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# **Intranasal immunization with phosphorylcholine suppresses allergic rhinitis in mice**

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Intranasal Immunization with Phosphorylcholine  
Suppresses Allergic Rhinitis in Mice

A short running title; PC suppresses allergic rhinitis in mice

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## Abstract

**Objectives/Hypothesis:** Intranasal immunization with phosphorylcholine (PC) is known to reduce IgE production. However, its effects on the occurrence of allergic rhinitis (AR) are unknown.

**Objectives:** This study was performed to evaluate the effects of PC-keyhole limpet hemocyanin (PC-KLH) and to examine the effects on the occurrence of AR in a murine model of AR.

**Study Design:** In vivo study using an animal model.

**Methods:** Forty-five female BALB/c mice were divided into 3 groups; those pretreated with intranasal administration of PC-keyhole limpet hemocyanin (PC-KLH) followed by intra-peritoneal sensitization and nasal challenge with ovalbumin (OVA) (Group A), those untreated with PC-KLH followed by sensitization and nasal challenge with OVA (Group B), and those untreated with PC-KLH or OVA as a control (Group C). Nasal symptoms, allergic inflammation in the nasal mucosa, OVA specific IgE production, and cytokine profile were compared among those three groups. Further, dendritic cells (DCs) were isolated from splenic

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cells and PC-KLH-stimulated IL-12p40 production was measured.

**Results:** The mice pretreated with PC-KLH showed lower allergic nasal symptoms and inflammation compared to untreated mice. The levels of total IgE and OVA-specific IgE in serum, and IL-4 production by nasal and splenic CD4<sup>+</sup> T cells were significantly reduced by PC-KLH pretreatment. Furthermore, IL-12p40 production by DCs was induced by PC-KLH in a dose-dependent manner.

**Conclusion:** Intranasal administration of PC-KLH suppressed allergic inflammation in nasal mucosa and antigen-specific IgE production by down regulating Th2-type immune response. Intranasal immunization with PC might be useful to prevent AR and upper airway bacterial infection.

**Key Words:** allergic rhinitis, phosphorylcholine, NALT, dendritic cells, IL-4, IL-12

**Level of Evidence:** NA

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## INTRODUCTION

The incidence of upper airway infections such as acute otitis media (AOM) is still high despite the production of several new antibiotics. Furthermore, the prevalence of antibiotic-resistant bacteria has increased in recent years. Pneumococcal conjugate vaccines have been used in many countries since the mid-1990s and the incidence of invasive pneumococcal infections has been dramatically reduced.<sup>1</sup> However, the vaccines only moderately protected against AOM due to AOM by non-vaccine pneumococcal strains and other bacteria such as *Haemophilus influenzae*.<sup>2,3</sup> Therefore, development of a broad-spectrum vaccine effective against most pathogens of upper airway infections is needed.

The candidates of the broad-spectrum vaccines are pneumococcal protein A (PspA) of *Streptococcus pneumoniae* (Spn),<sup>4</sup> P6 of nontypeable *H. influenzae* (NTHi).<sup>5</sup> phosphorylcholine (PC),<sup>6</sup> and so on. However, the effectiveness of PspA and P6 as a vaccine is limited to the strains of Spn and NTHi, respectively. In contrast, PC vaccine is effective to both Spn and NTHi, since PC is a common pathogen-associated molecular pattern and

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presents in the outer cell membrane of both gram-positive and negative bacteria.<sup>7</sup> The immunomodulatory effects of PC as a vaccine have been demonstrated, wherein intranasal vaccination with protein-linked PC confers protection to mice against a lethal intranasal challenge with Spn.<sup>8</sup> Previously, we showed that intranasal immunization with PC-keyhole limpet hemocyanin (KLH) induced PC-specific mucosal IgA as well as systemic immune responses and enhanced the clearance of most strains of Spn and NTHi from the nasal cavity, indicating that PC may be a potential broad spectrum vaccine.<sup>9</sup> Furthermore, intranasal immunization with PC-KLH plus cholera toxin (CT) or PC-KLH alone did not increase IgE production, although intranasal administration with CT alone remarkably increased IgE level.<sup>9</sup> These findings suggest that PC might suppress IgE production while maintaining the induction of PC-specific mucosal IgA immune responses.

