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Novel Methods for Development of a Neutrophilic Nasal Polyp Murine Model Using Lipopolysaccharide or Polyinosinic:polycytidylic acid

LPS 또는 poly(I:C) 자극을 이용한 호중구성 비폴립 마우스 모델 개발의 새로운 방법

2021년 8월

서울대학교 대학원

의학과 중개의학 전공

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이 논문을 의학박사 학위논문으로 제출함
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2021년 7월

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Abstract

Novel Methods for Development of a Neutrophilic Nasal Polyp Murine Model Using Lipopolysaccharide or Polyinosinic:polycytidylic acid

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Nasal polyps can be classified as eosinophilic and neutrophilic polyps, based on the type of immune cell infiltration and cytokines. While a murine model of eosinophilic nasal polyp was previously developed using ovalbumin (OVA) combined with *staphylococcal* enterotoxin B (SEB), a neutrophilic nasal polyp murine model was not well established. In addition, several factors including bacteria, viruses, or fungi have been considered to play potential roles in nasal tissue remodeling in the rhinosinusitis, but the immunological role of bacterial or viral stimuli triggering polyp development remains unclear. The present study aimed to establish a murine

model of neutrophilic nasal polyp and to compare the different immune responses to bacterial– and viral–derived stimuli using lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid [poly(I:C)]. BALB/c mice were sensitized and challenged with OVA and SEB, with or without systemic/local LPS, and it was determined whether systemic or local stimulation of LPS is essential for neutrophilic nasal polyp development. In addition, BALB/c mice were stimulated with poly(I:C) both systemically and locally. The consequent histopathological findings, cytokines, and serum immunoglobulins were analyzed according to the groups stimulated with LPS or poly(I:C). When mice were systematically and locally stimulated with LPS, neutrophilic infiltration and Th1/Th17 immune environment were predominantly induced in the nasal polyp. While a murine model of nasal polyp was well developed with no significant differences in polyp formations and epithelial disruptions among the experimental groups, the local cell recruitment patterns slightly differed in animals that received either LPS or poly(I:C). Additionally, the local immune environments generated by LPS or poly(I:C) stimulation varied. LPS stimulation induced a marked Th1/Th17 response and predominantly neutrophilic nasal polyp formations, whereas poly(I:C) induced a Th2–skewed environment in neutrophilic nasal polyp development. We developed the neutrophilic polyp murine model by dual systemic/local stimulation of LPS or poly(I:C). Overall, our findings show that both cell recruitment patterns and local immune environments induced by these two

stimuli differ, which may have implications in the physiopathology of rhinosinusitis with nasal polyp.

Keyword: Animal model, Nasal polyps, Neutrophils, Sinusitis, Lipopolysaccharide, Polyinosinic:polycytidylic acid.

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Introduction

Chronic rhinosinusitis with nasal polyp (CRSwNP) is a common chronic inflammatory disease of the paranasal sinuses. There are different phenotypes of nasal polyps based on the type of immune cell infiltration and cytokines. In Western patients, nasal polyps are characterized as Th2–dominant immune responses including the predominance of eosinophil infiltration with excessive expression of type 2 cytokines (1). In contrast, in Asian patients, neutrophilic nasal polyps associated with Th1 and Th17 responses are typically observed (2, 3). These differences between Western and Asian patients suggest that nasal polyp endotypes differ according to the patient ethnicity.

Several studies suggest that *staphylococcus aureus* colonization and immunoglobulin (Ig) E antibody formation to enterotoxins is associated with pathogenesis of nasal polyp formation and eosinophilic inflammation, which is in line with the superantigen hypothesis (4–6). Therefore, the first murine model of eosinophilic nasal polyps using ovalbumin (OVA) combined with *staphylococcal* enterotoxin B (SEB) was previously developed (7) and widely used in experimental studies (8–10). However, a neutrophilic nasal polyp murine model was not well established.

We hypothesized that high–dose SEB and lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid [poly(I:C)] induce neutrophilic nasal polyp

formation. In a previous study, a higher SEB dose induces greater neutrophilic infiltration with a higher level of interferon (IFN)–γ than a lower one (7). Another study also reported that a high SEB dose induces interleukin (IL)–17A expression in mice (11). In addition, LPS is a fundamental constitutive block of the gram–negative bacterial cell wall and a potent activator of the immune system via Toll–like receptor (TLR) 4 recognition and signaling (12). LPS is known to induce a strong airway inflammation and promote local neutrophil recruitment (13–15). A few studies have already reported LPS–mediated neutrophil induction using murine models (16, 17). In fact, a neutrophil–dominant rhinitis model has been established with OVA and LPS intraperitoneal injections and showed that neutrophil recruitment is dependent on IL–17 (16). Additionally, a neutrophilic nasal polyp model has been developed with continuous intranasal instillation of LPS, that shows promotion of enhanced Th1– and Th17–related cytokines through TLR4 signaling pathway (17). Furthermore, poly(I:C) is a synthetic viral analogue (18) used in experimental studies of immune responses to viral infections. These responses are initiated via TLR 3 signaling (after the recognition of single–strand DNA molecules) that plays a critical role in the initiation of antiviral immunity. Previous studies showed that poly(I:C) triggers neutrophilic inflammation in asthma (19) and rhinitis (20) murine models.

Nasal polyp formation results from a combination of individual susceptibility and environmental factors. In fact, a recent study suggests that

a diversity of environmental factors, such as microbiota and air pollution, can influence Th cytokine profiles in patients with CRSwNP (21). Although several factors, including bacteria and viruses, have been associated with nasal tissue remodeling and rhinosinusitis (22–24), the role of bacterial or viral stimuli in polyp development remains unclear.

The present study aimed to establish a murine model of LPS– or poly(I:C)–induced neutrophilic nasal polyps. In addition, we aimed to compare the different immune response according to the LPS and poly(I:C), which are bacterial– and viral–derived stimuli, respectively, in a murine model using OVA combined with SEB.

Preliminary Studies

Preliminary study I – Materials & Methods

Experimental protocol is summarized in Figure 1. Mice were randomly divided into four groups: Group A: negative control, Group B: positive control, Group C: LPS (10 μg) and SEB intranasal stimulation, and Group D: LPS (20 μg) and SEB intranasal stimulation. For the negative control group, 40 μL of phosphate–buffered saline (PBS) was dropped into their nasal cavity once a week for 15 weeks. CRSwNP was induced in the positive control group following a previously established protocol (7). Briefly, mice were first sensitized with 25 μg of OVA (grade V; Sigma–Aldrich, St. Louis, MO, USA) in complex with 2 mg of aluminium hydroxide gel adjuvant (alum) (Thermo Fisher Scientific, Rockford, IL, USA) via intraperitoneal injection on days 0 and 5. The mice were then challenged intranasally with 3% OVA diluted in 40 μL of PBS daily, from days 12 to 19, followed by three times a week thereafter for 12 consecutive weeks. Simultaneously, from week 5, considering the triweekly instillations, mice were intranasally challenged on a weekly basis with 500 ng of SEB (Product #122; List Biological Laboratories, Inc., Campbell, CA, USA). Mice were challenged intranasally with 10 μg or 20 μg of LPS (#L2880; Sigma–Aldrich) once a week for six weeks, followed by intranasal stimulation with 500 ng of SEB once a week for nine weeks. Each control group included 10 mice and each experimental group included 20 mice.

Mice (n = 3 for each checking point) in experimental groups were sacrificed and histopathological analyses were conducted after six weeks and 10 weeks to assess the development of polypoid lesions. Other mice were sacrificed after 15 weeks.

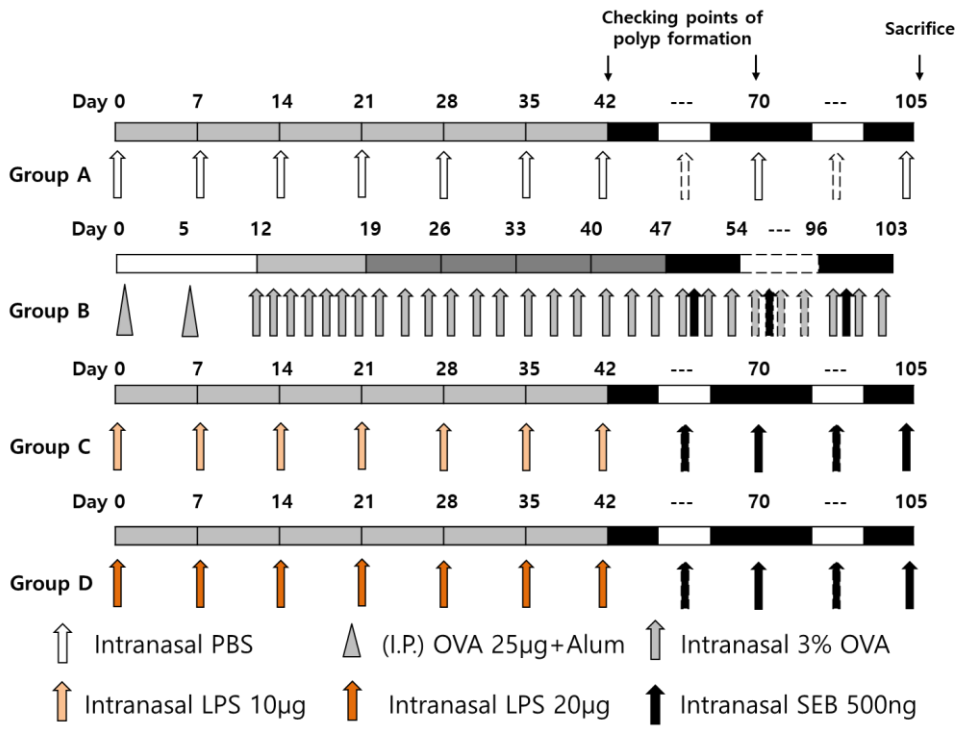


Figure 1. Experimental protocol (Preliminary study I).

