

Development of *Aspergillus* protease with ovalbumin-induced allergic chronic rhinosinusitis model in the mouse

Ji Heui Kim, M.D., Ph.D., Jong Sook Yi, M.D., Chang-Hoon Gong, B.S., and Yong Ju Jang, M.D., Ph.D.

ABSTRACT

Background: Chronic rhinosinusitis (CRS) is a multifactorial inflammatory disease. Particularly, eosinophilic CRS is often recalcitrant to treatment, so an appropriate animal model is required to evaluate the pathogenesis of, and to develop therapies for, recalcitrant eosinophilic CRS. This study aimed to improve the ovalbumin (OVA)-induced mouse model of eosinophilic/allergic CRS by combining OVA with *Aspergillus* protease, which is known to trigger allergic reactions in mouse lungs.

Methods: In a model of allergic CRS, mice were challenged intranasally with *Aspergillus* protease combined with OVA. Local and systemic responses were measured. Protease (0.54 U) from *Aspergillus oryzae*, prepared with or without OVA (75 micrograms), OVA alone, or saline, was administered intranasally to wild-type mice for 5 weeks. Sinonasal complex samples were evaluated histologically, and interleukin (IL)-4, IL-5, IL-6, macrophage inflammatory protein (MIP) 2, and tumor necrosis factor (TNF) alpha were measured in nasal lavage fluid. A differential white blood cell count was also performed.

Results: OVA alone induced minimal eosinophilic inflammation in sinonasal mucosa, while protease + OVA and protease alone induced moderate eosinophilic inflammation. Protease + OVA elevated eosinophil counts in blood comparable with controls, but not compared with OVA alone. Although IL-4, IL-5, IL-6, MIP-2, and TNF-alpha were increased in all study mice, the levels of IL-4 and IL-6 were higher in mice treated with protease + OVA than in mice treated with OVA alone. Protease alone excessively elevated the levels of IL-6, MIP-2, and TNF-alpha, not Th2 cytokines, compared with OVA alone and protease + OVA.

Conclusion: *Aspergillus* protease combined with OVA induced more severe allergic inflammation in sinonasal mucosa compared with OVA alone and similar eosinophilia. This model could be more relevant to recalcitrant eosinophilic CRS in humans than OVA-induced allergic CRS.

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Chronic rhinosinusitis (CRS) is not simply an infection, but a multifactorial inflammatory disease characterized by heterogeneous clinical features, histopathology, and therapeutic response.^{1,2} Several studies have examined the pathogenesis and pathophysiology of CRS using animal models such as rabbit and mouse.^{3–8} A mouse model of CRS has several advantages compared with a rabbit model, including the potential for genetic manipulation, use of various murine reagents, and lower housing costs.⁴ To date, inhalation of ovalbumin (OVA) or extracts of *Aspergillus fumigatus* were used to establish the allergic or eosinophilic CRS mouse models,^{4–8} and *Streptococcus pneumoniae* inoculation plus Merocel (Medtronic-Xomed, Jacksonville, FL) ostiomeatal obstruction was used for a mouse model of bacterial CRS.⁴

To mimic allergic or eosinophilic CRS, mice are sensitized intraperitoneally and challenged intranasally with OVA to induce allergic inflammation in the sinonasal cavity.^{4–6} In asthmatic mice, however, the inhalation of OVA alone does not elicit a broad spectrum of airway-allergic change or overcome airway tolerance if given in the absence of adjuvant.^{9,10} Moreover, OVA does not cause CRS in humans. Inhalation of fungi, including *Alternaria*, *Cladosporium*, and *Aspergillus*, is involved in human allergic-airway diseases such as asthma and allergic rhinitis.¹¹ In particular, activation of the protease-activated receptor (PAR) by proteases from fungi is responsible for allergic inflammation in the upper airway,

such as in rhinitis and nasal polyps (NPs).¹² Fungal proteases may be more specific triggers of allergic CRS. In an asthmatic mouse model sharing many histological and immunologic features with eosinophilic CRS,^{13,14} fungal proteases combined with OVA readily induced allergic inflammation, including an increase in airway eosinophils and Th2 cells. This model also showed termination of the induction of airway tolerance and the establishment of allergic lung disease.¹⁰

