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Role of NOD1-Mediated Signals in a Mouse Model of Allergic Rhinitis

Ji-Hyeon Shin, MD¹*, Soo Whan Kim, MD, PhD¹*, and Yong-Soo Park, MD, PhD¹

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Abstract

Objectives. The purpose of the present study was to investigate the effect of nucleotide-binding oligomerization domain 1 (NOD1), an innate immune sensor, on allergic inflammation and induction of regulatory T cells in a mouse model of allergic rhinitis. We also aimed to explore whether there were differences in the effect of NOD1 ligand according to the timing of administration.

Study Design. An in vivo study using an animal model.

Setting. Catholic Research Institutes of Medical Science.

Subjects and Methods. Forty BALB/c mice were divided into 4 groups: control, OVA, pre-NOD1, and post-NOD1. Ovalbumin (OVA) was used for sensitization and challenge. The pre-NOD1 group received NOD1 ligand intranasally before sensitization, whereas the post-NOD1 group received it after sensitization. The effects of allergic inflammation and regulatory T cells were compared among the groups.

Results. In the post-NOD1 group, serum OVA-specific IgE, eosinophil counts, interleukin (IL)-13 levels, and GATA-3 mRNA expression were significantly increased and Foxp3⁺ mRNA expression and CD4⁺ Foxp3⁺ T cells were decreased compared with the OVA group. In the pre-NOD1 group, Foxp3 mRNA expression and CD4⁺ Foxp3⁺ T cells were significantly decreased compared with the OVA group. Although not significant, the pre-NOD1 group showed increases in serum OVA-specific IgE, eosinophil counts, IL-13 levels, and GATA-3 mRNA expression compared with the OVA group.

Conclusion. The innate immune response through NOD1 enhances allergen-specific Th2 response and suppresses induction of regulatory T cells in a mouse model of allergic rhinitis, and the effects are different depending on the timing of exposure to NOD1 ligand.

Keywords

allergic rhinitis, mouse model, NLR, NOD1, regulatory T cells, Th2 cells

Allergic rhinitis is a symptomatic disorder of the nose induced by IgE-mediated inflammation after allergen exposure, and its symptoms include sneezing, itching, nasal obstruction, and rhinorrhea.¹ The prevalence of allergic rhinitis has rapidly increased, especially in developed countries.² This tendency could be explained by the “hygiene hypothesis” that infection and unhygienic contact may confer protection from development of allergic illness.³ The initial interpretation proposed a missing immune deviation of allergen-specific responses from type 2 helper T (Th2) cell-mediated immunity to type 1 helper T (Th1) cell-mediated immunity. More recently, the importance of insufficient regulatory T (Treg) cell activity has been emphasized.^{4,5} Many epidemiologic and experimental studies support this hypothesis.^{6–8}

However, there is increasing recognition that infectious agents could inversely promote the development of allergic disease.^{9–13} In a murine model, moderate but not severe bacterial infection induces allergic sensitization to inhaled antigen.⁹ A cohort study demonstrated that viral infection with atopy in infancy promoted later asthma.¹² These findings suggest that the relationship between microbial exposure and asthma pathogenesis may depend on several variables, including geographic location, genetics, and immune system interactions.¹³

These infectious agents contain pathogen-associated molecular patterns (PAMPs) recognized by pattern recognition receptors (PRRs). Several families of PRR, including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), RIG-I-like receptors (RLRs), and DNA receptors, play a crucial role in

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host defense. Among them, NLRs are a specialized group of intracellular receptors.^{14,15}

The most well-known members of NLR are NOD1 and NOD2. Both have been shown to be associated with allergic disorders and allergen-specific IgE levels.^{16–19} In addition, many studies have demonstrated that these ligands are involved in the induction of Th2 response.^{20–25} Particularly, NOD1 has been suggested to be involved in the pathogenesis of allergic diseases. Therefore, as TLR-based therapeutics such as TLR4 and TLR9 agonists are in development for allergy,^{26,27} we hypothesized that NOD1 also can be considered a target of treatment for allergic rhinitis.

In the present study, we investigated the effect of NOD1 ligand on allergic inflammation and the induction of Treg cells in a mouse model of allergic rhinitis. We also explored whether there were differences in the effect of NOD1 ligand according to the timing of its administration. For this purpose, we compared the effects of NOD1 ligand before and after sensitization.