Pneumococcal vaccines have been reported to reduce the frequency of acute asthmatic attacks and the risk of asthma-related hospitalizations.<sup>10,11</sup> Some investigators have demonstrated the inhibition of allergic inflammation in animal models of asthma by intra-tracheal immunization with whole killed Spn or intranasal administration of

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pneumococcal conjugate vaccine.<sup>12,13</sup> Kim and Shin et al reported that intranasal administration of pneumococcal conjugate vaccine and pneumococcal polysaccharide vaccine suppressed allergen-specific IgE production and attenuated allergic inflammation in a murine model of allergic rhinitis (AR) sensitized with *Dermatophagoides farinae* (Der f).<sup>14,15</sup> Since PC is a component of pneumococcal cell wall and polysaccharide, its presence might be associated with the suppression of allergen-specific IgE production and allergic inflammation in pneumococcal vaccine administration. In fact, a recent study showed that exposure of neonatal mice to PC-bearing pneumococci significantly reduced house dust mite (HDM)-specific IgE production and development of airway hyper-responsiveness during adult life.<sup>16</sup> However, the potential of PC to reduce allergen-specific IgE production and attenuate allergic inflammation is unknown. If PC is shown to suppress allergic inflammation, PC could be applicable for both preventing upper airway bacterial infection and allergic diseases such as AR.

In the present study, we investigated the effects of PC on allergen-specific IgE production and nasal symptoms by systemic and intranasal sensitization of mice that were induced with



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AR by ovalbumin (OVA). Furthermore, IL-4 production from CD4<sup>+</sup> T cells isolated from the spleen and nasopharynx-associated lymphoid tissue (NALT) of OVA-sensitized mice, and IL-12p40 production from splenic dendritic cells (DCs) stimulated with PC were examined, since IL-4 causes specific heavy chain class switching to IgE in Th2-stimulated B cell cultures and IL-12p40 produced from DCs is known to inhibit Th2-type immune responses.<sup>17,18</sup> Based on those results, the mechanism how intranasal PC immunization suppresses IgE production is discussed

## MATERIALS AND METHODS

### *Mice*

Six-week-old female BALB/c mice were purchased from CLEA Japan Inc. (Shizuoka, Japan) and maintained in the experimental animal facility of Kagoshima University under specific pathogen-free conditions. All mice used in this study were 7-12 weeks of age. The experimental protocol was approved by the Ethics Board of the Institute of Laboratory Animal Sciences of Kagoshima University.

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### ***Intranasal immunization with PC and induction of AR***

Female BALB/c mice were divided into 3 groups; those pretreated with intranasal administration of PC-keyhole limpet hemocyanin (PC-KLH) followed by intra-peritoneal sensitization and nasal challenge with ovalbumin (OVA) (Group A), those untreated with PC-KLH followed by sensitization and nasal challenge with OVA (Group B), and those untreated with PC-KLH or OVA as a control (Group C). Since five mice were included in each group and each experiment was repeated 3 times, totally forty-five mice were used in the experiment.

Group A mice were pretreated by intranasal administration of 100 µg of PC-keyhole limpet hemocyanin (KLH) (Biosearch, San Rafael, CA) diluted in 10 µl of phosphate buffered saline (PBS) or with 10 µl of PBS alone on days 0, 7, and 14 as previously described with some modification<sup>9</sup> (Fig 1). AR was then induced by OVA sensitization as previously described.<sup>19</sup>

In brief, mice were sensitized by intra-peritoneal injection of 25 µg of OVA (Sigma Chemical Co., St. Louis, MO) together with 1 mg of aluminum hydroxide hydrate gel (Alum) (LSL Co.,

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Tokyo, Japan) diluted in 300  $\mu$ l of PBS on days 21, 28, and 35. In order to induce AR symptoms, the sensitized mice were challenged by intranasal administration of 500  $\mu$ g of OVA diluted in 30  $\mu$ l of PBS for seven consecutive days from days 42 to 48. Group B mice received intra-peritoneal sensitization and nasal challenge with OVA without intranasal administration of PC-KLH. Group C control mice were pretreated with intranasal administration of PBS instead of PC-KLH and received no intra-peritoneal sensitization and nasal challenge with OVA.

Serum samples were collected on days 0, 21, 42, and 49 just after the provocation of AR in order to monitor total and OVA-specific IgE production. Then, the mice were sacrificed, and nasal passage tissues, NALT, and spleen were collected. Further, spleen and palatine plate tissue containing NALT were dissected from each mouse, and mononuclear cells were isolated as previously described with some modifications.<sup>20</sup> In brief, the spleen and NALT tissues were gently teased through a stainless steel screen and 100 $\mu$ m nylon mesh, and the collected cells were suspended in Ammonium-Chloride-Potassium (ACK) lysing buffer and incubated for five minutes at room temperature to remove red blood cells. The cells were then

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washed and centrifuged over a discontinuous Percoll gradient. Mononuclear cells at the interface of the 40% and 75% layers were collected and used for the purification of CD4<sup>+</sup> T cells. For the isolation of mononuclear cells from NALT and spleen, the cells were respectively collected from ten and five mice, and were pooled and processed in each experiment.