Eosinophilic CRS is distinguished by prominent accumulation of eosinophils and a marked increase in the expression of Th2 cytokines.¹³ One previous clinical study showed that the Lund-Mackay scores of preoperative computed tomography ≥ 7 were significantly more frequent in eosinophilic CRS with NPs than in eosinophilic CRS without NPs and noneosinophilic CRS with/without NPs.¹⁵ Eosinophilic inflammation in CRS is often recalcitrant to medical and surgical intervention,^{16,17} so an appropriate animal model is required to research the pathogenesis of, and to develop therapies for, recalcitrant eosinophilic CRS. Here, we propose the addition of protease from *Aspergillus oryzae*, a potent respiratory allergen, to the OVA-induced allergic CRS mouse model. This model may reflect more relevantly the pathogenesis of recalcitrant eosinophilic CRS in humans compared with the previous model using OVA alone.

METHODS

Mice and Allergens

BALB/c mice (6–7 weeks old) were purchased from Central Laboratory Animals (Seoul, Korea). Groups of mice were challenged intranasally with phosphate-buffered saline (PBS; three mice), OVA alone (three mice), protease from *A. oryzae* combined with OVA (five mice), and protease alone (five mice). In the OVA-alone group, mice were sensitized with an intraperitoneal injection of 25 μ g of OVA (grade V; Sigma, St. Louis, MO) precipitated in alum on days 0 and 4, followed by intranasal challenge (from day 7 to 11) with 75 μ g of

From the Department of Otolaryngology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

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Address correspondence to Yong Ju Jang, M.D., Ph.D., Department of Otolaryngology, Asan Medical Center, University of Ulsan College of Medicine, 88 Olympic-ro 43-gil, Songpa-gu, Seoul 138-736, Republic of Korea

E-mail address: jangyj@amc.seoul.kr

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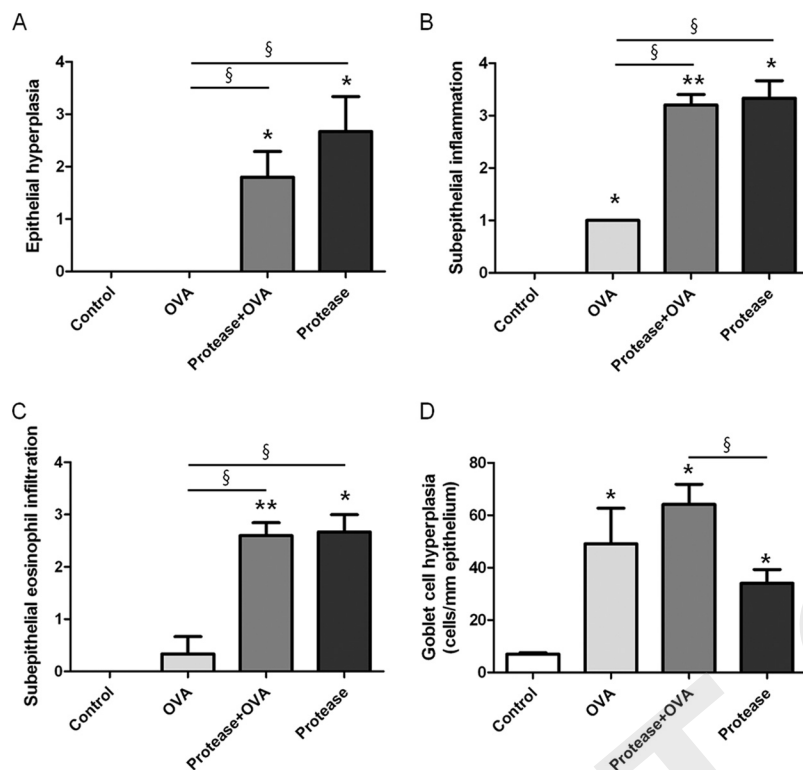


Figure 1. Comparison of histological findings between each group. The values of * $p < 0.05$ and ** $p < 0.01$, compared with the control; § $p < 0.05$.

OVA diluted in 30 μL of sterile PBS. The mice were then exposed to 75 μg of OVA intranasally three times a week for 5 weeks.

The protease + OVA mixture was prepared by centrifugation. A preliminary experiment was conducted to determine the appropriate dose of protease with OVA. Two mice at each dose received 75 μg of OVA mixed with 2.7, 1.35, and 0.54 U of protease from *A. oryzae* without presensitization of OVA, respectively. A dose of 0.54 U was established and used in the protease + OVA group. Mice were challenged intranasally with a mixture of 0.54 U of protease from *A. oryzae* (Sigma) and 75 μg of OVA diluted in sterile PBS to a total volume of 30 μL three times a week for 5 weeks.