Methods

Experimental Animals

Six-week-old female BALB/c mice (weight, 20–30 g; Orient Bio, Seoul, Korea) were used in this study. The experiment was performed with the approval of the Institutional Animal Care and Use Committee at the Catholic University of Korea (#2010-0151-02).

Production of the Allergic Rhinitis Model

Forty mice were randomized into 4 groups: control (n = 10), OVA (n = 10), pre-NOD1 (n = 10), and post-NOD1 (n = 10). As we had no a priori knowledge of the expected outcome in numerical value, no statistical sample size determination was performed. When we determined the sample size in this study, we referred to the previous studies.^{28,29} The design of this study was modified from previous reports.^{29–31} Because BALB/c mice produce a strong IgE response to ovalbumin (OVA), we used OVA as the allergen. The induction of allergic inflammation is summarized in **Figure 1**. Briefly, on days 7, 14, and 21, all mice except those in the control group were immunized by an intraperitoneal injection of 20 μ g OVA (grade V; Sigma-Aldrich, St Louis, Missouri) and 2 mg aluminum hydroxide (Sigma-Aldrich). Two weeks after sensitization, all sensitized mice were intranasally challenged with 200 μ g OVA for 5 consecutive days. The control group received phosphate-buffered saline (PBS) instead of OVA.

NOD1 Ligand Administration

Before sensitization, mice in the pre-NOD1 group intranasally received 0.01 mg NOD1 agonist (lauroyl- γ -D-glutamyl-meso-diaminopimelic acid [C12-iE-DAP]; InvivoGen, San Diego, California). After sensitization, mice in the post-NOD1 group intranasally received 0.01 mg NOD1 agonist (**Figure 1**).

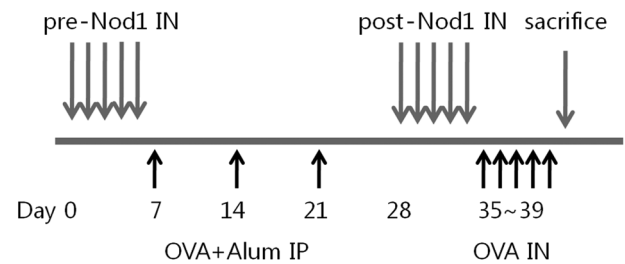


Figure 1. Schematic representation of administration of NOD1 ligand and induction of allergic inflammation. Al(OH)₃, aluminum hydroxide; IN, intranasal administration; IP, intraperitoneal injection; Nod, nucleotide-binding oligomerization domain; OVA, ovalbumin.

Evaluation of Allergic Symptoms

The number of sneezing and nose-rubbing motions during the 15 minutes after the final allergen challenge was recorded and compared among the experimental groups by blinded observers.

Nasal Lavage Fluid

Using ether anesthesia, thoracotomy was performed on the mice 24 hours after the final challenge. Nasal lavage fluid (NALF) was obtained after tracheal resection using 22-gauge catheters, inserted into the tracheal opening into the nasopharynx. The nasal passages were perfused with 1 mL PBS from the choana to the nostril, and NALF was collected from the nares. The NALF was centrifuged at 1500 rpm for 5 minutes, and the supernatant was aliquoted and stored at -70°C for detection of cytokines.

Nasal Mucosa Tissue Evaluation

After the collection of NALF, the mice were sacrificed and decapitated. The heads were fixed in 4% paraformaldehyde, decalcified with Calci-Clear Rapid (National Diagnostics, Atlanta, Georgia), dehydrated with alcohol, and embedded in paraffin blocks. The blocks were sliced into 4- μ m-thick sections and stained with hematoxylin and eosin to evaluate the number of eosinophils in the lamina propria. The average number of eosinophils was counted in 4 areas around the nasal septa by an individual blinded to the animals' group assignments.

Cytokines in NALF and OVA-Specific IgE in Serum

To evaluate the allergic reaction with the NALF (the supernatant), interferon (IFN)- γ for Th1 response and interleukin (IL)-13 for Th2 response were measured by enzyme-linked immunosorbent assay (ELISA) (Multiplex; Millipore, Billerica, Massachusetts). The serum OVA-specific IgE level was measured with an ELISA kit (Pharmingen, San Diego, CA).

