate the effect of macrophage-derived progranulin in allergen induced airway

inflammation, we intranasally gave 30 μg HDM extract to wild type and macrophage-derived progranulin deficient mice. The evaluation was performed at 16 h after administration of HDM extract.

To induce the adaptive immune responses to HDM allergen, we administered 30 μg HDM extract to wild type and macrophage-derived progranulin deficient mice twice a week for 4 weeks. The mice were sacrificed 24 h after final allergen challenge, and then immunological evaluations were done. In order to evaluate the responses after replenishment of progranulin in the progranulin deficient mice, depending on the timing of replenishment, recombinant mouse progranulin was administered either on day 0 and 1 (early, sensitization period) or on day 21 and 21 (late, challenge period).

The role of progranulin on the development of adaptive immune response to HDM allergen was again evaluated in the conventional alum model. We injected chicken egg ovalbumin (OVA) with aluminum hydroxide (alum) into the peritoneal cavity at day 0 and 14. After sensitization and boosting, we administered OVA alone at day 21 to 23. Mice were sacrificed 24 h after the last protein challenge, and evaluations were done. To check the cytokine production in the sensitization period, a single injection of OVA/alum mixture was given to wild type mice, and sacrifice and evaluation were performed at serial time-points.

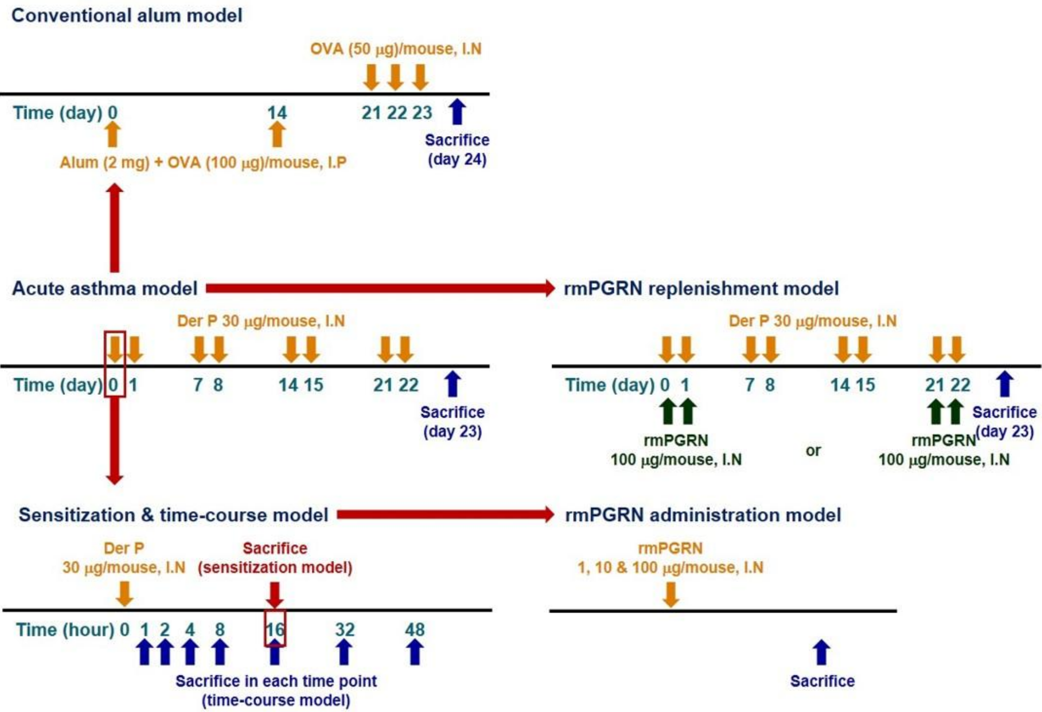


Figure 2. Protocol for the establishment of airway inflammation in animal models

2.5. Processing of BAL fluid, serum isolation and peritoneal fluid

To obtain the BAL fluids after sacrifice, we washed mouse airway with 1 ml of cold, sterile phosphate-buffered saline (PBS) as our previous study.[77] Next, we separated immune cells from BAL fluids by centrifugation at 4 ˚C, 5000 rpm for 10 minutes, and counted total cell with trypan blue after RBC lysis (StemCell Technologies, VC, Canada). For differential cell count, we counted 300 inflammatory cells after Diff-Quick staining. BAL fluid was stored at -20 ˚C for evaluation of cytokine production after days.

To collect the whole blood, we performed heart-puncture with 1 ml syringe, settle the cells in blood with centrifugation as in the processing of BAL fluid. Then we collected and saved the supernatant portion for evaluation of total antibody level in serum.

To collect the periotoneal fluids, we inserted 3 ml of fresh, cold PBS into peritoneal cavity with 5 ml syringe. After insertion, we shaked mouse for 10 seconds, and extracted 2 ml of periotoneal fluid – PBS mixture with syringe. Then, we processed peritoneal fluids as mentioned for processing of BAL fluids.

2.6. Single cell isolation from lung tissues and regional lymph nodes

To isolate the immune cells from lung tissues, we chopped lung tissues with razor-blade. Next, the tissues were incubated at 37˚C with 0.05% trypsin (GIBCO, Grand Island, NY, USA) and 200 unit/ml (GIBCO) of collagenase for 10 minutes for digestion. After digestion, tissues were grinded with plunger of syringe and cell strainer (BD Falcon, Bedford, MA,

USA), and incubated in 4˚C with RBC lysis buffer (StemCell Technologies). For isolation of immune cells from regional lymph nodes (LNs), we grinded tissues and incubated with RBC lysis buffer as in the single cell preparation from lung tissues described above.

2.7. Evaluation of immune responses of the single cells obtained from lungs and regional lymph nodes

Isolated cells were seeded as 1.0 x 10^6/ml concentration with 500 μl of RPMI (Welgene) in 24-well plates and cultured under 37°C condition with or without CD3 and CD28 antibodies (1 μg/ml each, eBioscience, San Diego, CA, USA). We collected the supernatant 12 h after T cell re-stimulation, and evaluated cytokine production with ELISA system.

2.8. Measurement of cytokines in BAL fluid/culture supernatant and immunoglobulin in serum

To evaluate the level of each cytokine in BAL fluid or culture supernatant, we purchased mouse ELISA Duoset (R&D systems, Minneapolis, MN, USA) and estimated the production of IL-4, IL-6, IL-12p70, IL-13, IL-17, IL-33, eotaxin, IFN-γ and TSLP in each sample following the guideline of manufacturer. In the case of immunoglobulin evaluation in serum, we isolated serum from mouse whole blood sample with centrifugation at 4°C, 5000 rpm for 10 min, and measured the level of total antibodies of IgE, IgG1 and IgG2c using mouse ELISA Quantitation set (Bethyl laboratories, Montgomery, TX, USA).

2.9. Evaluation of lung histology

Blood depleted lung tissues with cold, sterile-PBS were sectioned and stained with hematoxylin and eosin solution (H&E stain) after fixation in 4% para-formaldehyde solution. All sides were observed under light microscope.

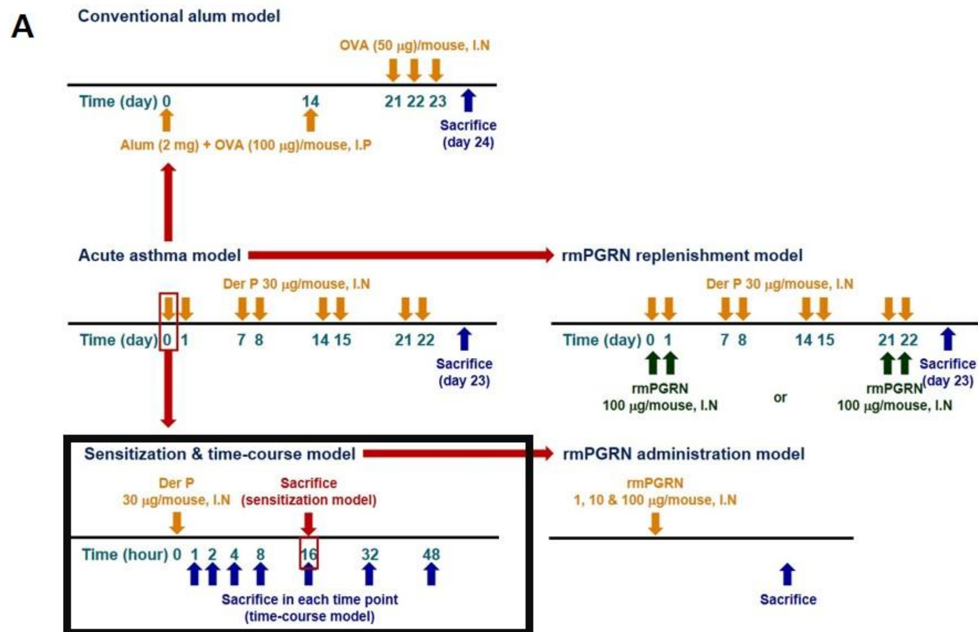
2.10. Statistical analysis

All of the data were presented as mean ± SEM with GraphPad Prism software (GraphPad, La Jolla, CA). One-way or two-way ANOVA with post-hoc comparison was used to determine the statistical significance. For comparison between two specific groups, the Student t test was performed. The p values < 0.05 were considered statistically significant.

3. Results

3.1. Airway inflammation and production of cytokines and progranulin was induced by HDM allergen exposure

First, to evaluate the effect of HDM allergen exposure to production of progranulin in airway, we measured the level of cytokine production, including progranulin, from BAL fluid at several time points after HDM exposure. (Fig 3A) The total number of inflammatory cells in BAL fluid peaked at 16 h and 32 h after HDM exposure. (Fig 3B) The production of type 2 cytokines, IL-4 and 13, were measured to be highest at 8 h (Fig 4A), while IL-6 and IL-12 started to peak at 4 h and 8h after HDM exposure. (Fig 3B) In particular, a significant rise in progranulin level was seen at 2 h with its peak at 4 h after HDM exposure. (Fig 4A, 4B) Also, the absolute amount of progranulin production was higher than other cytokines measured. (Fig 4A, 4B)



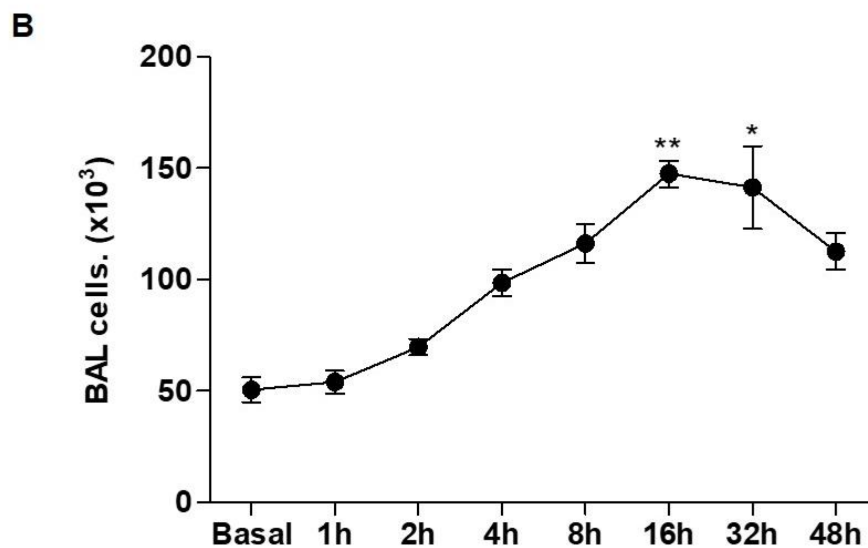


Figure 3. Airway inflammation was induced by intranasal administration of HDM allergen in wild type mice.

Animal model protocol for Der p stimulation in wild type mice (A; indicated in square box, blue arrows). The change in number of inflammatory cells in BAL fluid after Der p exposure (B). The experiment consisted of five mice. *P<0.05; **P<0.01 relative to the basal level

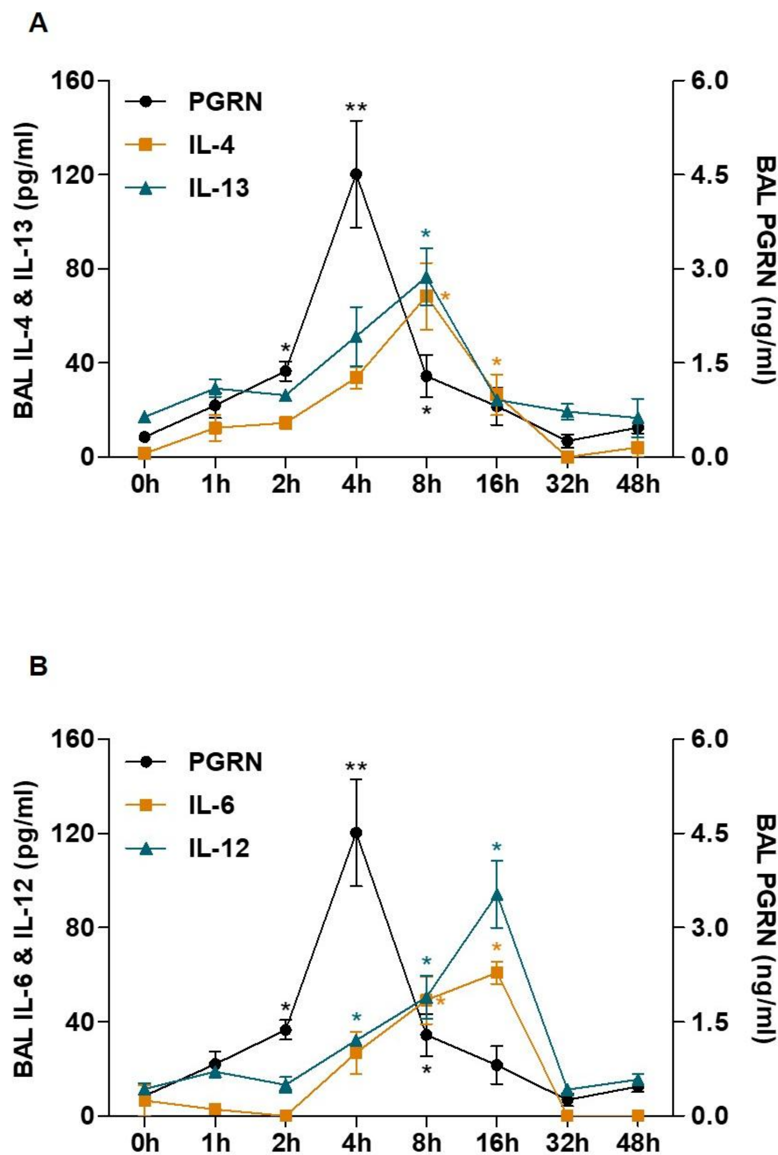


Figure 4. The production of progranulin in BAL fluid in HDM stimulated wild type mice was induced earlier than other pro-inflammatory cytokines, including type 2 cytokines. A single intranasal administration of Der p 30 μg in wild type mice was performed and the expression of progranulin and other pro-inflammatory cytokines were evaluated at serial time points. IL-4 and IL-13 expression profile in BAL fluid after intranasal administration of HDM allergen in relation to the progranulin (A). IL-6 and IL-12 expression profile compared with progranulin (B). Each set of experiment consisted of five mice. *P<0.05; **P<0.01 relative to its basal level.