The protease-alone and control groups were administered with 0.54 U of protease diluted in sterile PBS to a total volume of 30 and 30 μL of nasal saline solution at the same time intervals, respectively. The experiment was continued for 5 weeks, allowing sufficient time to induce chronic inflammatory changes in the CRS mouse model.^{7,8,18} All experiments were conducted according to the Guidelines of the National Institute of Health and were approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Science (2013-12-043).

Histological Analysis

Mice were killed and decapitated after being anesthetized. Skulls were fixed in 10% neutral-buffered formalin for 24 hours, followed by decalcification and embedding in paraffin. Paraffin-embedded sinonasal cavity tissues were sectioned coronally at a thickness of 4 μm and stained with hematoxylin and eosin, Congo red, and periodic acid-Schiff.^{4,5} Histological analysis was performed by a pathologist in the Department of Pathology, Asan Medical Center, who was blind to the group assignments. Epithelial hyperplasia, subepithelial inflammation, and subepithelial eosinophil infiltration were evaluated in hematoxylin and eosin and Congo red-stained sections. The grade of each parameter was scored on a scale of none (0), minimal (1), mild (2), moderate (3), to severe (4). Minimal was defined as barely detectable, mild as slightly detectable, moderate as easily detectable, and

severe as very evident.⁷ Goblet cells were evaluated in periodic acid-Schiff-stained sections and recorded as cells per millimeter of epithelium.

Nasal Lavage

A 22G catheter was inserted into the tracheal opening in the direction of the choana after a partial tracheal resection under anesthesia.⁴ Then, 0.5 mL of normal saline was gently introduced into the nasal cavity, and the fluid from the nostril was collected. Interleukin (IL)-4, IL-5, IL-6, macrophage inflammatory protein (MIP) 2, and tumor necrosis factor (TNF) α concentrations in nasal lavage fluid were measured with enzyme-linked immunosorbent assay kits (R&D systems, Minneapolis, MN) in accordance with the manufacturer's protocols. Because the nasal lavage fluid from OVA, protease + OVA, and protease groups showed similar stickiness to that from the control group, the additional procedure to dissolve the mucin was not performed.

White Blood Cell Counts

Blood samples were taken by cardiac puncture after the death of the mice. A differential cell count was then performed with Wright-Giemsa staining. The absolute numbers of lymphocytes, neutrophils, monocytes, and eosinophils were obtained as: (percentages of each leukocyte subtype in five randomly selected high-power fields) $\times$ (total number of cells recovered from the blood).

Statistical Analysis

Data are expressed as the mean $\pm$ SEM. IBM SPSS Statistics for Windows, Version 20.0 (IBM, Armonk, NY) was used to analyze data. All parameters were compared between the groups using Mann-Whitney *U* tests (two-tailed). A value of $p < 0.05$ was considered statistically significant.

