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Nasal Administration of Lipopolysaccharide Exacerbates Allergic Rhinitis through Th2 Cytokine Production from Mast Cells

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Abstract: Background: Microbial infection or exposure to endotoxin later in life exacerbates established asthma. Mast cells are involved in the exacerbation of asthma. This exacerbation involves a toll-like receptor (TLR)–mediated response of mast cells. In the clinical practice of otolaryngology, otolaryngologists experience an exacerbation of nasal congestion when infectious rhinitis develops in patients with allergic rhinitis, but the mechanisms are unknown. Therefore, this study investigated the effect of lipopolysaccharide (LPS) on allergic rhinitis using a mouse allergic rhinitis model. Methods: Female BALB/c mice, *TLR4* gene mutant C3H/HeJ mice or mast cell–deficient WBB6F1-$^{W/Wv}$ mice were sensitized intraperitoneally with ovalbumin (OVA)/alum, and were intranasal challenged with OVA and/or LPS. Nasal symptoms and histologic changes were examined. Cytokines in nasal tissue were examined by Western blot. The effects of LPS on degranulation and cytokine production of bone marrow–derived mast cells (BMMCs) were investigated. Results: Nasal administration of LPS together with the antigen exacerbated nasal symptoms, eosinophil infiltration of the nasal mucosa, and increased IL-5 production in the nasal mucosa. It was not observed in C3H/HeJ mice and WBB6F1-$^{W/Wv}$ mice. The addition of LPS increased the production of IL-5 from BMMCs in a dose-dependent manner, but no effect on degranulation was observed. Conclusions: Intranasal administration of LPS exacerbates allergic rhinitis through Th2 cytokine production from mast cells. This observation provides clues to the mechanism of exacerbation of allergic rhinitis caused by an infection in daily clinical practice.

Keywords: nasal allergy; LPS; toll-like receptor

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1. Introduction

Microbial infection exacerbates established asthma or contributes to the initial development of the clinical onset of asthma [1,2]. In particular, microbial infection or exposure to endotoxin early in life is considered to protect from the later development of asthma by stimulating the immune system toward a T-helper lymphocytes type 1 (Th1) response from Th2 response [3,4]. This is well known as the hygiene hypothesis. On the other hand, microbial infection or exposure to endotoxin later in life exacerbates established asthma [2].

In the last 20 years, it has been extensively elucidated that toll-like receptors (TLRs) are mammalian homologues of the *Drosophila* toll receptor and have a role in the innate recognition of bacteria. Furthermore, TLR2 and TLR4 are reported to be implicated in the recognition of various bacterial cell wall components [5].

Systemic administration of lipopolysaccharide (LPS) before sensitization inhibits Th2 response and suppresses the development of airway inflammation in murine asthma models [6]. On the contrary, administration of LPS with antigen in the reaction phase

exacerbates asthma. Administration of LPS has been shown to enhance the Th2 response through activation of mast cells in the reaction phase [7].

Otolaryngologists experience an exacerbation of nasal congestion when infectious rhinitis develops in patients with allergic rhinitis. The exacerbation of asthma caused by microbial infection has been well studied, but none for allergic rhinitis.

Therefore, the mechanism of exacerbation of allergic rhinitis caused by microbial infection was investigated using the mouse allergic rhinitis model.

2. Materials and Methods

2.1. Mice

Six-week-old female BALB/c mice, C3H/HeJ, C3H/HeN, WBB6F1-Kit^{W}/Kit$^{W\text{-}V}$, and WBB6F1-$^{+/+}$ mice were purchased from CLEA Japan (Meguro, Tokyo, Japan). C3H/HeJ mice are a nonresponder strain to LPS. WBB6F1-Kit^{W}/Kit$^{W\text{-}V}$ mice are deficient in mast cells. These mice were maintained under specific pathogen-free conditions and received an ovalbumin (OVA)-free diet at the laboratory of the animal research center of Shimane University. All mice were 6 to 7 weeks of age at the beginning of individual experiments. Animal care and experimental procedures were approved by the Animal Research Committee of Shimane University (approval code: IZ27-150, IZ30-76, and approval date: 1 March 2016–31 March 2021) and conducted according to the Regulations for Animal Experimentation at Shimane University.