Real-Time Polymerase Chain Reaction

Spleens were removed after sacrifice. Total RNA was extracted from splenic mononuclear cells using TRIzol reagent (Invitrogen, Carlsbad, California). The levels of T-bet (a transcription factor for Th21 cells), GATA-3 (a

transcription factor for Th2 cells), Foxp3 (a transcription factor for Treg cells), and β -actin (an internal control) mRNA expression of splenic mononuclear cells were determined by real-time polymerase chain reaction (PCR) using the ABI PRISM 7300 Real-Time PCR System (Applied Biosystems, Foster City, California) and SYBR Green PCR Master Mix (TaKaRa Bio Inc, Otsu, Shiga, Japan). The oligonucleotide primer sequences were as follows: T-bet forward primer, 5'-GCCAGGGAACCGCTTATA-3' and T-bet reverse primer, 5'-CCTTGTTGTTGGTGAGCTTTA-3'; GATA-3 forward primer, 5'-CTGGATGGCGGCAAAGC-3' and GATA-3 reverse primer, 5'-GTGGGCGGGAAGGTGAA-3'; Foxp3 forward primer, 5'-GAAAGCGGATACCA AATGA-3' and Foxp3 reverse primer, 5'-CTGTGAGGAC TACCGAGCC-3'; and β -actin forward primer, 5'-CCCAT CTACGAGGGCTAT-3' and β -actin reverse primer, 5'-CCCATCTACGAGGGCTAT-3'.³² The levels of these mRNAs expressions were analyzed using the ABI 7300 Sequence Detection System (Applied Biosystems). The results were normalized by β -actin expression and shown as the fold increase over the expression of the control group.

Flow Cytometry

For cell surface staining, 10^6 splenic mononuclear cells were incubated with FITC-conjugated anti-mouse CD4 (GK1.5) antibody (eBioscience, San Diego, California). For intracellular staining, cells stained with CD4 were incubated with fixation/permeabilization working solution, and Fc receptors were blocked with excess mouse Fc block. Then the cells were stained with PE-Cy5-conjugated anti-mouse Foxp3 (FJK-16s) antibody (eBioscience). CD4⁺ Foxp3⁺ T cells were analyzed using FACSCalibur (Becton Dickinson, San Jose, California).

Statistical Analysis

All mice survived until the study was completed, so the calculations were based on 40 mice. All measured parameters are expressed as means $\pm$ standard deviations. The differences among the groups were analyzed using Kruskal-Wallis analysis. If there were statistical significances, the ranked parameters were compared by one-way analysis of variance (ANOVA) and Bonferroni's multiple-comparison tests (PASW Statistics 18; SPSS, Inc, an IBM Company, Chicago, Illinois). A P value of $<.05$ was considered significant.

Results

Allergic Symptoms

The symptom scores were 6.1 ± 1.5 in the control group, 23.5 ± 8.3 in the OVA group, 24.6 ± 13.2 in the pre-NOD1 group, and 31.6 ± 18.1 in the post-NOD1 group. The scores in the control group were lower than those in the OVA ($P = .031$), pre-NOD1 ($P = .031$), and post-NOD1 groups ($P = .007$).

Serum OVA-Specific IgE

Serum OVA-specific IgE levels in the control group (0.55 ± 0.1 ng/mL) were lower than those in the OVA (4.43 ± 22.3 ng/mL, $P = .002$), pre-NOD1 (4.69 ± 1.1 ng/mL, $P = .002$), and

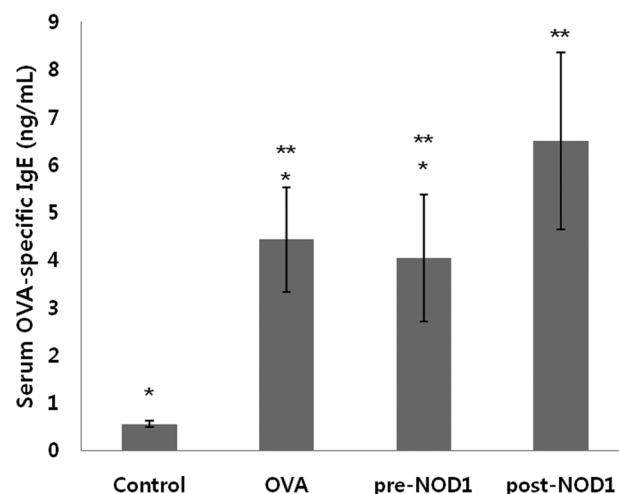


Figure 2. The levels of serum ovalbumin (OVA)-specific IgE. The error bars represent the standard deviation. * $P < .05$ vs the post-NOD1 group. ** $P < .05$ vs the control group.

post-NOD1 groups (6.50 ± 1.86 ng/mL, $P < .001$). Serum OVA-specific IgE levels were elevated in the post-NOD1 group compared with those in the OVA ($P = .01$) and pre-NOD1 groups ($P = .007$), indicating that NOD1 ligand can enhance OVA-specific IgE synthesis after sensitization (**Figure 2**).