### ***Assessment of AR symptoms***

Nasal symptoms of AR such as sneezing and nasal rubbing were observed on day 49 after nasal provocation with the same dose of OVA as used for the intranasal challenge in Group A and B mice. In Group C control mice, nasal symptoms were observed in the same manner without nasal challenge with OVA. The nasal symptom frequencies were counted for five minutes after nasal provocation by investigators blinded to the previous treatments.

### ***Histological analysis of nasal mucosa***

In order to examine eosinophil infiltration into the nasal mucosa and the increase in mucosal

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swelling, nasal passage tissues surgically removed from the mice were fixed in 4% paraformaldehyde for 16 hours at 4°C. The fixed tissues were then decalcified in EDTA solution for 10 days at 4°C and embedded in paraffin blocks. The samples were sliced in 5 µm thick coronal sections and the sections were stained with hematoxylin and eosin (H&E). The number of eosinophils and the thickness of nasal mucosa in the nasal septum were measured under high magnification (400×) microscope by investigators blinded to the previous treatments. The average number of eosinophils and thickness of the nasal mucosa in four different fields were calculated and compared between each group of mice.

***ELISA for total and OVA-specific IgE in serum***

Total IgE and OVA-specific IgE levels in serum were determined by sandwich enzyme-linked immunosorbent assay (ELISA) system using a Mouse IgE ELISA Quantitation Kit (BETHYL laboratories, Inc., Montgomery, TX) and a DS mouse IgE ELISA (OVA) Kit (Dainippon Sumitomo Pharma Co., Ltd. Tokyo, Japan), respectively. Assays were performed according to the manufacturer’s instructions.

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### ***Analysis of IL-4 and IFN- $\gamma$ production by CD4<sup>+</sup> T cells***

In order to purify CD4<sup>+</sup> T cells, mononuclear cells isolated from spleen and NALT were incubated with microbeads conjugated anti-CD4 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) at 4°C for 30 minutes. CD4<sup>+</sup> cells were sorted by auto-MACS (Miltenyi Biotec) and suspended at a density of  $2 \times 10^6$  cells/ml in complete RPMI medium containing 10% FBS, 5  $\mu$ M of 2-mercaptoethanol, 10 U/ml of penicillin, and 100  $\mu$ g/ml of streptomycin. For feeder cell preparation, the cells isolated from the spleen were incubated in Mitomycin C (50  $\mu$ g/ml) for 20 minutes after T cell depletion by auto-MACS and suspended in the medium at a density of  $2 \times 10^6$  cells/ml. CD4<sup>+</sup> T cells ( $1 \times 10^5$  cells/well) were cultured in the presence of 10  $\mu$ l/well of OVA (1 mg/ml) with feeder cells ( $1 \times 10^5$  cells/well) in round-bottom 96-well microculture plates for 72 hours. The concentrations of IL-4 and IFN- $\gamma$  in supernatants were measured by Mouse ELISA Kit (BioSource International Inc., CA) according to the manufacturer's instructions. The detection limit of mouse IFN- $\gamma$  and IL-4 using the kits were 9.4 pg/ml and 7.8 pg/ml, respectively.

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***Effects of PC on IL-12p40 production by DCs***

The effect of PC on DCs was examined by the use of CD11c<sup>+</sup> cells isolated from the spleen of five naïve mice. Splenic mononuclear cells isolated from each naïve mouse were incubated with microbeads conjugated with anti-CD11c mAb (Miltenyi Biotec) and CD11c<sup>+</sup> cells sorted by auto-MACS were used as DCs. The cells ( $2 \times 10^6$  cells/ml) were cultured with 1, 10, and 100 µg/well of PC-KLH or lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO) for 18 hours. The IL-12p40 concentration in the culture supernatants was measured by a cytokine ELISA kit (BioSource International Inc., CA).

***Statistical analysis***

The median numbers of nasal rubbing and sneezing were measured and statistically analyzed using the Mann-Whitney *U* test. The other data were expressed as mean ± standard error. Total and OVA specific IgE levels in serum, IL-4, IFNγ, and IL-12p40 levels were analyzed using the Student unpaired *t* test. A *p* value of <0.05 was considered as statistically

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significant.