2.2. Immunization Protocol and Treatment

Mice were intraperitoneally sensitized with 100 μg OVA mixed with 1 mg alum on day 0 and day 7. On days 21, 22, 23, 24, 25, 26, 27, and 28 after the first sensitization, sensitized mice were intranasally challenged with 400 μg OVA together with or without 4 μg LPS from *Pseudomonas aeruginosa* (Sigma-Aldrich, St. Louis, MO, USA) dissolved in phosphate-buffered saline (PBS) into the bilateral nostril.

2.3. Evaluation of Nasal Signs

Just after the final intranasal challenge with OVA with or without LPS on day 28, the mice were placed into an observation cage (one animal/cage) for about 10 min for acclimatization. The mice were placed into the observation cage again, and the number of sneezes was counted for 5 min by the method of Sugimoto et al. [8].

2.4. Histological Examination

Mice were killed 12 h after the final intranasal challenge with OVA with or without LPS on day 28. The heads were removed and fixed in 10% formaldehyde solution for 24 h at room temperature. The heads were decalcified in 5% formic acid for 36 h at room temperature and neutralized in 5% sodium sulfate solution for 12 h at room temperature after fixation. Coronal nasal sections were then stained with hematoxylin and eosin.

2.5. Western Blot Analysis

The nasal mucosal tissue was collected, frozen in liquid nitrogen, and then crushed with a homogenizer in PLC lysis buffer. Protein G beads was added with each primary antibody (anti-IL-5, anti-IL-10, or anti-IL-10 antibody, Pharmingen, Franklin Lake, NJ, USA) and incubated at 4 °C for 24 h using a rotator. It was washed with RIPAI buffer and Western blot was performed according to the manufacturer's instructions.

2.6. Preparation of Murine Bone Marrow–Derived Mast Cells

Bone marrow cells were obtained by flushing the femurs of BALB/c mice. The bone marrow cells were cultured at 37 °C in a RPMI-1640 medium, supplemented with 5 ng/mL IL-3, 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 U/mL penicillin, 50 μg/mL streptomycin, and 50 mM 2-mercaptoethanol. After

changing the medium every week for 4 weeks, the recovered populations were composed of >95% mast cells, as judged by flow cytometry of the expression of FcεRI and c-kit.

2.7. Measurement of Cytokine Productions from BMMCs

Bone marrow–derived mast cells (BMMCs) were cultured for 24 h with various concentrations of LPS (1 ng/mL–1000 ng/mL). As a positive control, BMMCs (1×10^6 cells/mL) were sensitized by incubating for 2 h at 37 °C with 0.5 μg/mL anti-DNP IgE antibody in complete RPMI medium with 10% FBS. The cells were washed and then stimulated with 10 μg/mL DNP-HSA. After culturing for 24 h, IL-5, IL-10, and IL-13 in the supernatant were measured by the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions.

2.8. Measurement of Degranulation of BMMCs

Degranulation rate was evaluated by β-hexosaminidase release assay. BMMCs were cultured for 30 min with various concentration of LPS (1 ng/mL–1000 ng/mL). As a positive control, BMMCs (1×10^6 cells/mL) were sensitized by incubating for 2 h at 37 °C with 0.5 μg/mL anti-DNP IgE antibody in complete RPMI medium with 10% FBS. The cells were washed and then stimulated with 10 μg/mL DNP-HSA for 30 min.

β-Hexosaminidase assay was conducted according to the method of Razin et al. [9]. Supernatant (50 μL) and pellet samples were incubated with 50 μL 1 mM p-nitrophenyl-*N*-acetyl-β-D-galactosaminide, dissolved in 0.1 M citrate buffer, pH 5.0, in a 96-well plate at 37 °C for 1 h. The reaction was stopped with 200 μL/well 0.1 M carbonate buffer, pH 10.5. The plate was read at 405 nm by a plate reader. The net percentage of β-hexosaminidase release was calculated as follows: β-hexosaminidase in supernatant/(β-hexosaminidase in supernatant + β-hexosaminidase in pellet) × 100.