3.2. Alveolar macrophage was the source of progranulin production at HDM allergen exposure

To investigate the source of progranulin which had increased after HDM allergen exposure, we stimulated the major resident cells of the airway, such as alveolar macrophage, NKT cell and epithelial cells, with HDM allergen. The production of progranulin was noted in the HDM allergen stimulated alveolar macrophage, with its level significantly higher at 16 h to 32 h compared with its amount at 1 h. Moreover, the amount of progranulin was far higher than those measured in HDM exposed epithelial cells and NKT cells. (Fig 5) A slight amount of progranulin was detected in HDM allergen exposed epithelial cells, however, its increment was not significant in relation with exposure time or HDM concentration. (Fig 5B) In NKT cell, the elevation of progranulin was not detected. (Fig 5C)

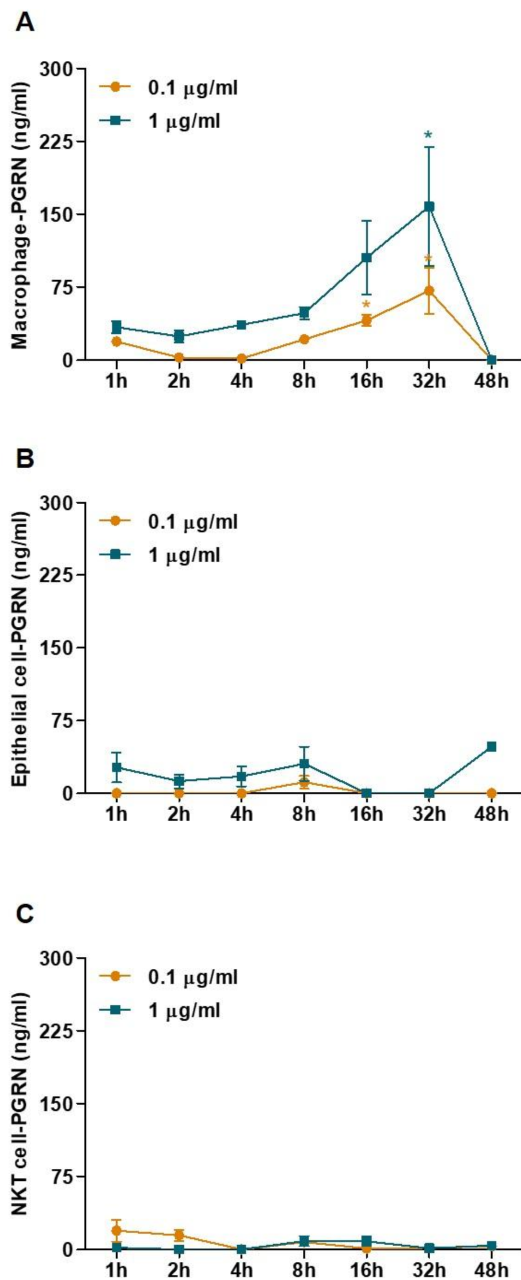
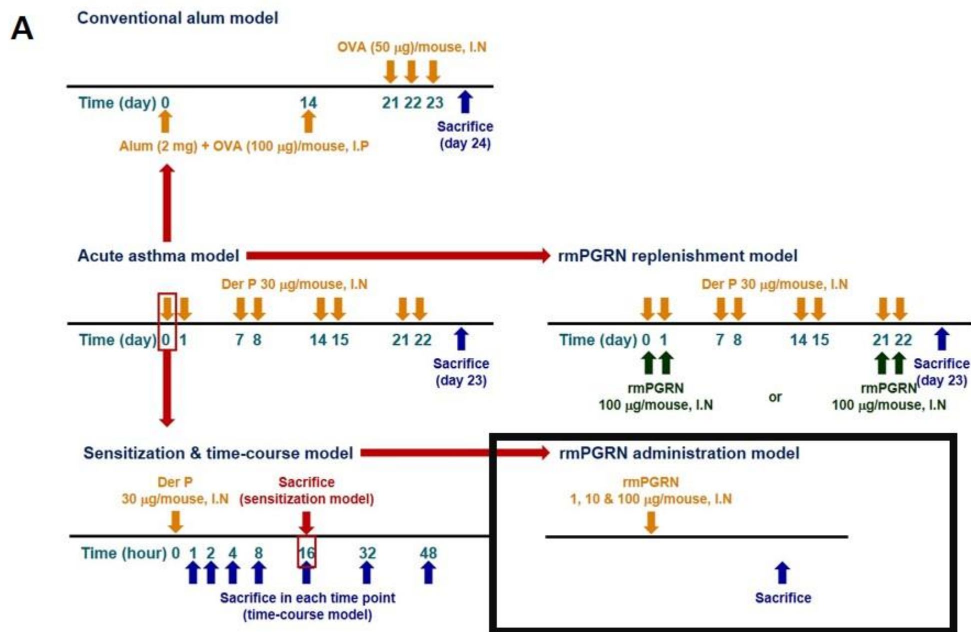


Figure 5. Progranulin was mainly produced by alveolar macrophages after HDM allergen exposure.

Comparison of the ability of progranulin production after HDM allergen stimulation in cell lines associated with innate immunity and airway structural cell line. Progranulin production after HDM allergen stimulation in alveolar macrophages (A), airway epithelial cells, (B) and NKT cell culture supernatant (C). All values are shown post subtraction with its media value measured at the corresponding time. *P<0.05 relative to the value at 1 h

3.3. Intranasal treatment of recombinant progranulin increased airway inflammation

We then questioned if progranulin itself had any effect on airway inflammation. Intranasal treatment of recombinant mouse progranulin was performed and we measured immunological parameters, such as inflammatory cells and cytokine production in BAL fluid. (Fig 6A) The number of total inflammatory cells in BAL fluid showed a significant increase at 16 h after treatment of 10 and 100 ng of recombinant mouse progranulin. (Fig 6B) In particular, there was a significant increase in the number of alveolar macrophages and neutrophils with treatment of 100 ng recombinant mouse progranulin. (Fig 6B) The effect of progranulin treatment was prominent on type 2 cytokines, IL-4, IL-13, IL-33 and TSLP, as the level of these cytokines in BAL fluid peaked with a significant increase after intranasal treatment of 100 ng progranulin. (Fig 7) On the other hand, we could not see any effect of progranulin on the production of IL-6 and IL-12p70. (Fig 8)



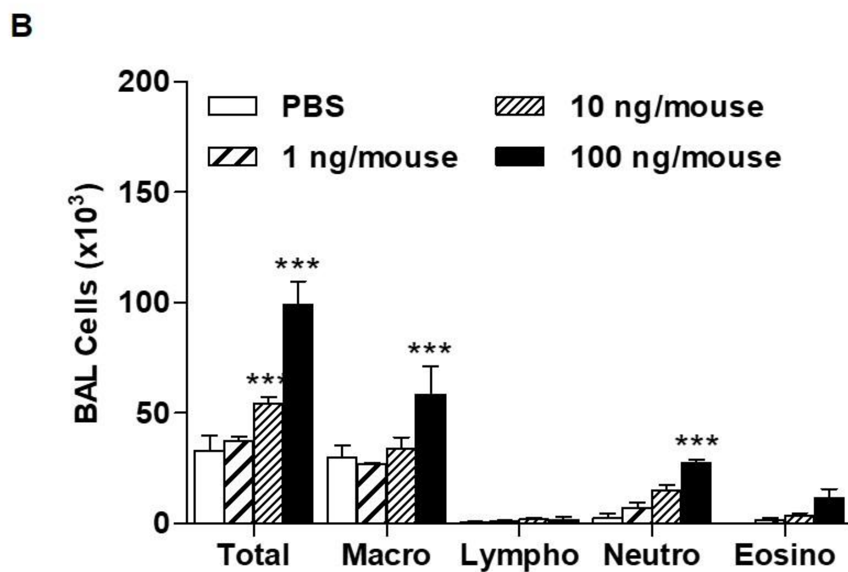


Figure 6. Airway inflammation was induced after intranasal administration of recombinant mouse progranulin.

Animal model protocol for recombinant progranulin administration in wild type mice, (A, indicated in square box), and the number of inflammatory cells and its composition in BAL fluid. (B) Mice were given with each dose of recombinant mouse progranulin. For all experiments, each group consisted of five mice. *P<0.05; **P<0.01; ***P<0.001 relative to PBS group

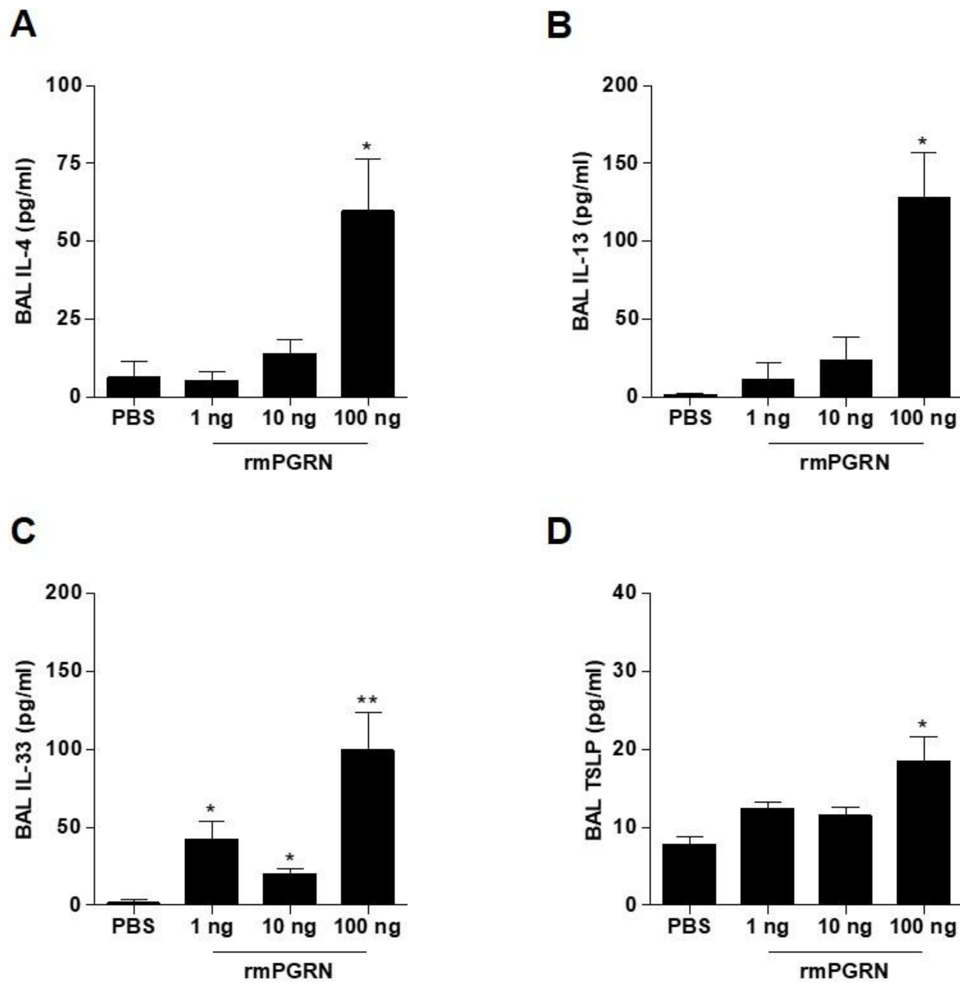


Figure 7. Intranasal administration of recombinant mouse progranulin induced type 2 cytokines in wild type mice.

The level of cytokine production in BAL fluid after intranasal administration of each dose of recombinant mouse progranulin. (A: IL-4, B: IL-13, C: IL-33, D: TSLP) For all experiments, each group consisted of five mice. *P<0.05; **P<0.01 relative to PBS group.

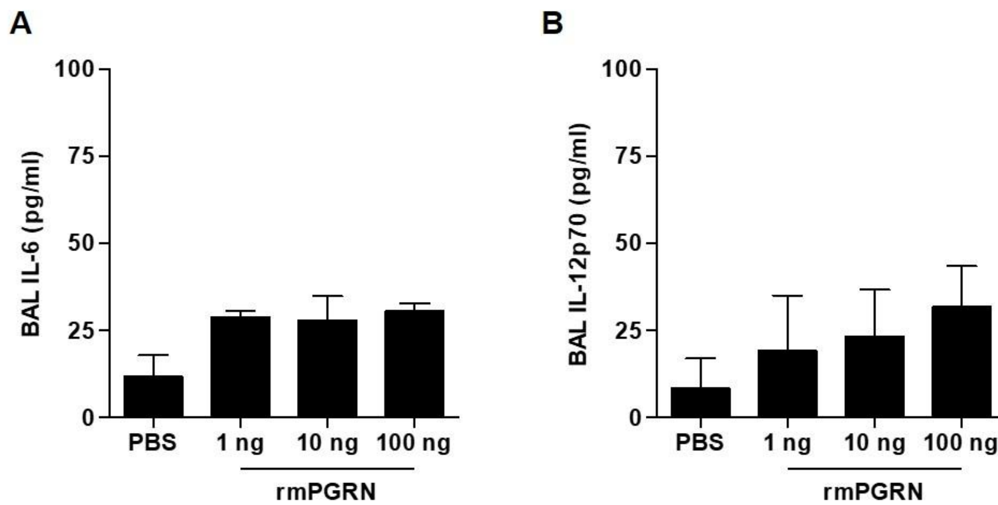


Figure 8. Recombinant mouse progranulin administration did not induce the production of IL-6 and IL-12 in BAL fluid of wild type mice.

The level of IL-6 and IL-12 production in BALF fluid after intranasal administration of each dose of recombinant mouse progranulin. (A: IL-6, B: IL-12p70) For all experiments, each group consisted of five mice. Cytokine levels were statistically compared relative to PBS group.

