의학박사 학위논문

Symbiotic microbiome *Staphylococcus aureus* from human nasal mucus modulates IL-33-mediated type 2 immune responses in allergic nasal mucosa

알레르기비염 코점막에서 분리 배양된 공생 미생물 황색포도상구균의 인터루킨-33 매개 제2형 면역 반응 조절 효과

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Symbiotic microbiome *Staphylococcus aureus* from human nasal mucus modulates IL-33-mediated type 2 immune responses in allergic nasal mucosa

지도교수 김 현 직

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전 영 진

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2021년 1월

위 원 장 이 재 서 (인)

부위원장 김 현 직 (인)

위 원 김 동 영 (인)

위 원 박 인 규 (인)

위 원 윤 상 선 (인)

Symbiotic microbiome *Staphylococcus aureus* from human nasal mucus modulates IL-33-mediated type 2 immune responses in allergic nasal mucosa

By

Yung Jin Jeon

A thesis submitted in partial fulfillment of the requirements for the Degree of the Doctor of Philosophy in Translational Medicine at Seoul National University College of Medicine

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Approved by Thesis Committee:

Professor Chae-Seo Rhee (Chairman)

Professor Hyun Jik Kim (Vice chairman)

Professor Dong-Young Kim

Professor In Kyu Park

Professor Sang Sun Yoon

Abstract

Symbiotic microbiome *Staphylococcus aureus* from human nasal mucus modulates IL-33-mediated type 2 immune responses in allergic nasal mucosa

Yung Jin Jeon

Department of Translational Medicine

Seoul National University College of Medicine

Background: The host-microbial commensalism can shape the innate immune responses in respiratory mucosa and the nasal microbiome also modulates front-line immune mechanisms in the nasal mucosa. Inhaled allergens encounter the host immune system first in the nasal mucosa, and microbial characteristics of nasal mucus directly impact the mechanisms of initial allergic responses in nasal epithelium. However, the roles of the nasal microbiome in allergic nasal mucosa remain uncertain.

Objectives: In this study, we aimed to determine the distribution of nasal microbiomes in the allergic nasal mucosa and elucidate the

interplay between nasal microbiome Staphylococcus species and T helper 2 (Th2) cytokines in allergic rhinitis (AR) models.

Methods: *Staphylococcus aureus* (AR-SA) and *Staphylococcus epidermidis* (AR-SE) were isolated from the nasal mucosa of patients with AR. The influence of nasal microbiome *Staphylococcus* species on allergic nasal mucosa was also tested with *in vitro* and *in vivo* AR models.

Results: Pyrosequencing data showed that colonization by *S. epidermidis* and *S. aureus* was more dominant in the nasal mucus of AR subjects. The mRNA and protein levels of IL-33 and TSLP were significantly higher in AR nasal epithelial (ARNE) cells which were cultured from nasal mucosa of AR subjects, and exposure of ARNE cells to AR-SA reduced IL-33 mRNA and secreted protein levels. Particularly, ovalbumin-driven AR mice inoculated with AR-SA by intranasal delivery exhibited significantly reduced IL-33 in their nasal mucosa. In the context of these results, allergic symptoms and Th2 cytokine levels were significantly downregulated after intranasal inoculation of AR-SA *in vivo* AR mice.

Conclusion: Colonization by *Staphylococcus* species was more dominant in the allergic nasal mucosa, and nasal commensal *S. aureus* from subjects with AR mediates anti-allergic effects by modulating IL-33-dependent Th2 inflammation. The results demonstrate the role of host-bacterial commensalism in shaping human allergic inflammation.

Keywords: *Staphylococcus aureus*; allergic rhinitis; interleukin-33; symbiosis; nasal microbiome

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List of Abbreviations

- AR: allergic rhinitis
- IgE: immunoglobulin E
- Th: T helper
- TSLP: thymic stromal lymphopoietin
- IL-: interleukin-
- PNS-CT: paranasal sinus computed tomography
- AST: allergic skin test
- MAST: multiallergen simultaneous test
- LB: Luria-Bertani
- NHNE: normal human nasal epithelium
- ARNE: allergic rhinitis nasal epithelium
- ALI: air - liquid interface
- AR-SA: *S. aureus* isolated from allergic nasal mucus
- AR-SE: *S. epidermidis* isolated from allergic nasal mucus
- DMEM: Dulbecco's modified Eagle's medium
- PBS: phosphate-buffered saline
- MOI: multiplicity of infection
- NAL: nasal lavage
- EDTA: ethylene diamine tetraacetic acid
- ELISA: enzyme-linked immunosorbent assay
- PCR: polymerase chain reaction
- WT: negative control group (wildtype mice)
- AR-OVA: positive control group (AR mice)

- SA-PBS: AR-SA infected WT mice
- SA-OVA: AR-SA infected AR mice
- OVA: ovalbumin
- HRP: horseradish peroxidase
- H&E: hematoxylin and eosin
- PAS: periodic acid-Schiff
- CFU: colony-forming unit
- GAPDH: glyceraldehyde phosphate dehydrogenase
- TTBS: tween-tris-buffered saline
- SD: standard deviation
- ANOVA: analysis of variance
- hpi: hours (hr) post of inoculation
- Tregs: regulatory T helper cells

Introduction

Allergic rhinitis (AR), an immunoglobulin E (IgE)- and T helper (Th)2-mediated inflammatory nasal disease, is caused by sensitized immune responses to inhaled allergens. This allergen-specific immune response is thought to arise from an imbalance in Th1-Th2 immune regulation that results in increased levels of Th2 cytokines.[1,2] Nasal epithelial cells exposed to external allergens induce Th2 inflammatory responses and Th2 inflammations proceed to the upper airway mucosa. The allergen-mediated inflammatory immune response begins with increased secretion of epithelial cell-derived cytokines, such as thymic stromal lymphopoietin (TSLP) and interleukin (IL)-33.[1] Epithelial cell-derived cytokines provide critical signals to innate and adaptive cell populations related to Th2 inflammation.[2,3] Allergic nasal epithelium might be responsible for the vast majority of allergic inflammation to inhaled allergens, and research about the regulation of epithelial cell-derived cytokines is needed to develop a more effective approach to the treatment of AR.[4] IL-33, which is produced by the airway epithelium and other cell types, is a key cytokine involved in allergic airway diseases and provides an essential axis for rapid immune responses and tissue homeostasis. Since the discovery of IL-33, tremendous progress has been made in understanding its biological functions, and the potential importance of IL-33 as a therapeutic target of AR has been suggested.[5,6]

Human mucosal surfaces are in direct contact with the external

environment and are susceptible to invasion and colonization by various allergens and pathogens.[7-9] Respiratory mucosa is constantly exposed to inhaled pathogens and allergens that directly impact mucosal immune mechanisms.[10,11] Studies on the interaction between the mucosal microbiome and the host increasingly consider the contribution of mucosal immune responses and specific microbiome-mediated protection from external pathogens to integrate environmental signals.[11] Inhaled allergens encounter the host immune system first in the nasal mucosa, and microbial characteristics of nasal mucus directly impact the mechanisms of initial allergic responses in nasal epithelium.[9,12,13] Insights into the microbiota and dysbiosis of allergic nasal mucosa provide fundamental information regarding susceptibility to allergens and their relation to allergic inflammation, including induction of epithelial cell-derived cytokines such as IL-33 which are are central regulators of type 2 immunity.[14-16] There is considerable evidence that the microbiota contributes to responses of the mucosal immune system and protects against pathogenic bacterial or viral infection. Recent studies have sought to identify how the commensal microbiota regulates sensitization tolerance to allergens in asthma.[9,17,18] However, our knowledge of microbial composition in allergic nasal mucus is limited, and the responses of the nasal microbiome to inhaled allergens have not been comprehensively examined in the nasal mucosa.

Based on our previous evidence of microbial distribution in healthy nasal mucus, we assessed the microbial composition in allergic nasal

mucus and subsequently investigated whether nasal commensal bacteria in allergic nasal mucus contributes to Th2 inflammatory responses in nasal epithelium. The present study identified *Staphylococcus* species, including *Staphylococcus epidermidis* and *Staphylococcus aureus*, as the most abundant constituents in allergic nasal mucus. Particularly, *S. aureus* isolated from the nasal mucus of AR subjects accelerated the reduction of IL-33 in AR nasal epithelium. Furthermore, allergic nasal commensal *S. aureus* prevented allergic inflammation *in vivo* in mice by reducing IL-33-related Th2 immune responses in the nasal mucosa. Overall, our study presents evidence that *Staphylococcus* species colonization is significantly increased in the nasal mucus of AR subjects. In addition, the administration of *S. aureus* isolated from human allergic nasal mucus suppressed allergic inflammation in nasal mucosa by reducing IL-33 secretion.