## RESULTS

### *Intranasal immunization with PC attenuates AR symptoms*

After intranasal challenge with OVA, the mice sensitized with intra-peritoneal administration of OVA started to show nasal symptoms such as nasal rubbing and sneezing. The nasal symptoms in Group B mice showed increasingly severe symptoms day by day, and the median numbers of nasal rubbing and sneezing in this group of mice were significantly greater than that of Group C mice at day 49. (Fig. 2) In contrast, the median numbers of nasal rubbing and sneezing in Group A mice were remarkably decreased and significantly lower than that of Group B mice, although the frequencies were significantly higher than that of Group C mice.



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***Intranasal immunization with PC suppresses total and OVA-specific IgE production***

Total and OVA-specific IgE levels were increased in Group A and B mice after intra-peritoneal sensitization and intranasal challenge with OVA. (Fig. 3) The increase of total IgE levels in Group B mice was remarkable and the levels in this group of mice on day 49 were significantly higher than that of Group A and Group C mice. Although the increase in total IgE levels in Group A mice was mild, the levels on day 49 were significantly higher than that of Group C mice. In contrast, OVA-specific IgE levels were remarkably increased after intranasal challenge with OVA in Group A and Group B mice when compared to Group C mice. However, the production of OVA-specific IgE in Group A mice was significantly lower than that of Group B mice, though the levels were significantly higher than that of Group C mice.

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## ***Intranasal immunization with PC reduces eosinophilic inflammation***

Marked eosinophil infiltration into the nasal mucosa was observed after intranasal challenge with OVA in Group B mice. The numbers of eosinophils in this group of mice were significantly higher than that of Group A and Group C mice. (Fig 4A) In Group A mice, the numbers of eosinophils were almost similar to that of Group C mice.

Swelling of the nasal mucosa was also observed after intranasal challenge with OVA in Group B mice. The thickness of the nasal mucosa in this group of mice was significantly greater than that of Group A and Group C mice (Fig. 4B). There was no significant difference between Group A and Group C mice.

## ***Intranasal immunization with PC suppresses IL-4 production from CD4<sup>+</sup> T cells***

IL-4 was produced by CD4<sup>+</sup> T cells isolated from NALT and spleen in all groups of mice.

However, IFN- $\gamma$  was not detected in any group. The level of IL-4 produced from cells

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isolated from NALT of Group B mice was significantly higher than that of Group A and Group C mice. There was no significant difference between Group A and Group C mice. (Fig. 5A) IL-4 production from splenic CD4<sup>+</sup> T cells was also significantly higher in Group B mice than in Group A and Group C mice. (Fig. 5B) There was also a significant difference between Group A and Group C mice.

***PC induces the production of IL-12p40 from splenic DCs***

IL-12p40 production by CD11c<sup>+</sup> cells isolated from the spleen was increased by PC-KLH stimulation and LPS in a dose-dependent manner (Fig. 6). Although the levels of IL-12p40 produced by PC-KLH stimulation were significantly lower than that by LPS at each concentration, the results suggest that similar to LPS, PC has a potential to induce IL-12p40 production from DCs.

**DISCUSSION**

The present study demonstrated that intranasal pretreatment with PC suppresses nasal

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symptoms and allergic inflammation of the nasal mucosa in a murine model of AR. The inhibitory effects of intranasal immunization with PC on nasal symptoms are similar to those reported by previous studies, in which nasal symptoms in a murine AR model induced by sensitization with Der f were reduced by intranasal pretreatment with pneumococcal polysaccharide vaccine and pneumococcal conjugate vaccine.<sup>14,15</sup> Furthermore, in the present study, the levels of serum IgE and eosinophil infiltration into the nasal mucosa were significantly decreased by intranasal PC pretreatment similar to the experiments using pneumococcal vaccines.<sup>14,15</sup> Since eosinophils enhance hyper-responsiveness and vascular permeability,<sup>21</sup> the suppression of nasal symptoms and mucosal swelling observed in those studies might be due to decreased eosinophil infiltration into the nasal mucosa.

Th2-type immune responses in NALT is characterized by IL-4 and IL-13 production and these are known to be the key factors in AR induction.<sup>19</sup> Our study showed that IL-4 production in NALT and spleen as well as allergic inflammation in nasal mucosa and serum OVA-specific IgE levels were significantly lower in mice pretreated with PC than in mice without PC pretreatment. These results suggest that IL-4 expression in NALT and spleen play

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a critical role for AR induction and that intranasal administration of PC affects the production of IL-4 in NALT and spleen. In contrast, IFN- $\gamma$  production from NALT and splenic CD4<sup>+</sup> T cells was not detected in our study. The same results were observed in the experiments using pneumococcal vaccines<sup>14,15</sup>, although the administration of killed Spn before OVA sensitization significantly enhanced IFN- $\gamma$  production from mediastinal lymph node T cells.<sup>12</sup> Further, it has been reported that regulatory T cells are associated with the suppression of Th2-type immune response by pneumococcal vaccines.<sup>13,14,15</sup> Although PC is a T cell-independent antigen and does not induce regulatory T cells, conjugation to protein carrier such as KLH allows the involvement of T cells,<sup>22</sup> indicating that intranasal administration of PC-KLH might have induced regulatory T cells and suppressed IL-4 production. These findings suggest that the suppression of allergic inflammation by PC is associated with decreased Th2-type immune response, but not with increased Th1-type response.