2.9. Northern Blot Analyses

RNA was isolated with Trizol from BMMCs stimulated with LPS for 24 h. Northern blots with 5 to 10 μg of RNA were hybridized to a *GATA3* cDNA probe. Normalization of RNA loading was done with a probe for GAPDH.

2.10. Statistical Analysis

Statistical analysis of the primary data was made using JMP. Data were presented as mean ± standard deviation. The one-way ANOVA and parametric independent samples "*t*" test were applied to evaluate the number of sneezes. Dunnet's test was applied to evaluate the degranulation rate of BMMCs and cytokine production from BMMCs to compare with negative or positive control A value of $p < 0.05$ was taken as significant.

3. Results

3.1. Nasal Administration of LPS Together with the Antigen Exacerbates Nasal Allergy via TLR4 of Mast Cells

To determine the role of LPS in the effector phase of nasal allergy, OVA-sensitized BALB/c mice were intranasally challenged with OVA together with or without LPS on days 21, 22, 23, 24, 25, 26, 27, and 28. The number of sneezes by each mouse after the final nasal challenge was counted. The number of sneezes was significantly greater in mice administrated LPS intranasally together with OVA (Figure 1A). Eosinophil infiltration into the nasal mucosa was increased in mice receiving LPS intranasally (Figure 1B). Nasal instillation of LPS increased the production of IL-5 in the nasal mucosa (Figure 1C).

To prove whether the aggravation of nasal allergy because of nasal administration of LPS is mediated by TLR4, similar experiments were performed using C3H/HeJ mice, which are TLR4 gene mutant mice, and C3H/HeN mice, which are their wild type. The number of sneezes (Figure 2A), eosinophil infiltration into nasal mucosa (Figure 2B), and IL-5 production in nasal mucosa (Figure 2C) were aggravated in C3H/HeN mice, not in C3H/HeJ mice, after the final intranasal administration of LPS together with OVA.

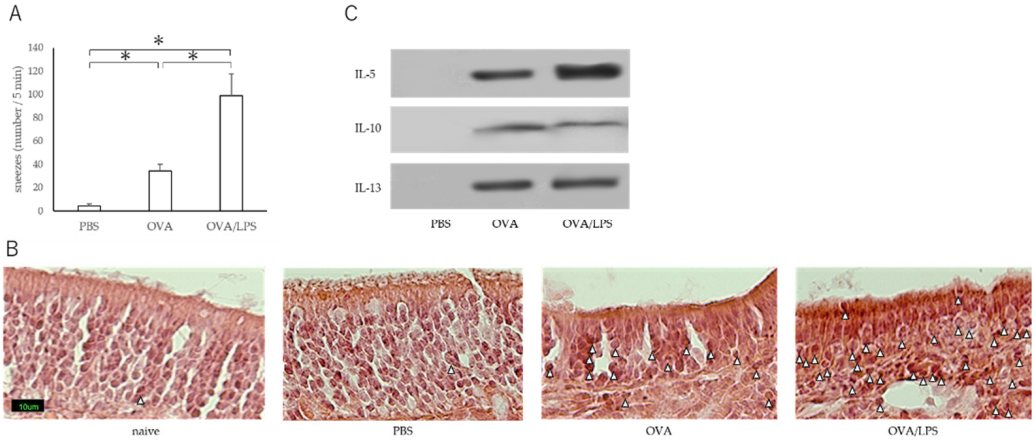

Figure 1. Allergic symptom, eosinophil infiltration, and cytokine production in nasal mucosa after nasal challenge with PBS, OVA, or OVA and LPS in OVA-sensitized BALB/c mice. (**A**), The number of sneezes after challenge with PBS, OVA, or OVA and LPS. (**B**), Nasal coronal sections stained with hematoxylin and eosin. The black bar indicates 10 μm. The arrow heads indicate infiltrating eosinophil. (**C**), Cytokines in nasal mucosa evaluated by Western blot. In each experiment, seven mice were used. * $p < 0.5$.