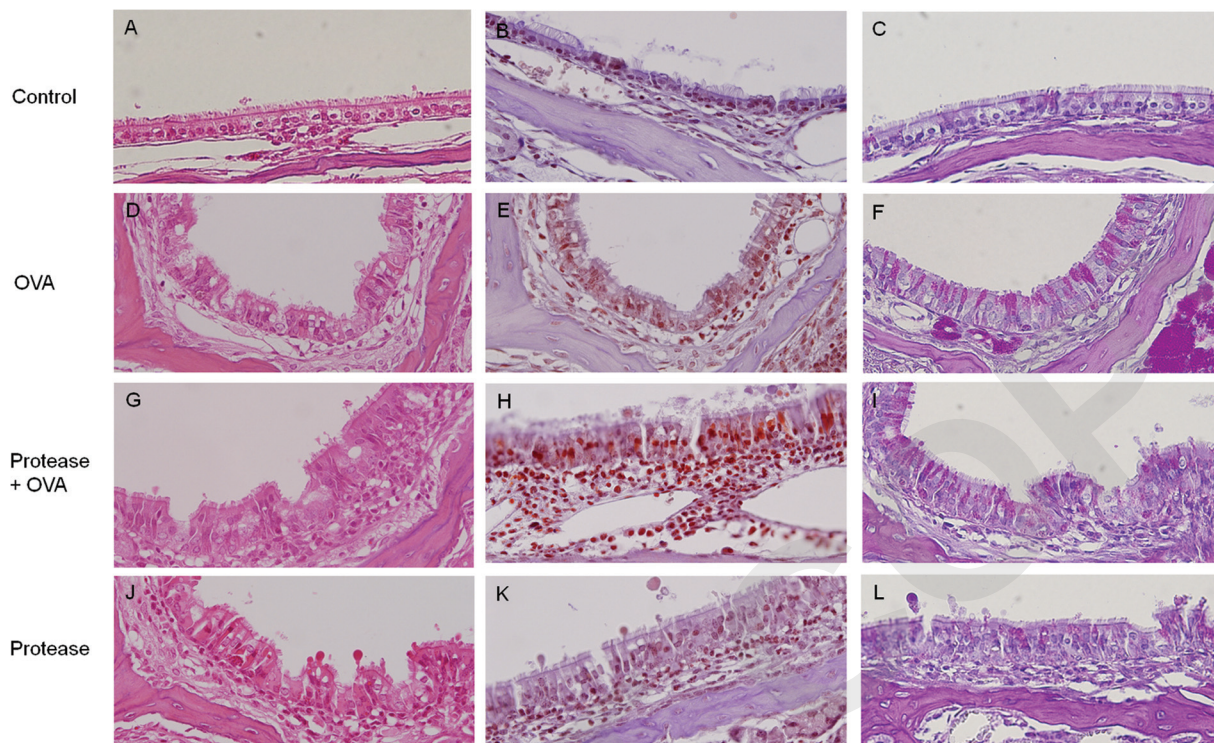


Figure 2. Epithelial hyperplasia, inflammatory cell infiltration, and goblet cell hyperplasia in different groups. (A, D, G, and J) Representative photographs of hematoxylin and eosin (B, E, H, and K) Congo red, and (C, F, I, and L) periodic acid-Schiff staining in (A–C) control, (D–F) OVA, (G–I) protease + OVA, and (J–L) protease groups (original magnification, $\times 400$).

RESULTS

Mortality

Two mice, one challenged with 75 μg of OVA mixed with 2.7 U of protease and one with 75 μg of OVA mixed with 1.35 U of protease, died during the preliminary experiment to determine protease dose with OVA. Histological findings from the surviving mice showed moderate eosinophilic inflammation, hyperplasia, and subepithelial inflammation regardless of protease dosage. Consequently, 0.54 U of protease is sufficient for the development of eosinophilic CRS. However, two of the five mice challenged with 0.54 U of protease alone died, indicating that administration of protease alone may be lethal.

Chronic Sinonasal Inflammation

Figures 1 and 2 show the histology and scoring of the different groups. Epithelial hyperplasia was detected only in the protease + OVA and protease groups. Subepithelial inflammation and eosinophil infiltration were observed to be greater in the OVA, protease + OVA, and protease groups than in the control group ($p < 0.05$ for all). However, their severity was higher in the protease + OVA and protease-alone groups than in the OVA-alone group ($p < 0.05$ for all). Hyperplasia of goblet cells was more prominent in the OVA, protease + OVA, and protease groups than in the control group ($p < 0.05$ for all). Goblet cell hyperplasia was lower in the protease group than in the protease + OVA mice ($p = 0.034$).

In the nasal lavage fluid, IL-4, IL-5, IL-6, MIP-2, and TNF- α were significantly increased in the OVA, protease + OVA, and protease groups compared with the control group ($p < 0.05$ for each). However, some cytokines were more highly elevated in the protease + OVA and protease groups than in the OVA group. The levels of IL-4 and IL-6 were significantly higher in the protease + OVA group than

in the OVA group ($p < 0.05$ for each), and the levels of IL-6, MIP-2, and TNF- α were significantly greater in the protease group than in the OVA group ($p < 0.05$ for each; Fig. 3).

Differential Cell Counts in the Blood

Total leukocyte, lymphocyte, neutrophil, and eosinophil counts were significantly increased in the OVA, protease + OVA, and protease groups compared with the control group ($p < 0.05$ for each). However, there were no differences in the total leukocyte, lymphocyte, neutrophil, and eosinophil counts of the protease + OVA and protease groups compared with the OVA group (Fig. 4).