Preliminary study I – Results

Nasal polypoid lesion or epithelial disruption was not noted in the mice of the experimental groups when LPS was challenged intranasally for six weeks and when SEB was stimulated for an additional four weeks (Figure 2). Even after 15 weeks, there was no polypoid lesion or epithelial disruption in groups C and D, while nasal polyps and epithelial disruptions

were noted in group B (Figure 3).

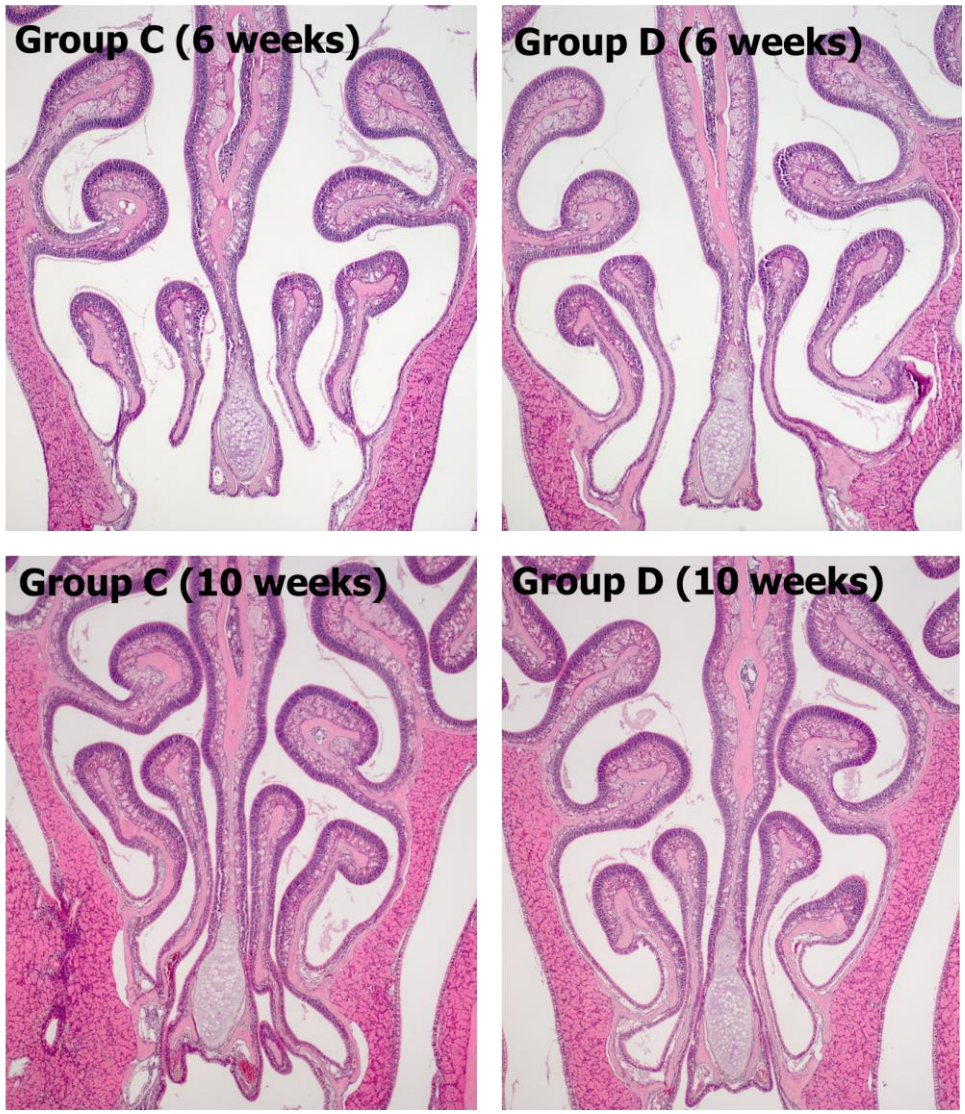


Figure 2. Histopathological results for checking the formation of nasal polypoid lesions in experimental groups (Hematoxylin & Eosin staining, x40). Mice in Group C were stimulated with six weeks of 10 μg LPS plus four weeks of SEB intranasally, Mice in Group D were stimulated with six weeks of 20 μg LPS plus four weeks of SEB intranasally. There was no polyp formation and epithelial disruption at each checking point.

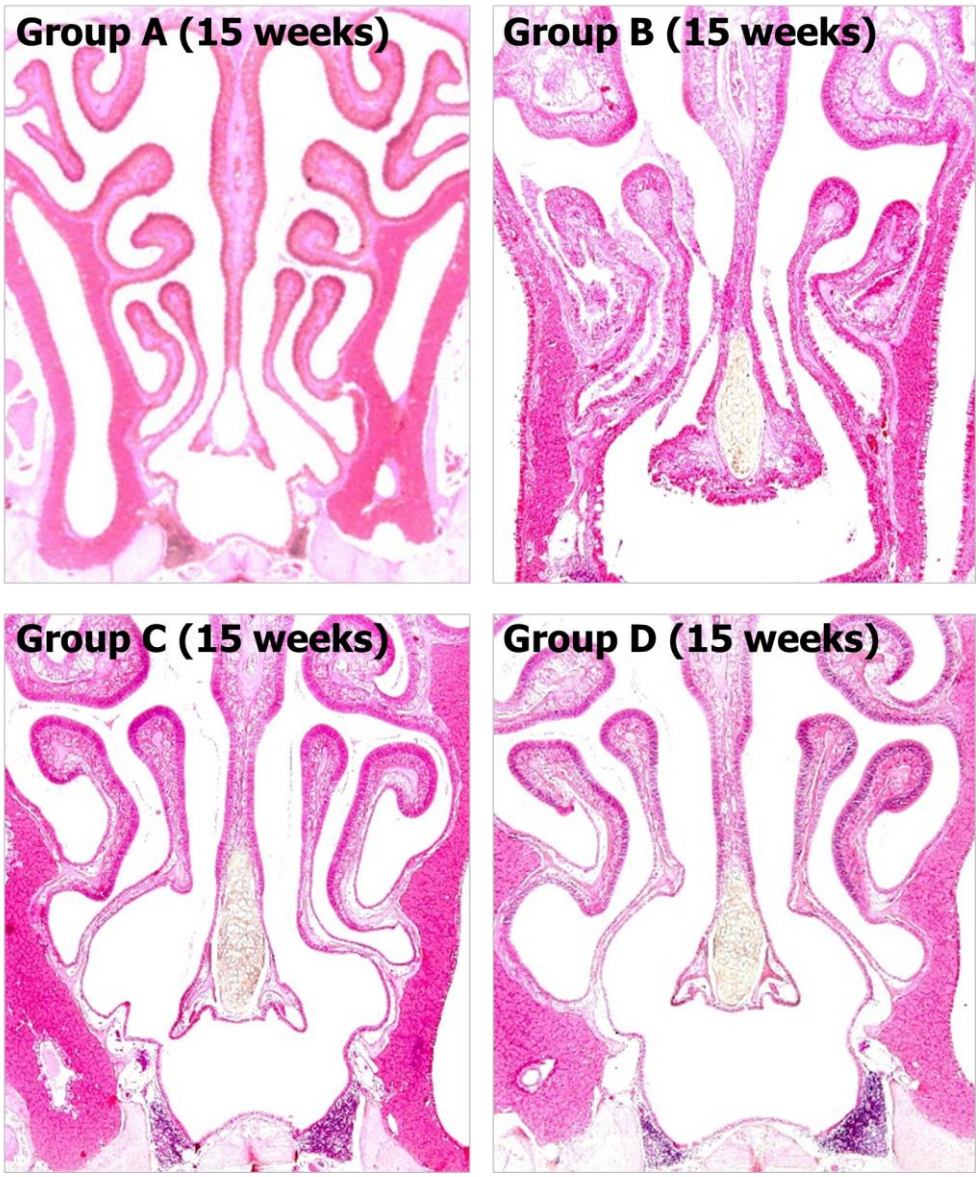


Figure 3. Histopathological results according to the groups (Hematoxylin & Eosin staining, x40). Group A: PBS, Group B: 3% OVA intraperitoneally and intranasally + SEB intranasally, Group C: LPS 10 μg (6 weeks) + SEB 500 ng (9 weeks) intranasally, Group D: LPS 20 μg (6 weeks) + SEB 500 ng (9 weeks) intranasally. There was no polypoid lesion and epithelial disruption in groups C and D, while nasal polyps and epithelial disruptions were noted in group B.

Preliminary study I – Discussion

The hypothesis of our first preliminary study was that intranasal stimulation with LPS and high dose SEB would induce neutrophilic nasal polyps in BALB/c mice. Several studies were reviewed to determine the route, concentration, frequency, and duration of LPS stimulation. First, we selected intranasal stimulation of LPS. In a previous asthma murine model discussing the development of airway hyperreactivity, local LPS administration switched the airway inflammation from eosinophilic to a neutrophilic, whereas systemic LPS inhibited airway inflammation (15). Second, we divided the experimental group as 10 μg and 20 μg of LPS intranasal stimulation. Although an asthma murine model was induced by nasal stimulation with 20 μg LPS (15), in another murine model, intranasal stimulation of 10 μg LPS induced acute lung injury (25). Finally, mice were challenged intranasally with LPS once a week for six weeks and followed by intranasal stimulation with SEB once a week for nine weeks. Although we could not identify any clear evidence for frequency and duration of LPS stimulation, this study aimed to develop novel methods for neutrophilic nasal polyp murine model. However, there were no nasal polypoid lesions or epithelial disruption in the mice of the experimental groups under this protocol. The dose, frequency, and duration of LPS stimulation had to be changed in the experimental protocol.