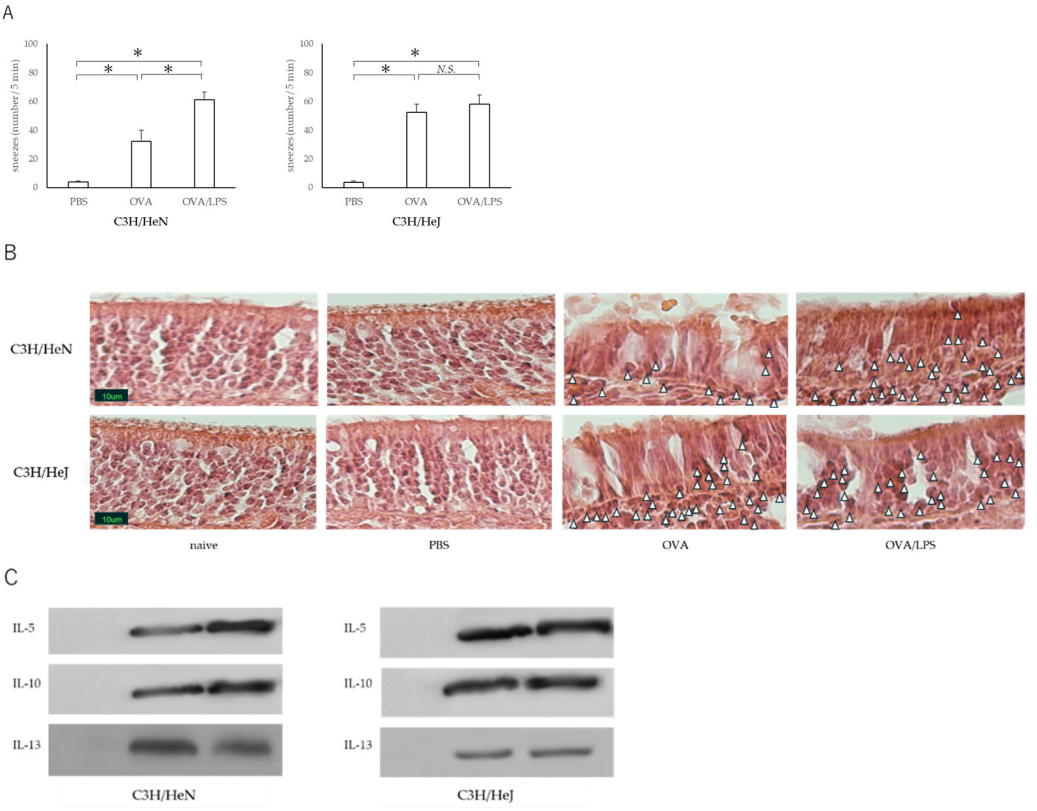

Figure 2. Allergic symptom, eosinophil infiltration, and cytokine production in nasal mucosa after nasal challenge with PBS, OVA, or OVA and LPS in OVA-sensitized C3H/HeN or C3H/HeJ mice. (**A**), The number of sneezes after challenge with PBS, OVA, or OVA and LPS. (**B**), Nasal coronal sections stained with hematoxylin and eosin. The arrow heads indicate infiltrating eosinophil. (**C**), Cytokines in nasal mucosa evaluated by Western blot. In each experiment, seven mice were used. * $p < 0.5$.

To find out whether mast cells are involved in this reaction, similar experiments were performed using WBB6F1-Kit^{W}/Kit$^{W\text{-}V}$ mice, which are deficient in mast cells, and WBB6F1-$^{+/+}$ mice, which are their wild type. Eosinophil infiltration into nasal mucosa

(Figure 3A) and IL-5 production in nasal mucosa (Figure 3B) were aggravated in WBB6F1-$^{+/+}$ mice, not in WBB6F1-Kit^{w}/Kit^{w-v} mice after the final intranasal administration of LPS together with OVA.