DISCUSSION

We successfully established a mouse model of allergic CRS induced by protease from *A. oryzae* combined with OVA, which showed more severe eosinophilic inflammation and more excessive secretion of IL-4 in the sinonasal tissue compared with administration of OVA alone.

Fungi have been generally considered to be inhalant allergens and are also CRS-causing agents.¹¹ Immunophenotyping of noninvasive fungal rhinosinusitis showed a predominant T-cell population, which may be induced by fungal-activated eosinophils.¹⁹ One previous study reported that intranasal application of antifungal agents significantly reduced the inflammatory sinonasal mucosa thickening,²⁰ although topical antifungal agents had no significant effect on activation markers of nasal inflammatory cells in another study of CRS.²¹ A mouse model of eosinophilic CRS using mycelial extracts of *A. fumigatus* was previously developed.^{7,8} The mechanism of *Aspergillus*-induced eosinophilic CRS was plausibly explained as allergic inflammation by fungal protease. One *in vitro* study indicated that production of IL-6 and IL-8 and morphological changes in A549 cells incubated with various concentrations of *A. fumigatus* was dependent

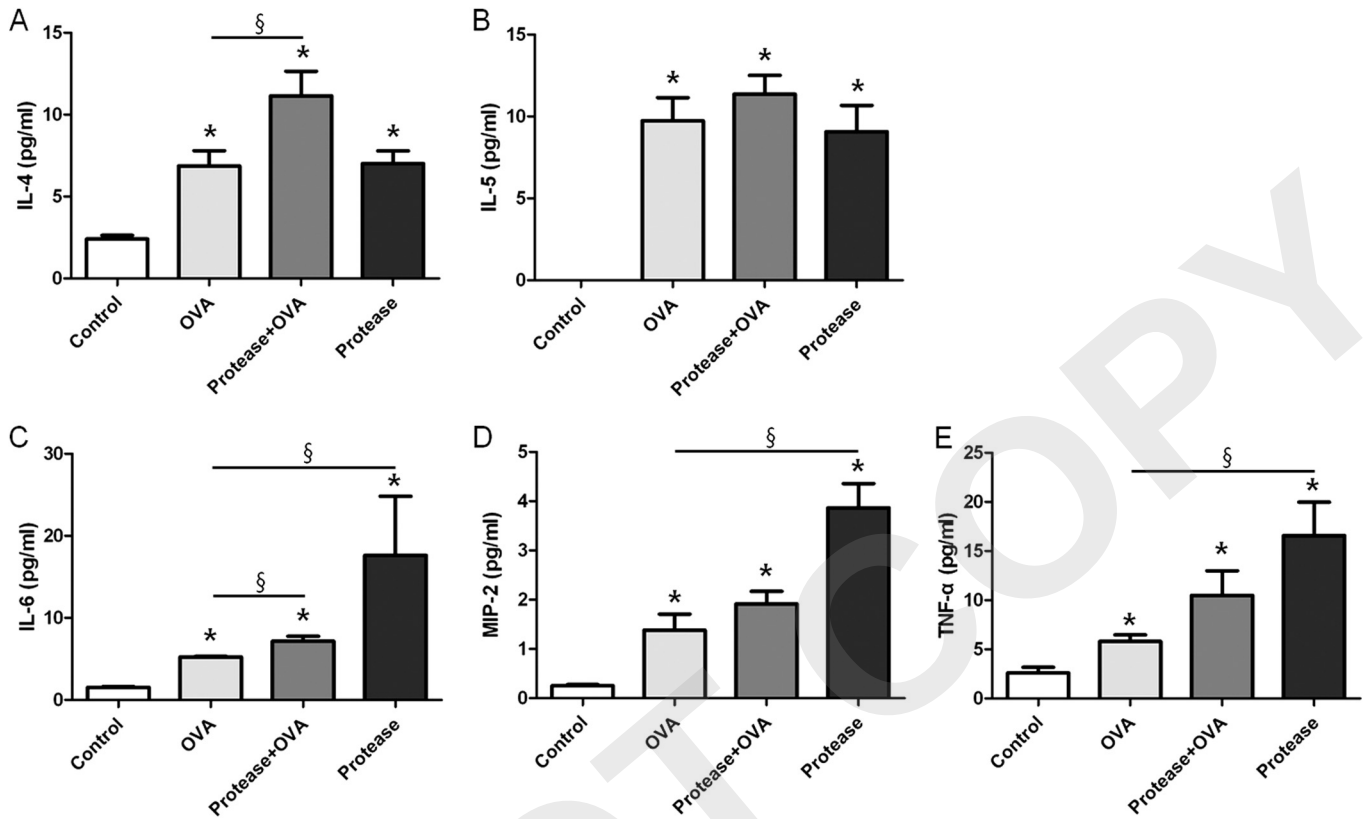


Figure 3. Cytokines in the nasal lavage fluid. The value of * $p < 0.05$, compared with the control; § $p < 0.05$.