Preliminary study II – Materials & Methods

Dose, frequency, and duration were altered for the experimental protocol for LPS stimulation (Figure 4). Mice were randomly divided into four groups with 10 mice per group. For the negative and positive control groups, the same method was performed as preliminary study I. Mice in experimental groups were challenged intranasally with LPS (20 μg in group C and 50 μg in group D) three times a week for 10 weeks. Additionally, the mice were challenged weekly with SEB (500 ng) after LPS stimulation for 10 weeks.

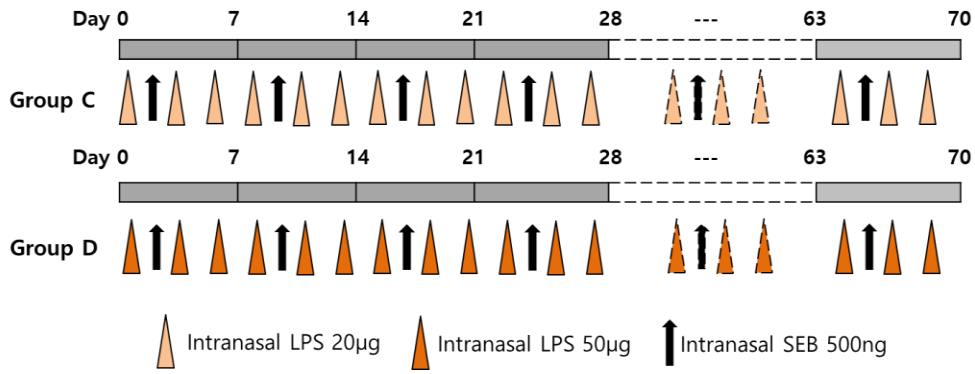


Figure 4. The changed protocol in experimental groups (Preliminary study II).

Preliminary study II – Results

After 10 weeks, neutrophils were increased in submucosa and neutrophilic exudates were observed in the sinonasal cavities, however, there was still no polyp formation in groups C and D (Figure 5).

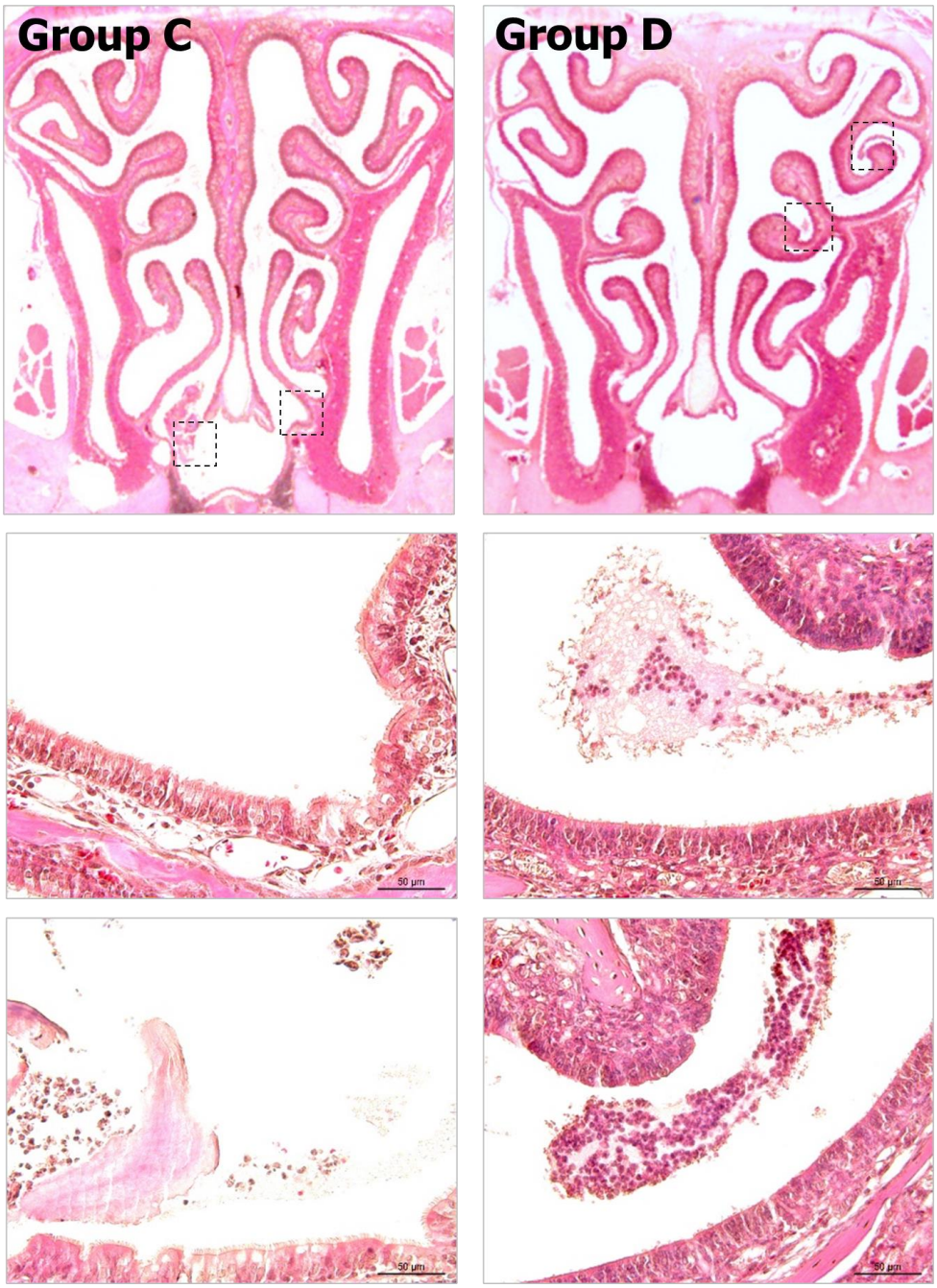


Figure 5. Histopathological results in experimental groups (Hematoxylin & Eosin staining, x40 & x400). Group C: LPS 20 µg three times a week & SEB 500 ng once a week intranasally, Group D: LPS 20 µg three times a week & SEB 500 ng once a week intranasally. There was no polyp formation, but some neutrophil inflammation was observed.

Preliminary study II – Discussion

In preliminary study I, the doses of LPS stimulation were selected as 10 μg and 20 μg considering the risk of acute lung injury. However, since polyp formation and neutrophilic inflammation were not observed, the dose of LPS stimulation was increased to 20 μg and 50 μg, respectively, and the frequency of stimulation was changed from once a week to three times a week. This was referenced in the method of a previously developed murine model of neutrophilic nasal polyps by stimulating LPS into the nostrils of mice three times a week for three months (17). However, there was no polyp formation in experimental groups (preliminary study II) despite increasing the dose and frequency of LPS stimulation. Instead, an LPS local stimulation dose of 50 μg was selected because the mice lived well without other problems and showed neutrophilic inflammation. Based on the preliminary results, we concluded that local LPS stimulation alone would not induce neutrophilic nasal polyp formation. However, a previous study reported that systemic LPS inhibited airway inflammation (15), and another study showed that intraperitoneal injection of LPS induced neutrophilic inflammation (16). In an LPS–induced neutrophil dominant rhinitis model, BALB/c mice were sensitized with OVA and 10 μg of LPS on days 0, 1, 2, 7, and 14 (total 40 μg of LPS) and challenged intranasally with OVA (16). We modified the experimental protocol to determine whether systemic or local stimulation of LPS was essential for nasal polyp development.

Furthermore, we planned the experimental methods to change eosinophilic inflammation to neutrophilic inflammation by additional stimulation of LPS or poly(I:C), based on the pre–established eosinophilic nasal polyp murine model.

Materials & Methods

Experimental animals

Four–week–old BALB/c mice (weighing 20–25 g) were used as the experimental animals. Animals were kept in a special pathogen–free biohazard containment facility maintained at 22–24°C and 50–60% humidity. All animal experiments were performed in compliance with the Seoul National University Animal Care and Use Committee guidelines and approved under the reference code SNU–170203–3–2.

Experimental groups and sensitization/challenge protocols

The general experimental layout is summarized in Figure 6. Mice were randomly divided into six groups having 10 mice each: Group A: negative control, Group B: positive control, Group C: systemic LPS stimulation, Group D: local LPS stimulation, Group E: both systemic and local LPS stimulation, and Group F: both systemic and local poly(I:C) stimulation. CRSwNP was induced in positive control group, following a previously established protocol (7). Briefly, mice were first sensitized with 25 μg of OVA (grade V; Sigma–Aldrich) in complex with 2 mg of alum (Thermo Fisher Scientific) via intraperitoneal injection on days 0 and 5. The mice were then challenged intranasally with 3% OVA diluted in 40 μL of PBS daily, from days 12 to 19, followed by three times a week thereafter for 12 consecutive weeks. Simultaneously, from week 5, considering the triweekly

instillations, mice were intranasally challenged on a weekly basis with SEB (500 ng) (Product# 122; List Biological Laboratories). Additionally, 20 μg of LPS was added to the OVA sensitization for the experimental groups C and E and 50 μg of LPS was added to OVA challenge for the experimental groups D and E. In experimental group F, 20 μg of poly(I:C) (#P1530; Sigma–Aldrich) was added each to the OVA sensitization and local challenge. In parallel, PBS was always administered to the negative control animals via the same administration routes.