3.4. Progranulin induced production of type 2 cytokines from NKT and lung epithelial cells

To identify the source of type 2 cytokine production in response to intranasal treatment of recombinant progranulin, we checked cytokine production after stimulation of recombinant mouse progranulin in NKT cells and epithelial cells. In NKT cell, the results showed that IL-4 concentration peaked at 1, 2 and 4 h (Fig 9A) and IL-13 concentration peaked at 8 and 16 h. (Fig 9B) In epithelial cells treated with recombinant progranulin, both IL-33 and TSLP production were increased, IL-33 at 32 h after progranulin treatment (Fig 10A) while TSLP at 8 and 16 h. (Fig 10B) The level of IL-4, IL-33 and TSLP increased with progranulin treatment in a dose dependent manner, while IL-13 did not. (Fig 9, 10)

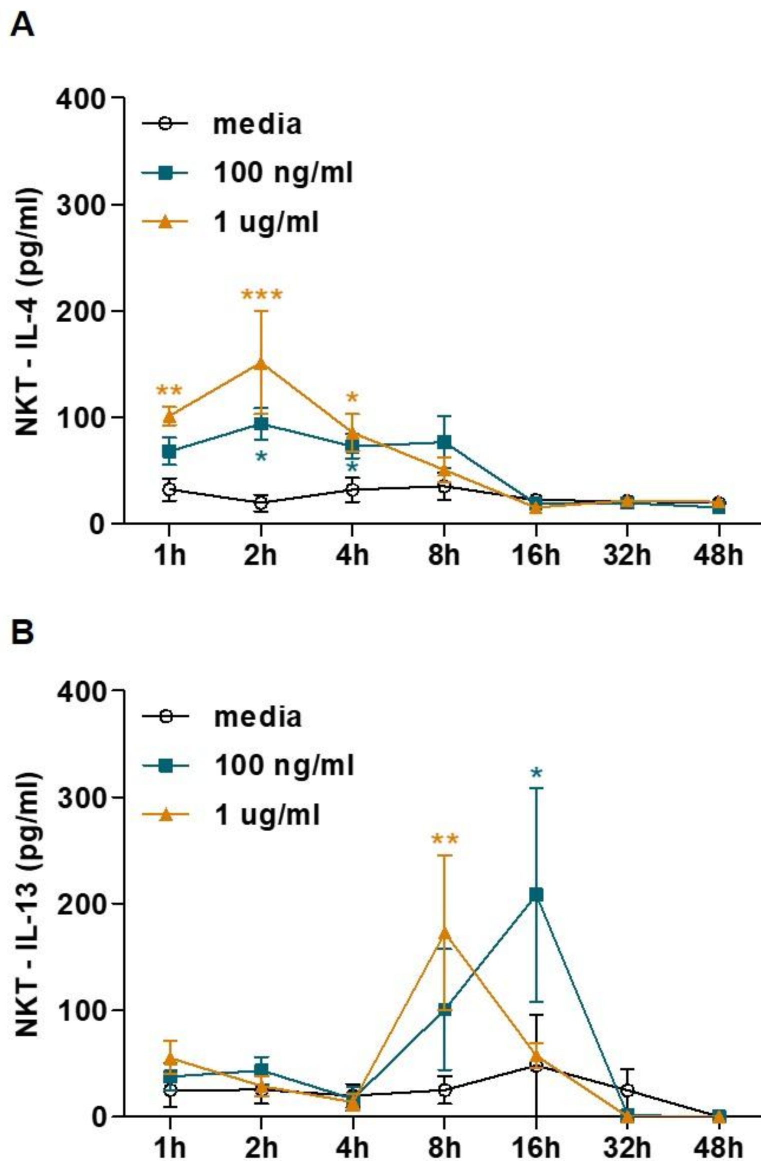
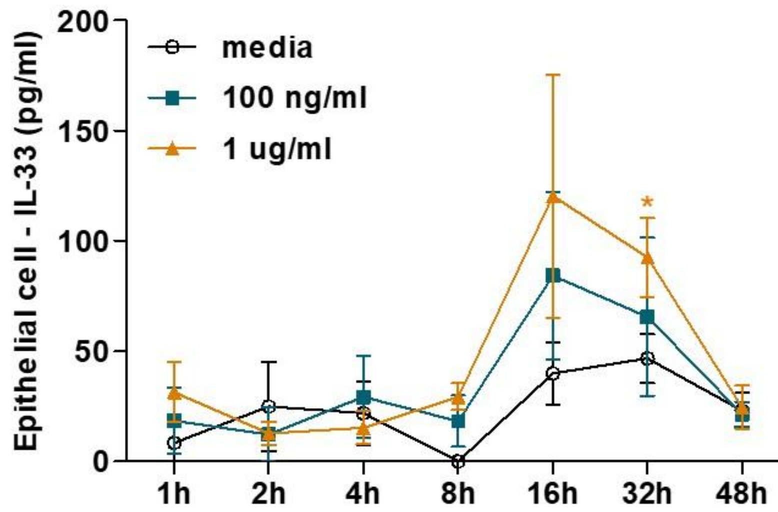


Figure 9. IL-4 and IL-13 were induced by stimulation of recombinant mouse progranulin in NKT cells.

The level of cytokine production level in NKT cell culture supernatant after stimulation with each dose of recombinant mouse progranulin. (A: IL-4, B: IL-13) *P<0.05; **P<0.01; ***P<0.001 relative to media value at each time

A



B

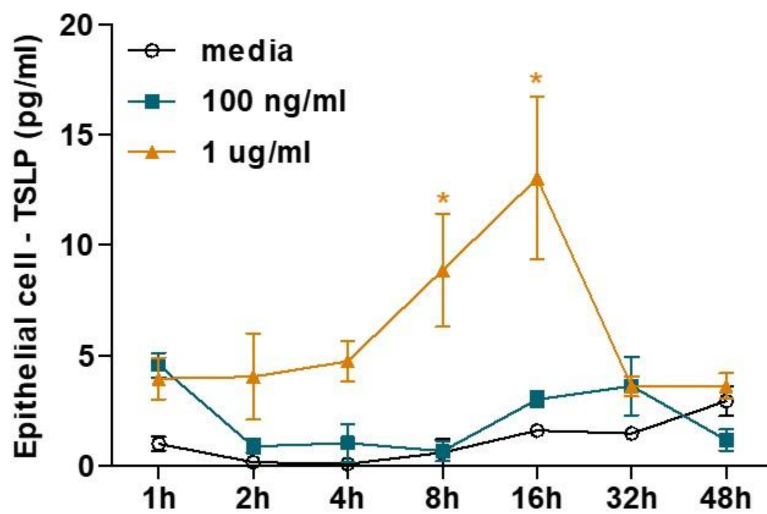


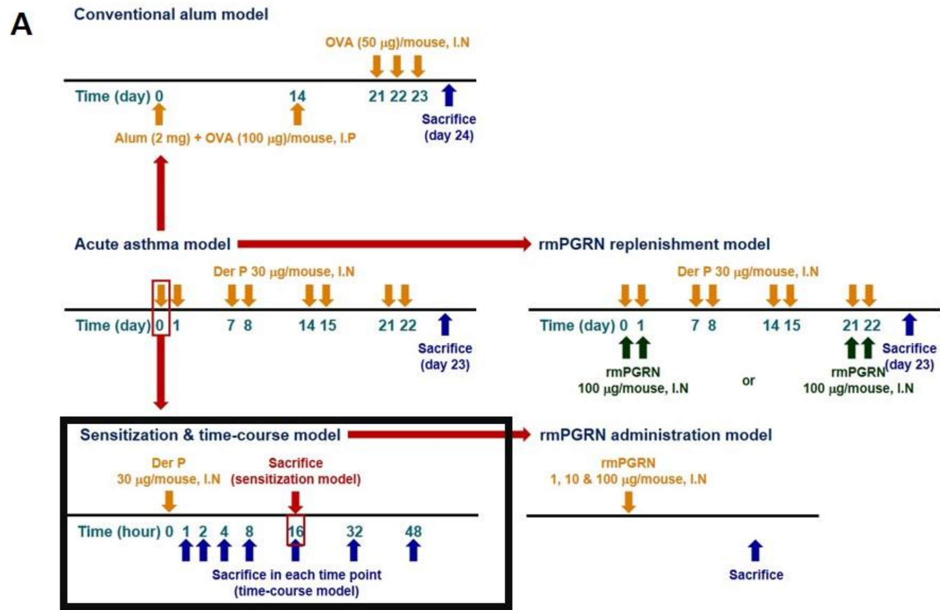
Figure 10. IL-33 and TSLP were induced by stimulation of recombinant mouse progranulin in epithelial cells.

The level of cytokine production in epithelial cell culture supernatant after stimulation with each dose of recombinant mouse progranulin. (A: IL-33, B: TSLP) *P<0.05 relative to media value at each time.

3.5. Effect of macrophage-derived progranulin on the development of innate immune response induced by HDM allergen stimulation

In the above experiments, we identified that the major source of progranulin by HDM allergen stimulation was alveolar macrophage, and progranulin induced type 2 cytokine production, such as IL-4, IL-13, IL-33 and TSLP from NKT or epithelial cells. Next, to evaluate the effect of macrophage-derived progranulin on the development of innate immune response to HDM allergen, we evaluated various immunological parameters in macrophage-derived progranulin deficient mice 16 h after a single HDM allergen sensitization. (Fig 11A) The results revealed that the inflammatory cells in BAL fluid were significantly lower in the progranulin deficient mouse compared with wild type, and the inflammatory cell that showed such prominent difference was the macrophage. (Fig 11B) The type 2 cytokines, IL-4, IL-13, IL-33 and TSLP in the BAL fluid increased with allergen exposure in the wild type mice, however, the production of these cytokines were much lower in the progranulin deficient mice. (Fig 12) IL-6 production also increased with allergen exposure but the level did not differ between wild type and progranulin deficient mice. (Fig 13A) IL-12 production was minimal with HDM exposure hence the difference was insignificant between wild type and progranulin deficient mice. (Fig 13B) The cytokine production in the tissue of the lung was measured with cells treated with PMA/Ionomycin, and the level of IL-4 and IL-13 increased with HDM exposure in the wild type mice, while the levels were lower in the progranulin deficient mice. (Fig 14) IL-6, IL-12, IL-33 and TSLP levels did not show any significant changes in the cells

of wild type and progranulin deficient mice treated with PMA/Ionomycin. (data not shown)



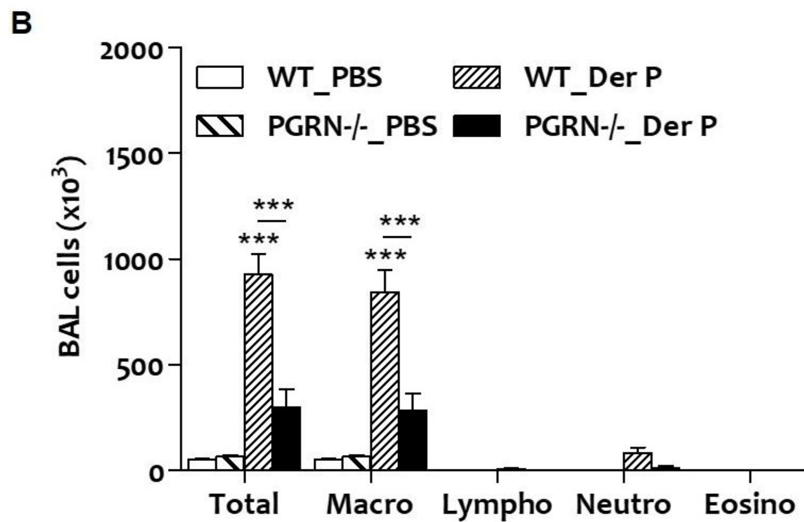


Figure 11. Inflammatory cells in BAL fluid were decreased in macrophage-derived progranulin deficient mice after a single HDM allergen sensitization.

Animal model protocol for single Der p stimulation in wild type mice and macrophage-derived progranulin deficient mice (A, indicated in square box, red arrow). The number of inflammatory cells and its composition in BAL fluid (B). For all experiments, each group consisted of five mice. ***P<0.001 relative to PBS group. WT, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

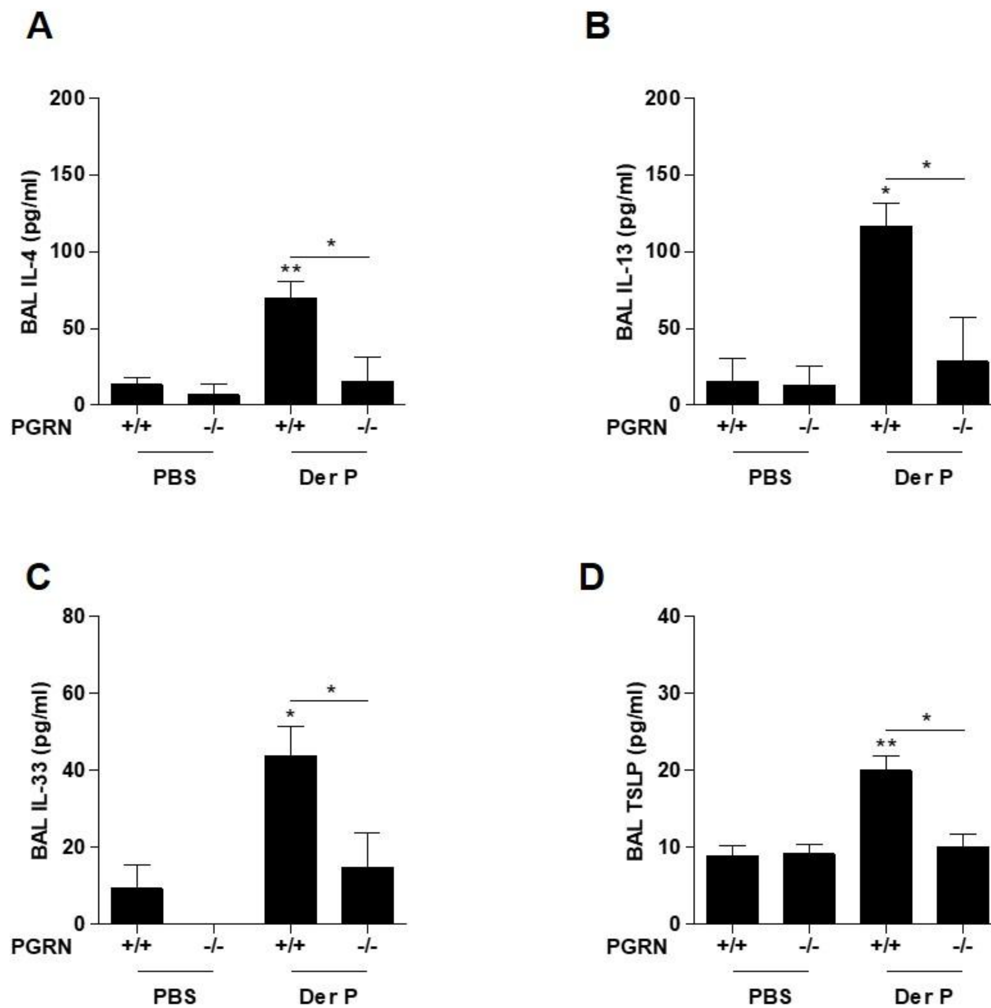


Figure 12. Type 2 cytokine expression in BAL fluid was down-regulated in the macrophage-derived progranulin deficient mice after single HDM allergen sensitization. The expression of type 2 cytokines BAL fluid was measured 16 h after a single HDM allergen sensitization (sensitization period), in wild type mice and in macrophage-derived progranulin deficient mice. (A: IL-4, B: IL-13, C: IL-33 and D: TSLP) For all experiments, each group consisted of five mice. *P<0.05; **P<0.01 relative to PBS group, and compared between wild type and macrophage-derived progranulin deficient mice. PGRN+/+, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