Materials and Methods

Participant recruitment and sample collection

We recruited 17 patients referred to the Department of Otorhinolaryngology primarily for septal surgery in Seoul National University Hospital (Seoul, Republic of Korea) between October 2017 and September 2018, and in Gyeongsang National University Hospital (Jinju, Republic of Korea) between June 2019 and October 2019. This study was approved and monitored by the Institutional Review Board (IRB) of Seoul National University College of Medicine (No. 1709-049-883) and Gyeongsang National University Hospital (No. 2019-05-004). All subjects who participated in the sampling of nasal mucosa provided written informed consent. The septal deviation was diagnosed with the intranasal endoscope and paranasal sinus computed tomography (PNS-CT), and the subjects did not show any clinical or imaging findings of sinusitis. To confirm AR, they underwent an allergic skin test (AST) or a multi-allergen simultaneous test (MAST) for the detection of allergens and specific IgE. Twenty subjects were classified into healthy subjects and fifteen were diagnosed with AR. The mean age of the subjects was 35.2 ± 7.1 years and there was no significant difference between AR and healthy subjects. Mucus and/or 1×1 ㎠ sized nasal mucosa from the middle turbinate of human subjects was collected and assessed for quality under general anesthesia.

Characterization of *Staphylococcus* species in human allergic nasal mucosa

To isolate bacterial colonies, the mucus was placed in Luria-Bertani (LB) plates. After 1 day of incubation, bacterial colonies were obtained from LB plates. The species of each colony was identified using GS-FLX 454 pyrosequencing with 16S rRNA gene amplification, as described previously.[19] *Staphylococcus epidermidis* and *S. aureus* strains from five individual subjects with AR were used in the study.

Cell culture

Normal human nasal epithelial (NHNE) and allergic rhinitis nasal epithelial (ARNE) cells were each cultured from five subjects using an air-liquid interface method. Cells were used 14 days after the creation of the air-liquid interface (ALI). *Staphylococcus aureus* (AR-SA) and *Staphylococcus epidermidis* (AR-SE) isolated from allergic nasal mucus were inoculated as described previously.[19] Briefly, passage-2 NHNE cells (1×10^5 cells/culture) were seeded in 0.5㎖ of culture medium on Transwell clear culture inserts (24.5㎜, with a 0.45㎜ pore size; Costar Co., Cambridge, MA). Cells were cultured in a 1:1 mixture of basal epithelial growth medium and Dulbecco's Modified Eagle's Medium (DMEM) containing previously described supplements. Cultures were grown submerged for the first nine days. The culture medium was changed on day 1, and every other day thereafter. An ALI was created on day 9 by removing the apical

medium and feeding the cultures from the basal compartment only. The culture medium was changed daily after the initiation of the ALI. We added antibiotics (such as 1% penicillin and streptomycin) into the media for subculture, and we also added the antifungal agent Fungizone® (1㎖/1000㎖ media; Life Technologies, Grand Island, NY) after filtering the media. During the last seven days, a basal compartment feeding medium without antibiotics or antifungals was used for the incubation of NHNE cells with *Staphylococcus* species. All experiments described here used cultured nasal epithelial cells taken 14 days after the creation of the ALI.

Isolated *Staphylococcus aureus* strain and *Staphylococcus epidermidis* strain from a patient with allergic rhinitis (AR-SA and AR-SE) were used to induce acute bacterial infection in NHNE and ARNE cells. The AR-SA and AR-SE strains were maintained in a -80°C deep freezer until required and were plated and grown overnight at 37°C on LB agar plates (DifcoTM LB agar, Miller base; Becton Dickinson and Company, Franklin Lakes, NJ). A single colony of AR-SA and AR-SE was grown in 1㎖ of LB medium (DifcoTM LB broth, Miller base; Becton Dickinson and Company, Franklin Lakes, NJ) for 24h, in a 37°C incubation shaker. 10㎖ of the fresh LB medium was added to 100㎕ of the AR-SA and AR-SE culture. This mixture was grown for 2h to reach the log phase. Passage-2 fully differentiated NHNE cells were either mock-infected (phosphate-buffered saline, PBS) or inoculated with AR-SA and AR-SE strains to the apical side of ALI at a multiplicity of infection

(MOI) of 0.25. After inoculation, the cells were incubated at 37°C in 5% CO2. At the designated times post-inoculation, the cell lysates, and culture supernatants were collected. The AR-SA and AR-SE cultures were diluted with the non-antibiotic feeding medium to adjust the amount to 300㎕ and inoculated to the apical compartment of each NHNE and ARNE cell culture wells. NHNE and ARNE cells were infected with AR-SA and AR-SE cultures for 0, 2, 8, 24, and 48 hrs.

Murine inoculation model

Animal experiments were approved by the Institutional Animal Care and Use Committees of Seoul National University Hospital (No 2016-1470) and the research methods were carried out in accordance with the approved guidelines. Four-week-old female wildtype (WT) BALB/C mice (Orient, Gyeonggi, Republic of Korea) were maintained under specific-pathogen-free conditions, and all mice were housed in a temperature-controlled environment with a 12-hour dark/light cycle. For infections, AR-SA and AR-SE ($3.2x10^6$ CFU in 30㎕ PBS) were inoculated into WT mice by intranasal delivery. Mice were euthanized and sacrificed by cervical dislocation and by intramuscular injection of a high dose of a mixture of 10mg/kg xylazine (Bayer, Puteaux, France) and 5mg/kg ketamine (Merial, Lyon, France) according to the reviewed protocol. Death was verified when no heartbeat was detected. There were no mice without euthanasia. When mice were euthanized by injection, a cervical dislocation was also performed to

ensure that the mice were dead.

After euthanizing the mice, nasal lavage (NAL) fluid was obtained from the nasal cavity by lavaging with 1000μl 0.5mM ethylene diamine tetraacetic acid (EDTA) in PBS. The NAL fluid was used for enzyme-linked immunosorbent assay (ELISA) for measuring secreted protein levels. Mouse nasal tissue was also harvested for real-time polymerase chain reaction (PCR),

The mice were divided into four groups. The negative control group (WT) was sensitized and challenged with PBS, the positive control group (AR-OVA) was sensitized and challenged with ovalbumin (OVA), the SA-PBS group consisted of AR-SA infected mice sensitized and challenged with PBS, and the SA-OVA group consisted of AR-SA infected mice sensitized and challenged with OVA. The schedule for allergen sensitization and the intranasal challenge is summarized in Figure 7. Briefly, the AR-OVA and SA-OVA groups were sensitized by intraperitoneal injection of 25㎍ OVA mixed with 2㎎ alum on days 0, 7, and 14 and then challenged by intranasal treatment of 100㎍ OVA for 7 consecutive days, from days 22 to day 28. The WT-PBS and SA-PBS groups were injected intraperitoneally and challenged intranasally with PBS following the same schedule.

Serum levels of total and OVA-specific IgE

AR mouse serum samples were stored at -70℃ before use for measurements of total and OVA-specific IgE, as described

previously.[20] Briefly, total serum IgE was measured by ELISA using an anti-mouse IgE capture monoclonal antibody (BD Pharmingen, San Diego, CA) and horseradish peroxidase (HRP)-conjugated anti-mouse IgE (Southern Biotechnology, Birmingham, AL). To detect OVA-specific IgE, 96-well immune plates were coated with 100㎍/㎖ of OVA in carbonate-bicarbonate buffer. After the serum samples had been incubated for 2h, biotin-conjugated rat anti-mouse IgE monoclonal antibody (BD Pharmingen) and streptavidin-HRP (BD Pharmingen) were used to detect OVA-specific IgE levels.

Histologic analysis

Mice heads were fixed in 10% formalin, decalcified, and embedded in paraffin wax. Nasal tissues were sectioned and stained with hematoxylin and eosin (H&E) for inflammatory cell counting, Sirius red stain for eosinophil counting, and periodic acid-Schiff (PAS) stain to indicate secretory cells of the nasal epithelium.

Colony count

Bacterial samples were serially diluted ten-fold with PBS. Next, 10 ㎕ of each diluted sample was plated on an LB agar plate. The plates were incubated for 24 h at 37°C. The numbers of AR-SA and AR-SE colonies growing on the agar surface were counted manually. Bacterial growth was reported based on the colony-forming units (CFUs) for each sample.

Real-time PCR and RNA preparation

Levels of transcripts encoding human IL-33 and TSLP, mouse IL-4, IL-5, IL-13, and IL-33, or *femA*s specific for *S. aureus* (*femA-SA*) and *S. epidermidis* (*femA-SE*) were determined using real-time PCR. Total RNA was isolated using TRIzol (Life Technology, Seoul, Republic of Korea) and cDNA was synthesized from 3μg of RNA with random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Perkin Elmer Life Sciences, Waltham, MA, and Roche Applied Science, Indianapolis, IN). Amplification was performed using the TaqMan Universal PCR Master Mix (PE Biosystems, Foster City, CA) according to the manufacturer's protocol. Briefly, amplification reactions had a total volume of 12μl and contained 2μl of cDNA (reverse transcription mixture), oligonucleotide primers (final concentration of 800nM), and TaqMan hybridization probe (200nM). Real-time PCR probes were labeled at the 5' end with carboxyfluorescein (FAM) and at the 3' end with the quencher carboxytetramethylrhodamine (TAMRA). To quantify the cellular viral level and host gene expression, cellular RNA was used to generate cDNA.