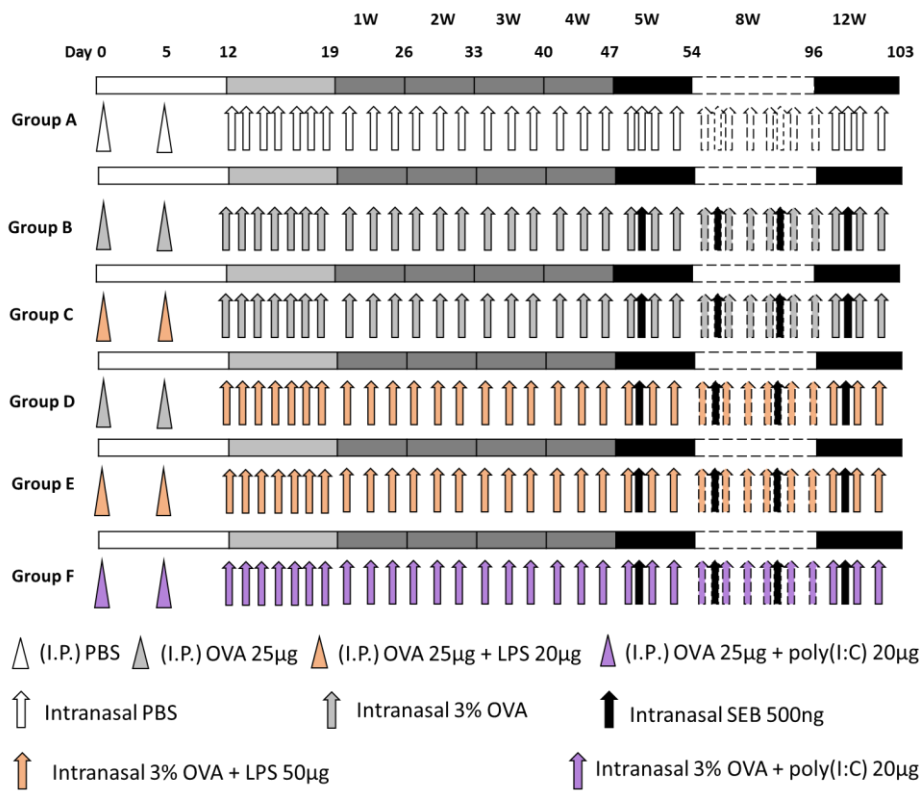


Figure 6. Experimental protocol.

Histopathological analysis

For the histopathological analysis, the heads of 5 mice per group were collected. Detailed experimental procedures have been previously described (26, 27). Different protocols were used to evaluate the degrees of inflammation, polyp formations, and epithelial disruptions. Hematoxylin and Eosin staining was used for the observation of tissue architecture and cellularity; Sirius red (Polysciences Inc., Warrington, PA, USA) staining, for eosinophils; anti–neutrophilic antibody (NIMP–R14; Abcam, Cambridge, UK) staining, for neutrophil; Giemsa (Sigma–Aldrich) staining, for mast cells; and Periodic acid-Schiff (Sigma–Aldrich) staining, for goblet cells.

The numbers of positive cells were determined in five high–power fields (HPF; x400) by two independent observers who were blinded to the group assignment. If the examiners had a disagreement, a consensus was reached by reviewing the specimen under a multi–head microscope by our research team. Nasal polyps were defined as distinct mucosal bulges with neutrophilic infiltration and/or microcavity formation (6). The results of inflammatory and secretory cells were expressed as cells per HPF.

Cytokine expression analysis

The nasal mucosa of the remaining five mice in each group was carefully taken out using a curette. Total RNA was isolated from the nasal mucosa samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA).

Complementary DNAs (cDNAs) were synthesized using amfiRivert Platinum cDNA Synthesis Master Mix (GenDEPOT, Katy, TX, USA). For the analysis of cytokine including IL–4 (Mm 00445259_m1), IL–5 (Mm 00439646_m1), IL–13 (Mm00434204_m1), IFN–γ (Mm99999071_m1), IL–17A (Mm00439618_m1), MMP–9 (Mm00442991_m1), IL–25 (Mm00499822_m1), IL–33 (Mm00505403_m1), thymic stromal lymphopoietin (TSLP) (Mm00498739_m1), and glyceraldehyde–3–phosphate dehydrogenase (GAPDH) (Mm99999915_g1), predeveloped assay reagent kits of primers and probes were purchased from Applied Biosystems (Foster City, CA, USA). Amplification of cDNAs was performed in MicroAmp optical 96–well reaction plates (Applied Biosystems) with TaqMan Universal PCR Master Mix (PE Biosystems, Foster City, CA, USA), using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). GAPDH was used as an endogenous control.

Quantification of serum total and OVA–specific IgE levels

Serum samples from mice were obtained at the time of sacrifice. Total and OVA–specific serum IgE levels were measured by enzyme–linked immunosorbent assay (ELISA) as described previously (28). Briefly, for the analysis of total IgE, serum samples were added to the 96–well plates along with purified mouse IgE isotype (#557079, BD Biosciences) used as a standard. For the analysis of OVA–specific IgE, serum samples were added

to the OVA (100 μg/mL in 0.05 M carbonate–bicarbonate buffer)–coated plates 96–well flat–bottom plates. After following the outlined protocols, plates were then washed three times and developed with 100 μL per well of 3,3´,5,5´–tetramethylbenzidine (TMB) (#52–00–00, KPL). The reaction was terminated by the addition of 1N HCL (50 ul/well). Optical density was measured in a microplate reader at 450 nm. Total IgE levels were determined by interpolation from a standard curve.

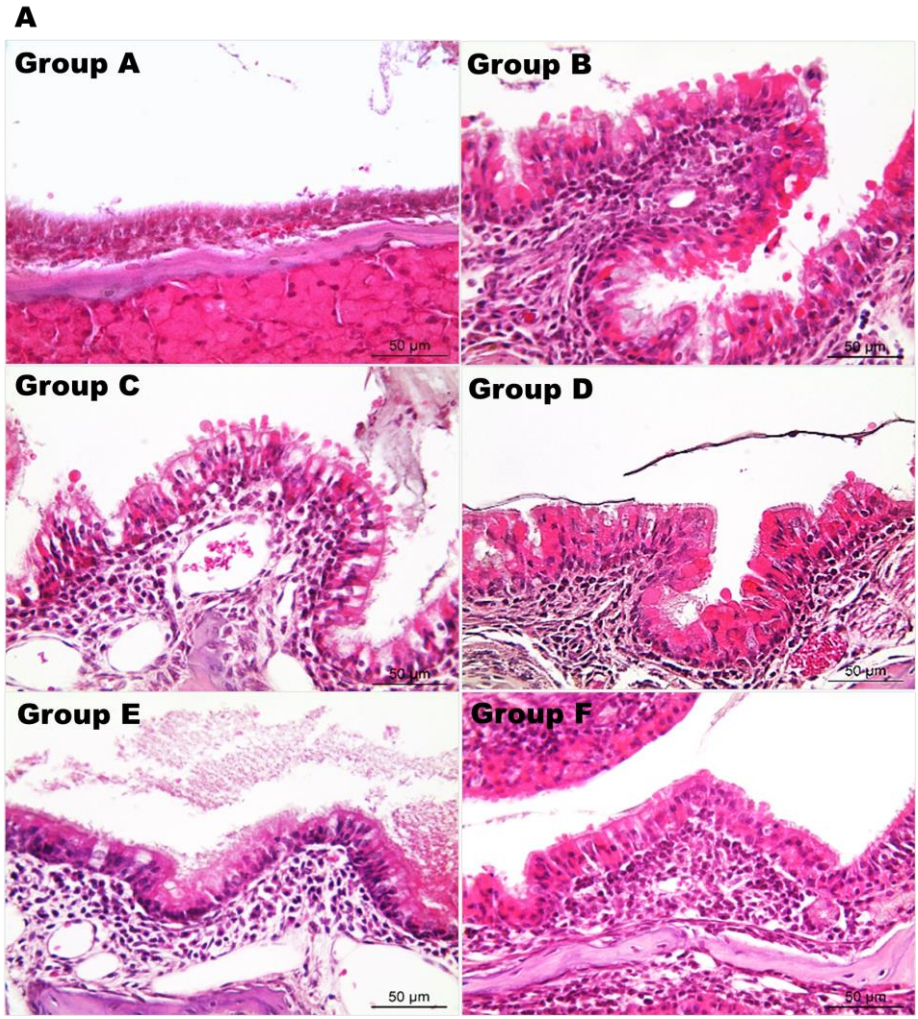
Statistical Analyses

The data are represented as means ± standard deviation. Illustrative figures were generated using Prism version 8.0 (GraphPad Software Inc., La Jolla, CA, USA) and Microsoft's PowerPoint 365. Statistical analysis was performed using SPSS 24.0 software (IBM, Armonk, NY, USA). Mann–Whitney U and Kruskal–Wallis tests were used to compare differences between two, or more than two groups, respectively. Statistical significance was given by a P value inferior to 0.05.

Results

Histopathological results

As expected, the development of polyps and epithelial disruptions were observed in the positive control group (group B), compared with the negative control group (group A) (Figure 7A). Additionally, these morphological alterations were also observed in all experimental groups (systemic LPS: group C, local LPS: group D, systemic and local LPS: group E, systemic and local poly(I:C): group F). Moreover, no significant differences in polyp formations (Figure 7B; all $P > 0.05$) and epithelial disruptions (Figure 7C; all $P > 0.05$) were detected among groups B, C, D, E, and F. Additionally, in groups B, C, D, E, and F, inflammatory cell infiltrates were observed to a greater extent than that in the negative control group.