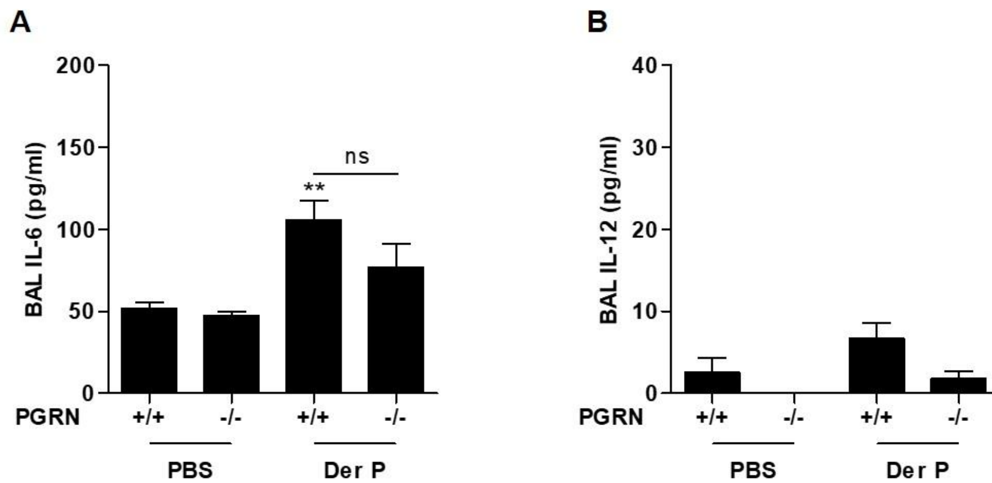


Figure 13. Macrophage-derived progranulin deficiency did not influence the levels of IL-6 and IL-12 after a single HDM allergen sensitization.

Changes of IL-6 and IL-12 production by HDM allergen stimulation in wild type mice and macrophage derived progranulin deficient mice. (A: IL-6 and B: IL-12p70) For all experiments, each group consisted of five mice. **P<0.01 relative to PBS group, ns; no significant different between wild type and macrophage-derived progranulin deficient mice. PGRN+/+, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

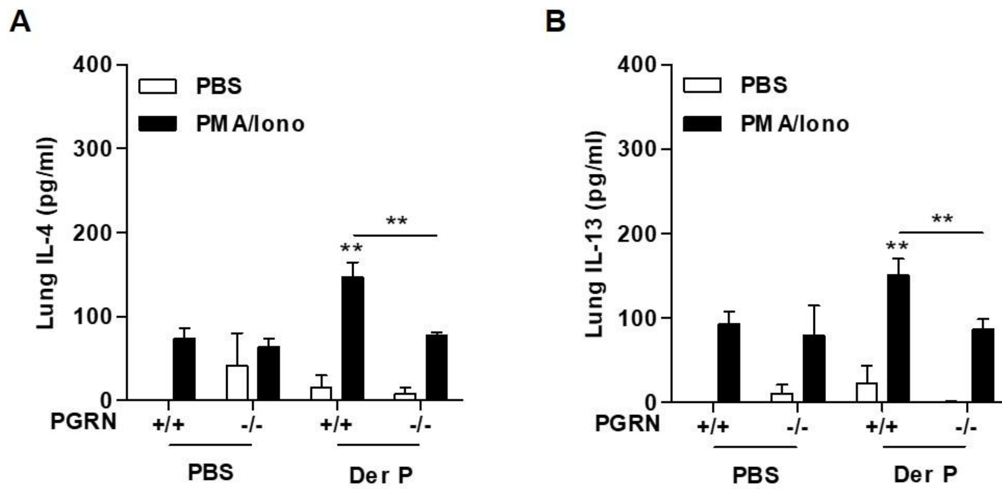


Figure 14. The expression of IL-4 and IL-13 were decreased in the lung tissue of macrophage-derived progranulin deficient mice after a single HDM allergen sensitization.

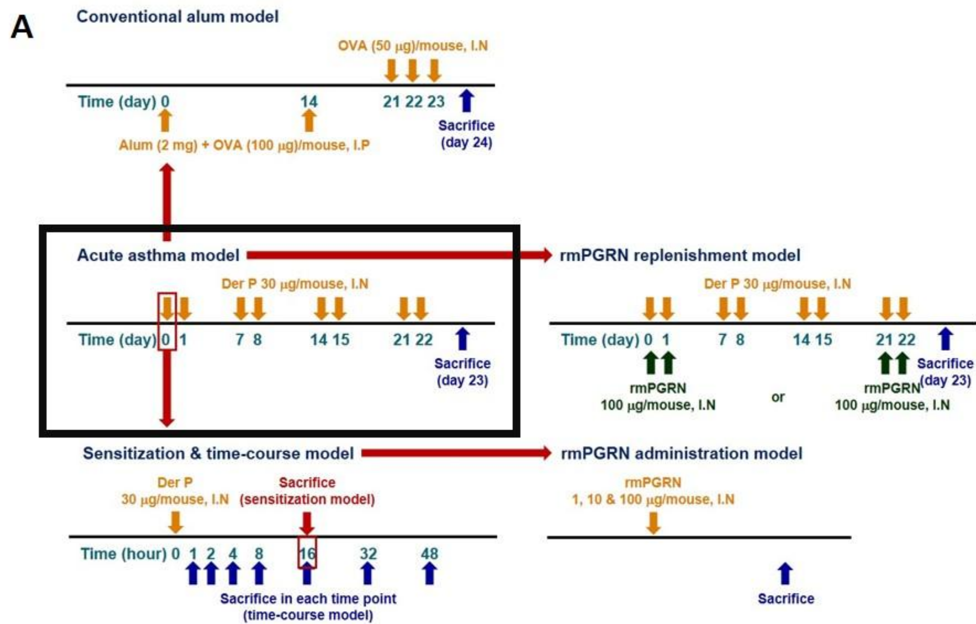
Changes of type 2 cytokines, IL-4 and IL-13, in wild type and macrophage-derived progranulin deficient mice after stimulating with PMA/Ionomycin (A: IL-4 and B: IL-13). The results were calculated by dividing with total amount of protein. For all experiments, each group was consisted of five mice. **P<0.01 relative to PBS group, and compared between wild type and macrophage-derived progranulin deficient mice. PGRN+/+, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

3.6. Effect of macrophage-derived progranulin on the development of adaptive immunity induced by HDM allergen stimulation

Next, to evaluate the effect of macrophage-derived progranulin on the development of adaptive immune response to HDM allergen, we evaluated the immunological parameters in macrophage-derived progranulin deficient mice after multiple administrations of HDM for two weeks. (Fig 15A) When evaluated 24 h after the final HDM allergen administration, there was a significant increase in neutrophils and eosinophils in the BAL fluid of wild type mice. However, the recruitment of these cells were significantly decreased in macrophage-derived progranulin deficient mice. (Fig 15B) In tissue analysis, the decrease in inflammation was prominent in the macrophage-derived progranulin deficient mice. (Fig 16d) The type 2 cytokines, IL-4, IL-13 and eotaxin were increased in the BAL fluid of wild type mice after allergen exposure, while these cytokine levels were decreased in the macrophage-derived progranulin deficient mice. (Fig 17) There was no difference in the level of IL-17 between the wild type and progranulin deficient mice, (Fig 18A) and IP-10 concentration was higher in the progranulin deficient mice than in the wild type mice. (Fig 18B) The serum IgE level also showed an increase after HDM exposure in the wild type mice, while the level was lower in the progranulin deficient mice. (Fig 19A) The serum IgG1 levels did not differ between the wild type and progranulin deficient mice (Fig 19B) and serum $IgG2_C$ was increased in the progranulin deficient mice, similar to the results of BAL IP-10. (Fig 19C)

When we stimulated T cells from the lung and regional LNs with anti-CD3/CD28

antibodies, IL-4 was increased in the wild type mice, while it was decreased in the progranulin deficient mice. (Fig 20A, 20D) IL-17 level increased in both wild type and progranulin deficient mice after stimulation, but the levels did not differ between the wild type and the progranulin deficient mice. (Fig 20B, 20E) IFN-γ was higher in the progranulin deficient mice than in the wild type mice. (Fig 20C, 20F)



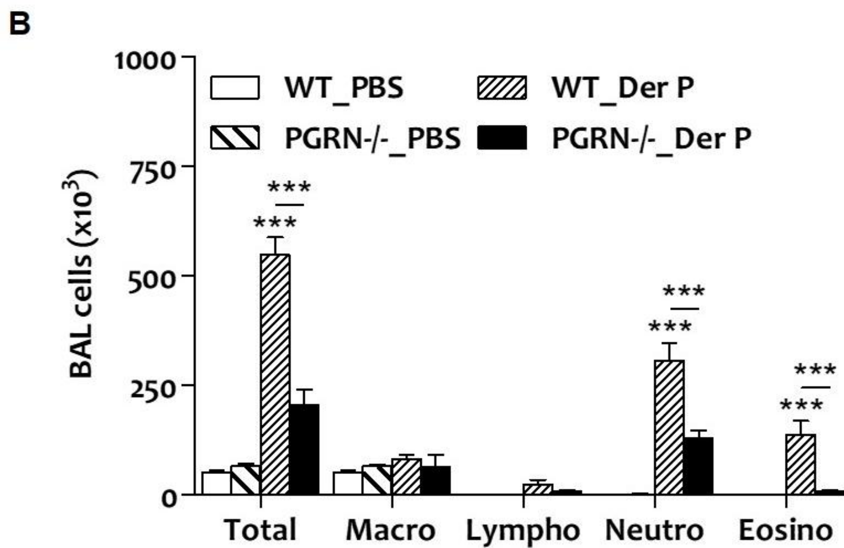


Figure 15. Inflammatory cell recruitment was decreased in macrophage-derived progranulin deficient mice after multiple administrations of HDM allergen.

Animal model protocol for multiple Der p stimuli in wild type mice and macrophage-derived progranulin deficient mice (A, indicated in square box). The number of inflammatory cells and its composition in BAL fluid (B). *** P<0.001 relative to PBS group, and compared between wild type and macrophage-derived progranulin deficient mice after Der p stimulation. WT, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

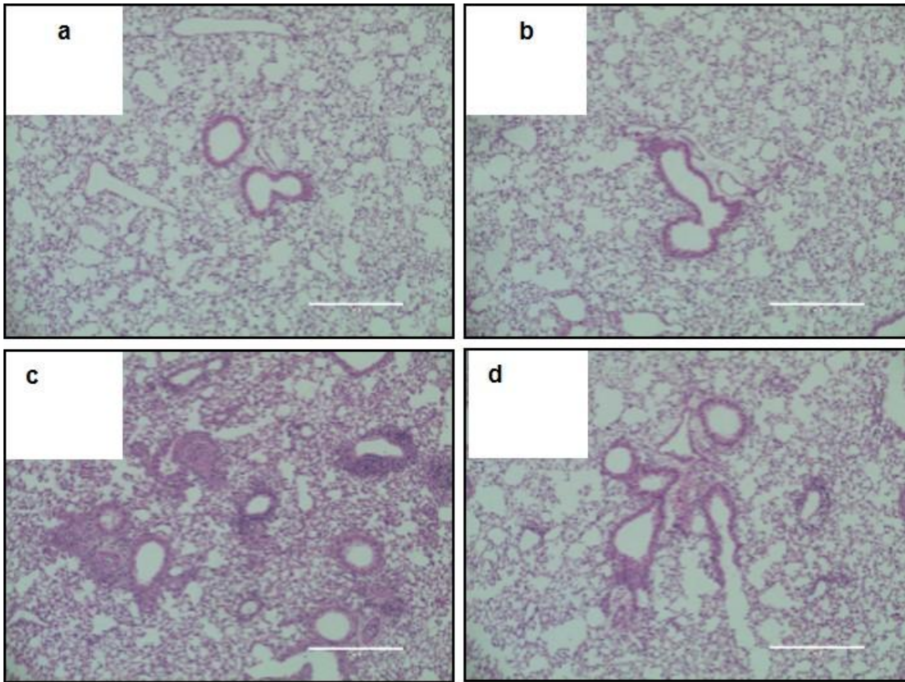


Figure 16. Tissue inflammation of the lung was down-regulated in macrophage-derived progranulin deficient mice after multiple administrations of HDM allergen.

Hematoxylin and eosin (H&E) staining, 100x magnification

a: wild type_PBS; b: PGRN -/-_PBS; c: wild type_Der p; d: PGRN -/-_Der p

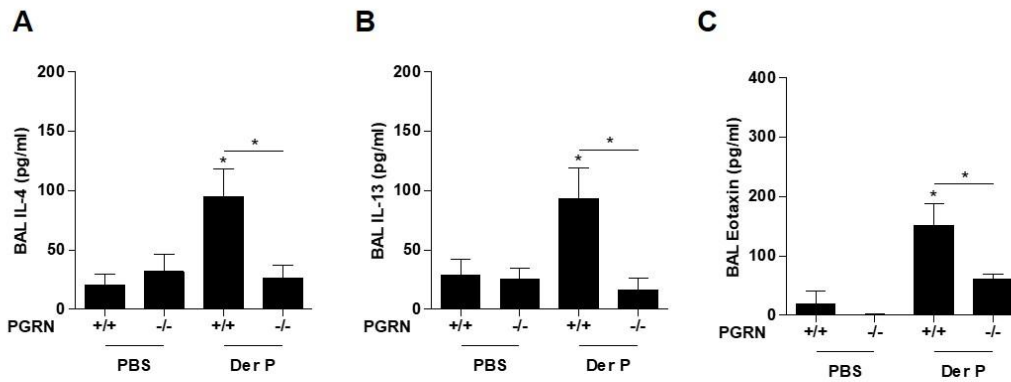


Figure 17. Type 2 cytokine levels were down-regulated in macrophage-derived progranulin deficient mice after multiple administrations of HDM allergen.