To clarify the mechanism on how intranasal PC immunization could suppress Th2-type immune responses, splenic CD11c<sup>+</sup> DCs were co-cultured with PC-KLH and the production of IL-12p40 was examined. IL-12p40 is known to differentiate naïve T cells to Th1-type cells

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and to inhibit Th2-type immune responses.<sup>23</sup> Results showed that IL-12p40 production by PC stimulation was dose-dependent similar to that of LPS. TLR4 and MyD88 signaling pathways play an important role in LPS-stimulated IL-12 production from DCs.<sup>24</sup> The binding of PC to TLR4 has been also demonstrated using PC-containing glycoprotein ES-62, a secretory product of parasitic nematodes. However, ES-62 acts to bias the immune response toward Th2-type and induces low levels of IL-12 production by macrophages and dendritic cells.<sup>25</sup> On the other hand, a synthetic oxidized molecule containing PC induced IL-8 by binding to TLR4 in the same manner as LPS,<sup>26</sup> indicating that protein components conjugated with PC play an immunomodulatory role in cytokine production. This might explain in part why parasite nematode induces a Th2-type immune response while Spn administration reduces Th2-type responses despite PC being present in both microorganisms.<sup>12,25</sup> Taking these findings together, it can be speculated that KLH coupling with PC might be associated with increased IL-12 production from splenic DCs.

## CONCLUSION

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In conclusion, the present study has demonstrated that intranasal pretreatment with PC-KLH suppresses allergic inflammation in the nasal mucosa of a murine AR model induced by OVA sensitization. Significantly reduced nasal symptoms, eosinophil infiltration and nasal mucosa swelling, and IL-4 production from CD4<sup>+</sup> T cells were observed in mice pretreated with PC-KLH, and IL-12 production from DCs was induced by PC-KLH. Those results obtained by the present study has the limitation to apply for human trial, since PC-KLH is not available to human and AR induced by OVA-sensitization is not observed in human. While further studies are needed by developing PC-conjugate which can be applied to human and establishing AR model sensitized with Der f, our findings suggest that intranasal immunization with PC might be useful in preventing AR and upper airway bacterial infection.

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FIGURE LEGENDS

Fig. 1. Experimental protocol of pretreatment with PC and induction of AR. For AR induction, mice were sensitized by intraperitoneal injection of OVA together with Alum on days 21, 28, and 35. The sensitized mice were then challenged by intranasal administration of OVA for seven consecutive days from days 42 to 48 to induce AR. Prior to intraperitoneal sensitization with OVA, mice were pretreated with intranasal administration of PC-KLH or PBS on days 0, 7, and 14. Nasal symptoms of AR were observed on day 49 after nasal provocation with OVA. The mice were then sacrificed for further analysis.

Fig. 2. Nasal symptoms of AR induced by intranasal challenge with OVA. The frequencies of nasal rubbing (A) and sneezing (B) per five minutes after intranasal OVA challenge were significantly reduced in mice pretreated with PC-KLH, but significantly higher than that of control mice. The results are expressed as median. \*,  $p<0.05$  by the Mann-Whitney  $U$  test.

Fig. 3. Total and OVA-specific IgE levels in serum. The levels of total IgE (A) and

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OVA-specific IgE (B) in serum were increased after intraperitoneal sensitization and intranasal challenge with OVA. The levels in mice pretreated with PC-KLH were significantly lower than that of mice without PC-KLH pretreatment, but significantly higher than that of control. The results are expressed as the mean  $\pm$  standard error (S.E.). \*\*,  $p < 0.05$

Fig. 4. Histological analysis of nasal mucosa after intranasal challenge with OVA. (A) The numbers of eosinophils infiltrating into the nasal mucosa were significantly reduced by intranasal pretreatment with PC-KLH to the same levels in control. (B) When the thickness of the nasal mucosa was measured as the distance between the nasal septum cartilage and maxillary bone nasal ridge, mucosal swelling was also decreased by intranasal pretreatment with PC-KLH to the same levels in control. The results are expressed as the mean  $\pm$  standard error (S.E.). \*,  $p < 0.05$

