Materials and Methods

Participant recruitment

We recruited 8 subjects referred to the Department of Otorhinolaryngology primarily for septoturbinoplasty in Seoul National University Hospital (Seoul, Korea) and Gyeongsang National University Hospital (Jinju, Korea) between September 2020 and January 2021. The Institutional Review Board of Seoul National University College of Medicine (No. 1709-049-883) and Gyeongsang National University Hospital (No. 2019-05-004) provided approval for this study and all participants provided written informed consent. All of them underwent a multi-allergen simultaneous test (MAST) to detect allergens and specific IgE levels to confirm whether subjects experienced atopic traits (Table 1). AR is defined based on the presence of allergen-specific IgE and documentation of the relationship between allergens and typical symptoms such as runny nose, stuffy nose, itching, and/or sneezing [1]. All participants did not have any history of smoking, asthma, other sinonasal diseases, or taking intranasal or systemic corticosteroids before sampling. Four subjects who did not have AR participated in nasal mucosal swabs to isolate nasal commensal *S. epidermidis*. In addition, intranasal mucosal brushing was done around the middle turbinate for the culture of normal (NHNE) and AR nasal epithelial cells (ARNE) in 4 healthy subjects and 4 AR patients during septoturbinoplasty under Propofol total intravenous anesthesia (TIVA) general anesthesia.

Characterization and isolation of S. epidermidis in human nasal mucus

Mucus from the middle turbinate of healthy subjects was collected individually using sterile 3M Quick swabs (3M Microbiology Products, St. Paul, MN, USA)

from four subjects using a rigid 0-degree endoscope in an operating room. For bacterial colony isolation, the mucus was plated on Lysogeny Broth (LB) plates. After two days of incubation, bacterial colonies were obtained from the LB plates, and the species of each colony was identified using GS-FLX 454 pyrosequencing and 16S rRNA gene amplification, as described previously [2].

Cell culture and inoculation with human nasal commensal S. epidermidis

NHNE and ARNE cells were cultured from four healthy subjects and four AR patients (Table 1) using an air-liquid interface (ALI) method [3]. Human nasal commensal *S. epidermidis* was inoculated with NHNE and ARNE cells as previously described [2]. Briefly, the isolated commensal *S. epidermidis* was plated and grown overnight at 37°C on LB plates. A single colony of *S. epidermidis* was grown overnight at 37°C in 1 ml of LB liquid medium using an incubation shaker. The mixture of commensal *S. epidermidis* culture was diluted with the non-antibiotic feeding medium to adjust the amount to 300 µl and inoculated into the apical compartment of each NHNE and ARNE cell culture well. NHNE and ARNE cells were incubated for 0, 2, 8, 24, and 48 hours at 37°C in 5% CO_2.

Bulk *RNA sequencing* (bulk-seq)

NovaSeq 6000 platforms (Illumina, San Diego, CA, USA), and preliminary sequencing results were converted to FASTQ files using the Cell Ranger pipeline (10× Genomics). We followed the 10× Genomics standard sequence protocol by trimming the barcode and unique molecular identifier end to 26 bp and the mRNA

end to 98 bp. Then, the FASTQ files were aligned to the human reference genome (GRCh38). Subsequently, Cell Ranger was applied for preliminary data analysis to generate a file that contained a barcode table, a gene table, and a gene-expression matrix. We used WinSeurat version 2.1 (Ebiogen Inc., Seoul, Korea), based on Seurat version 3, for quality control, analysis, and exploration of bulk-seq data. Data mining and graphic visualization were performed using ExDEGA (Ebiogen Inc.). Gene Ontology (GO) enrichment was evaluated using EnrichR analyses based on the cutoff criteria (adjusted $p < 0.05$, fold change ≥ or < 1.5 and normalized data (log2) ≥ or < 2.0). The original bulk-seq data of the NHNE cells with *S. epidermidis* inoculation are available (data accessible at NCBI Gene Expression Omnibus database, accession GSE167509).