Eosinophil Counts in Lamina Propria

Figure 3 shows the infiltration of eosinophils in the nasal mucosa. The number of eosinophils in the lamina propria was 0.2 ± 0.1 in the control group, 54.4 ± 11.3 in the OVA group, 61.6 ± 14.6 in the pre-NOD1 group, and 81.4 ± 4.6 in the post-NOD1 group. The eosinophil count in the control group was lower than that in the OVA ($P = .014$), pre-NOD1 ($P = .002$), and post-NOD1 ($P < .001$) groups. The post-NOD1 group had a significantly higher eosinophil count than the OVA group ($P = .009$). These results show that NOD1 ligand can promote eosinophil infiltration in the nasal mucosa after sensitization.

Cytokine Analysis of NALF

Interferon- γ levels in NALF were 0.7 ± 0.4 pg/mL in the control group, 0.5 ± 0.2 pg/mL in the OVA group, 0.7 ± 0.3 pg/mL in the pre-NOD1 group, and 1.2 ± 0.5 pg/mL in the post-NOD1 group. No significant differences were observed among the groups.

Interleukin-13 levels in NALF were 6.2 ± 0.3 pg/mL in the control group, 13.5 ± 11.7 pg/mL in the OVA group, 19.0 ± 12.4 pg/mL in the pre-NOD1 group, and 33.7 ± 6.9 pg/mL in the post-NOD1 group. The level of IL-13 in the post-NOD1 group was higher than that in the control ($P = .023$) and OVA groups ($P = .034$), indicating that NOD1 ligand increased the production of IL-13 after sensitization.

Real-Time PCR Analysis

T cells express the transcription factors T-bet, GATA-3, and Foxp3, representing Th1, Th2, and Treg cells, respectively.

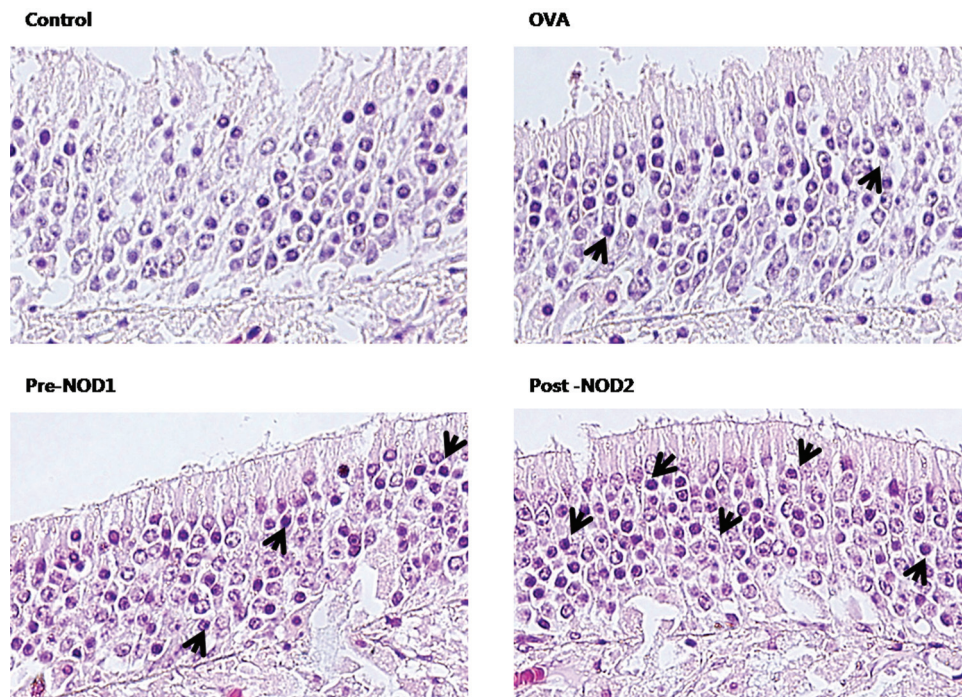


Figure 3. Infiltration of eosinophils (arrows) in the nasal mucosa of each study group. Control group, ovalbumin (OVA) group, pre-NOD1 group, and post-NOD1 group. (Hematoxylin and eosin stain; original magnification, $\times 400$.)