Fig. 5. Production of IL-4 from CD4<sup>+</sup> T cells isolated from NALT and spleen. (A) The level of IL-4 produced from CD4<sup>+</sup> T cells isolated from NALT was significantly higher in mice



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without PC-KLH pretreatment than in mice pretreated with PC-KLH and in control. (B)

Splenic CD4<sup>+</sup> T cells also produced significantly higher levels of IL-4 in mice without PC-KLH pretreatment compared to mice pretreated with PC-KLH and control. There was also a significant difference between mice pretreated with PC-KLH and control. The results are expressed as the mean  $\pm$  standard error (S.E.). \*, p<0.05

Fig. 6. Production of IL-12p40 from splenic DCs by stimulation with PC-KLH and LPS.

IL-12p40 production from splenic CD11c<sup>+</sup> cells was increased by stimulation with PC-KLH and LPS in a dose-dependent manner (n=5). The levels of IL-12p40 produced by PC-KLH stimulation were significantly lower than that by LPS at each concentration. Splenic DCs were isolated from five naïve mice and the results are expressed as the mean  $\pm$  standard error (S.E.). \*, p<0.05

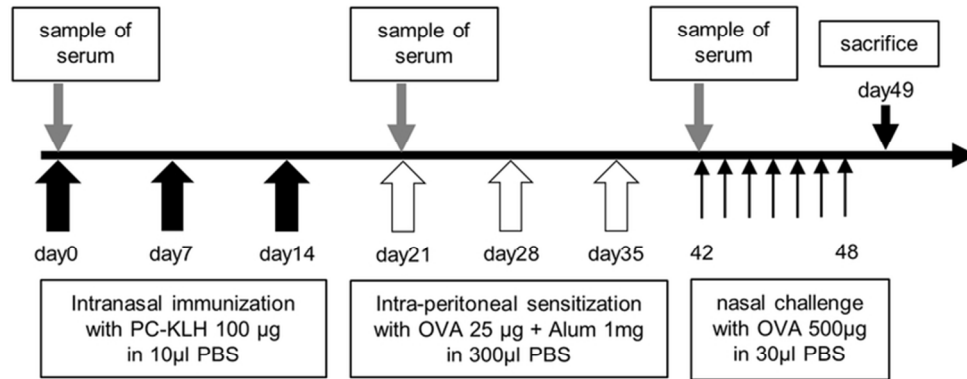


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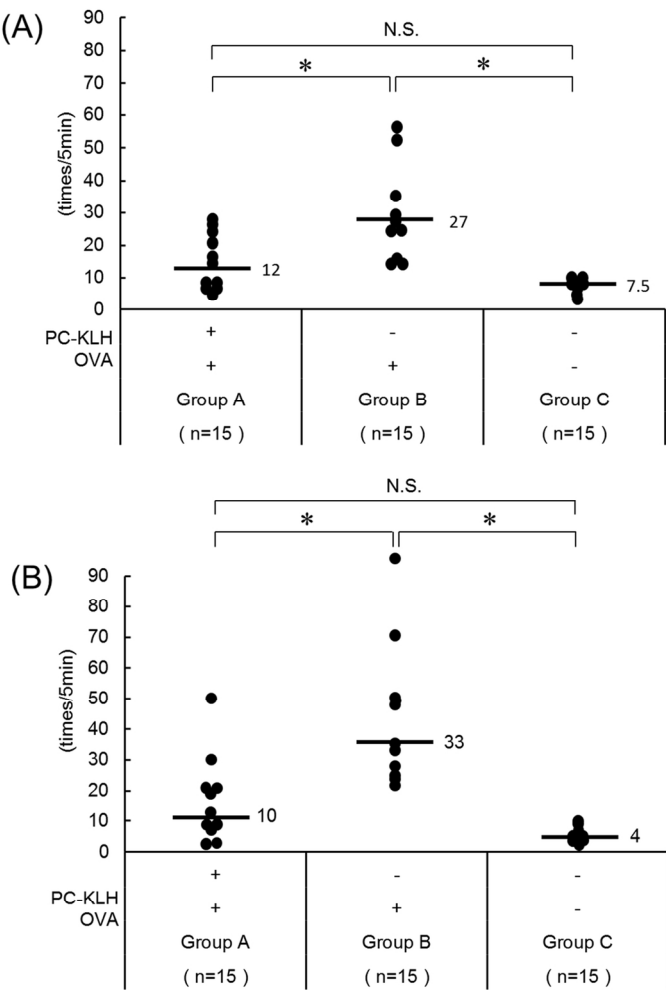