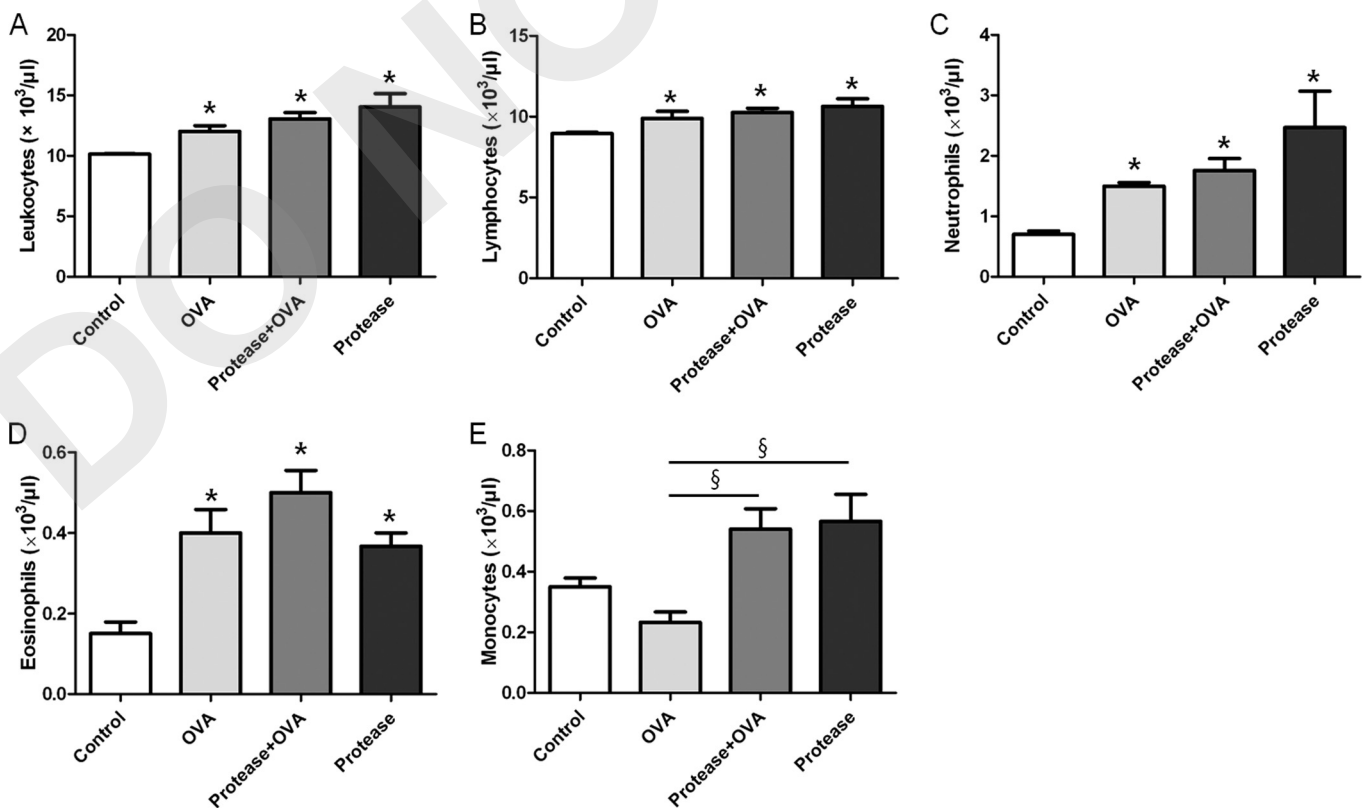


Figure 4. Comparison of white blood cell counts between the different study groups. The value of * $p < 0.05$, compared with the control; § $p < 0.05$.

on protease activity.²² Consequently, eosinophilic CRS induced by *A. fumigatus* extracts was probably generated by protease from *Aspergillus*. In accordance with this hypothesis, our results showed eosinophilic sinonasal inflammation and elevation of proinflammatory cytokines IL-6, TNF- α , and neutrophil chemokine MIP-2, the functional murine analog of human IL-8, in mice with CRS induced with protease + OVA.