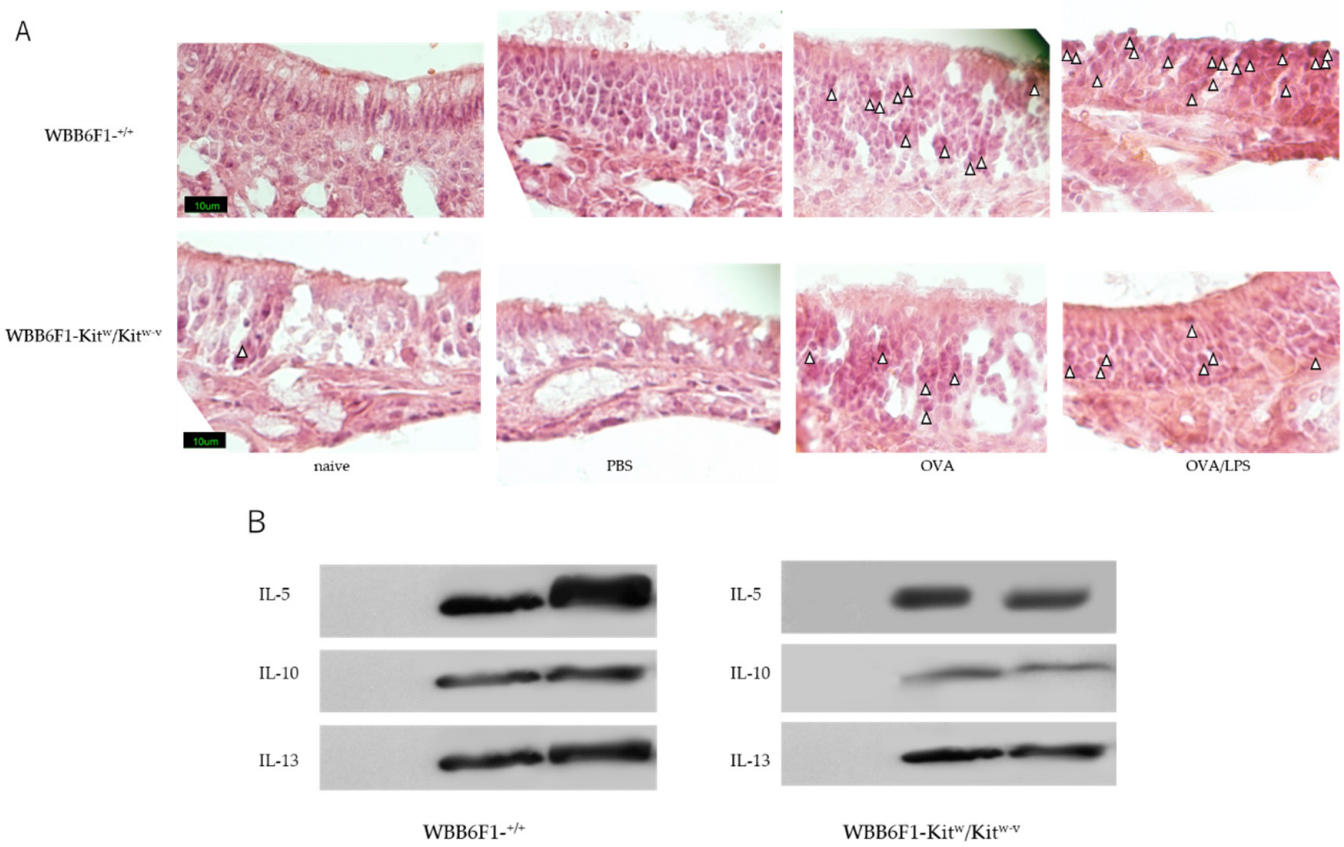

Figure 3. Eosinophil infiltration and cytokine production in nasal mucosa after nasal challenge with PBS, OVA, or OVA and LPS in OVA-sensitized WBB6F1-$^{+/+}$ or WBB6F1-Kit^{w}/Kit^{w-v} mice. (**A**), Nasal coronal sections stained with hematoxylin and eosin. The arrow heads indicate infiltrating eosinophil. (**B**), Cytokines in nasal mucosa evaluated by Western blot.