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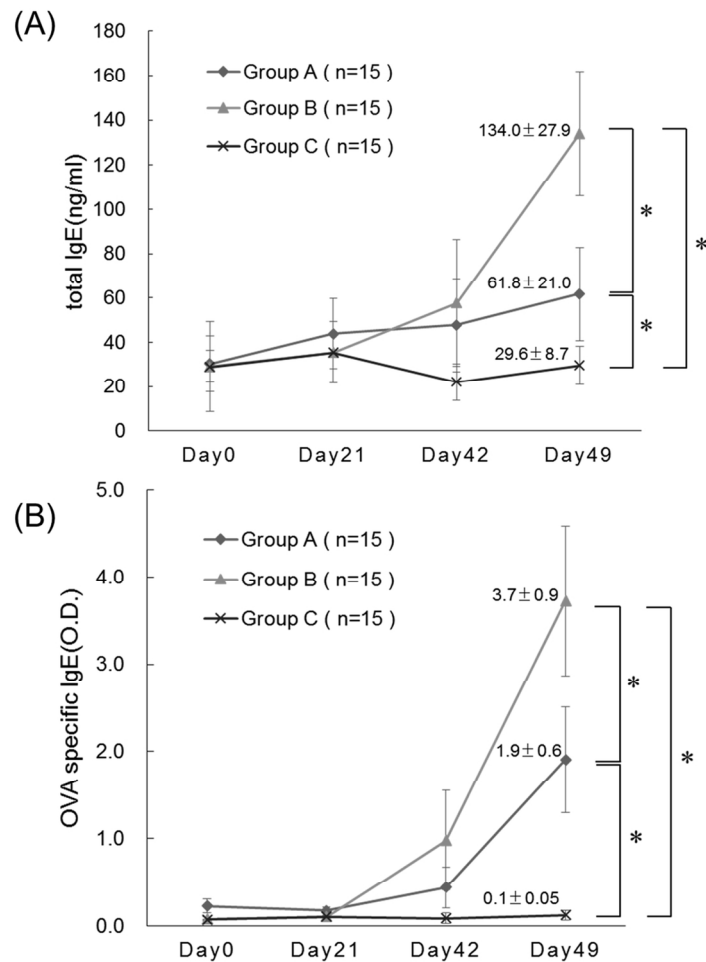


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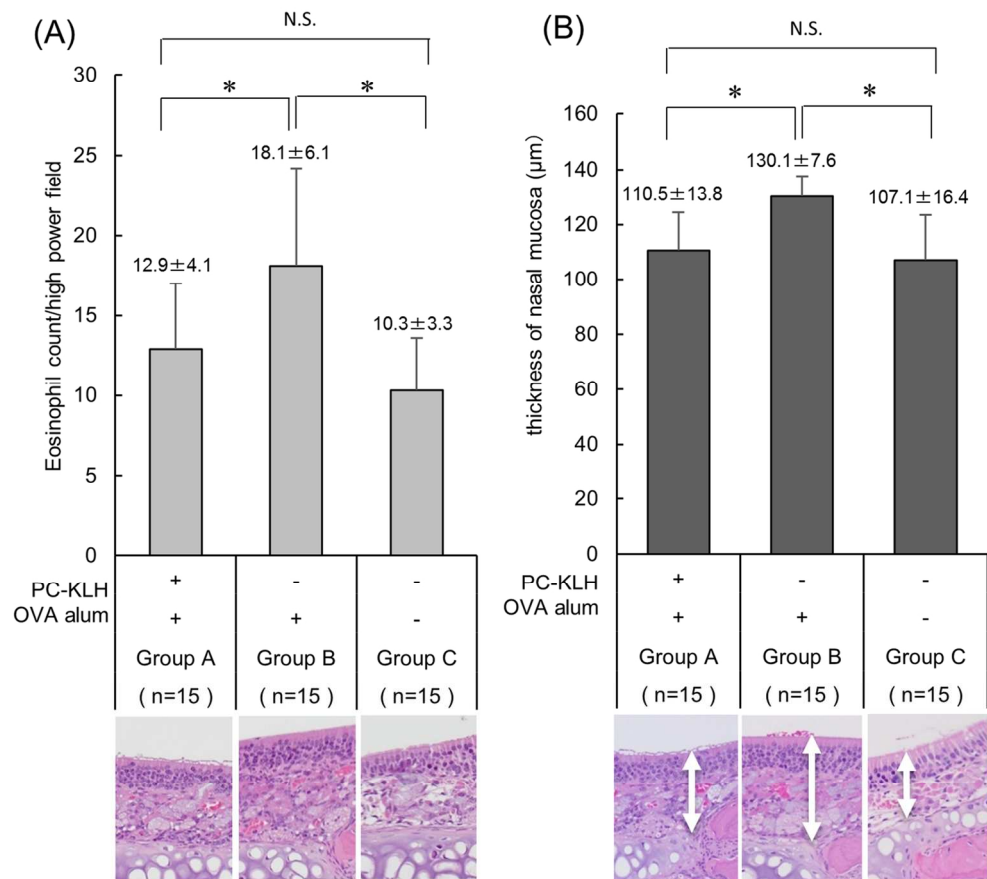