A
Group A
Group B
Group C
Group D
Group E
Group F
50 μm
B
Number of polyp formation
0
2
4
6
8
*
Group A
Group B
Group C
Group D
Group E
Group F

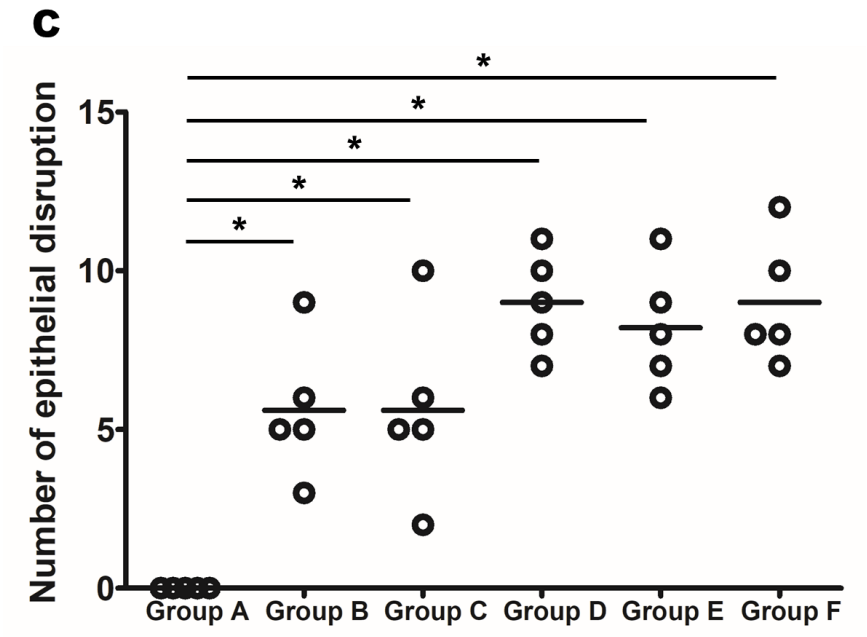
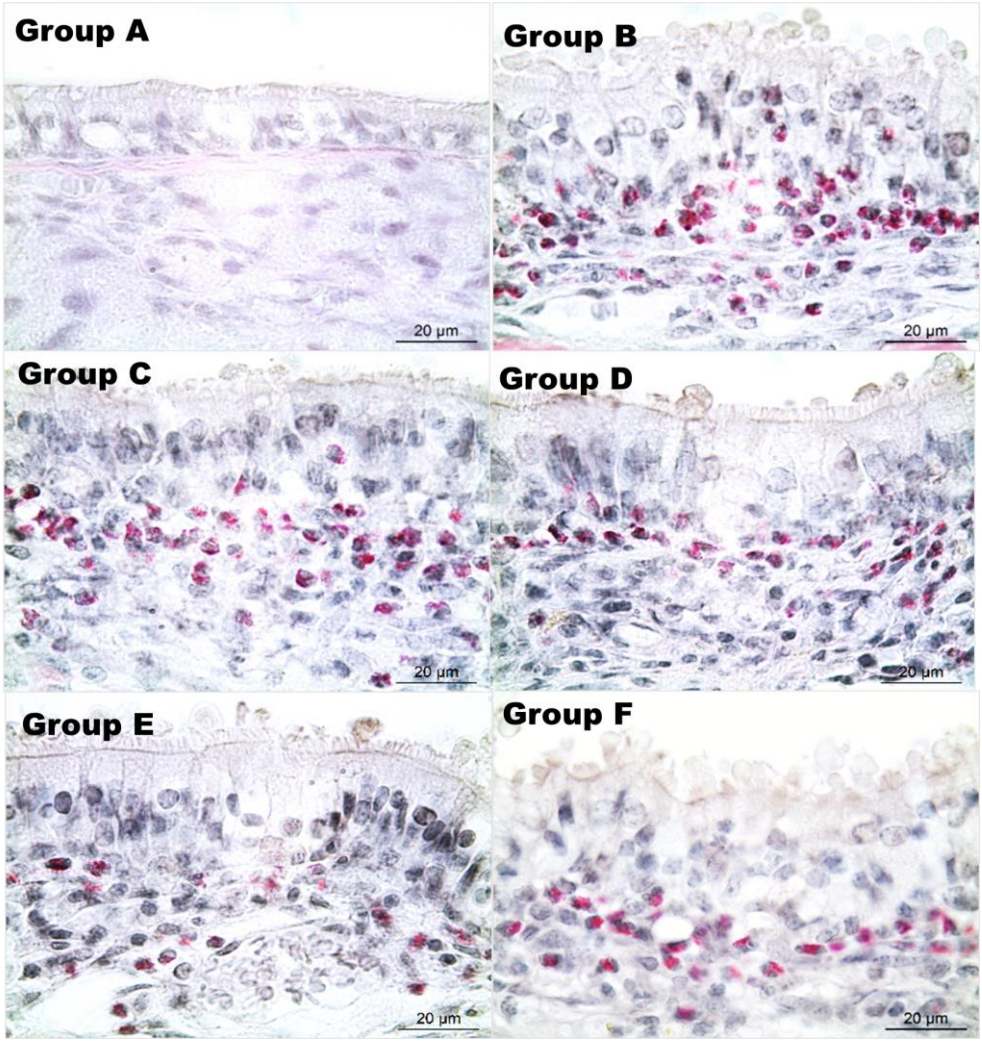


Figure 7. Histopathological results of the development of nasal polyps and epithelial disruptions. Nasal polyps and epithelial disruptions were observed in all experimental groups. (A) Representative Hematoxylin & Eosin staining (x400), (B) Polyp formations, and (C) Epithelial disruptions according to the groups. Group A: negative control, Group B: positive control, Group C: systemic LPS stimulation, Group D: local LPS stimulation, Group E: systemic and local LPS stimulation, Group F: systemic and local poly(I:C) stimulation. * $P < 0.05$

To determine whether systemic or local stimulation of LPS is essential for nasal polyp development, we divided the experimental groups (C, D, and E). With respect to cellularity, some differences were detected in the LPS and positive control groups. Animals that received systemic LPS stimulation (group C) showed no significant difference in levels of eosinophil (Figures. 8A and C; P = 0.886) and neutrophil (Figures. 8A and B; P = 0.801) infiltrations compared with the positive control group (group B). By contrast, animals that received local LPS stimulation (group D) and both systemic/local LPS stimulation (group E) showed lower levels of eosinophil (Figures. 8A and C; P = 0.016 in group D; P = 0.009 in group E) and higher neutrophil (Figs. 8B and C; P =0.018 in group D; P = 0.009 in group E) infiltrations, respectively, than those in the positive control group (group B).

To compare the different immune responses of bacterial– and viral–derived stimuli in nasal polyp development, we administered LPS and poly(I:C) additionally. Mice that received poly(I:C) (group F) showed higher levels of neutrophil infiltration than that in the positive control group (group B) (Figures. 8A and C; P = 0.027); however, although they showed decreased eosinophil counts, the difference between groups B and F was not significant (Figures. 8A and B; P = 0.095). Mast (all P > 0.05) and goblet cells (all P > 0.05) infiltrations showed no differences between groups B, C, D, E, and F (Figure. 8C).