The AR-SA and AR-SE levels were monitored using a real-time PCR for the factor essential for the expression of methicillin resistance (*femA*) genes specific for *S. aureus* and *S. epidermidis*. The forward and reverse primers and probes used for real-time are listed in Table 1. Primers for human IL-33 and TSLP, mouse IL-4, IL-5, IL-13, and IL-33 were purchased from Applied Biosystems

(Foster City, CA). Real-time PCR was performed using the PE Biosystems ABI PRISM® 7700 Sequence Detection System. Thermocyling parameters were as follows: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. All real-time PCR assays were quantitative and utilized plasmids containing the target gene sequences as standards. All reactions were performed in triplicate, and all real-time PCR data were normalized to the level of the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH, 1×10^6 copies) to correct for variations between samples.

Table 1. Real-time PCR primers and TaqMan probes for *femA*s specific for *Staphylococcus aureus* (*femA*-SA) and *Staphylococcus epidermidis* (*femA*-SE).

Target Genes	Sequence
femA-SA	5′-TGCCTTTACAGATAGCATGCCA-3′
	5′-AGTAAGTAAGCAAGCTGCAATGACC-3′
	5′-JOE-TCATTTCACGCAAACTGTTGGCCACTATG-BHQ1-3′
femA-SE	5′-CAACTCGATGCAAATCAGCAA-3′
	5′-GAACCGCATAGCTCCCTGC-3′
	5′-JOE-TACTACGCTGGTGGAACTTCAAATCGTTATCG-BHQ1-3′

Protein isolation and Western blot

IL-33 levels were assessed by western blotting using the human IL-33 antibody (AF3625, R&D Systems, Minneapolis, MN). Briefly, total protein lysates were harvested in RIPA lysis buffer (Thermo Fisher Scientific, Wilmington, DE). The cell lysates (25μg per lane, as measured by a BCA protein assay purchased from Thermo Fisher Scientific) were electrophoresed in 10% SDS gels and transferred to polyvinylidene difluoride membranes in tris-buffered saline (TBS; 50mM Tris-Cl, pH 7.5, and 150 mM NaCl) for one hour at room temperature. Each membrane was incubated overnight with the primary antibody in tween-tris-buffered saline (TTBS; 0.5% Tween-20 in TBS) at 4°C. After washing with TTBS, each blot was incubated for one hour at room temperature with secondary anti-rabbit or anti-mouse antibody (Cell Signaling, Beverly, MA) in TTBS. Expression was detected using an enhanced chemiluminescence system (Amersham, Little Chalfont, UK).

Quantification of secreted cytokines

Secreted mouse IL-33 (DY3626) was quantified using the DuoSet® ELISA kit from R&D Systems according to the manufacturer's instructions. To quantify IL-33, the NAL fluid of a murine inoculation model was collected. The working range of the assay was 15.6-1000 pg/mL.

Statistical analyses

At least three independent experiments were performed with cultured cells from each donor, and data are expressed as mean ± standard deviation (SD) of three independent experiments. Groups were compared by the Mann-Whitney U test, and differences among treatment groups were evaluated by analysis of variance (ANOVA) with a post hoc test. A p-value of < 0.05 was considered statistically significant. All statistical analyses were performed with GraphPad Prism software (version 5; GraphPad Software, Inc., La Jolla, CA).

Results

Characterization of bacterial communities in allergic nasal mucus

The microbial compositions of middle turbinate mucus in human subjects with AR (N = 17) were evaluated by analyzing cultured bacterial colonies and 16S rRNA gene sequencing, as previously described.[6] Based on at least 97% sequence identity, 112 bacterial species were discovered in the middle turbinate mucus of subjects with AR. *Staphylococcus epidermidis, S. aureus*, *Enterobacter aerogenes, Corynebacterium accolens, Micrococcus luteus, Corynebacterium pseudodiphtheriticum,* and *Klebsiella pneumoniae* were among the most commonly detected species. *S. epidermidis* demonstrated the highest abundance, accounting for 58.38% of mapped sequences, and *S. aureus* exhibited the largest increase in presence of microbial distribution in allergic nasal mucus (highest value of AR 14.29% vs mean value of healthy nasal mucus 5.61%[6]) (Figure 1).

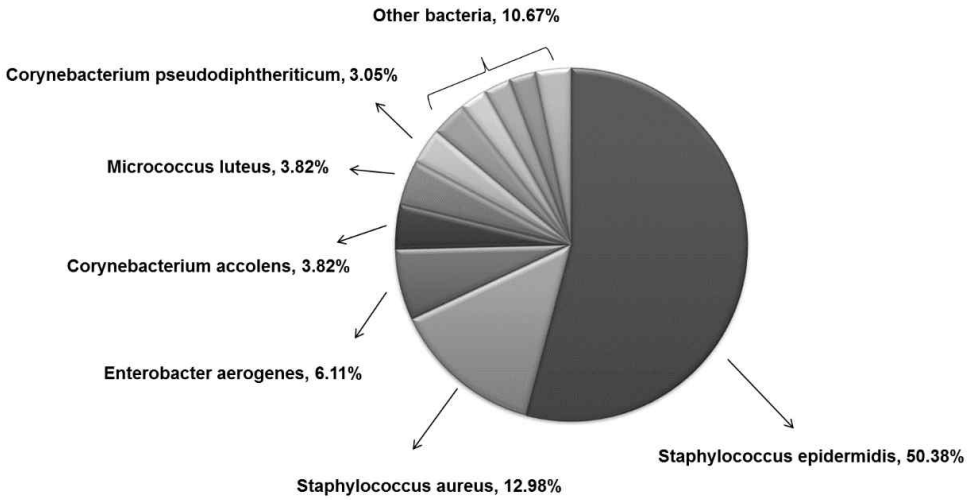

Figure 1. Distribution of bacterial colonies in the nasal mucus of subjects with allergic rhinitis. Bacterial species cultured from mucus samples obtained from middle turbinates of subjects with allergic rhinitis (N = 17) were identified via 16S rRNA gene sequencing. The distribution of the 112 identified bacterial species is presented in the graph.

The proportion of *S. epidermidis* and *S aureus* in allergic nasal mucus varied widely among subjects with AR to a maximum of 79.1%. The lowest abundance of *Staphylococcus* species in the nasal mucus of subjects with AR was 48.9% (Figure 2). We then questioned the role of the nasal commensal organisms *S. epidermidis* (AR-SE) and *S. aureus* (AR-SA) in allergic nasal mucus. Both AR-SE and AR-SA strains were isolated from four AR subjects to assess the mechanistic link between dominant allergic nasal commensals and pathogenesis of AR in the nasal mucosa.

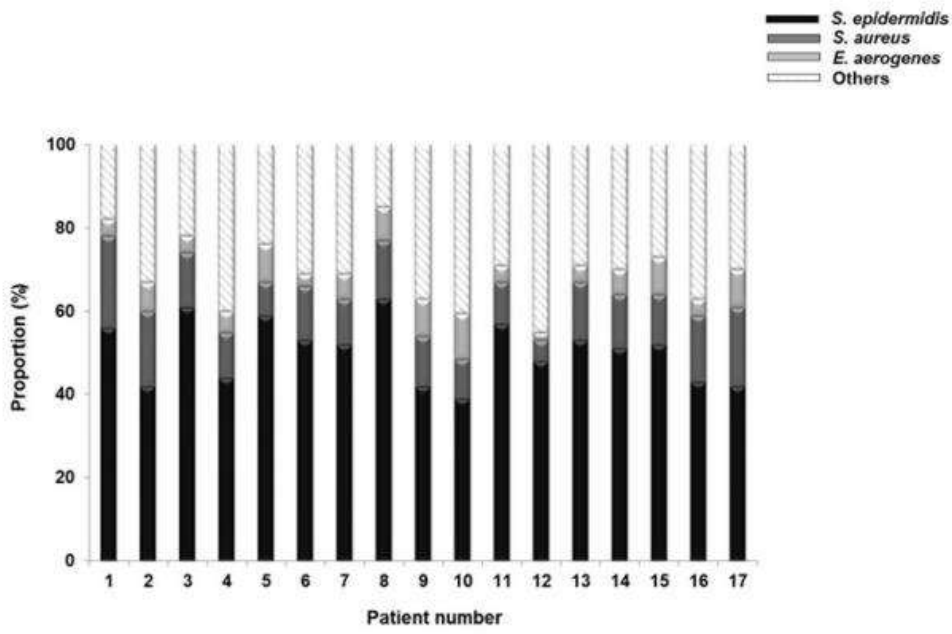

Figure 2. The relative abundance of nasal commensal, such as *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Enterobacter aerogenes*, and others, in each allergic rhinitis subject. The bar graph presents the relative abundance of nasal commensal organisms of 17 allergic rhinitis subjects at the species level.