Kheradmand and colleagues used protease from *A. oryzae* with OVA every 4 days for five total challenges to induce experimental Th2 cell-dependent allergic lung disease in the mouse.¹⁰ Protease from *A. oryzae* with OVA yielded induction of airway hyperresponsiveness, bronchoalveolar lavage eosinophilia, and histological changes of mouse lung, unlike both inactivated protease and OVA alone without alum. In addition, IL-4-producing cells and eosinophils were detected in lungs of mice receiving active *Aspergillus* protease but not OVA alone. These results were consistent with our findings that protease from *A. oryzae* combined with OVA generated more severe chronic eosinophilic sinonasal inflammation, including epithelial hyperplasia, subepithelial eosinophil infiltration, and goblet cell hyperplasia, than OVA alone. Furthermore, significantly more IL-4, a predominant Th2 cytokine, was produced in the sinonasal mucosa of mice receiving protease with OVA than in those receiving OVA alone. Goblet cell hyperplasia may be associated with IL-17A⁺ inflammatory cells, which could be important to mucus secretion in CRS with NPs, including eosinophils.²³ These results suggest that our model could be regarded as more relevant to recalcitrant eosinophilic CRS in humans compared with a previously established mouse model of OVA-induced allergic CRS.

Interestingly, in the present study there were no differences in the counts of total leukocytes in blood, including eosinophils, between the mice challenged with protease + OVA and those with OVA alone. This finding indicates that protease + OVA may cause more severe eosinophilic sinonasal inflammation locally, but similar systemic eosinophilia compared with OVA alone. One previous study reported that when allergenic protease from *A. oryzae* was added to OVA, but not OVA alone, it induced recruitment of Th2 cells and eosinophils to the lung independently of the signal transducer and activator of transcription 6 (STAT6), which was thought to be required for homing of Th2 cells to the lung.²⁴ Similarly, instillation of protease from *A. oryzae* combined with OVA into the sinonasal cavity may induce recruitment of IL-4-producing Th2 cells to the sinonasal tissue through an unrecognized innate immune signaling mechanism.

Unexpectedly, repetitive administration of protease alone was relatively lethal to mice in our study, despite the very low dose. The very high levels of IL-6, MIP-2, and TNF- α in their nasal lavage fluid reflected the occurrence of serious airway injury from protease alone. Moreover, the lungs of mice receiving protease alone sustained damage, revealing abundant inflammatory cell infiltrates and disrupted bronchoalveolar structure (data not shown). These *in vivo* results support the past *in vitro* studies, showing that large amounts of proteases produced by *Aspergillus* directly induce IL-6 and/or IL-8 production and subsequent epithelial cell detachment.¹² IL-6 is known to induce the differentiation of Th17 cells and have some role in skewing to Th2 response through suppression of regulatory T cells.^{25,26} Th17 cells appeared to play a critical role in eosinophil migration into the airway in mice after repeated *A. fumigatus* challenge.²⁷ In the present study, although the protease group showed higher levels of IL-6 and lower levels of IL-4 compared with the protease + OVA group, two groups seemed to show similar severity of eosinophilic inflammation. Therefore, our results suggest that IL-6-induced up-regulation of Th17 response may be associated with the pathogenesis of eosinophilic CRS.

The protease + OVA-induced eosinophilic CRS model in the mouse offers some advantages. The cost of protease from *Aspergillus* is much lower than extract of *Aspergillus*. Mixing protease and OVA reduces the stickiness of OVA, which allows easy inha-

lation and ameliorates the lethality of protease alone. PAR-mediated eosinophilic inflammatory and Th2-dominant immune responses in the sinonasal tissue could be specifically investigated and may be modulated through the direct stimulation of PAR by *Aspergillus* protease. Furthermore, this CRS model may be helpful for exploring potential therapeutic agents for recalcitrant eosinophilic CRS.

CONCLUSION

We developed an eosinophilic CRS model induced by protease from *Aspergillus* combined with OVA in the mouse. The high levels of IL-4 in the nasal lavage fluid show the usefulness of this model in studying the pathogenesis of eosinophilic CRS associated with a typical Th2-skewed inflammation.

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