A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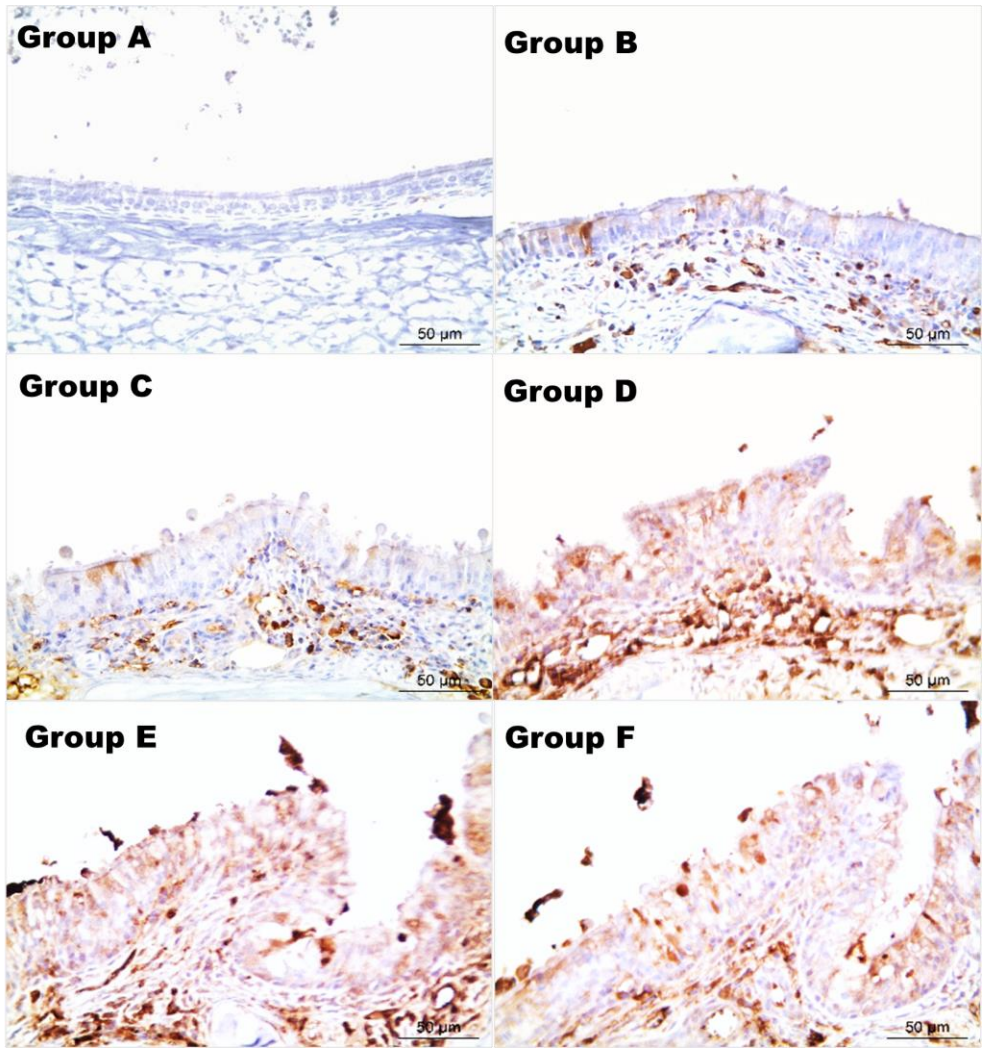
B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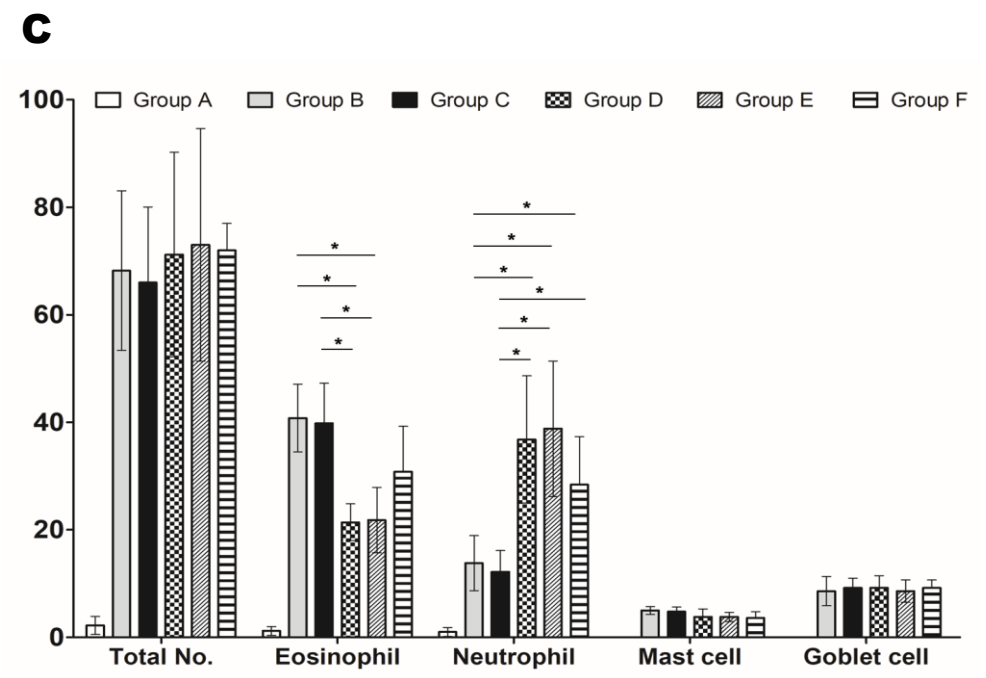


Figure 8. Histopathological results of inflammatory cell infiltrations according to the groups. Group D and E shows less eosinophil than group B. Group F shows decreased eosinophil counts, but the difference between groups B and F was not significant. In group D, E, and F, there are more neutrophil than group B. (A) Sirius red staining for eosinophils, (B) Anti–neutrophil antibody staining, and (C) Inflammatory cell infiltrate profile. Group A: negative control, Group B: positive control, Group C: systemic LPS stimulation, Group D: local LPS stimulation, Group E: systemic and local LPS stimulation, Group F: systemic and local poly(I:C) stimulation. * $P < 0.05$

Cytokines in the nasal mucosa

Tissue cellularity and immune tissue–environment are closely related (29). To understand if the above–mentioned differences observed for infiltrating cells would translate to distinct tissue environments, we analyzed expression

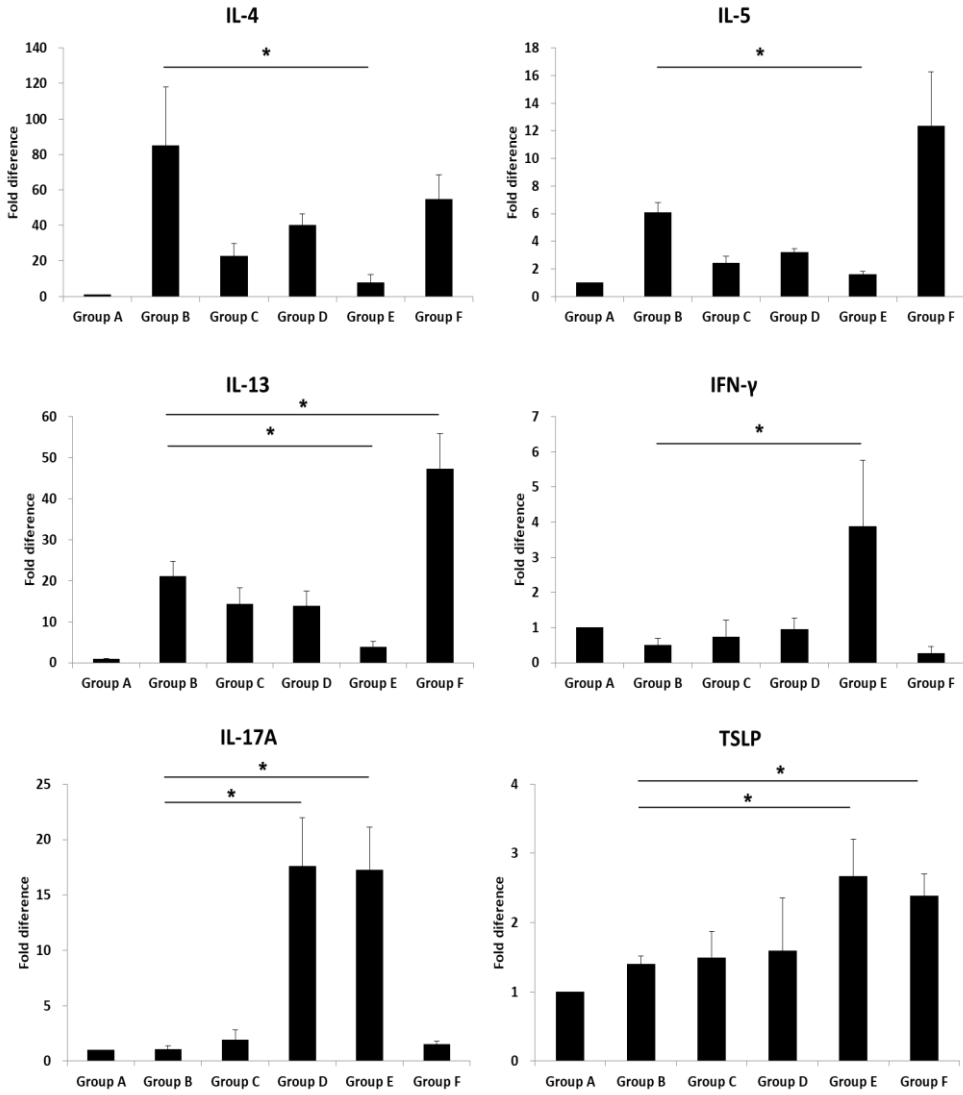
of cytokines in the nasal mucosa. Figure 9 represents the overall cytokines in the nasal mucosae of different groups. Animals that received both systemic and local LPS stimulation (group E) showed a significant upregulation of IFN–γ (P = 0.014) and IL–17A (P = 0.014) expression, along with significantly lower IL–4 (P = 0.016), IL–5 (P = 0.016), and IL–13 (P = 0.016) mRNA levels, compared with that in the positive control group. In contrast, animals that received systemic LPS stimulation (group C) showed no different IL–4 (P = 0.121), IL–5 (P = 0.129), IL–13 (P = 0.223), IL–17A (P = 0.376), and IFN–γ (P = 0.530) mRNA expression compared with that in the positive control group. In animals that received local LPS stimulation (group D), only IL–17A mRNA levels showed a significant upregulation (P = 0.029), compared with that in the positive control group. Furthermore, the IL–33 (P = 0.009) and TSLP (P = 0.028) mRNA levels were significantly increased only in group E (compared with that in the positive control animals). Additionally, no changes in IL–25 expressions were detected in the LPS groups compared with that in the positive control animals (P = 0.117).

In contrast, mice that received both systemic and local poly(I:C) stimulation showed a significantly increased IL–13 mRNA expression in the nasal mucosa (P = 0.047) compared with that in the positive control group. In the poly(I:C) group, the expressions of IL–4 (P = 0.016) and IL–5 (P = 0.047) were increased and that of IFN–γ (P = 0.101) and IL–17A (P = 0.101) were not different when compared with the negative control group; however,

the mRNA expressions of IL–4 (P = 0.690), IL–5 (P = 0.421), IFN–γ (P = 0.730), and IL–17A (P = 0.175) showed no significant differences compared with that in the positive control group. Furthermore, while the IL–25 (P = 0.009) and TSLP (P = 0.016) mRNA levels were significantly increased, IL–33 mRNA levels showed no significant differences (P = 0.754) in poly(I:C) groups (compared with that in the positive control group). Additionally, no changes in MMP–9 gene expression were detected in the experimental groups with that in the positive control animals (all P > 0.05).

Serum total and OVA–specific IgE levels

Allergy and IgE levels are intricately related (30). As antibody production may be influenced by the immune environment (31), we sought to understand if the reported differences in the nasal cavity cytokine expression would affect systemic IgE levels. The animals that received systemic LPS stimulation with or without local stimulation showed significantly lower serum levels of total (P < 0.001 in group C; P = 0.009 in group E) and OVA–specific IgE (P < 0.001 in group C; P = 0.014 in group E) compared with that in the positive control. However, in mice receiving only local LPS stimulation (group D) and receiving both systemic and local poly(I:C) stimulation (group F), the serum total (P = 0.051 in group D; 0.056 in group F) and OVA–specific IgE (P = 0.935 in group D; 0.686 in group F) levels were similar to those observed in positive control animals (Figure 10).