Inoculation of nasal commensal *S. aureus* suppressed IL-33 secretion in ARNE cells

The kinetics of expression of epithelial cell-derived cytokines including IL-33 and TSLP genes, protein production, and secreted protein levels were studied in ARNE cells after inoculation with dominant nasal microbiome AR-SE and AR-SA in the human allergic nasal mucosa. Cultured ARNE cells from five subjects with AR were inoculated with AR-SA and AR-SE at an MOI of 0.25, and cell lysates and supernatants were harvested at 0, 2, 8, 24, and 48 hr post of inoculation (hpi). The mRNA levels of *femA-SA* and *femA-SE* genes were then measured using real-time PCR. The results showed that *femA-SA* and *femA-SE* mRNA levels increased significantly from 8 hr after inoculation, with the highest expression observed at 48 hpi (Figure 3A, 3B). In addition, AR-SA colony counts from the supernatants of ARNE cells were significantly increased at 8 hpi (1.05×10^{8} ± 7.07×10^{6} CFU/mL) and were the highest at 48 hpi (1.28×10^{9} ± 3.20×10^{8} CFU/mL) (Figure 4A). AR-SE colony counts from the supernatants of ARNE cells were also significantly increased at 8 hpi (1.35×10^{8} ± 2.12×10^{7} CFU/mL) and were the highest at 48 hpi (1.85×10^{9} ± 3.53×10^{8} CFU/mL) (Figure 4B).

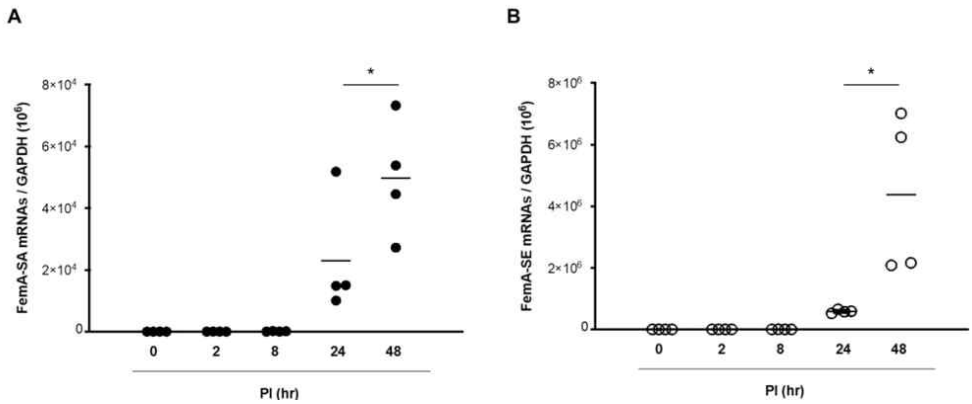

Figure 3. The allergic rhinitis nasal epithelial cells from four allergic rhinitis subjects were inoculated with nasal commensal, *Staphylococcus aureus* and *Staphylococcus epidermidis* at a multiplicity of infection (MOI) of 0.25. The mRNA levels of *femA* genes specific for *S. aureus* (A) and *S. epidermidis* (B), normalized to cellular GAPDH transcript levels, were monitored by real-time PCR for 48 hr post-infection. $*p < 0.05$ compared with control ARNE cells.

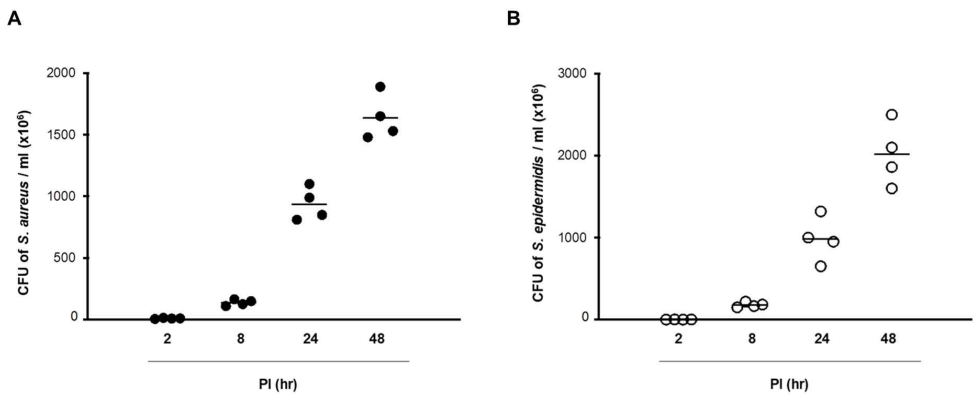

Figure 4. Colony counts of supernatants in cultured allergic rhinitis nasal epithelial cells inoculated with nasal commensal *Staphylococcus aureus* (A) and *Staphylococcus epidermidis* (B) from allergic rhinitis subjects were performed. Results are presented as mean ± standard deviation from five independent experiments.

Then, mRNA levels of IL-33 and TSLP were measured to analyze the effects of nasal commensal *Staphylococcus* species inoculation on the expression of epithelial cell-derived cytokines which play essential role in type 2 immunity in ARNE cells. Although IL-33 mRNA levels were also slightly attenuated in AR-SE inoculated ARNE cells at 48 hpi, IL-33 mRNA levels of ARNE cells decreased more significantly after inoculation of AR-SA from 8 hpi, and the lowest level was observed in AR-SA-inoculated ARNE cells at 48 hpi (Figure 5A, 5B). TSLP mRNA levels did not significantly change in ARNE cells inoculated with AR-SA or AR-SE (Figure 5C, 5D).

We then tested whether *S. aureus* targets IL-33 gene and protein expression in ARNE cells and compared expression levels in ARNE cells with those of AR-SA-inoculated NHNE cells. The IL-33 mRNA levels of ARNE cells were significantly higher than those of NHNE cells regardless of AR-SA inoculation (mean value of ARNE 1.21×10^8 vs mean value of NHNE 3.82×10^6). Reduced IL-33 gene expression was only observed in AR-SA inoculated ARNE cells until 48 hr after inoculation (mean value: 3.21×10^3, Figure 6A). Western blot analysis similarly revealed that full-length IL-33 protein levels gradually decreased until 48 hr in AR-SA-inoculated ARNE cells, and AR-SA did not show any IL-33 reduction in NHNE cells (Figure 6B). The secreted protein levels of IL-33 in the supernatants of ARNE cells were measured using ELISA and compared with those of NHNE cells after AR-SA inoculation. The secreted protein concentration of IL-33 was significantly higher in the supernatants of ARNE cells (mean

value of ARNE: 3693.2 pg/ml vs mean value of NHNE: 231.4 pg/ml) and secreted protein concentration of IL-33 was reduced in the supernatants of AR-SA-inoculated ARNE cells until 48 hr after inoculation (mean value: 103.5 pg/ml, Figure 6C). Based on these findings, nasal microbiome *S. aureus* isolated from the nasal mucus of subjects with AR reduced epithelial cell-derived cytokine, IL-33 gene expression, and protein production in allergic nasal epithelium.