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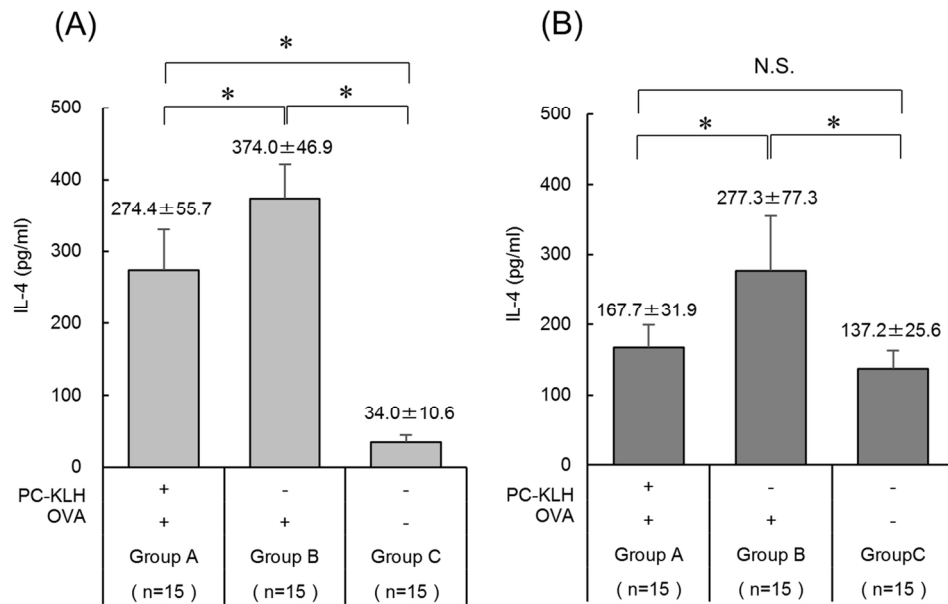


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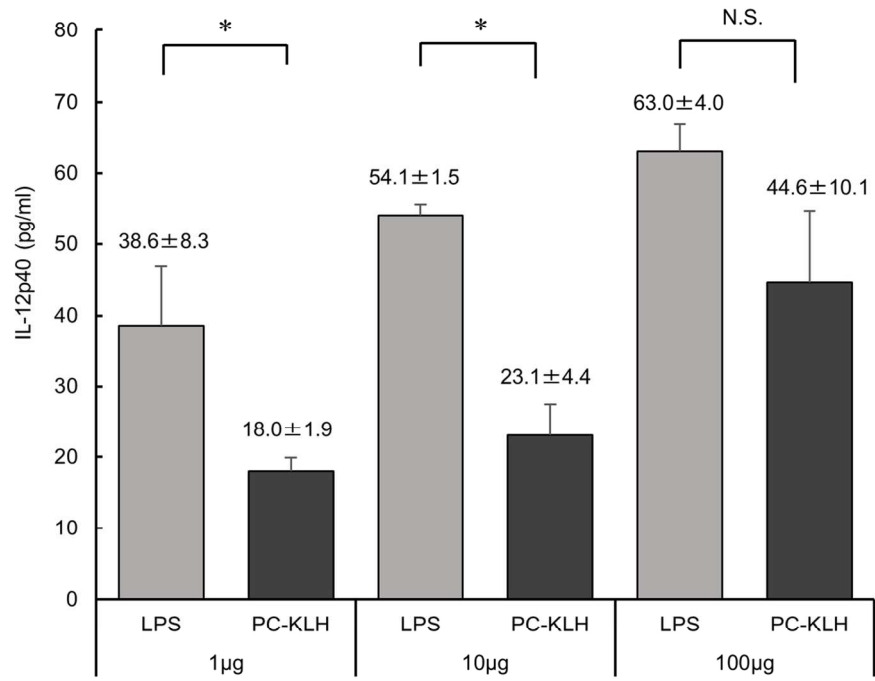


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