IL-4
Fold diference
140
120
100
80
60
40
20
0
*
Group A
Group B
Group C
Group D
Group E
Group F
IL-5
Fold diference
18
16
14
12
10
8
6
4
2
0
*
Group A
Group B
Group C
Group D
Group E
Group F
IL-13
Fold diference
60
50
40
30
20
10
0
*
*
Group A
Group B
Group C
Group D
Group E
Group F
IFN-γ
Fold diference
7
6
5
4
3
2
1
0
*
Group A
Group B
Group C
Group D
Group E
Group F
IL-17A
Fold diference
25
20
15
10
5
0
*
*
Group A
Group B
Group C
Group D
Group E
Group F
TSLP
Fold diference
4
3
2
1
0
*
*
Group A
Group B
Group C
Group D
Group E
Group F

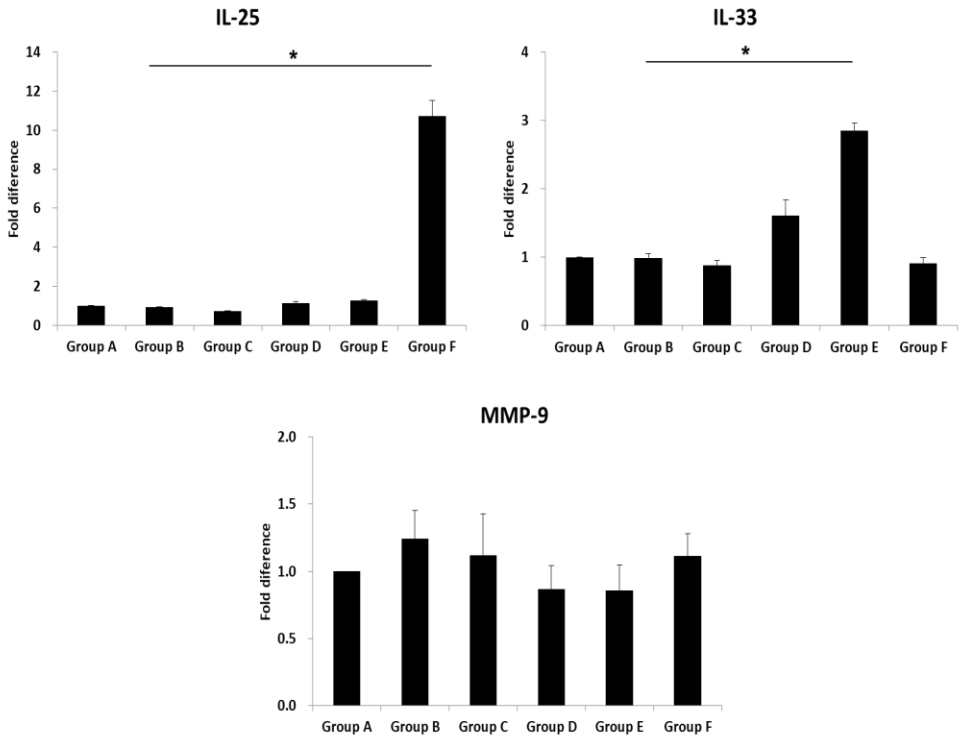


Figure 9. Inflammatory cytokines profile of the nasal mucosa according to the groups. Group E shows a significant upregulation of IFN–γ, along with significantly lower IL–4, IL–5, and IL–13 mRNA levels. Group D and E shows a significant upregulation IL–17A expression. IL–33 significantly increased in group E. IL–25 increased in group F. TSLP mRNA levels were increased in both group E and F. Group A: negative control, Group B: positive control, Group C: systemic LPS stimulation, Group D: local LPS stimulation, Group E: systemic and local LPS stimulation, Group F: systemic and local poly(I:C) stimulation. * $P < 0.05$

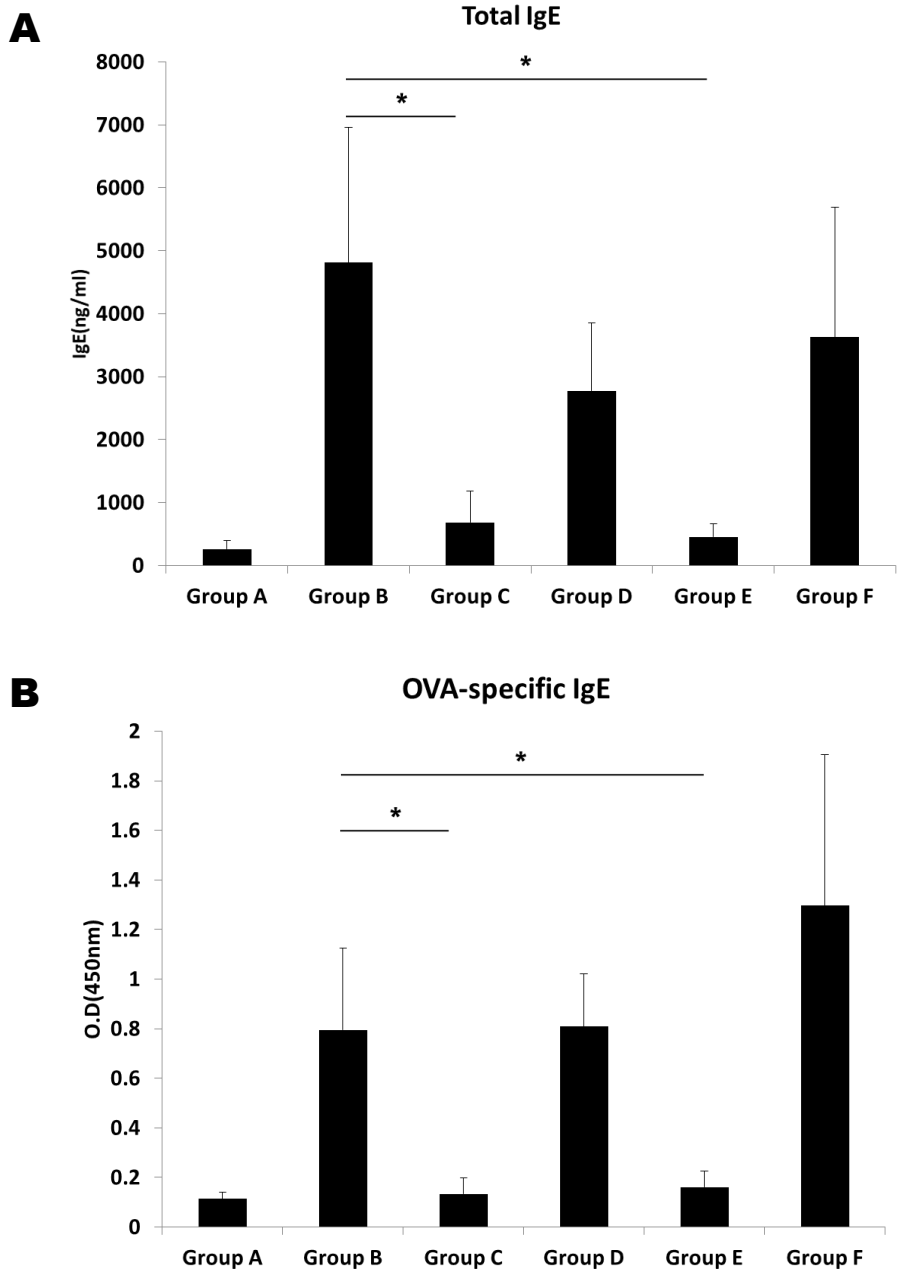


Figure 10. Serum total IgE (A) and OVA–specific IgE (B) according to the groups. Serum total IgE and OVA–specific IgE level were significantly decreased in groups C and E. Group A: negative control, Group B: positive control, Group C: systemic LPS stimulation, Group D: local LPS stimulation, Group E: systemic and local LPS stimulation, Group F: systemic and local poly(I:C) stimulation. * $P < 0.05$

Discussion

In this study, we established the neutrophilic polyp murine model using LPS or poly(I:C) stimulation. In addition, we determined that these two bacterial– and viral–derived stimuli induce different responses with respect to both cell recruitment patterns and local immune environments in nasal polyp development.

The establishment of an appropriate murine model of CRSwNP is extremely important to determine new therapies to prevent nasal polyp formation. Wang *et al*. established a murine model of LPS–induced neutrophilic nasal polyps by continuous intranasal instillation of LPS alone (17), which reproduced the dominant Th1/Th17 responses observed in Asian patients (21). However, in our preliminary studies, no polyp formation was observed after continuous intranasal administration of 10, 20, or 50 μg of LPS, although there was a difference in mouse strain between a previous study by Wang *et al*. using a Th1–biased mouse strain (C57BL/6) and our study using a Th2–biased mouse strain (BALB/c). BALB/c mice are generally preferred for studies on allergic immune responses, particularly in the upper nasal airway, while C57BL/6 mice are used in several studies, that primarily focused on the lower airway (32–34). Therefore, we attempted to evaluate whether systemic or local stimulation of LPS was essential for polyp formation and to establish a murine model of neutrophilic nasal polyps with high reproducibility in BALB/c mice.