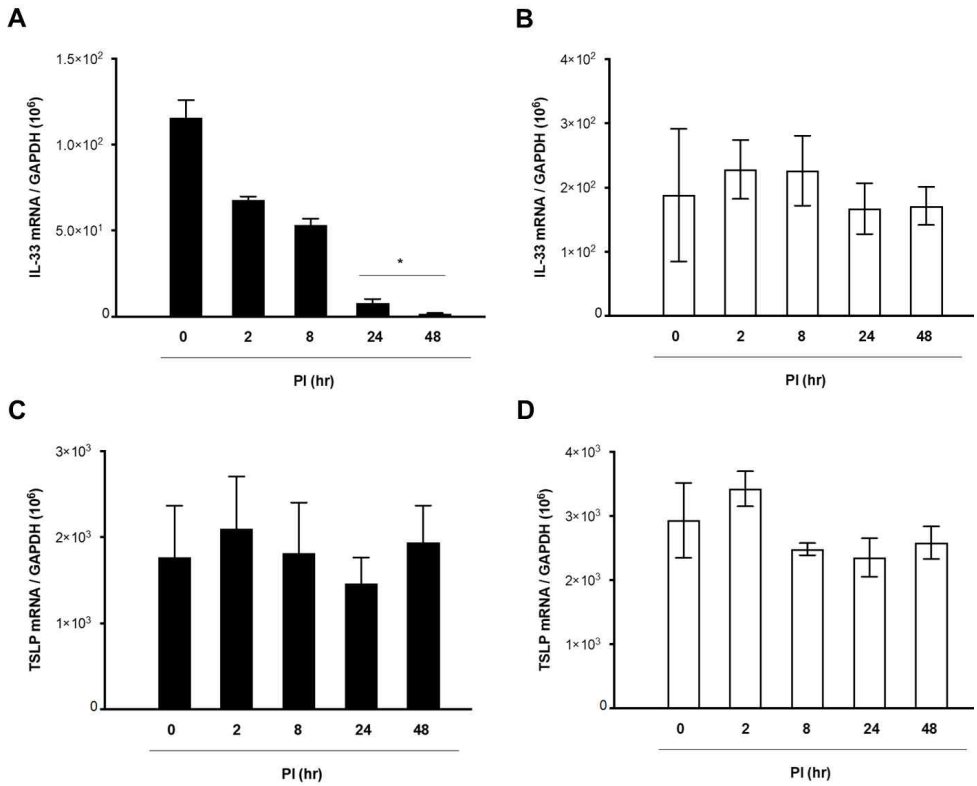

Figure 5. Allergic rhinitis nasal epithelial cells were inoculated with nasal commensal *Staphylococcus aureus* (AR-SA) and *Staphylococcus epidermidis* (AR-SE) from subjects with allergic rhinitis at the MOI of 0.25. Levels of mRNAs encoding epithelial cell-derived cytokines including IL-33 and thymic stromal lymphopoietin (TSLP) were monitored by real-time PCR (IL-33 mRNA in AR-SA-inoculated ARNE cells (A) and AR-SE-inoculated ARNE cells (B), TSLP mRNA in AR-SA-inoculated ARNE cells (C) and AR-SE-inoculated ARNE cells (D). $*p < 0.05$ compared with control ARNE cells.

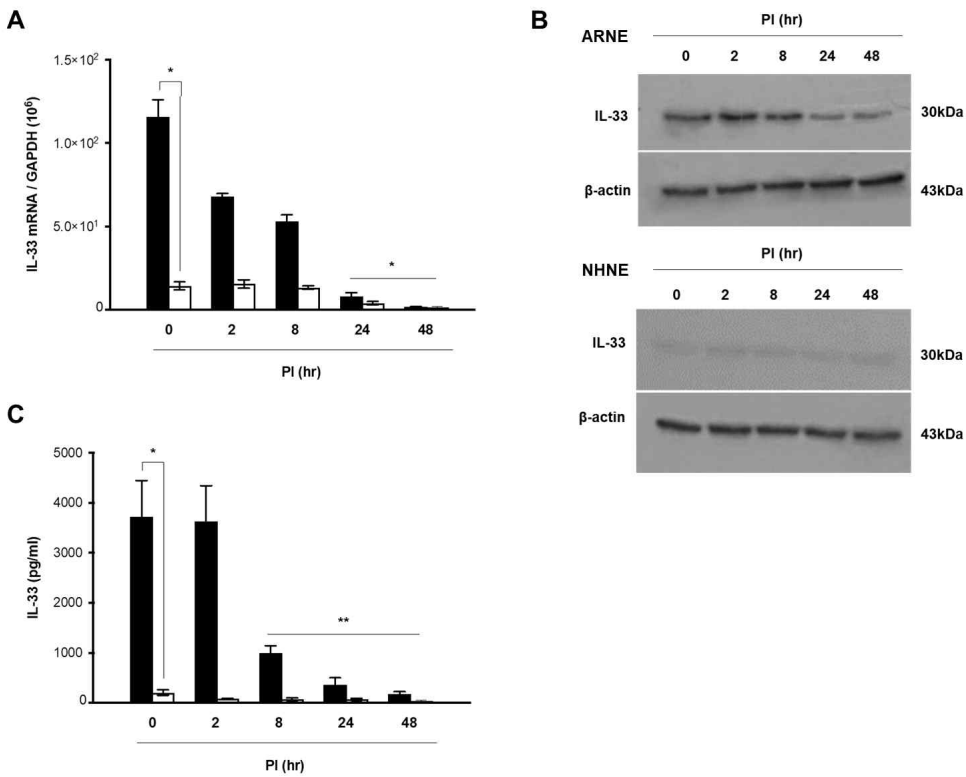

Figure 6. IL-33 mRNA and protein levels of normal human nasal epithelial (NHNE, white bar) and allergic rhinitis nasal epithelial (ARNE, black bar) cells inoculated with AR-SA were analyzed by real-time PCR (A) and Western blot (B), respectively. Secreted IL-33 protein levels of NHNE (white bar) and ARNE (Black bar) cells inoculated with nasal commensal *Staphylococcus aureus* (AR-SA) were analyzed by ELISA (C). Results are presented as mean ± standard deviation (SD) from five independent experiments and western blot results are also representative of five independent experiments.. $*p < 0.05$ comparing levels between NHNE and ARNE cells.

Pretreatment with human nasal microbiome *S. aureus* suppresses allergic inflammation *in vivo*

Considering the *in vitro* effect of nasal commensal *S. aureus* on IL-33 expression in ARNE cells, we next assessed the anti-allergic effect of *S. aureus* using an *in vivo* murine model of AR. BALB/C mice were sensitized and challenged with OVA (AR-OVA, N=5). BALB/C mice (N=5) were inoculated with human nasal microbiome *S. aureus* at 2 days (day 19, 20) following nasal microbiota depletion (day 15, 16, 17) using 30 μl of an antibiotic cocktail (vancomycin, neomycin, ampicillin, and metronidazole) (Figure 7). Nasal symptoms such as sneezing and rubbing, total serum IgE, OVA-specific IgE, serum eosinophil count, and histologic findings of nasal mucosa were compared for WT and AR-OVA mice depending on human nasal AR-SA.

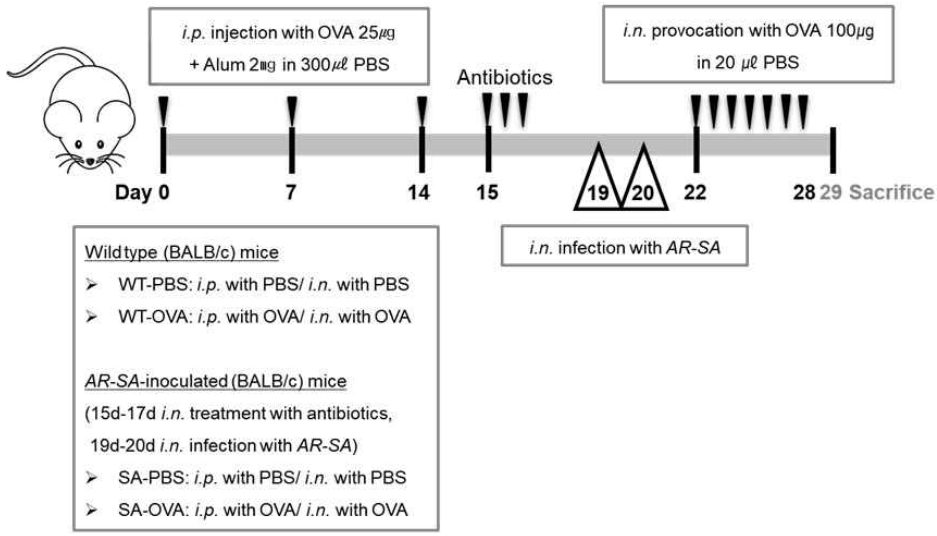

Figure 7. The experimental protocol for development of the allergic mice using BALB/C. Mice were sensitized by intraperitoneal injection of ovalbumin mixed with aluminum hydroxide on days 0, 7, and 14. Daily OVA intranasal challenge was performed from days 22 to 28 (OVA/OVA). Human nasal *Staphylococcus aureus* (AR-SA) (3.2×10^6 CFU/30 μl PBS) was inoculated at indicated time points (day 19, 20).

First, *S. aureus* mRNA levels were measured in *in vivo* nasal mucosa to confirm the effective colonization of AR-SA in WT mice and AR-OVA mice after inoculation. Real-time PCR results revealed that *femA* mRNA levels and colony counts of S. aureus were significantly higher in WT and AR-OVA mice with *S. aureus* inoculation than in WT and AR-OVA groups without inoculation (Figure 8A, 8B).

The difference in allergic symptoms of *in vivo* AR mice was clearly observed depending on inoculation with *S. aureus*-inoculated AR-OVA mice. The frequencies of sneezing and nasal rubbing in the positive control group were 9.98 ± 2.31 (WT mice) and 17.01 ± 1.43 (AR-OVA), respectively. These scores were significantly reduced in AR-OVA mice with human nasal microbiome AR-SA inoculation (1.35 ± 0.61, p = 0.018, and 4.60 ± 3.14, p = 0.010, respectively) (Figure 9A, 9B). Serum levels of total IgE (Figure 9C) and OVA-specific IgE (Figure 9D) were significantly lower in AR-OVA mice compared with the WT group.