The evaluation of the cytokine levels in BAL fluid was performed 24 h after last allergen challenge, sacrificed on day 23. (A: IL-4, B: IL-13 and C: eotaxin) For all experiments, each group consisted of five mice. *P<0.05 relative to PBS group and compared between wild type and macrophage-derived progranulin deficient mice. PGRN+/+, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

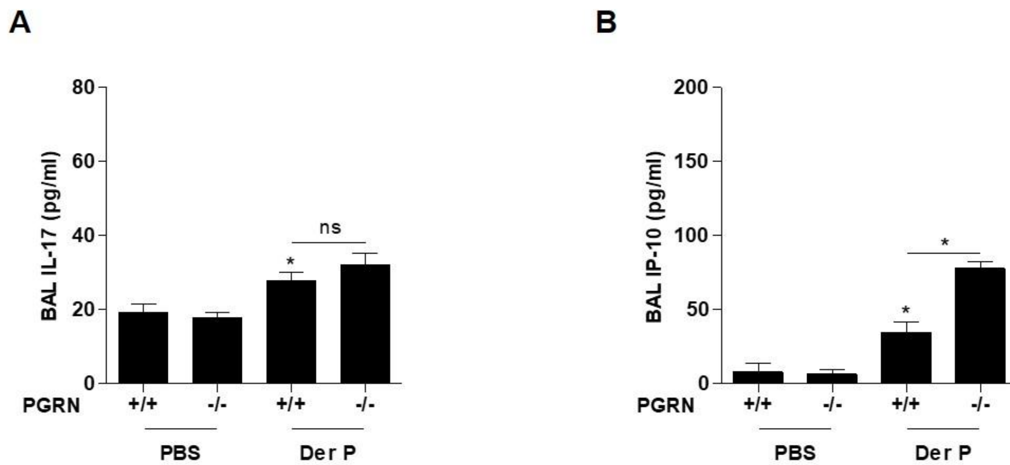


Figure 18. The absence of macrophage-derived progranulin affects the production of IL-17 and IP-10 after multiple administrations of HDM allergen.

The evaluation of the cytokine levels in BAL fluid was performed 24 h after last allergen challenge, sacrificed on day 23. (A: IL-17 and B: IP-10) For all experiments, each group consisted of five mice. *P<0.05 relative to PBS group and compared between wild type and macrophage-derived progranulin deficient mice. PGRN+/+, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

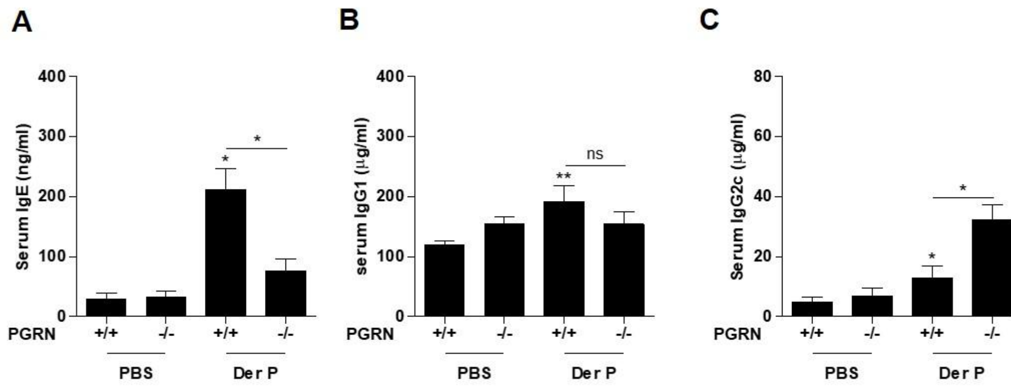


Figure 19. The absence of macrophage-derived progranulin affects the induction of serum immunoglobulin after multiple administrations of HDM allergen.

The effect of macrophage-derived progranulin to induction of serum immunoglobulin was measured with ELISA. (A: IgE, B: IgG1 and C: IgG2c) For all experiments, each group consisted of five mice. *P<0.05; **P<0.01 relative to PBS group and compared between wild type and macrophage-derived progranulin deficient mice. PGRN+/+, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

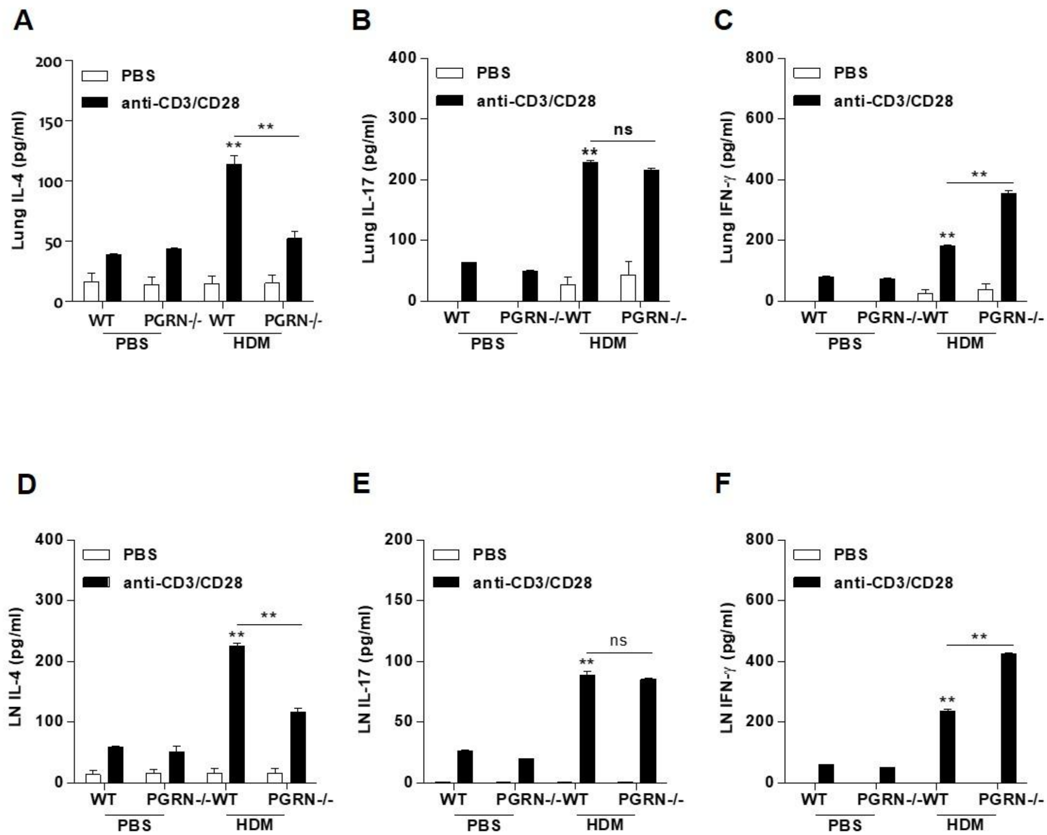


Figure 20. Induction of T_H2 cell development with HDM allergen challenge was down-regulated in macrophage-derived progranulin deficient mice.

Evaluation of each cytokine in lung and draining lymph node cells after T cell stimulation with anti-CD3/CD28 antibodies. (Upper, A-C: Lung IL-4, IL-17 and IFN-γ; lower, D-F: lymph node IL-4, IL-17 and IFN-γ) For all experiments, each group consisted of five mice. **P<0.01 relative to PBS group and compared between wild type and macrophage-derived progranulin deficient mice. WT, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

3.7. Progranulin supplement in the sensitization period restored down-regulated Der p specific airway inflammation in macrophage-derived progranulin deficient mice

In the subsequent experiment, we investigated whether replenishment of progranulin could restore the development of airway inflammation and the relevant cytokine production in macrophage-derived progranulin deficient mice after HDM exposure. We administered recombinant progranulin at different time points, in the sensitization and challenge period. (Fig 21A) In the macrophage-derived progranulin deficient mice, the recruitment of inflammatory cells in BAL fluid was decreased compared to wild type mice, and the decline was prominent in eosinophils in particular, as seen from previous experiments. The intranasal replenishment of progranulin in the sensitization period of HDM allergen resulted in the restoration of the inflammatory cell infiltration, especially the eosinophils to a similar level that was seen in the wild type mice. However, we could not see any increase of inflammatory cell in the group that progranulin supplement was performed in the challenge period of HDM allergen. (Fig 21B) The histologic findings also showed that inflammation was less prominent in the macrophage-derived progranulin deficient mice with Der p stimulation (Fig 22b), but it was restored to a similar extent when progranulin was supplemented in the sensitization period (Fig 22c) The levels of IL-4, IL-13 and eotaxin measured from the BAL fluid were decreased in the macrophage-derived progranulin deficient mice and they were restored with progranulin supplement in the sensitization period of Der p stimulation. (Fig 23) The timing of progranulin replenishment had no effect on the IL-17 production (Fig 24A), and the increased IP-10 level

in the progranulin deficient mice was decreased with progranulin supplement in the sensitization period. (Fig 24B) Serum IgE level showed a similar pattern with the type 2 cytokines (Fig 25A), while serum IgG1 had no change in all conditions (Fig 25B) and serum IgG2c showed opposite results with that seen from serum IgE. (Fig 25C)

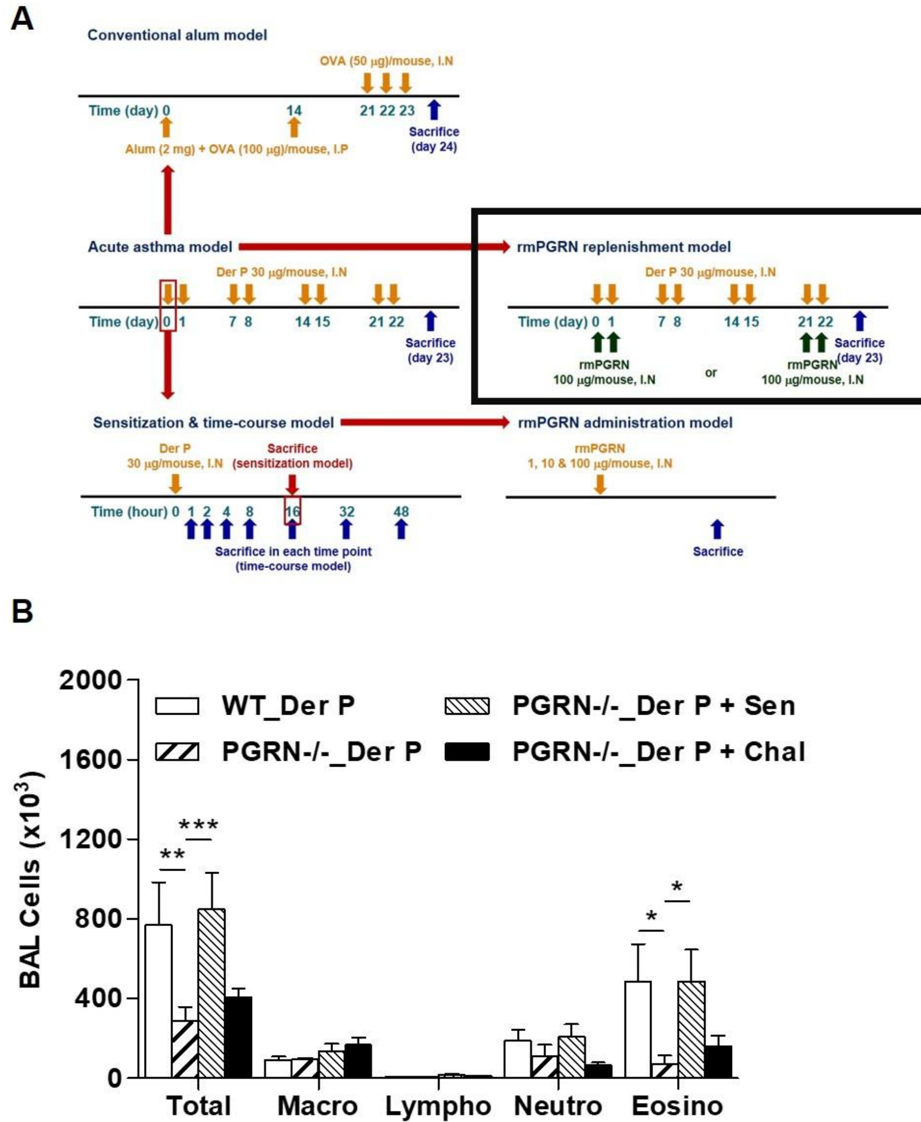


Figure 21. Reduced airway inflammatory cell recruitment in macrophage-derived progranulin deficient mice was restored by replenishment of progranulin during sensitization period.

Animal model protocol for recombinant progranulin replenishment model (A, indicated in square box). The number of inflammatory cells and its composition in BAL fluid (B). For all experiments, each group consisted of five mice. *P<0.05; **P<0.01; ***P<0.001 compared between wild type and macrophage-derived progranulin deficient mice, and between macrophage-derived progranulin deficient mice and those supplemented with recombinant mouse progranulin. WT, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

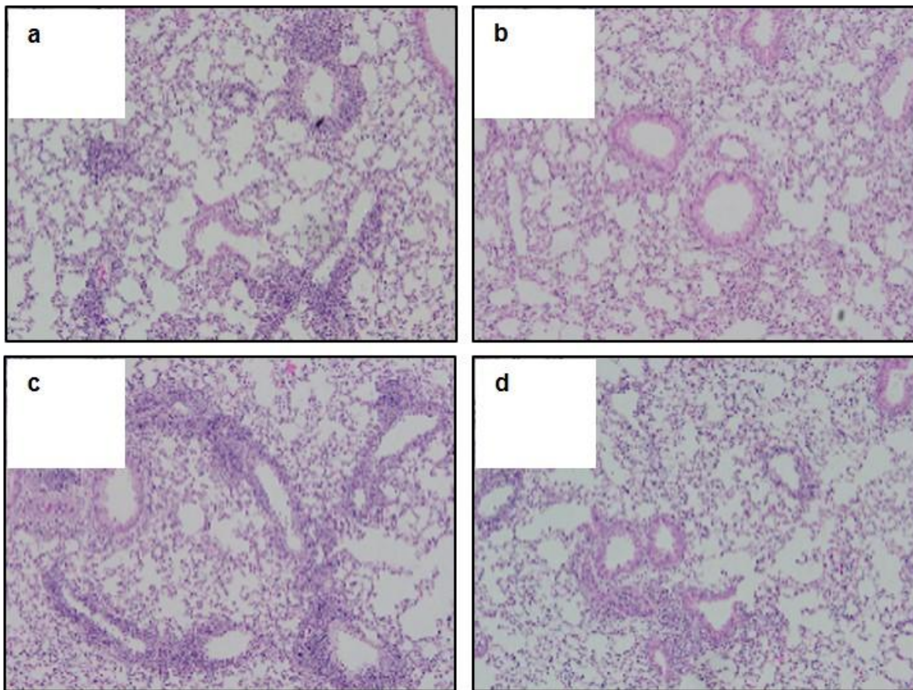


Figure 22. Down-regulated Der p specific airway inflammation in macrophage-derived progranulin deficient mice was restored by replenishment of progranulin during sensitization period.