3.2. LPS Does Not Exacerbate Mast Cell Degranulation but Promotes TH2 Production from Mast Cells via Expression of GATA3 Gene

Whether LPS affects its degranulation was investigated using BMMCs. Addition of LPS did not cause mast cell degranulation (Figure 4A). When LPS was added to mast cells, Th2 cytokine production was observed (Figure 4B). When the expression of GATA3 gene having a binding capacity to the promoter region of the IL5 gene was examined by northern blotting, the expression of GATA3 gene was enhanced in a dose-dependent manner of LPS (Figure 4C).

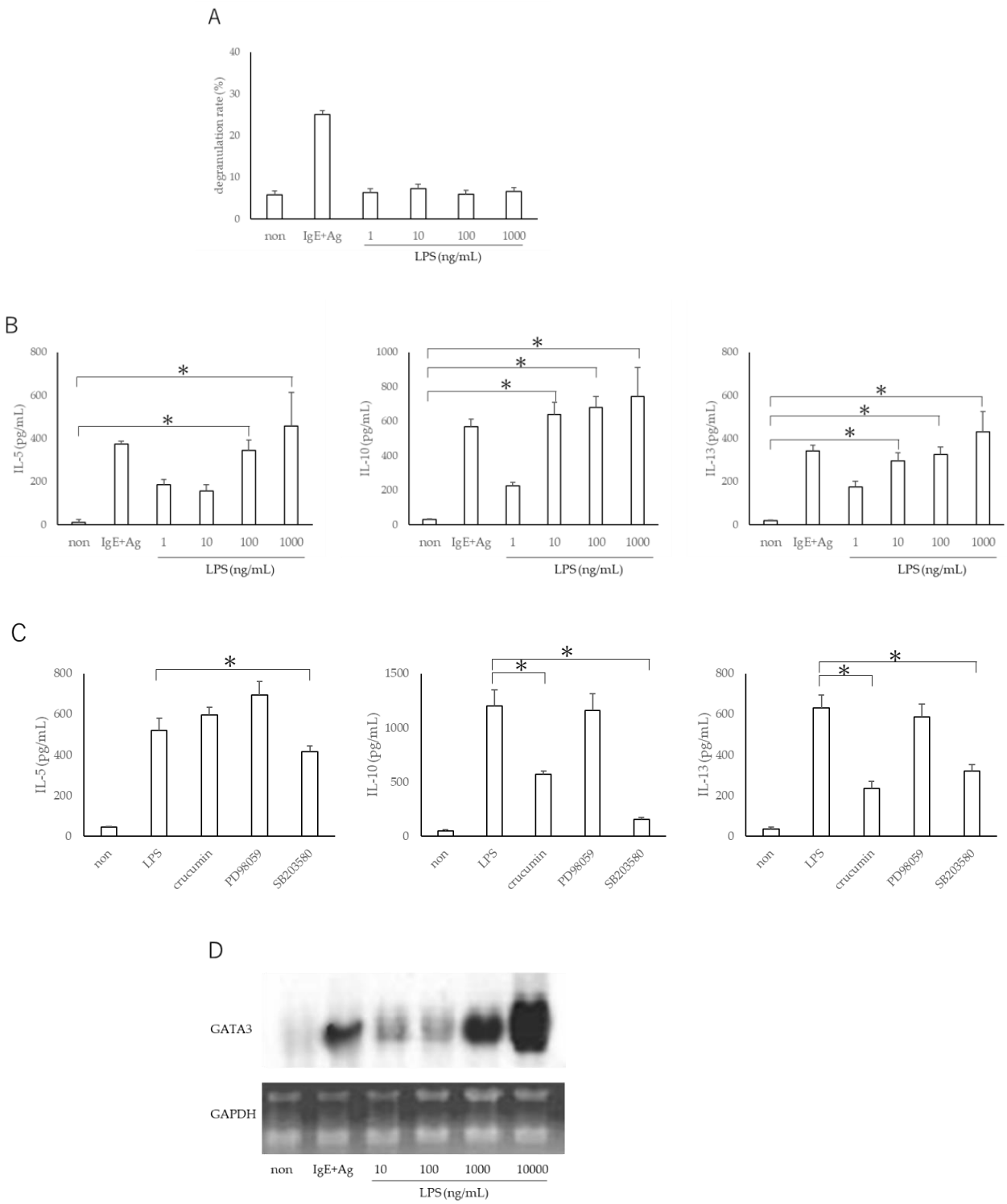

Figure 4. The effect of LPS on mast cell degranulation, Th2 cytokine production, and expression of *GATA3* gene. (**A**), Degranulation rate of BMMCs stimulated with LPS evaluated by β-hexosaminidase release assay. (**B**), Cytokine productions of BMMCs stimulated with LPS evaluated by ELISA. (**C**), Effect of MAP kinase inhibitor on cytokine production of BMMC stimulated with LPS (1000 ng/mL) evaluated by ELISA. curcumin: JNK inhibitor (50 mMol), PD98059: ERK inhibitor (30 mMol), SB203580: p38 inhibitor (30 mmol). (**D**), Expression of *GATA3* gene of BMMCs stimulated with LPS evaluated by Northern blot. * $p < 0.5$.

4. Discussion

The relationship between the onset or exacerbation of asthma and infectious diseases or environmental endotoxin (LPS) is well researched [8]. Childhood asthma and allergies are called the epidemic of the 21st century, because of a sharp rise in prevalence from the middle of the 20th century in high-income countries. Microbial infection or exposure to endotoxin early in life is considered to protect from the later development of asthma [3,4]. Asthma rates are rapidly rising in urban areas of low- and middle-income countries [9]. In mice experiments, farm dust or lipopolysaccharide treatment induced the expression of A20, a regulator of TLR-induced inflammation in respiratory epithelium, which is instrumental in reducing allergic airway inflammation [10]. Microbial infection or exposure to endotoxin later in life exacerbates established asthma. The causes of this exacerbation are suggested as follows: (1) by increasing the severity of the airway inflammation; (2) by increasing the susceptibility to rhinovirus-induced colds; and (3) by causing chronic bronchitis and emphysema with the development of irreversible airway obstruction after chronic exposure of adults [2]. Respiratory tract infections and common colds account for more than half of the causes of fatal asthma in Japan. In the clinical practice of otolaryngology, otolaryngologists experience an exacerbation of nasal congestion when infectious rhinitis develops in patients with allergic rhinitis. Unlike asthma, it is not fatal, so the cause of the exacerbation of allergic rhinitis because of infection is unknown.