Eosinophils and secretory cells of nasal mucosal tissue were detected by Sirius red and PAS staining. Eosinophil infiltration in the submucosa of AR-OVA mice was significantly higher than that in *S. aureus*-inoculated AR-OVA mice (Figure 10A). Fewer PAS-stained secretory cells were observed in nasal mucosal tissue of *S. aureus*-inoculated AR-OVA mice than in AR-OVA mice (Figure 10B). Collectively, these data suggest that intranasal inoculation of human microbiome *S. aureus* improved allergic inflammation in an *in*

vivo AR model. In addition, nasal microbiome isolated from allergic nasal mucus reduced Th2 inflammation of *in vivo* nasal mucosa.

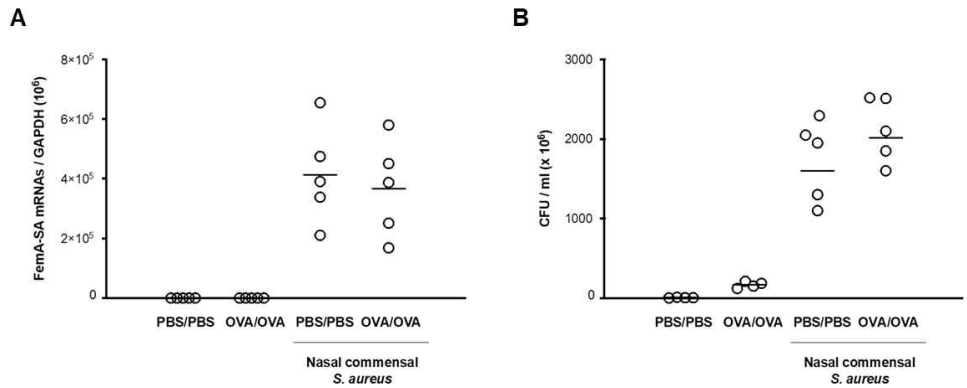

Figure 8. The mRNA levels of *femA* gene, specific for *Staphylococcus aureus* and normalized to cellular GAPDH transcript levels, were monitored by real-time PCR (A). Colony counts (B) of nasal lavage fluid were performed.

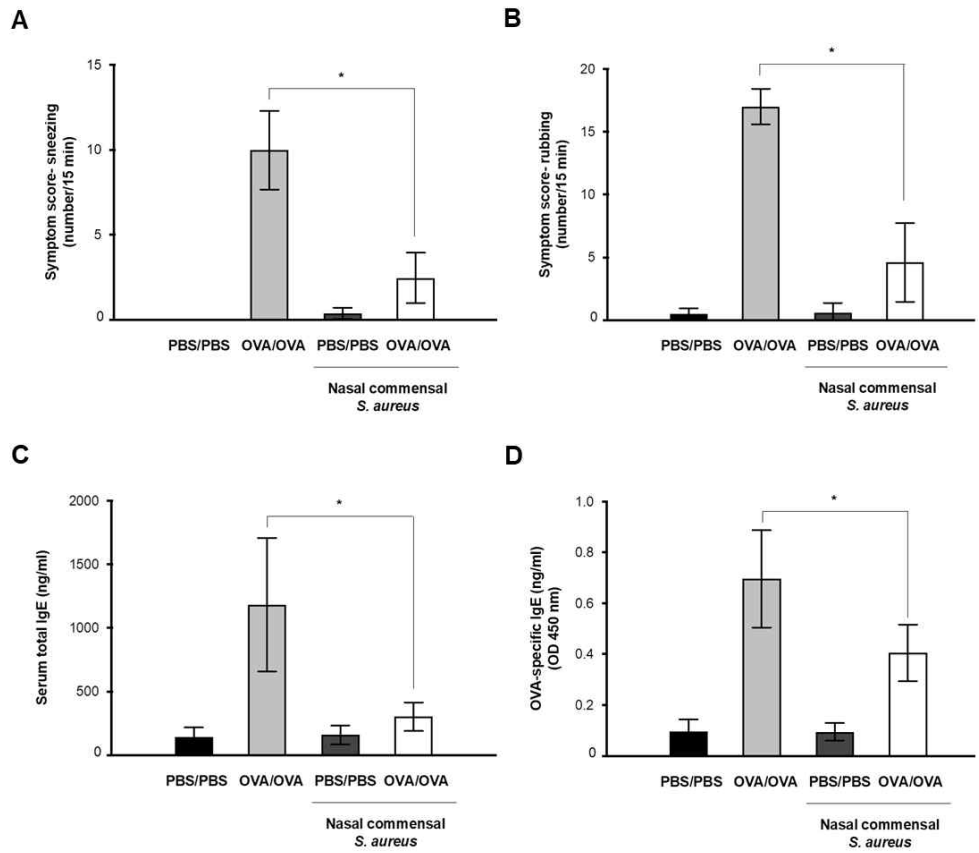

Figure 9. Frequencies of sneezing (A) and rubbing (B) events were assessed over a 15 min period after OVA provocation. Serum levels of total IgE (C) and OVA-specific IgE (D) were significantly lower in SA-OVA mice than in AR-OVA mice. Results are presented as mean ± standard deviation (N = 5). $*p < 0.05$.

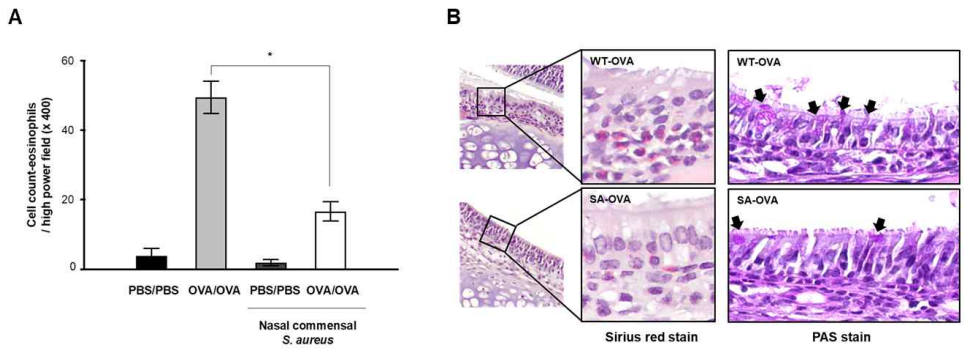

Figure 10. Histologic findings in the nasal mucosa of each group (×400 magnification) with Sirius red staining for eosinophils (A) and periodic acid-Schiff (PAS) staining for secretory cells (B). Results are presented as mean ± standard deviation (N = 5) and histologic finding is representative of nose sections from five mice. $*p < 0.05$.

Finally, mRNA expression of IL-4, IL-5, and IL-13 was measured to evaluate changes in Th2 cytokine-regulated inflammation of *in vivo* nasal mucosa after *S. aureus* inoculation. As expected, intranasal inoculation of AR-SA significantly reduced the mRNA levels of IL-4, IL-5, and IL-13 in *in vivo* nasal mucosa of AR-OVA mice compared to AR-OVA mice without AR-SA inoculation (Figure 11A, 11B and 11C). Levels of IL-33 mRNA in mouse nasal mucosa were then determined by real-time PCR. Secreted IL-33 protein levels in NAL fluid were also determined with ELISA in *in vivo* AR mice depending on human nasal *S. aureus* inoculation. Real-time PCR results revealed a higher level of IL-33 mRNA in the nasal mucosa of AR-OVA mice that was significantly attenuated in the nasal mucosa of AR-OVA mice with AR-SA inoculation (Figure 12A). In addition, OVA-induced secreted IL-33 protein level was significantly decreased in AR-OVA mice with inoculation of human nasal microbiome *S. aureus* (Figure 12B). These findings demonstrate that the nasal mucosa of AR mice had significantly reduced Th2 cytokines in response to human nasal microbiome *S. aureus*. *S. aureus*-regulated anti-allergic immune responses might be involved in IL-33 downregulation of the nasal mucosa in the *in vivo* AR model.