This study showed that dual systemic/local LPS stimulation was critical in neutrophilic nasal polyp formation in a murine model of allergic rhinosinusitis. In addition, there were different results between mice that were only systematically stimulated and those locally stimulated with LPS. Increased neutrophil infiltrations and IL–17 levels were only observed in mice where LPS stimulation was added locally. We found that IgE production was only inhibited in mice systemically stimulated with LPS. Exposure to LPS showed heterogeneous effects on eosinophilic inflammation (15, 16, 35, 36). While some previous studies have reported that the intraperitoneal injection of LPS alone induced neutrophilic inflammation (16), others have demonstrated that systemic LPS administration, concomitantly with the OVA challenge, inhibited airway eosinophilic inflammation, whereas local LPS induced a strong airway inflammation with predominance of neutrophils (15). The route, concentration, frequency, and duration of LPS exposure are thought to determine whether LPS down- or up-regulates Th2-mediated allergic responses. Furthermore, when TLR ligand stimulated systematically or locally, it could be because due to differences in their responses as the stimulated cells may be immune or mucosal epithelial cells, respectively. TLR mRNA and protein expression is generally detected in airway mucosal epithelial cells (37).

LPS predominantly induced neutrophilic infiltration in the nasal polyp and Th1/Th17 immune environment. Our observation is consistent with

previous studies reporting that LPS regulates the secretion of IL–17 in a variety of cell types through TLR4–mediated signaling (17, 38, 39). Importantly, TLR4 has been shown to play critical roles in regulating the migration, activation, and life span of neutrophils (39, 40). Therefore, the present data may provide further supporting evidence for the relationship between LPS–TLR4 crosstalk, IL–17 expressions, and neutrophil infiltrations.

Although the role of viral infections in CRSwNP development is not clear, herein we showed that the TLR3 agonist and viral analogue poly(I:C) promoted a Th2–skewed environment in neutrophilic nasal polyp development. Furthermore, data analysis hypothesizes that this is a consequence of the secretion of TSLP and IL–25. In fact, previous *ex vivo* studies reported that viral stimulation of polyp–derived epithelial cells enhanced the Th2 immune responses via the release of TSLP and IL–25 from epithelial cells (41, 42). However, in this *in vivo* study, the Th2–like nature was not as striking. This may be due to the model that was used. Administering a high SEB dose in an OVA–induced allergic chronic rhinosinusitis murine model was shown to induce high neutrophilic infiltration levels associated with increased expression of IFN–γ (7). Although poly(I:C) stimulation in this context resulted in a distinct environment, we still observed a considerable amount of neutrophil infiltration and some proinflammatory cytokine secretion, which may indicate that our stimulatory conditions might not have completely

counteracted the strong SEB–induced effect. Furthermore, in asthma (19) and rhinitis animal models (20), contrary to the above–mentioned *ex vivo* models (40, 41), poly(I:C) was shown to promote significant mucosal neutrophil infiltration together with a mixed Th1/Th2 environment.

In the present study, IL–33 was upregulated by LPS, but not by poly(I:C) stimulation. IL–33 is thought to be the most probable triggering factor for Th2 immune responses in the mucosal tissues (43). However, some studies also suggest that IL–33 has a role in neutrophil recruitment during infection (44, 45). In fact, a recent study reported that IL–33 plays a crucial role in the pathogenesis of neutrophilic inflammation in Asian patients with CRSwNP (46). However, this may not be a universal fact, as another recent study has reported that IL-33 expression is strongly influenced by geographically variable environmental factors (47). Among these are infections caused by different agents. In line with this, and based on our data, it could be considered that bacterial–, but not viral–derived, stimuli promote IL–33 secretion.

MMP–9 is known to degrade collagen IV, which is the main component of the basement membrane that provides structural support to epithelial and endothelial cells (48). Consequently, MMP–9 secretion is thought to increase the microvasculature leakiness, promoting the transmigration of inflammatory cells and stromal oedema. Importantly, previous findings suggest that MMP–9 is involved in the pathophysiology of nasal polyps (49). It has been shown that both TLR–4 (50) and TLR–3

(51) mediated signaling promote MMP–9 expressions. However, in our study, no significant differences were detected in the MMP–9 expression levels between animals that received LPS and poly(I:C) (TLR–4 and TLR–3 ligands, respectively) and the experimental positive controls. Again, this may be due to the choice of murine polyp model. Importantly, the fact that we did not observe any significant differences in the nasal polyp formation between these groups aligns with the comparable MMP–9 expression levels determined.

The relevance of murine models in human diseases has been questioned because human conditions cannot be fully mimicked or actually developed differently in mice. Inbred mouse strains have limited genetic diversity and may not reflect the responses generated in genetically polymorphic human populations (52). Furthermore, there are some limitations in the murine model in that mice have small sinus cavities and that the maxillary sinuses are not completely enclosed by the maxilla (53). However, murine models are invaluable *in vivo* models for examining a variety of human diseases that cannot be possible via *in vitro* experiments using the middle or inferior turbinate mucosa. In addition, rabbit models are sometimes considered superior to mice because rabbits have well pneumatised sinus cavities, and their morphological features are highly similar to that of the human sinonasal epithelium, as opposed to that of mice (54). However, rabbit models are unsuitable to explore the underlying immunopathological mechanisms related to sinus diseases. In contrast,

murine models are generally considered suitable for investigating the sinus disease and have been widely applied for studies understanding the nasal polypogenesis and molecular immune responses in CRSwNP (55). Most recently, Kim *et al*. showed that the nasal polyp murine model demonstrates enhanced B cell responses reminiscent of B cell responses in human nasal polyp (56).

This study has some limitations. First, it is difficult to definitively know whether these are the effects of LPS or poly(I:C) stimulation because various stimulants have been stimulated over a long period of time in this murine model. Second, we did not investigate the relationship of the immune response between this murine model and human nasal polyps. Further studies are needed to confirm that this murine model reproduces the immune responses observed in Asian patients with neutrophilic nasal polyps. Furthermore, if blocking TSLP, IL–25, or IL–33 activity reduces nasal polyp formation using this neutrophilic murine model, this could be used as a therapeutic strategy to improve clinical outcomes of patients with neutrophilic nasal polyps.

Conclusions

We developed the neutrophilic polyp murine model by systemic and local stimulation of either LPS or poly(I:C). This study shows that the administration of LPS or poly(I:C), as bacterial– and viral–derived components, respectively, in a murine model of allergic rhinosinusitis with nasal polyp formation leads to the development of different inflammatory profiles but does not influence nasal polyp formation itself. While LPS induced a predominant Th1/Th17 environment, poly(I:C) contributed towards a Th2–skewed environment. Therefore, our data may have implications in the physiopathology of CRSwNP with a known complex etiology.

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초 록

LPS 또는 poly(I:C) 자극을 이용한 호중구성 비폴립 마우스 모델 개발의 새로운 방법

위 지 혜

의학과 중개의학 전공

서울대학교 대학원

서론: 비강 폴립은 침윤된 면역세포의 유형과 사이토카인에 따라 호산구성과 호중구성으로 분류할 수 있다. 호산구성 비강 폴립의 마우스 모델은 이미 난알부민과 포도상 구균 장독소 B (SEB)으로 감작하여 개발되었지만, 호중구성 비강 폴립의 마우스 모델은 아직까지 잘 확립되어 있지 않다. 또한 박테리아, 바이러스, 또는 진균을 포함한 여러 요인이 비강 조직의 재형성 및 비부비동염에 중요한 역할을 하는 것으로 알려져 있고, 박테리아 유래성분인 LPS와 합성 바이러스 유사체인 poly(I:C)가 호중구성 면역반응을 유발한다고 알려져 있다. 이에 본 연구에서는 LPS 또는 poly(I:C)를 이용하여 새로운 호중구성 비폴립 마우스 모델을 개발하고, 박테리아와 바이러스 자극에 따른 면역반응을 비교하고자 하였다.

방법: 이전에 개발된 BALB/c 마우스에 난알부민과 SEB로 감작된 호산구성 비폴립 모델을 기반으로 LPS 전신 자극, LPS 국소 자극, LPS

전신/국소 자극을 추가로 하여 호중구성 폴립의 생성 유무를 관찰하여 전신 또는 국소 자극이 호중구성 비폴립 형성에 필수적인지 여부를 결정하였다. 또한 poly(I:C)를 전신/국소 자극을 주어 LPS군과 조직병리학적 소견, 사이토 카인 및 혈청 면역 글로불린 등 면역반응을 비교분석하였다.

결과: LPS 전신 및 국소 자극을 모두 주었을 때 호중구 침윤이 유도되며 Th1/Th17 면역 반응을 보이는 비폴립이 형성된 것을 관찰할 수 있었다. 또한 LPS와 poly(I:C)를 전신 및 국소 자극을 준 경우 모두 호중구성 비폴립 마우스 모델이 개발되었지만, 면역 세포의 패턴 및 사이토카인의 발현이 서로 다르게 나타났다. LPS 자극은 IL-4, IL-5, IL-13은 감소하고 IL-17A, IFN-γ는 증가한 반면, poly(I:C) 자극은 호산구성 폴립모델인 양성 대조군의 IL-4, IL-5, IL-17A, IFN-γ에 비해 유의한 차이를 보이지 않았으며, LPS 자극에는 TSLP와 IL-33가 poly(I:C) 자극에는 TSLP와 IL-25가 증가하였다.

결론: 난알부민과 SEB로 감작된 호산구성 비폴립 모델에서 LPS 또는 poly(I:C)의 추가적인 전신 및 국소 자극은 호중구성 비폴립을 형성하여 새로운 마우스 모델을 개발할 수 있었다. LPS는 Th1/Th17 반응이 현저한 반면 poly(I:C)는 Th2에 치우친 서로 다른 면역반응을 보여, 이는 박테리아와 바이러스 자극이 호중구성 비폴립을 동반한 비부비동염의 병태생리학에 영향을 미칠 수 있음을 보여주었다.

주요어: 동물 모델, 비폴립, 호중구, LPS, poly(I:C), 부비동염.
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