T-bet mRNA expression was decreased in the OVA, pre-NOD1, and post-NOD1 groups compared with the control group, although without significance (**Figure 4A**).

GATA-3 mRNA expression in the control group was lower than that in the OVA ($P = .017$), pre-NOD1 ($P = .003$), and post-NOD1 groups ($P < .001$). In the post-NOD1 group, GATA-3 mRNA expression increased markedly compared with that in the OVA ($P = .003$) and pre-NOD1 groups ($P = .022$) (**Figure 4B**).

Foxp3 mRNA expression in the control group was elevated compared with that in the OVA ($P = .002$), pre-NOD1 ($P < .001$), and post-NOD1 groups ($P < .001$). In the post-NOD1 group, Foxp3 mRNA expression was decreased compared with that in the OVA ($P < .001$) and pre-NOD1 groups ($P < .001$). The pre-NOD1 group showed lower Foxp3 mRNA expression than the OVA group ($P < .001$) (**Figure 4C**).

These results demonstrate that NOD1 ligand enhanced the induction of Th2 cells and inhibited the induction of Treg cells after sensitization. It also decreased the induction of Treg cells before sensitization.

Flow Cytometric Analysis of $CD4^+$ $Foxp3^+$ T Cells from Splenic Mononuclear Cells

Cells were sorted based on their level of Foxp3 expression and whether they expressed CD4. The percentages of $CD4^+$ $Foxp3^+$ T cells as a proportion of total splenic mononuclear cells were $6.5\% \pm 2.4\%$ in the control group, $1.9\% \pm 0.9\%$ in the OVA group, $0.8\% \pm 0.5\%$ in the pre-NOD1 group, and $0.7\% \pm 0.4\%$ in the post-NOD1 group. The percentage of these cells in the control group was higher than that in

the OVA ($P = .003$), pre-NOD1 ($P < .001$), and post-NOD1 groups ($P < .001$). The pre-NOD1 ($P = .001$) and post-NOD1 groups ($P = .001$) had lower percentages of these cells than did the OVA group, indicating that NOD1 ligand decreased the induction of $CD4^+$ $Foxp3^+$ T cells both before and after sensitization (**Figure 5**).

Discussion

The innate immune system constantly monitors the host's environment. Recognition of invading microorganisms, the primary tasks of the innate immune system, is achieved through innate immune sensors.³³ Innate microbial sensors recognize molecular structures that they use to distinguish between microbes and the host environment. These microbe-specific molecular structures are called PAMPs, and the receptors that recognize them are called PRRs.^{15,34}

The most well-known members of NLR are NOD1 and NOD2. NOD1 is a cytosolic sensor for detecting D-glutamyl-meso-diaminopimelic acid (DAP), which is a fragment of peptidoglycan. NOD2 is a well-characterized member of the NLR family that detects muramyl dipeptide (MDP) from peptidoglycan.^{15,34,35}

Several genetic studies have reported that NOD1 and NOD2 are associated with allergic disorders. A population-based study reported that NOD2 single-nucleotide polymorphisms (SNPs) were significantly associated with susceptibility to develop atopic diseases.¹⁶ The NOD1 gene is on chromosome 7p14 in a region genetically linked to atopy. A previous study reported that NOD1 polymorphism was strongly associated with the presence of asthma and