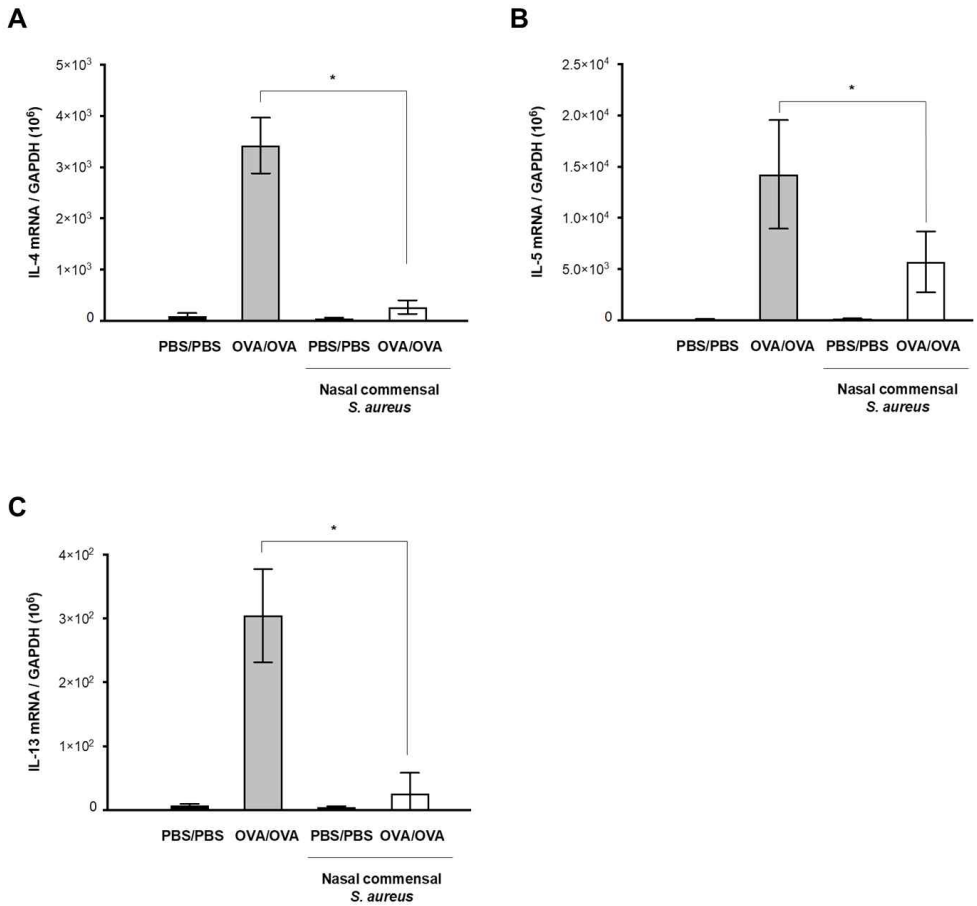

Figure 11. The mRNA levels of interleukin (IL)-4 (A), IL-5 (B), IL-13 (C) were measured by real-time PCR. Results are presented as mean ± standard deviation (N = 5). $*p < 0.05$.

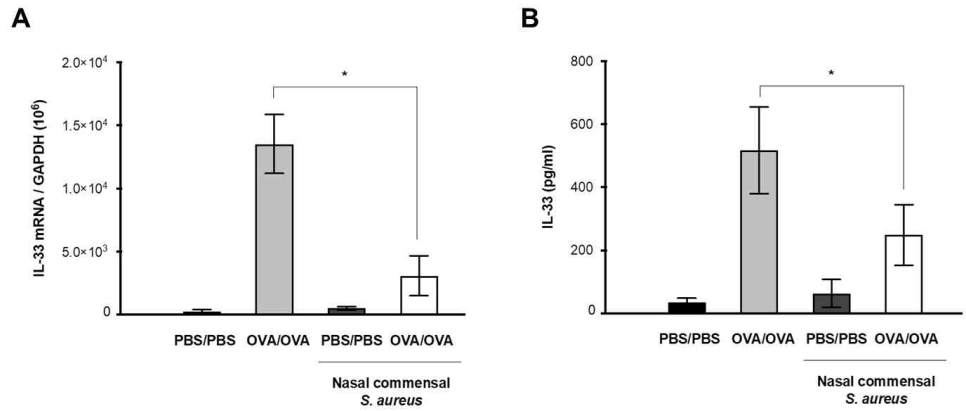

Figure 12. Interleukin (IL)-33 mRNA levels were measured by real-time PCR (A). Secreted IL-33 protein levels secreted from nasal mucosa were measured by ELISA (B) using nasal lavage fluid. Results are presented as mean ± standard deviation (N = 5). $*p < 0.05$.

Discussion

Here, we showed that there was significantly more colonization by *S. epidermidis* and *S. aureus* in the nasal mucus of subjects with AR compared to healthy nasal mucus. In addition, inoculation of human nasal microbiome *S. aureus* to ARNE cells and an OVA-sensitized AR murine model reduced Th2 cytokine-regulated allergic inflammation by suppressing the epithelial cell-derived cytokine IL-33. Our findings imply that the intranasal administration of *S. aureus* is a potential strategy for improving allergic inflammation by regulating epithelial cell-derived cytokines. Inoculation of *S. aureus* isolated from nasal mucus of subjects with AR might be a new treatment for controlling allergic inflammation caused by inhaled allergens in the nasal epithelium.

Respiratory epithelium is the first target organ for environmental allergens, and recent work has highlighted the critical role of the respiratory epithelium as a barrier that restricts host exposure to allergens.[11,21-23] Like intestinal symbiotic commensal organisms, the microbiome that colonizes respiratory mucus is thought to have critical immune functions in the respiratory tract. There may be a functional connection between respiratory epithelial cells that contact inhaled allergens and the respiratory microbiome. There is increasing interest in compositional and predicted functional differences in respiratory mucus microbiomes of allergic diseases. The importance of the respiratory microbiome, especially with respect to Th2

cytokine-regulated immune responses, has been increasingly recognized.[24-28] Understanding compositional changes in the microbiome and identifying dominant microbial species in respiratory mucus might be essential for developing new therapeutic approaches for allergic respiratory diseases. The nasal mucosa is also a key player in immunologic defenses that protect the respiratory tract and is responsible for filtering inhaled allergens from direct exposure to pressurized airflow.[22,23]

Diverse evidence suggests that microbes reside in the nasal mucus secreted from epithelial cells. Our previous study revealed that the most dominant nasal microbiome, *S. epidermidis*, increased immunity against influenza viral lung infection through IFN-λ amplification at the level of the nasal epithelium. The findings provide evidence that the nasal microbiome strengthens the innate immune responses of the host to protect the respiratory tract from viral pathogens that it is exposed to.[17] We extended this paradigm to the nasal microbiome in the field of allergic diseases and noted marked changes in the microbial composition of nasal mucus in subjects with AR. The distribution of both *S. epidermidis* and *S. aureus* was significantly increased in AR nasal mucus to constitute over 60% of the identified nasal microbiome. Among nasal microbiomes of AR nasal mucus, *S. aureus* colonization increased the most compared to normal mucus. Thus, we focused on the contributions of *S. epidermidis* and *S. aureus* to the pathogenic mechanism of AR and synergism of *Staphylococcus* species colonization with Th2-related allergic reaction

in the nasal mucosa. Both *S. epidermidis* and *S. aureus* are well-known human pathogens that may cause serious opportunistic infections of the respiratory tract. However, *Staphylococcus species* are commonly detected in the normal microflora of the skin, intestine, and upper airway. In certain circumstances, *Staphylococcus species* can invade tissues and act as disease-causing pathogens. The factors that determine the difference between pathogenic and commensal conditions are still unknown. The upregulation of the *sdr*C, *fnb*A, *fhu*D, *sst*D, and *hla* genes has been reported to occur in *S. aureus*, which acts as a pathogen compared to the commensal microbiome.[29] Many studies implicate the role of *Staphylococcus* species as symbiotic commensal organisms that are also involved in allergic diseases.[30-32] Patients with allergic eczema are more likely to have skin colonized with *S. aureus* than healthy control subjects, and disease severity is associated with *S. aureus* colonization. The dominant distribution of *S. aureus* in the upper respiratory tract of patients with asthma and aspirin sensitivity has been reported up to 87.5%, and higher nasal *S. aureus* colonization is significantly related to asthma.[30,33] In addition, patients with AR are more frequently colonized with nasal *S. aureus* or sensitized to *S. aureus* enterotoxins than healthy control subjects.[32]

Therefore, we hypothesized that *Staphylococcus* species aggravate allergic immune responses in the nasal epithelium, inoculation of *Staphylococcus* colonies dysregulates Th2 inflammation, and epithelial cell-derived cytokines regulating type 2 immunity of *in vitro* and *in*

vivo AR models would be elevated. We investigated the definite function of *Staphylococcus* species in allergic nasal mucus by isolating colonies of *S. epidermidis* and *S. aureus* from human nasal mucus of subjects with AR. Interestingly, the results differed from what was initially expected. Nasal commensal *S. aureus* isolated from the nasal mucus of subjects with AR reduced IL-33 expression in both ARNE cells and the OVA-driven AR mice model. Conversely, *S. epidermidis* isolated from the nasal mucus of subjects with AR did not induce significant changes in epithelial cell-derived cytokines such as IL-33 or TSLP. *S. aureus* induced reduction of IL-33 expression in nasal epithelium had a crucial impact on the downstream activities of epithelial cell-derived cytokines and suppressed allergic airway inflammation, eosinophil infiltration, and induction of Th2 cytokines such as IL-4, IL-5, and IL-13. This study demonstrates that colonization by *S. epidermidis* and *S. aureus* is highly increased in the nasal mucus of subjects with AR and particularly, inoculation of isolated nasal commensal *S. aureus* suppresses Th2 cytokine-dependent allergic inflammation by regulating epithelial cell-derived cytokines, especially IL-33, in *in vitro* and *in vivo* AR models.