Murine inoculation model with human nasal commensal S. epidermidis

Animal experiments were approved by the Institutional Animal Care and Use Committees of Gyeongsang National University (No. GNU-201007-M0065) and the research methods were carried out by the approved guidelines. Five-week-old male wild-type (WT) BALB/C mice (Orient, Busan, 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. Mice were divided into four groups depending on *S. epidermidis* administration and OVA sensitization, which were the negative control group (WT), AR group (AR-OVA), *S. epidermidis*-administrated WT mice group, and *S. epidermidis*-administrated AR-OVA mice group. AR-OVA mice were sensitized and challenged with OVA as previously described [4]. Briefly, BALB/C mice were sensitized by intraperitoneal injection of 25 ug OVA mixed with

2㎎ alum on Day 0, 7, and 14 and then challenged by intranasal treatment of 100㎍ OVA for 7 consecutive days, from Day 22 to Day 28. At the end of the protocol, mice were euthanized by intramuscular injection of a high dose of a mixture of 10mg/kg xylazine (Bayer, Puteaux, France) and 5mg/kg ketamine (Merial, Lyon, France). If no heartbeat was detected, death was confirmed. When the mice were euthanized by injection, cervical dislocation was also performed to confirm that the mice were dead. Nasal symptoms such as sneezing and rubbing for 15 min were measured and total and OVA-specific IgE levels were analyzed using a serum sample. Nasal lavage (NAL) fluid was obtained from the nasal cavity by lavaging with 200μl 0.5mM ethylene diamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS). The NAL fluid was used for absolute colony count and enzyme-linked immunosorbent assay for measuring secreted protein concentration. Nasal tissue was harvested for real-time PCR and histologic analysis.

Real-time PCR

Human nasal commensal *S. epidermidis* colonization was monitored using real-time PCR to assess factors that were essential for methicillin-resistant (*femA*) gene expression specific for *S. epidermidis*. The *S. epidermidis* mRNA was monitored using real-time PCR for the *femA* gene with forward and reverse primers and probe 5'-CAACTCGATGCAAATCAGCAA-3', 5'-GAACCGCATAGCTCCCTGC-3', and 5′-JOE-TACTACGCTGGTGGAACTTCAAATCGTTATCG-BHQ1-3′, respectively. Levels of transcripts encoding human *IL-33* and *GATA3* and mouse *IL-4, IL-5, IL-13, GATA3, IL-33, TSLP*, *MLKL*, and *RIPK3* were determined using real-time PCR. All primers were purchased from Applied Biosystems (Foster City, CA, USA).

Western blot

Human primary IL-33 antibody was purchased from R&D Systems (AF3625; Minneapolis, MN, USA). Human primary antibodies for RIPK3 (PA5-19956), phospho-MLKL (Ser358, PA5-105678), total MLKL (PA5-34733), and CASP3 (9H19L2) were purchased from Invitrogen (Waltham, MA, USA).

Absolute colony count

Bacterial samples were serially diluted 10-fold with phosphate-buffered saline (PBS). Next, 10 µl of each diluted sample was plated onto an LB agar plate and incubated for 24 hours at 37°C. The number of *S. epidermidis* colonies growing on the agar surface was counted manually. Bacterial growth was reported based on the colony-forming units (CFUs) for each sample.

Serum levels of total and OVA-specific IgE

Serum samples of the murine model were stored at -70℃ before use for measurements of total and OVA-specific IgE, as described previously [4].

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 mucosa.

Quantification of secreted cytokines

Secreted human IL-33 (DY3625B) and mouse IL-33 (DY3626) were quantified using the DuoSet® ELISA kit from R&D Systems according to the manufacturer's instructions.

Statistical analyses

At least three independent experiments were performed with cultured cells from each donor, and data are expressed as the 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 < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 25.0 software (IBM, Chicago, IL, USA).

References

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