The mammalian TLR family consists of 13 members and recognizes specific patterns of microbial components, called pathogen-associated molecular patterns. TLR2 recognizes lipoprotein, lipopeptide, peptidoglycan, and lipoteichoic acid from Gram-positive bacteria. TLR4 recognize LPS from Gram-negative bacteria [5]. Generally, the cause of acute rhinosinusitis is that the virus first infects and then develops into bacterial inflammation. Most of the causative bacteria are *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* [11]. Since *S. pneumoniae* is a Gram-positive bacterium, its cell component is recognized by TLR2. Since *H. influenzae* and *M. catarrhalis* are Gram-negative bacteria, its cell component is recognized by TLR4.

Murakami et al. demonstrated that LPS activates mast cells to exacerbate asthma in a mouse asthma model [7]. LPS activated mast cells and exacerbated allergic rhinitis in the reaction phase using a mouse allergic rhinitis model in the present study. However, there is a difference in terms of expressions of TLR in upper and lower respiratory tracts. Acute nasal inflammatory diseases are infectious, have a bacterial etiology, and cause inflammatory responses elicited by nasally pathogenic exposure. In these responses, nasal epithelial cells are thought to play important roles as the initial point of contact with pathogens. The same is true for lower respiratory tract infections. Both TLR2 and TLR4 are expressed in the epithelial cells of the lower respiratory tract, but only TLR2 is expressed in the epithelial cells of the nasal mucosa [12]. TLR4 is reported to be expressed in the nasal epithelial cells of mice [13] or humans [14]. Neutrophil inflammation occurs when TLR2 agonist is administered to the nose of mice [15], but intranasal administration of LPS does not cause nasal inflammation (Supplementary Figure S1). LPS causes neutrophil inflammation in the lungs when administered intratracheally [16]. Xu et al. reported that intranasal administration of TLR4-short hairpin RNA (shRNA) inhibits murine allergic rhinitis by regulating the NF-κB pathway [13]. TLR4 in the nasal mucosa is thought to have a mechanism that enhances allergic rhinitis.