Hematoxylin and eosin (H&E) staining, 100x magnification

a: wild type_Der p; b: PGRN -/-_Der p; c: PGRN -/-_Der p + progranulin replenishment in the sensitization period; d: PGRN -/-_Der p + progranulin replenishment in the challenge period

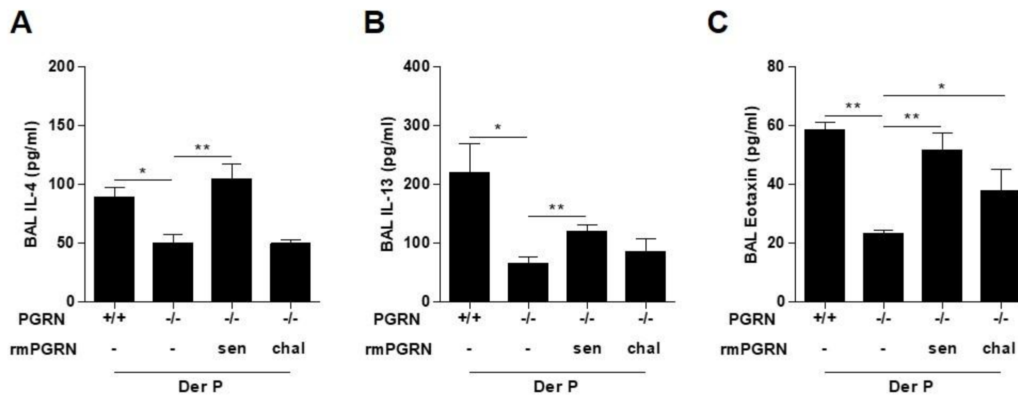


Figure 23. Down-regulated expression of type 2 cytokines in macrophage-derived progranulin deficient mice was restored by replenishment of progranulin during sensitization period.

The evaluation of the cytokine levels in BAL fluid was performed 24 h after last allergen challenge, sacrificed on day 23. (A: IL-4, B: IL-13 and C: eotaxin) For all experiments, each group consisted of five mice. *P<0.05; **P<0.01 compared between groups. PGRN+/+, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice; sen, progranulin replenishment in the sensitization period; chal, progranulin replenishment in the challenge period.

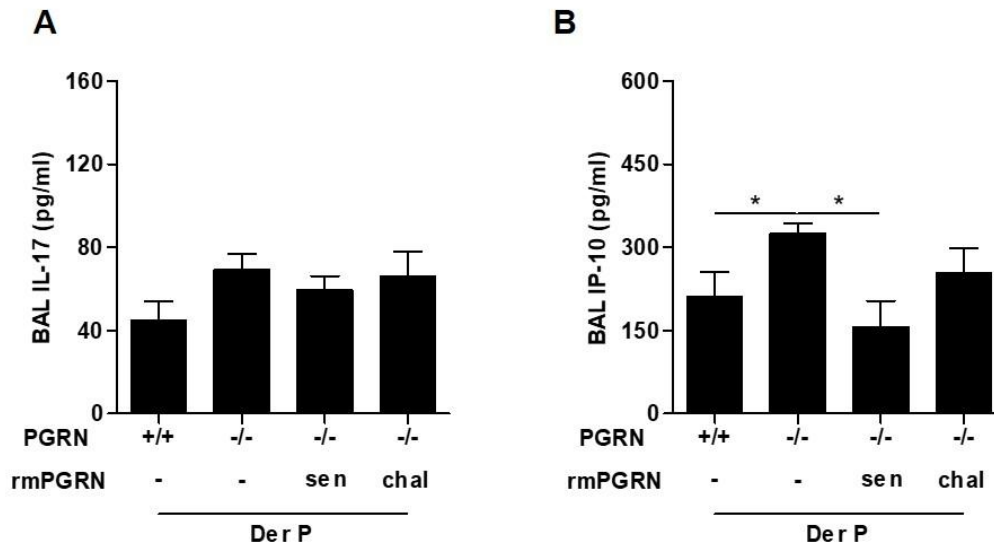


Figure 24. Progranulin replenishment in the sensitization period of Der p affects IL-17 and IP-10 levels in macrophage-derived progranulin deficient mice.

The evaluation of the cytokine levels in BAL fluid was performed 24 h after last allergen challenge, sacrificed on day 23. (A: IL-17 and B: IP-10) For all experiments, each group consisted of five mice. *P<0.05 compared between groups. PGRN+/+, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice; sen, progranulin replenishment in the sensitization period; chal, progranulin replenishment in the challenge period.

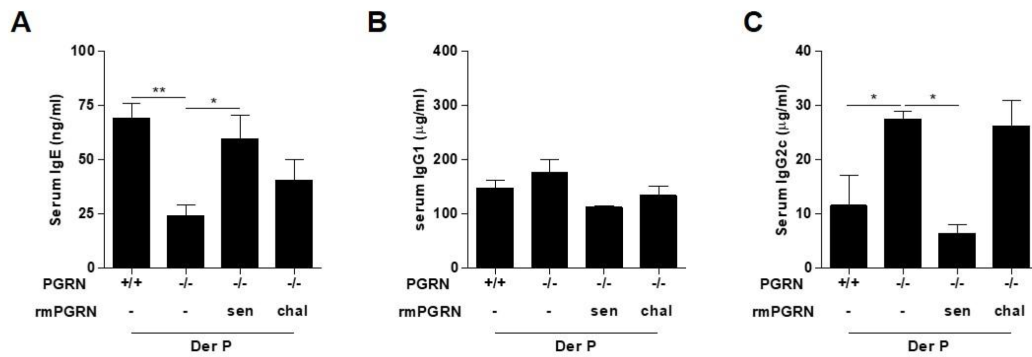
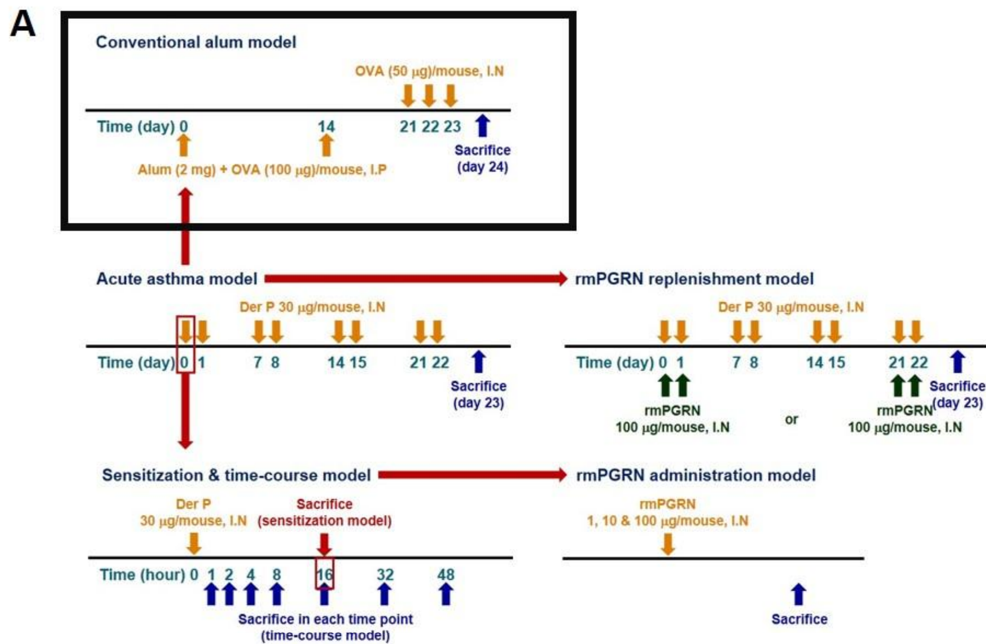


Figure 25. Progranulin replenishment in the sensitization period of Der p affects the induction of serum immunoglobulin in macrophage-derived progranulin deficient mice. The effect of macrophage-derived progranulin to induction of serum immunoglobulin was measured with ELISA. (A: IgE, B: IgG1 and C: IgG2c) For all experiments, each group consisted of five mice. *P<0.05; **P<0.01 compared between groups. PGRN+/+, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice; sen, progranulin replenishment in the sensitization period; chal, progranulin replenishment in the challenge period.

3.8. Effect of the progranulin on the development of adaptive immune response in conventional alum model

With the above results, we found that macrophage-derived progranulin deficiency led to the down-regulation of T_H2 immune response to allergen. To confirm these findings, we tested our hypothesis in another T_H2 animal model, the conventional alum model. (Fig 26A) The recruitment of eosinophils in the BAL fluid was seen in the wild type mice, which was significantly decreased in the progranulin deficient mice. (Fig 26B) The cellular infiltrations of the lung tissue showed the same pattern as the infiltration was increased in the OVA/alum stimulated wild type mice (Fig 27c) and was decreased in the same model of progranulin deficient mice. (Fig 27d) Also, the type 2 cytokines in the BAL fluid, IL-4, IL-13 and eotaxin were increased in the asthma model of wild type mice while a significant decline in these levels were seen in the progranulin deficient mice. (Fig 28) There no change in IL-17 and IP-10 concentration was higher in the progranulin deficient mice. (Fig 29) Serum IgE was also decreased in the asthma model of progranulin deficient mice (Fig 30A), while serum IgG1 showed no difference (Fig 30B) and serum IgG2c higher in the progranulin deficient mice. (Fig 30C) The cells were separated from the LNs and were then stimulated with CD3/CD28 to evaluate the T cell response, and results showed that the IL-4 concentration was increased in the wild type mice and was decreased in the progranulin deficient mice. (Fig 31A) IL-17 level did not differ between the wild type and the progranulin deficient mice (Fig 31B), and IFN-γ was increased in the progranulin deficient mice. (Fig 31C)



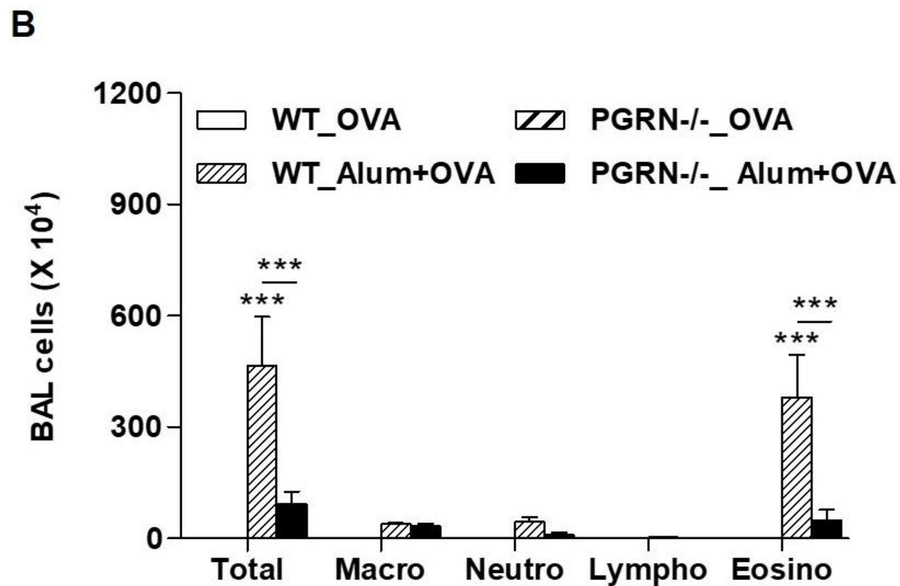


Figure 26. Recruitment of inflammatory cells was reduced in macrophage-driven progranulin deficient mice after OVA/alum stimulation.

Animal model protocol for generation of conventional alum model (A, indicated in square box). The number of inflammatory cells and its composition in BAL fluid (B). For all experiments, each group consisted of five mice. ***P<0.001 compared between indicated groups. WT, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

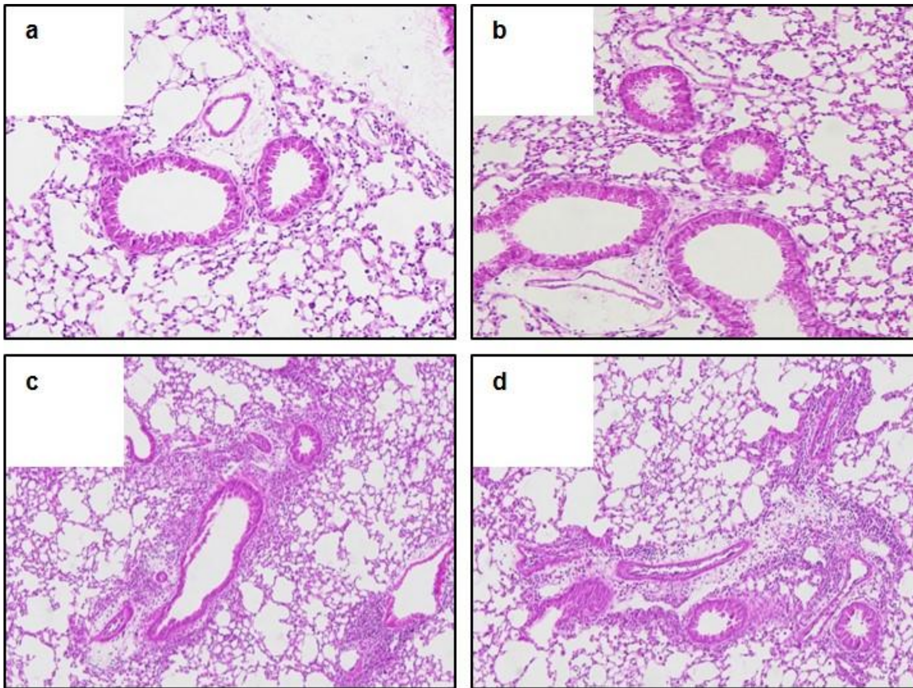


Figure 27. Ova specific airway inflammation was down-regulated in macrophage-driven progranulin deficient mice.