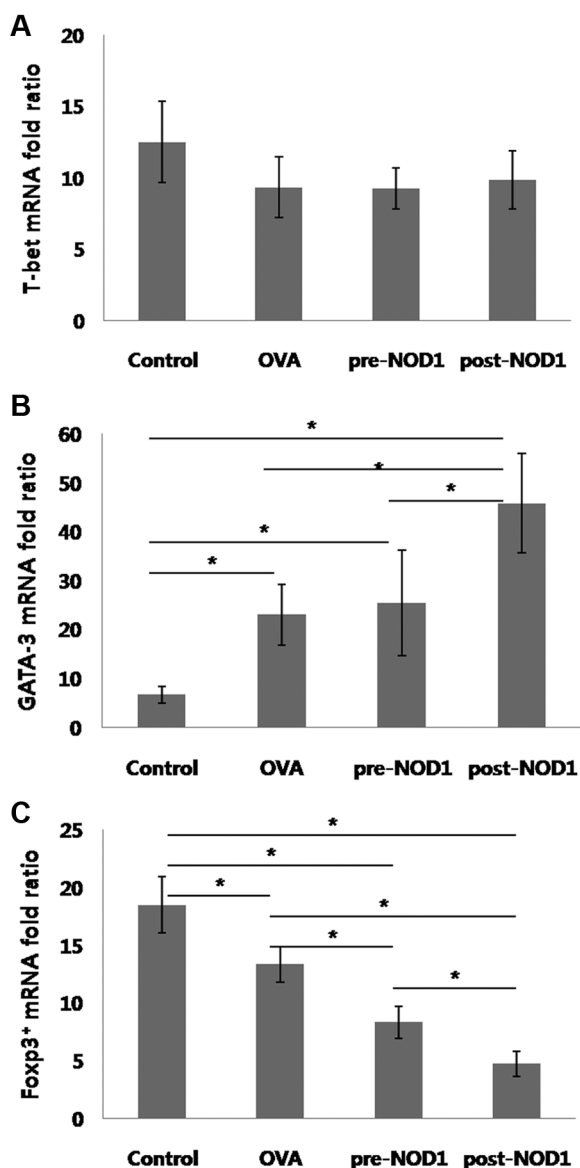


Figure 4. Quantitative analysis of T-bet (A), GATA-3 (B), and Foxp3 (C) mRNA expression by real-time polymerase chain reaction from splenic mononuclear cells. The results were normalized by β -actin expression. The error bars represent the standard deviation. OVA, ovalbumin. * $P < .05$.

high IgE levels.¹⁷ In a population-based study, NOD1 polymorphisms were significantly associated with atopic eczema and asthma.¹⁸ In another study, a very common polymorphism in NOD1 significantly contributed to the lower frequency of allergies in children heavily exposed to microbial products.¹⁹ These results show that bacterial stimuli via NOD1 might regulate Th2 polarization and IgE levels as well as susceptibility to allergic disease.³⁶ In a recent genome-wide meta-analysis, NOD1 SNPs were associated with allergic rhinitis or IgE sensitization to grass.³⁷

In the present study, serum OVA-specific IgE, tissue eosinophil counts, IL-13 levels in NALF, and GATA-3 mRNA

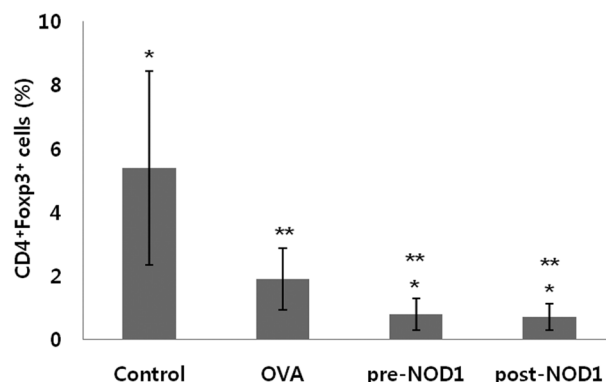


Figure 5. Flow cytometric analysis of CD4⁺ Foxp3⁺ T cell subsets. The percentages of CD4⁺ Foxp3⁺ T cells as a proportion of total splenic mononuclear cells. RU, the upper right quadrant, which represents CD4⁺ Foxp3⁺ T cells. The error bars represent the standard deviation. * $P < .05$ vs the OVA group. ** $P < .05$ vs the control group.

expression in the post-NOD1 group were increased compared with those in the OVA group, indicating that innate immunity via NOD1 may increase allergen-specific Th2 responses in allergen-sensitized subjects. Moreover, in contrast to the hygiene hypothesis, exposure to NOD1 before sensitization increased Th2 responses but to a lesser degree than those of the post-NOD1 group. These results may partially explain how acute upper airway infection exacerbates nasal symptoms in patients with allergic rhinitis.