Mast cells play an important role in type 1 allergic reactions, but they have a protective function against enterobacterial infections. BMMCs express TLR 2, 4, 6, and 8 but not TLR5, and produce inflammatory cytokines (IL-1β, TNF-α, IL-6, and IL-13) by LPS stimulation [17]. The data of the present study showed BMMCs produced Th2 cytokines via TLR4 stimuli. It has been reported that stimulation of LPS derived from *E. coli* does not induce degranulation of BMMCs [17]. Our study using LPS derived from *P. aeruginosa* showed a similar result. Mast cells migrate to the nasal epithelium from the mucosal connective tissue in allergic rhinitis patients [18]. It is possible that allergic rhinitis was exacerbated by LPS, activating mast cells migrated to the nasal epithelium and enhancing Th2 cytokines.

Cytokine production from mast cells is mediated by MAP kinase, both via FC ε receptor and TLR [19]. Cytokine production by LPS stimulation of BMMC, the production of IL-5, IL-10, and IL-13, was suppressed by the p38 inhibitor, and the production of IL-10 and IL-13 was suppressed by the JNK inhibitor. We investigated the gene expression of *GATA3*, which has the ability to bind to the promoter region of the *IL5* gene [20]. LPS stimulation enhanced *GATA3* gene expression in a dose-dependent manner. The present study showed that the mechanism of *IL5* production from mast cells stimulated with LPS depended on p38 and *GATA3*.

The major effects of TLR4 are activation of antigen-presenting cells and enhancement of antigen presentation; it determines the direction of naive T-cell differentiation. In fact, vaccines containing TLR ligands as adjuvants are in clinical use. In allergic rhinitis, drugs containing TLR ligands are used clinically in subcutaneous immunotherapy [21], and clinical studies are being conducted in sublingual immunotherapy [22]. The mechanism of immunotherapy for allergic rhinitis is the induction of regulatory T cells. A preliminary experiment of this study was conducted using 40 μg LPS, which is the limit dose that can be dissolved, but in that case, the symptoms of allergic rhinitis were alleviated and the exacerbation of mucosal eosinophil infiltration was not observed. Depending on the amount of antigen and LPS, there may be effects like immunotherapy. This study focuses on mast cells, not antigen presentation or T cells. Further studies are needed on antigen presentation or T-cell function.

This study data explained one of the mechanisms by which bacterial acute rhinosinusitis exacerbates allergic rhinitis. When patients with allergic rhinitis develop bacterial acute rhinosinusitis in daily clinical practice, it may be necessary to step up the treatment of allergic rhinitis.

5. Conclusions

Intranasal administration of LPS exacerbates allergic rhinitis through Th2 cytokine production from mast cells. This observation provides clues to the mechanism of exacerbation of allergic rhinitis by infection in daily clinical practice.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/allergies1040020/s1, Figure S1: H&E stain of coronal section of head of mice, intranasal administrated with 4 μg LPS for 7 days.

Author Contributions: N.A., I.M. and T.F. have done all experiments together and produced data. H.K. made this project and supervised all experimental processes with his immunological background. T.S. supervised all experiments from a clinical point of view. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Animal care and experimental procedures were approved by the Animal Research Committee of Shimane University (approval code: IZ27-150, IZ30-76, and approval date: 1 March 2016–31 March 2021).

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Conflicts of Interest: We have no conflict of interest to submit this manuscript.

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