Hematoxylin and eosin (H&E) staining, 100x magnification

a: wild type_PBS p; b: PGRN -/-_PBS; c: wild type_OVA/alum; d: PGRN -/-_OVA/alum

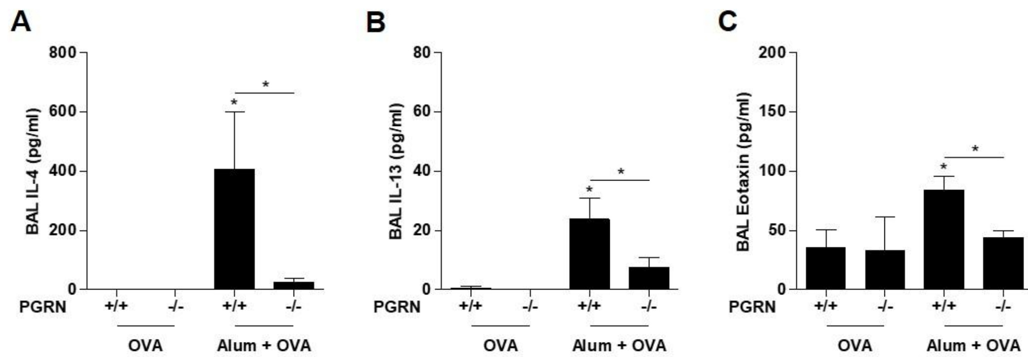


Figure 28. Type 2 cytokine levels were down-regulated in macrophage-driven progranulin deficient mice after OVA/alum stimulation.

The expression of type 2 cytokines BAL fluid was measured on day 24 of OVA/alum stimulation, in wild type mice and in macrophage-derived progranulin deficient mice. (A: IL-4, B: IL-13 and C: eotaxin) For all experiments, each group consisted of five mice. *$P<0.05$ relative to OVA group, and compared between wild type and macrophage-derived progranulin deficient mice. PGRN+/+, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

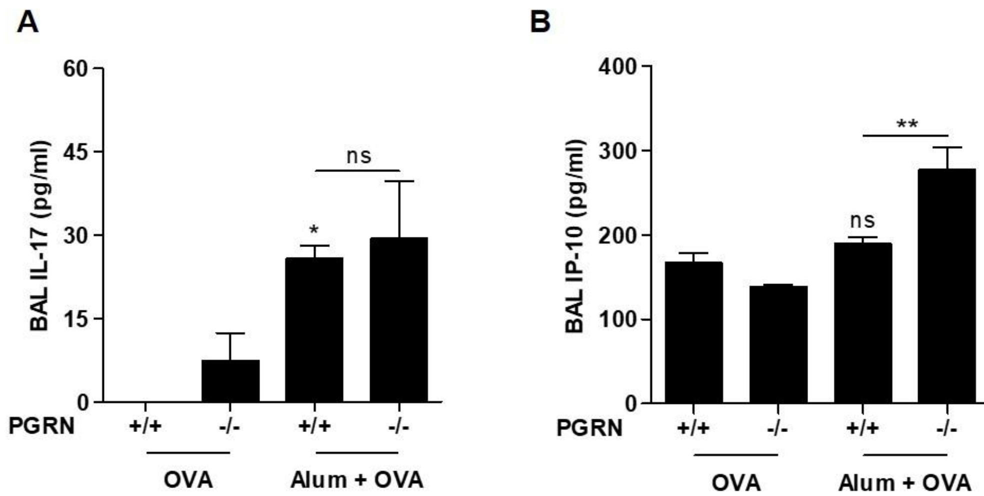


Figure 29. The absence of macrophage-derived progranulin affects the production of IL-17 and IP-10 after OVA/alum stimulation.

The evaluation of the cytokine levels in BAL fluid was performed on day 24 of OVA/alum stimulation. (A: IL-17 and B: IP-10) For all experiments, each group consisted of five mice. *P<0.05; **P<0.01 relative to OVA group and compared between wild type and macrophage-derived progranulin deficient mice. PGRN+/+, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

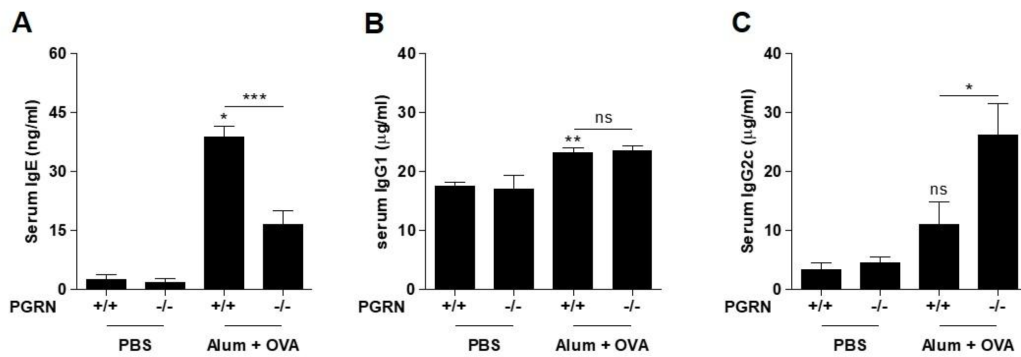


Figure 30. The absence of macrophage-derived progranulin affects the induction of serum immunoglobulin after OVA/alum stimulation.

The effect of macrophage-derived progranulin to induction of serum immunoglobulin was measured with ELISA. (A: IgE, B: IgG1 and C: IgG2c) For all experiments, each group consisted of five mice. *P<0.05; **P<0.01; ***P<0.001 relative to PBS group and compared between wild type and macrophage-derived progranulin deficient mice. PGRN+/+, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

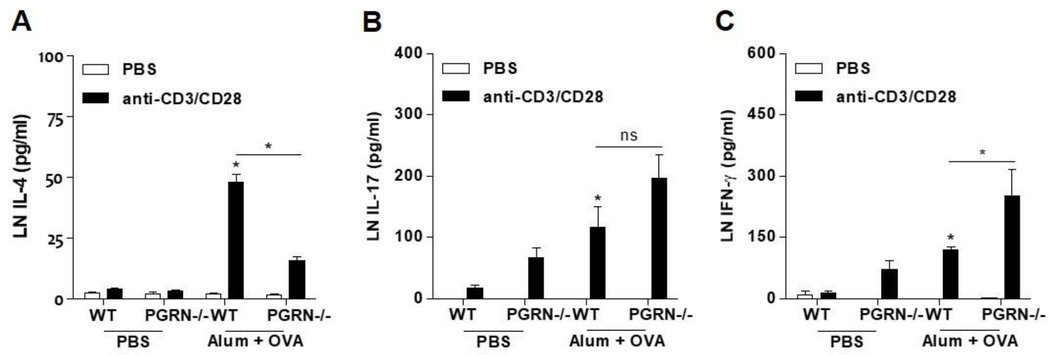


Figure 31. Induction of T_H2 cell development with OVA/alum stimulation was down-regulated in macrophage-driven progranulin deficient mice.

Evaluation of each cytokine in draning lymph node cells after T cell stimulation with anti-CD3/CD28 antibodies. (A: IL-4, B: IL-17 and C: IFN-γ) For all experiments, each group consisted of five mice. *$P<0.05$ relative to PBS group and compared between wild type and macrophage-derived progranulin deficient mice. WT, wild-type mice; PGRN-/-, macrophage-derived progranulin deficient mice.

3.9. Effect of OVA/alum stimulation on the induction of progranulin in peritoneal cavity

To clarify whether progranulin is produced in response to OVA/alum into the peritoneum, we evaluated the immune responses in the peritoneal cavity at serial time points after a single injection of OVA/alum. The inflammatory cells in the peritoneal fluid significantly increased after 8 h of injection which peaked at 16-32 h. (Fig 32A) In the peritoneal washing, IL-4 and IL-13 started to increase significantly at 4 h after injection (Fig 32B), while IL-6 started at 2 h but to a very small extent and no change was seen in IL-12. (Fig 32C) However, progranulin showed a steep rise at 2 h after injection and persisted over 48 h. (Fig 32B, 32C) We stimulated peritoneal macrophage with alum adjuvant directly to identify the source of progranulin, and we observed a large amount of progranulin in a dose dependent manner with the alum concentration. (Fig 32D)

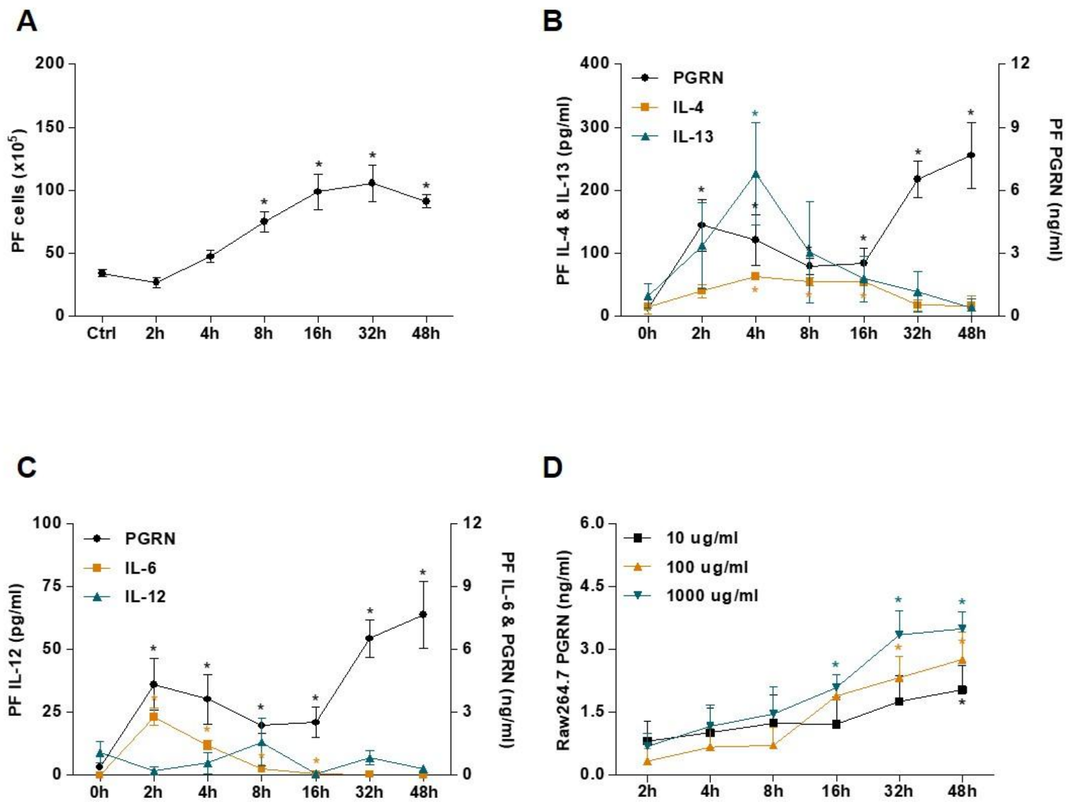


Figure 32. Intraperitoneal injection of alum induced increase in inflammatory cells, type 2 cytokine production along with progranulin.

The change in number of inflammatory cells in peritoneal fluid. (A) The change in IL-4, IL-13 and progranulin in peritoneal fluid. (B) The change in IL-6, IL-12 and progranulin in peritoneal fluid. (C) Progranulin production in peritoneal macrophage by stimulation of alum. (D) For all experiments, each group consisted of five mice. *P<0.05 relative to baseline value.

4. Discussion

In this present study, we demonstrated that progranulin is a molecule produced from alveolar macrophage in response to allergen exposure which in turn induced type 2 cytokine production from NKT and epithelial cells. In the absence of macrophage-derived progranulin in the airway, both innate and adaptive immune response were not switched on appropriately after HDM allergen exposure and replenishment of progranulin in the macrophage-derived progranulin deficient mice during sensitization period of HDM restored both immune responses.

This is the first mechanistic study of progranulin in asthma, which had first started with the idea if it could be a breakthrough for T_H2-low, T_H1 and/or T_H17 high subtype of asthma. Despite the efforts to develop new drugs that target T_H1 and/or T_H17 dominant asthma, for example, anti-TNF-α agents and anti-IL-17 monoclonal antibodies[45], none have documented significant success to date.[78] Recent studies on progranulin have suggested that it is a key regulator of inflammation and it mediates its anti-inflammatory effect by blocking TNF-α binding to its receptors.[69] The role of progranulin in the airway was evaluated in a series of studies, and we could learn that progranulin was induced by noxious stimuli (eg. lipopolysaccharide, cigarette-smoke extract) in BAL fluid and progranulin showed protective effect on development of subsequent injury. *Ungurs et al* have suggested that progranulin is an antagonistic substrate for proteases that lead to neutrophil degranulation.[74] These studies brought us to perform a preliminary study with serum samples from asthma patients, in which

we have suggested that progranulin may be an indicator of airflow obstruction and neutrophilia in asthma.[76]

Similar to the previous studies[73,75], progranulin was induced by HDM allergen exposure in BAL fluid and it was preceded by other cytokines, IL-4, IL-13, IL-6 and IL-12. Recombinant mouse progranulin was administered intranasally to determine the order of progranulin and cytokine production, and as a result, we found a prominent increase in the level of T_H2 cytokines, IL-4, IL-13, IL-33 and TSLP upon progranulin administration. Interestingly, there was no change in the level of IL-6 and IL-12 after administration of recombinant progranulin alone, and we could infer that progranulin itself may not directly affect T cell polarization to T_H1 or T_H17 cells, instead may induce polarization to T_H2 cells.

Progranulin is expressed in a wide range of tissues and cell types, which include epithelial cells, macrophages, fibroblasts and immune cells including T cells and dendritic cells.[71] The major resident cells of the airway consist of alveolar macrophage and NKT cells as the immune cells and epithelial cells as the structural cells, which are known to be the gatekeeper of the immune surveillance in the airway.[79] Thus, the production of progranulin was evaluated in these cells in this present study. We excluded other cell lines such as fibroblasts which also exist in the airway, as we did not consider them as the frontline barrier that react to allergen exposure. We found that progranulin was mainly produced from alveolar macrophage when stimulated with HDM allergen, while a relatively smaller amount of progranulin was measured from epithelial cells and a scarce amount from NKT cells. This served as the background for

our subsequent experiments determining the role of macrophage-derived progranulin in the development of immune responses after HDM allergen stimulation.

Of note, the expression of progranulin after HDM allergen stimulation in animal model was highest at 4 h. However, in the experiment with cell lines – macrophage, epithelial cell, and NKT cell – determining which one is the major source of progranulin production, the expression of progranulin was highest at 32 h. The difference can be explained by the different experimental settings as the latter one had not been performed in an animal model, in which progranulin from various sources could have accrued to reach its peak earlier.

It is known that type 2 cytokines are mainly produced from NKT cells and airway epithelial cells when the airway immune system respond to allergen sensitization.[80-82] As the next step, we aimed to identify the source of the cytokines produced after allergen sensitization by giving the same treatment of recombinant mouse progranulin to each cell type, NKT cell and epithelial cell. We found that IL-4 and IL-13 were produced from NKT cell, which are known as important cytokines in the development of allergen specific T_H2 cell in adaptive immune responses.[83] Also, IL-33 and TSLP were induced from epithelial cell, which are known to amplify local T_H2 immune responses by stimulating ILC2.[80,82] Although we did not confirm the direct effect of progranulin on amplification of T_H2 immune responses via stimulation of ILC2, we have shown that progranulin induced production of type 2 cytokines and that it could be associated with allergen specific T_H2 immune response.