Many studies have demonstrated that NOD1 and NOD2 ligands are involved in the induction of Th2 responses. NOD2 triggers a antigen-specific immune response with Th2-type polarization in a murine model.²⁰ However, co-stimulation of dendritic cells (DCs) with NOD2 and TLR ligands results in both Th1 and Th2 response or only Th1 response.^{20,21} NOD1-mediated signal enhances TLR-mediated IL-12 release and priming of Th1 and Th17 responses. However, the NOD1-specific agonist FK156, when applied alone, elicits priming of antigen-specific T- and B-cell immunity with Th2 response.²² Cholera toxin fails to activate NOD1-deficient mouse bone marrow mast cells (BMMCs) induced to release IL-4 but increases the release of IL-4 from BMMCs with a normal NOD1 gene.²³ Recently, 2 studies exploited the effect of NOD1 and NOD2 stimuli on Th2 response and innate cytokines in murine models.^{24,25} One study showed that only NOD2, not NOD1, prevented tolerance to inhaled allergens and induced innate cytokines in the lung. NOD2 activation blocks induction of CD4⁺ Foxp3⁺ Treg cells and promotes IL-4⁺ CD4⁺ T cells.²⁴ However, in this study, NOD1 ligand enhanced Th2 responses and inhibited the induction of Treg cells. These different results may be caused by the differences in the strain of animal, NOD1 ligand, administered dose of NOD1 ligand, and treatment protocols, among other factors.²⁴ Another study demonstrated that Th2 induction upon NOD1 and NOD2 activation depended on thymic stromal

lymphopoietin (TSLP) production by stromal cells and the upregulation of OX40 ligand on DCs.²⁵ In a human study, eosinophils were activated by NOD1 ligand and NOD2 ligand, and these responses were augmented in the presence of IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF), indicating that both NOD ligands constitute a link between respiratory infection and exacerbation of allergic diseases.³⁸

In addition, we found that NOD1 ligand inhibited the induction of Treg cells. Foxp3 mRNA expression levels and CD4⁺ Foxp3⁺ T cells in both the post-NOD1 group and the pre-NOD1 group were decreased each compared with those in the OVA group. Several studies have reported the effects of NOD1 and NOD2 on Treg cells.^{39,40} *Brugia malayi*, the parasite, induced a significantly higher production of Th1 and Th17 cytokines and decreased the induction of Treg cells in patients with lymph edema compared with asymptomatic individuals. It also induced significantly higher expression of NOD1 and NOD2 mRNA cells in patients with lymph edema compared with asymptomatic individuals.³⁹ As previously mentioned, NOD2 activation prevents airway tolerance by inhibiting Treg cells.²⁴

Finally, we found that the Th2 response was stronger in the post-NOD1 group than in the pre-NOD1 group. A previous study using a mouse model infected with *Chlamydomonas pneumoniae* demonstrated airway sensitization via TLR. Airway sensitization depended on the severity and timing of infection.⁹ In other studies, microorganisms via TLR showed potential to treat asthma by altering the Th1 and Th2 responses. However, their efficacy in treating asthma depends on several factors, including timing, dose, anatomic site, and duration, as well as host characteristics.^{41,42} We suggest that our results, similar to those of these previous studies, might be caused by the timing of exposure to NOD1 ligand and/or the different immunologic conditions of mice in the 2 groups. Further studies are needed to identify the underlying mechanisms of these differences according to the timing of exposure to NOD1 ligand.

In summary, this study may partially explain how acute upper airway infection exacerbates nasal symptoms in patients with allergic rhinitis. Also, in the future, NOD1 agonists and/or antagonists can be considered new treatments for preventing or alleviating allergic rhinitis in clinical settings.

However, there are some limitations in the present study. First, OVA, which has been most commonly used as an allergen in a mouse model, is not a common respiratory allergen in humans. Second, to strengthen our conclusion, a study with a larger number of mice might be needed. Third, we need to study the mechanisms of how several factors, including the timing and duration of exposure to NOD1, the dose of NOD1, and host characteristics, can influence the allergic inflammation. Finally, for uses in a clinical setting, further studies in humans might be also needed.

Conclusion

Our results demonstrate that the innate immune responses through NOD1 after allergen sensitization enhance the allergen-specific Th2 response and suppress the induction of Treg cells in a model of allergic rhinitis. NOD1 administered before allergen sensitization also showed a tendency to increase Th2 responses and clearly inhibited the induction of Treg cells. Therefore, we suggest that the innate immune response through NOD1 can affect adaptive immunity and that the outcome can be altered according to the timing of exposure to NOD1 ligand.

Author Contributions

Ji-Hyeon Shin, designed study, analyzed data, wrote article; **Soo Whan Kim**, designed study, analyzed data, wrote article; **Yong-Soo Park**, collected data, revised article.

Disclosures

Competing interests: None.

Sponsorships: None.

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