There are studies on the effect of the gut microbiome and bacterial administration on Th2 inflammation in AR. Gut microbiome organisms such as *Lactobacilli* and *Bifidobacteria* have recently been found to be effective in murine models of allergic airway inflammation. Animal studies have suggested the effects of gut commensal bacteria on

allergen-induced airway responses, and administration of microbiome strains or bacterial components played a protective role in allergic airway inflammation.[34] Chronic obstructive pulmonary disease and neutrophilic exacerbation in asthma are associated with *Proteobacteria*, while eosinophilic exacerbation is associated with *Bacteroides* in sputum.[35] Antibiotic treatment can be used as a therapeutic for asthma. *Mycoplasma* and *Chlamydophila* pneumonia are often found in the respiratory tracts of patients with severe asthma, antibiotic treatment with Macrolide may attenuate allergic inflammation.[35,36] There is growing evidence that timely exposure colonization by microbiome is important to attenuate airway allergic immune responses by promoting regulatory Th cells (Tregs) and enhancing dendritic cells expression.[35,37] Microbial dysbiosis can induce Th2/Th17 cell polarization, leading to high IgE secretion, disrupting the epithelial integrity, and promoting excessive mucus secretion. Microbial dysbiosis contributes to exaggerating allergic responses. T cells originated from lymphoid progenitor cells appear in different subsets and functionalities, and flow cytometry can identify the specific phenotype.[38] So we did the flow cytometry to visualize and demonstrate the differentiation of cytokine-based subsets of T cells. Although the experiment was conducted by collecting the nasal mucosa of *in vivo* nasal mucosa of 5 mice in each group, the amount of sample was too small to obtain meaningful results through the experiment unfortunately and further researches will be needed to reveal this.

The nasal mucosa is a key player in immunologic responses to inhaled allergens, and we presumed that local microbiome distribution in the nasal mucus of subjects with AR might influence allergic inflammation. We wonder how nasal *Staphylococcus* species colonization is more abundant in AR nasal mucus and whether this plays a critical role in regulating Th2 immunity and allergic diseases. We surmise that allergic inflammation in the nasal mucosa of subjects with AR is thought to provide a better environment for *Staphylococcus* species to colonize and inoculation of *Staphylococcus* species adapted to AR nasal mucus contributed to reduced Th2 inflammatory responses in the allergic nasal mucosa. These findings propose that manipulation of the microbiome or inoculation of a dominant nasal microbiome might be a therapeutic strategy for AR.

Among various epithelial cell products, three epithelial cell-derived cytokines, IL-33, IL-25, and TSLP, have critical functions in the pathophysiology of allergic airway diseases. In the present study, we repeated the experiments to measure the IL-25 mRNA level using real-time PCR and the concentration of secreted IL-25 using ELISA in the ARNE cells inoculated with AR-SE and AR-SA and murine inoculation model. But all values of IL-25 were undetermined that usually refers to below detection limit of the reactions and lower than the minimum value of assay range. Therefore, we considered the IL-25 expression was too low to be detectable by real-time PCR and EISA and just focused on measuring the levels of IL-33 and TSLP. IL-33 has been regarded as an 'alarmin' and is released to activate

the immune system through first-line cells.[39] IL-33, predominantly expressed by tissue cells such as epithelial cells, fibroblasts, and endothelial cells, can be induced in allergic inflammatory conditions. By binding suppression of tumorigenicity 2 (ST2), IL-33 activates target cells to produce Th2 cytokines that regulate local and systemic allergic inflammation.[6,39] Both IL-33 and ST2 are highly expressed in the nasal mucosa of subjects with AR, suggesting that the IL-33/ST2 axis may be a key mechanistic link in the pathogenesis of allergic nasal diseases.[6,38] We suggest that reduction of IL-33 is a critical option for controlling inflammation in the nasal mucosa of patients with AR, and research to identify an effective modulator of IL-33 may improve the possibility of treating AR. We found that nasal commensal *S. aureus* isolated from subjects with AR preferentially modulated IL-33 expression in both ARNE cells and the nasal mucosa of an OVA-driven AR mice model. The current data indicate that isolation of *S. aureus* from the nasal mucus of subjects with AR and culturing it for intranasal inoculation is a promising therapeutic option for restricting IL-33/ST signaling pathways in AR nasal mucosa. Although *S. aureus* may cause respiratory infections, isolation of *S. aureus* from the adapted environment of AR nasal mucus reduced Th2 inflammatory responses by suppressing IL-33 expression in allergic nasal epithelium.

Conclusions

Our study increases understanding of whether the nasal microbiome of subjects with AR reduces Th2 inflammation to protect AR nasal epithelium from IL-33-mediated inflammation. We showed that abundant *S. aureus* colonization enhances resistance to allergic inflammation in the human nasal epithelium by reducing IL-33 expression in the nasal mucosa. Thus, intranasal delivery of human nasal commensal *S. aureus* represents a potential therapeutic approach for preventing and treating allergic inflammation via the reduction of epithelial cell-derived cytokine, especially IL-33.

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국문 초록

알레르기비염 코점막에서 분리 배양된 공생 미생물 황색포도상구균의 인터루킨-33 매개 제2형 면역 반응 조절 효과

서울대학교 대학원
의학과 중개의학 전공
전 영 진

배 경: 호흡기 공생 미생물총은 숙주 - 미생물 간 상호작용을 통하여 숙주 점막의 선천 면역 반응에 영향을 준다. 특히 코점막 공생 미생물총은 해부학적 위치 때문에 알레르기항원 흡입 시에 최전선에서 면역 반응 조절에 기여한다. 코로 흡입된 알레르기항원은 코점막의 상피세포를 자극하여 사이토카인 분비를 유도하여 일련의 면역 반응이 일어나게 된다. 제2형 보조 T (Th2) 사이토카인 발현이 증가함으로써 제2형 면역 반응 즉, 알레르기 반응을 증폭시킨다. 알레르기비염은 제2형 면역 반응의 대표적인 질환이다. 이때 코점막 공생 미생물총이 숙주의 알레르기 반응 기전에 직접적인 영향을 줄 수 있고, 알레르기 반응을 억제하는 것이 알레르기 질환 치료에 필수적일 것이다. 코점막 공생 미생물총이 숙주 점막 면역의 항상성 유지와 면역 관용에 기여하는 것으로 생각되나 그 기전이 아직 분명하게 밝혀지지 않았다.

목 적: 본 연구에서 알레르기비염 코점막의 미생물총 분포를 분석하고, 코점막 공생 미생물총의 상피세포 유래 사이토카인 분비 조절 및 제2형 면역 반응 중재 역할을 규명하고 하고자 하였다.

방 법: 알레르기 코상피세포 배양 모델과 알레르기비염 동물 모델을 제작하여, 알레르기비염 코점액에서 분리 배양된 공생 미생물 황색포도상구균 (AR-SA)과 표피포도구균 (AR-SE)을 처치하였다. 제2형 면역 반응에 관여하는 상피 유래 사이토카인 발현 변화를 분석하고, Th2 사이토카인, 면역글로불린 E 등의 분비 변화를 조사하였다. 또한 증상 변화와 조직학적 소견을 관찰하여 제2형 면역 반응 조절 여부를 평가하였다.

결 과: 16S 리보솜 RNA pyrosequencing 통해 코점막 공생 미생물총의 분포를 분석해보았을 때, 알레르기비염 코점막에서 정상 코점막과 비교하여 황색포도상구균와 표피포도구균의 분포가 유의하게 우세했다. 알레르기 코상피세포에서 정상 코상피세포보다 인터루킨-33과 TSLP 발현이 유의하게 높았으며, 알레르기 코상피세포에 AR-SA를 처치할 경우 인터루킨-33의 발현이 현저히 감소하였다. 알레르기비염 동물 모델에서 코를 통해 AR-SA를 처치하였을 때도 인터루킨-33의 발현이 현저하게 감소하였으며, 알레르기 증상 및 면역글로불린 E, Th2 사이토카인 분비가 유의하게 감소하였다.

결 론: 알레르기비염 코점막 공생 미생물총에서 포도상구균 종의 분포가 상당히 우세하였다. 특히 공생 미생물 황색포도상구균은 알레르기비염 코상피세포의 인터루킨-33의 발현을 억제하여 제2형 면역 반응을 저하시킴으로써 항알레르기 효과를 보일 수 있음을 증명하였다.

주요어: 황색포도상구균, 알레르기비염, 인터루킨-33, 공생, 미생물총

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