We now demonstrated the effect of macrophage-derived progranulin in the airway milieu

after allergen exposure. Initially, innate immune response was evaluated in wild type mice and macrophage-derived progranulin deficient mice after a single HDM allergen sensitization. We found a marked decrease in the number of inflammatory cells and production of type 2 cytokines (IL-4, IL-13, IL-33 and TSLP) in BAL fluid of progranulin deficient mice compared with wild type after sensitization. Particularly, there was a prominent decrease in the number of macrophages and there was also a decrease in the number of neutrophils in the allergen sensitized progranulin deficient mice but the change was statistically insignificant. This may be due to the setting of a single sensitization with which evaluated early response at 16 h. The absence of progranulin did not influence the production of IL-6 and IL-12, similar to the findings obtained from the BAL fluid after recombinant mouse progranulin administration in wild type mice. We could only find changes in the production of IL-4 and IL-13 from the cells separated from lung tissue after stimulation of PMA/Ionomycin and we consider this is because the process of cell separation did not include epithelial cells. Collectively, the absence of macrophage-derived progranulin mitigated the inflammatory response to allergen sensitization, and the production of IL-6 and IL-12 may solely be the output of HDM allergen sensitization, independent of the presence of progranulin.

Taken together, our in vitro cell experiment and in vivo experiment of a single Der p sensitization, we showed that IL-4 and IL-13 were induced from NKT cell and this process was driven by the macrophage-derived progranulin after HDM sensitization. According to previous studies, NKT cell is a key producer of IL-4 and IL-13 in the airway, and exists

abundantly in about 20-40% of T cells from asthmatic lung.[84,85] Many studies underscored the importance of NKT cell in the development of Th2 airway inflammation using knock-out mice, however, the absence of NKT cell did not result in the elimination of airway inflammation perfectly.[86] Recently, a new cell population was discovered, innate lymphoid cells, ILCs. Among ILCs, ILC2 is known to be a potent producer of Th2 cytokines, such as IL-5 and IL-13, and were found in mucosa-related immune system.[80,87] Studies showed that allergen exposure of the airway induced IL-25 and IL-33, and stimulation of ILC2 by these cytokines resulted in enormous production of IL-5 and IL-33 in early sensitization period.[88,89] These evidence indicates that ILC2 is also important in fostering Th2 milieu by production of IL-5 and IL-13. And subsequent studies revealed that IL-4 was also induced by ILC2[90,91]. Although we showed that NKT cell is important in the development of Th2 milieu upon HDM stimulation and subsequent expression of progranulin by producing IL-4 and IL-13, we can assume that the cascade of these events may not have resulted owing solely to the NKT cells, but ILCs may also have taken part. This assumption certainly needs further studies to be proven.

The next part of our study was to demonstrate the effect of macrophage-driven progranulin on the adaptive immune response, after multiple Der p sensitizations. Again, the absence of progranulin resulted in a decreased inflammatory response to repeated allergen exposure, but this time prominent in the number of neutrophils and eosinophils. The results showed that the type 2 cytokines (IL-4, IL-13 and eotaxin) in BAL fluid, serum IgE levels were measured

lower in the progranulin deficient mice, and T cells from the lung tissue and the regional LNs also demonstrated a lower level of IL-4. Overall, these results verify that the absence of progranulin leads to a mitigated T_H2 response to allergen exposure. However, IL-17 measured from BAL fluid and from T cells of the lung and regional LNs and serum IgG1 did not show any change and IP-10 measured from BAL, serum IgG2c and IFN-γ from T cells were increased in the absence of progranulin. This may be explained by the effect of IL-6 and IL-12 induced by HDM exposure and possibly as a consequence of T_H2-low environment induced by the absence of progranulin, thus inducing T_H1-dominant milieu.

To verify the role of progranulin more specifically in the adaptive immune response after allergen exposure, we supplemented progranulin in the progranulin deficient mice at different time points, sensitization and challenge period. The Der P specific inflammatory responses were restored with progranulin supplement in the sensitization period as the type 2 cytokine levels (IL-4, IL-13 and eotaxin) in BAL fluid and serum IgE levels were increased. However, we could not see any augmentation of T_H2 immune response when progranulin was supplemented in the challenge period. Interestingly, eotaxin was also increased with progranulin supplement in the challenge period. We considered that this may have resulted from direct stimulation of epithelial and immune cells, rather than by the process of Der p specific adaptive immune response. Another point discovered was that progranulin supplement in the sensitization period lowered the level of IP-10 in BAL fluid and serum IgG2c that had been increased with progranulin deficiency. Overall, we have demonstrated the importance of

macrophage-derived progranulin induced by HDM allergen exposure, specifically in the sensitization period, on the development of allergen specific T_H2 adaptive immune response.

There are several methods of generating an animal model of asthma, which include HDM allergen administration as shown in the above experiments and the conventional OVA/alum model that utilizes peritoneal injection of aluminum hydroxide and ovalbumin. To test our results in the conventional OVA/alum model, we induced an asthma model in the macrophage-derived progranulin deficient mice and in wild type mice. As a result, we found a mitigated ovalbumin-specific T_H2 adaptive immune response in the progranulin deficient mice. Similar to previous findings, IL-17 and serum IgG1 levels were similar and other markers of T_H1 immune response (IP-10 in BAL, serum IgG2c, IL-17/IFN-γ from T cells of regional LNs) were increased in the progranulin deficient mice. Moreover, intraperitoneal progranulin was increased when exposed to OVA/alum and the source of progranulin was again found to be intraperitoneal macrophage. Collectively, our results were confirmed again in the OVA/alum model that macrophage-derived progranulin is important in the generation of T_H2 immune response.

We obtained interesting results most of what was not expected from our assumptions based on studies of acute lung injury and COPD. On HDM allergen exposure, progranulin was produced mainly from macrophage and was more associated with T_H2 inflammatory response, sequentially enhancing production of type 2 cytokines in BAL fluid and tissue inflammation on histopathology. In our asthma model generated by HDM and OVA/alum, progranulin did

not show a protective effect on airway inflammation. Progranulin is composed of repeats of a cysteine-rich motif and can be digested into granulin peptides by MMP-9, 12, and 14, neutrophil elastase and proteinase 3,[71] which stimulates IL-8 expression to enhance neutrophilic inflammation[53]. The digestion into granulin peptides depend on the environment that it exists, and therefore, progranulin can express either of the two contrasting effects; pro-inflammatory or anti-inflammatory.[71] In this experiment, we used a recombinant mouse progranulin in its full form, however, we are not yet aware of its final form when it starts its function in the airway on HDM allergen exposure. Moreover, the restricted condition of inducing asthmatic condition with Der p antigen in a single dose that led us to conclude the source of progranulin to be alveolar macrophage could have been another explanation. If we were to stimulate epithelial cells to secrete progranulin, probably in a larger amount and within a different cytokine milieu, progranulin could have demonstrated the opposite functions. In this regard, further studies are needed to simultaneously evaluate the expression of granulin and progranulin as well as the proteinases likely to exist in the setting of different levels of HDM allergen exposure.

In future studies, the cytokines evaluated for innate immune response should include TNF-α, IL-1, IL-2, and reactive oxygen species. Progranulin knockout mice, other than macrophage-specific progranulin deficient mice should be adopted to investigate the development of asthma to better define its role in the pathogenesis of allergic asthma. We also need to extend our study to a neutrophilic asthma model to better understand the biology of

progranulin in asthma and to understand the results of this present study.

Collectively, progranulin was induced by HDM allergen exposure from alveolar macrophage, which sequentially induced type 2 cytokine production from NKT and epithelial cells. The presence of macrophage-derived progranulin in the airway was important for the generation of T_H2 immune response on HDM allergen exposure, especially during the sensitization period. The clinical significance of our findings needs further laboratory research. (Fig 33)

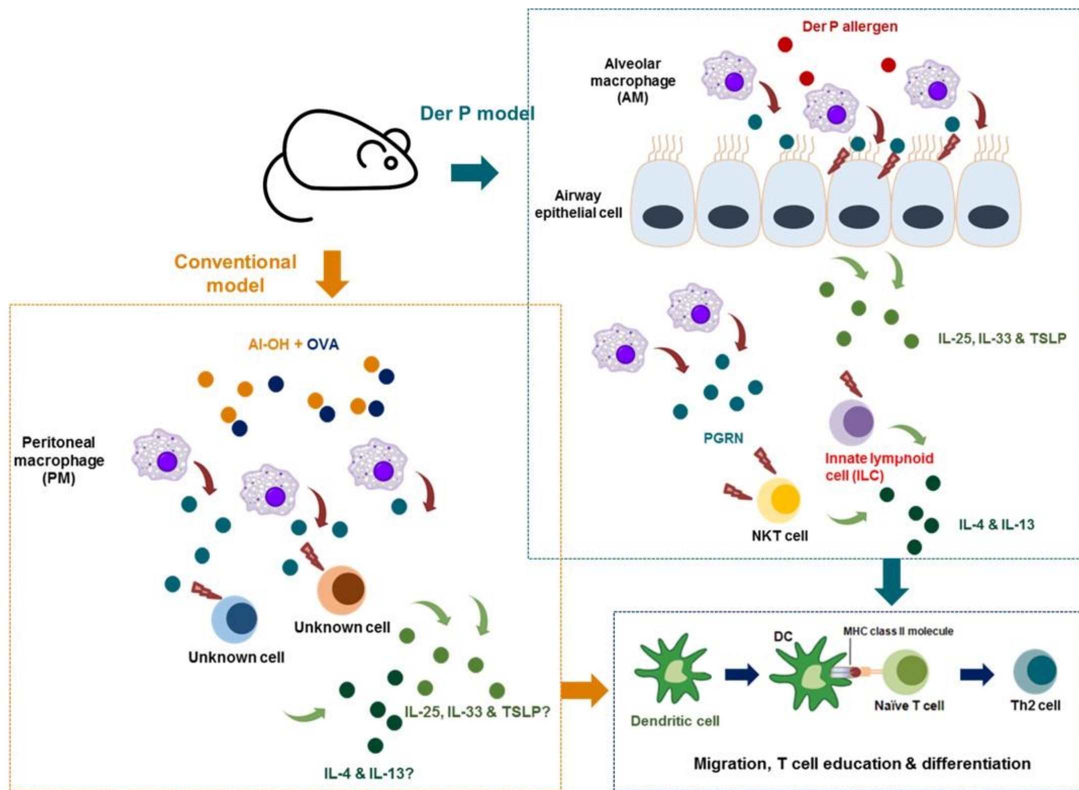


Figure 33. Experimental summary and proposed mechanism of development of T_H2 immune response by macrophage-derived progranulin.

Allergen stimulation with either Der p or OVA/alum induced progranulin secretion from macrophage. Macrophage-derived progranulin then induced production of IL-4 and IL-13 from NKT cells and IL-33 and TSLP from airway epithelial cells, possibly further enhanced through ILC2 stimulation. Subsequently, these cytokines are expected to elicit naïve T cells to polarize to T_H2 cells.

5. Conclusion

In conclusion, our current findings indicate that macrophage-derived progranulin has a role in the induction of T_H2 immune response after HDM allergen exposure, especially progranulin produced in the early sensitization period of HDM stimulation. The clinical significance of our findings needs further laboratory research.

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7. Korean abstract

알레르기성 기도염증에서 대식세포 유래 progranulin 의 역할

기관지 천식은 호흡기에 발생하는 대표적인 기도 질환으로 만성 염증과 가역적 기도 폐쇄와 기도 과민성이 관찰된다. 기존의 천식 치료는 염증 반응을 비특이적으로 억제하는 흡입 스테로이드제가 근간이 되고 있다. 하지만, 이러한 비특이적인 염증의 억제로 증상이 잘 조절되지 않는 난치성 천식 환자군이 드러남에 따라 천식은 한 가지 병인으로 설명 가능한 한 가지 질환이 아닌, 다양한 병태 생리와 그에 상응하는 임상 표현형을 가지는 heterogeneous 한 질병이라는 인식이 널리 받아들여지고 있다. 이에 새로운 천식 치료 전략으로 병태 생리에 기반하여 특정 염증 반응을 억제하는 생물학적 치료가 주목받기 시작하였다.

저자는 progranulin 을 천식의 새로운 생물학적 치료의 바이오마커 후보로 제시하게 되었다. 그 근거로, 급성 폐손상 및 폐기종 동물 모델에서 progranulin 이 항염증 역할을 가진다는 것이 밝혀진 바 있고, 천식 환자를 대상으로 한 임상 연구로부터 progranulin 농도가 기도 폐쇄의 정도와 혈액 호중구 수치와 연관성을 보였다는 점을 들었다. 하지만, 알레르기 천식에서 progranulin 이 정확히 어떤 역할을 하는지, 어떤 기전을 통해 기능을 수행하는지 알려진 바가 없어, 본 연구에서는 천식에서 progranulin 의 면역학적 역할 및 기전을 알아보고자 하였다.

먼저, 알레르겐 노출 시 progranulin 에 어떠한 영향이 있는지 알아보기 위해, 집먼지 진드기 추출물을 마우스 모델에 비강 주입하였고 기관지세척액에서 측정한 progranulin 농도가 type 1, type 2 사이토카인에 선행하여 상승하는 것을 확인하였다. Progranulin 이

주로 생성되는 세포는 대식세포임을 확인하였고, recombinant mouse progranulin 을 마우스 모델에 비강 주입 시 대식세포, 호중구, type 2 사이토카인 증가를 보였다. Type 2 사이토카인은 NKT 세포 및 기도 상피세포로 분비됨이 확인되었다.

대식세포 유래 progranulin 의 역할을 알아보기 위해 대식세포 유래 progranulin 의 생성이 저하되는 유전자 조작 마우스를 이용하였다. 대식세포 유래 progranulin 생성 저하 마우스에서는 집먼지 진드기 자극이 선천 면역 및 후천 면역 반응을 유도하지 못했고, recombinant mouse progranulin 을 집먼지 진드기 자극의 초기 단계인 감작 시기에 보충하였을 때 두 가지 면역 반응이 모두 발생하게 됨을 확인하였다.

따라서, 대식세포 유래 progranulin 은 집먼지 진드기 자극에 따르는 T_H2 면역 반응의 생성에 중요한 역할을 하며, 추가적인 연구를 통해 구체적인 면역학적 기전을 밝혀야 할 것이다.

핵심어: progranulin, 대식세포, 천식